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The evolution and molecular bases of seasonal
coat color variation in hares: gene expression
and the shared contribution of ancestral
polymorphism and introgression

Ana Mafalda Sousa Ferreira

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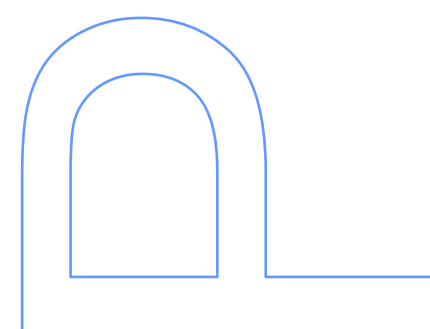
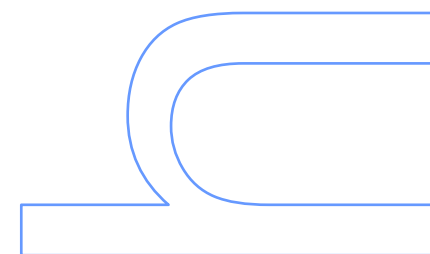
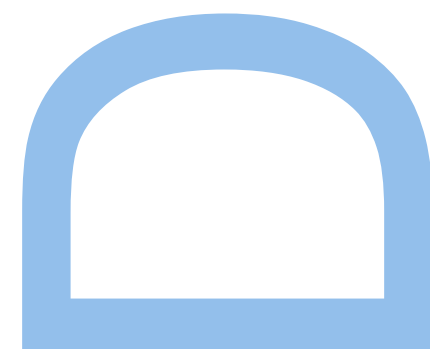
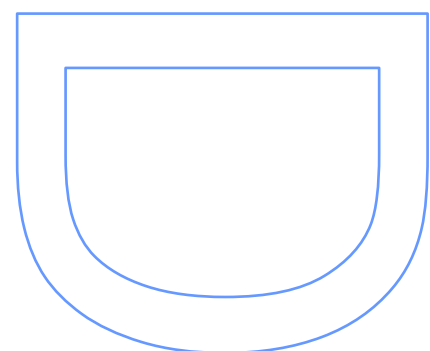
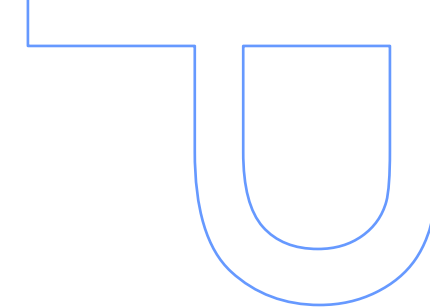
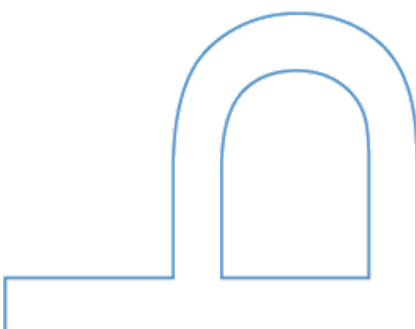
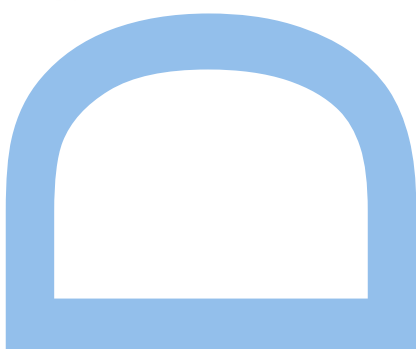


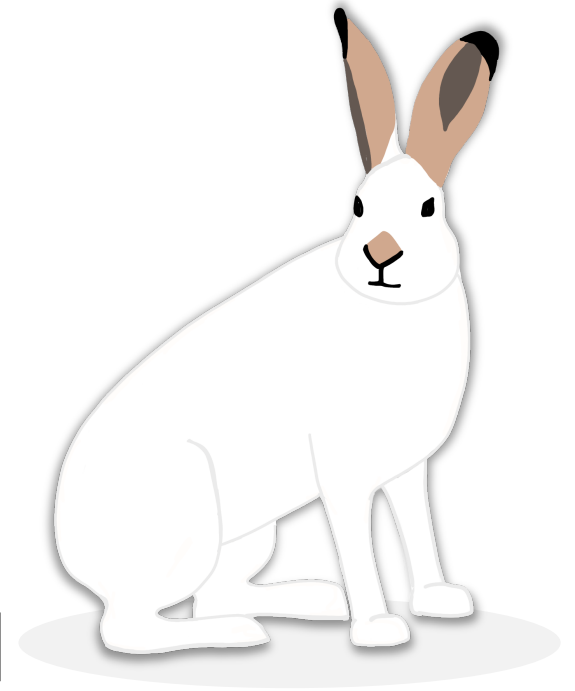
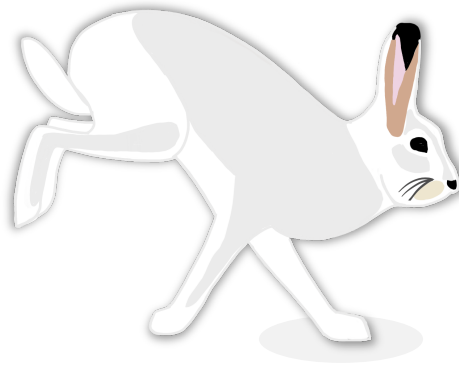
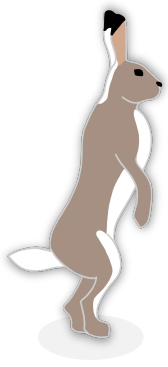
The evolution and molecular bases of seasonal coat color change variation in hares: gene expression and the contribution of ancestral polymorphism and introgression

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Tese de Doutoramento apresentada à
Faculdade de Ciências da Universidade do Porto
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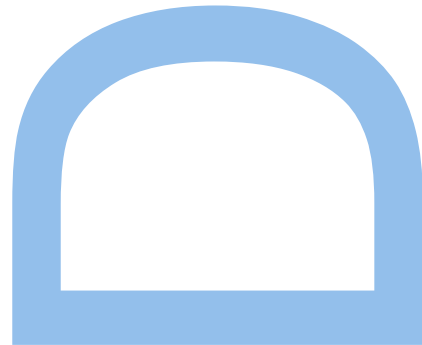
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gene expression and the shared contribution of ancestral polymorphism and introgression



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Doctoral Program in Biodiversity, Genetics and Evolution
Departamento de Biologia, Faculdade de Ciências da Universidade do Porto
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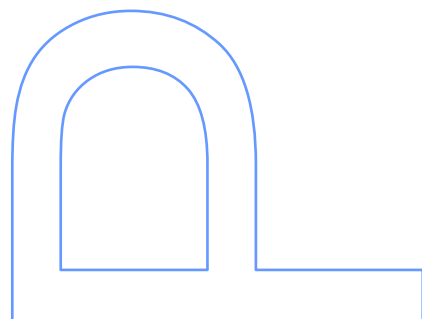
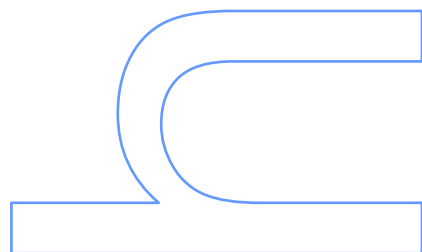
Orientadores

Dr. José Melo-Ferreira, Investigador Auxiliar, Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO-InBIO)

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Coorientador

Prof. Dr. Paulo Célio Alves, Professor Associado, Faculdade de Ciências da Universidade do Porto





Cochetopa Dome (left) and surrounding plains, Colorado, United States of America, January 2018. Photo: Mafalda S. Ferreira

“Establishing yourself as a scientist takes an awfully long time. The riskiest part is learning what a true scientist is and then taking the first shaky steps down that path, which will become a road, which will become a highway, which will maybe someday lead you home. A true scientist doesn’t perform prescribed experiments; she develops her own and thus generates wholly new knowledge. This transition between doing what you’re told and telling yourself what to do generally occurs midway through a dissertation. In many ways, it is the most difficult and terrifying thing that a student can do, and being unable or unwilling to do it is much of what weeds people out of Ph.D. programs.”

Hope Jahren, in “Lab Girl”

"In the plains of British Columbia, Nebraska, Kansas and parts of Colorado white-tailed jackrabbits, Lepus townsendii, molt into a winter pelage only slightly paler than their dark summer coat [...] and elsewhere they are nearly pure white in winter. [...] in the San Luis Valley, we have observed only dark-colored white-tails in winter, and to the north, in the central Colorado mountains, we have observed only white individuals in winter. [...] The polytypic variation in winter coats of white-tails from the Cochetopa Dome area suggests that this population has evolved as a result of secondary inter-gradation. [...] Simple Mendelian inheritance, one gene of a pair dominant to its allele, could adequately describe the genetic basis for winter coat colors for rabbits and weasels in areas where both white and dark winter coats are found. However, the polytypic winter coats of white-tails from the Cochetopa area suggests that incomplete dominance of 2 or more alleles or pairs of genes are involved in the inheritance of the ability to acquire varied winter coat colors."

Hansen & Bear, 1963,
Journal of Mammology

Foreword

In compliance with the no. 2 of article 4 of the General Regulation of Third Cycles of the University of Porto and with the article 31 of the Decree-Law no. 74/2006, of March, with the alteration introduced by the Decree-Law no. 230.2009, of 14 September, the results of previously published work were totally used and included in some of the chapters of this dissertation. As this work was performed in collaboration with other authors, the candidate clarifies that she participated in obtaining, interpreting, analyzing and discussing the results, as well in the writing the published forms.

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To my supervisor at the University of Montana, Dr. Jeffrey M. Good, I thank you for receiving me in your lab during all these years. Thank you for pushing me to think critically, to always thrive to do more and advance our field with our insights. Thank you for your kind, inspirational and supportive words along the way.

To both of you, I thank you deeply for being outstanding scientists and role models. I aspire one day to think as clearly and sharply as both of you, to develop my own ideas and guide others as I have watched you do all these years. Our discussions and your input were vital for my work and my scientific development. All your supportive words, acknowledgements of my progress and good work, and excitement for the research we developed together always encouraged me to continue. Thank you!

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Summary

With climate change threatening the survival of natural populations worldwide, it is pressing to understand how species adapt to a changing environment and use this knowledge to direct conservation measures that promote adaptation in the future. By studying the evolution of fitness-relevant traits, we can determine the molecular and evolutionary constraints of adaptation, what sources of genetic variation allow faster and effective adaptation, or how adaptive mutations are established and maintained. The work developed in this thesis used genome-wide sequencing data and phylogenomic, population genomic and transcriptomic analyses to tackle some of these questions in the context of the rapid divergence of hares (genus *Lepus* spp). In particular, we focused on seasonal coat color change, a fitness-relevant trait that involves an alternation between a brown summer coat and a white winter coat and allows camouflage in at least 18 mammal and bird species, including six hare species inhabiting seasonally snow-covered environments. We first studied the evolutionary processes at play during the rapid divergence of the genus *Lepus* and determined the importance of ancient introgression in the diversification of hares and possibly for local adaptation of extant lineages, including seasonal coat color changing species. Then, we tackled the genetic basis and evolutionary history of seasonal coat color change in a declining species, the white-tailed jackrabbit, using the genomics of adaptation to inform conservation strategies. Finally, we dissected the regulatory mechanisms underlying seasonal coat color change in hares, establishing a regulatory model for this seasonal flexible trait.

In the first manuscript, we studied the evolutionary history of hares, a widespread group of 32 species that colonized all major biomes of the Northern Hemisphere and Africa in the last 4-6 million years. We used whole-exome data to infer and date the relationships of 15 hare species representing the global distribution of *Lepus*. By studying patterns of phylogenetic discordance and hybridization, we revealed substantial levels of shared polymorphisms among species, which we show resulted not only from incomplete lineage sorting but also from introgressive hybridization events occurring since the origin of the divergence of the genus. Furthermore, this work revealed that seasonal coat color change evolved in two separate hare lineages that were connected by ancestral hybridization. We studied the genomic impact of this ancestral event of hybridization and detected introgression of regions with potential adaptive significance to highly seasonal environments, including genes involved in circadian rhythm regulation (*ARNTL2*), pigmentation (*TCF4*), and thermoregulation (*EBF2*). The results of this work revealed the importance of studying

signatures of hybridization in a phylogenetic context, which allows disentangling signatures of past and recent hybridization layered across the divergence of a clade. Due to our comparative approach, we revealed an important and overlooked role of ancestral hybridization to extant levels of standing polymorphism that may contribute to adaptation.

In the second manuscript, we studied the genetic basis and evolutionary history of continuous winter coat color polymorphism (white to brown gradient) in the white-tailed jackrabbit (*L. townsendii*), a declining North American hare species. We used a predictive model to show that the distribution of winter coat color is correlated with snow cover duration and thus likely allows camouflage across a landscape with varying snow conditions. Using spectrophotometry reflectance data, whole-genome data and mass spectrometry genotypes for 74 individuals from a transition zone in coat color in Colorado, United States of America, we associated large effect additive interactions among pigmentation genes *EDNRB* and *CORIN* to coat color variation, with a possible epistatic contribution of *ASIP*. We used comparative analysis with whole-genome data from 10 hare species, to show that variation at *EDNRB* and *CORIN* was maintained throughout 4-6 million years of divergence, while the *ASIP* brown allele originated from introgression with black-tailed jackrabbits. These results reinforce the importance of introgressive hybridization to the evolution of winter coat color polymorphisms in hares but reveal a novel role of ancestral balanced polymorphisms to survival in heterogeneous snow cover environments. We further predicted a 60-year shift in climatic conditions that will favor a 3.2-fold increase in the range occupied by winter brown phenotypes, from ~15% of the current distribution to ~49% in the 2080s. Therefore, the genetic variation underlying polymorphic winter coloration that has persisted past-selective events and is readily available in current winter brown populations will more likely seed an adaptive response to the predicted shift in selective pressures, allowing the long-term persistence of the species. However, the success of this adaptive response depends on variation standing in declining winter brown populations that must be protected to ensure the long-term viability of white-tailed jackrabbits. This work demonstrates how evolutionary processes can help define conservation priorities to promote adaptation to climate change.

In the third manuscript, we studied the transcriptional landscape underlying autumn molt from brown to white coat in the Alpine mountain hare. We detected the differential expression of 632 genes between three stages of molt – “brown” (early molt), “intermediate” and “white” (late molt) – with known functions in circadian rhythm, pigmentation and hair growth cycle regulation. Comparing the function of these genes with well described gene expression changes of the hair growth cycle, we were able to associate early molt to anagen

and late molt to the transition to catagen. These changes largely recapitulate the findings in snowshoe hares, suggesting that the regulatory mechanisms underlying seasonal coat color change are largely conserved across species. Important differences suggest seasonal variation in melanocyte differentiation and integration of nutritional cues. This work contributes to the establishment of a regulatory model of seasonal coat color change that is important to contextualize the functional architecture and evolution of this seasonal flexible phenotype.

Overall, the studies in this thesis contribute to a better understanding of the evolutionary mechanisms driving phenotypic diversification across a group of closely related and rapidly diverging species. Our results underscore the important role of ancestral polymorphism and introgressive hybridization for levels of shared standing genetic variation among species and local adaptation. The evolution of winter-coat color polymorphism across hares is a clear example, where ancestral variation in one species and introgressed variation in three species is linked to adaptation to heterogeneous snow cover conditions. The shallow and recent divergence among hares likely contributes to shared mechanisms of adaptive evolution, from similar regulatory mechanisms underlying seasonal molts, to the repeated involvement of pigmentation pathway genes in local adaptation and to the recurring role of introgressive hybridization at the origin of adaptive alleles. Our work also bridges evolutionary biology and conservation, as seasonal coat color change is a critical trait that allows increased survival in boreal and temperate environments with different winter snow cover, and we link standing genetic variation underlying adaptive winter coat color to the persistence of species in the face of rapid environmental change. We therefore provide a significant contribution towards the mechanisms of adaptive evolution and underscore the importance of studying past and present mechanisms of adaptation to predict how species may survive in the face of present and future selective challenges.

Keywords

Ancestral polymorphism, Introgressive hybridization, Ancestral hybridization, Seasonal coat color change, Adaptive evolution, Rapid divergence, Genomics.

Sumário

Numa altura em que as alterações climáticas ameaçam a sobrevivência das populações naturais, é de vital importância compreender como é que as espécies se adaptam a alterações no meio ambiente de forma a direcionar estratégias de conservação que visam promover adaptação no futuro. Ao estudar a evolução de fenótipos adaptativos, podemos compreender melhor os constrangimentos moleculares e evolutivos do processo adaptativo, quais as fontes de variação genética que resultam em adaptação rápida e eficaz, e como é que as mutações adaptativas surgem e são mantidas nas populações. A investigação desenvolvida nesta tese usou dados de sequenciação genómica e métodos filogenómicos, de genómica das populações e de transcriptómica para responder a estas perguntas no contexto da divergência rápida das lebres (género *Lepus* spp.). Em particular, focou-se na mudança sazonal da cor da pelagem, um fenótipo adaptativo que consiste na alternância entre uma coloração castanha no verão e branca no inverno, permitindo que pelo menos 18 espécies de mamíferos e aves, incluindo seis lebres, mantenham a camuflagem durante o ano em ambientes sazonalmente cobertos de neve. Em primeiro lugar, estudámos os processos evolutivos que ocorreram durante a divergência rápida do género *Lepus* e determinamos a importância da hibridização ancestral durante a diversificação das lebres e possivelmente para a adaptação local de espécies atuais, incluindo as espécies que mudam de cor. Depois, focámo-nos na evolução e base genética da mudança sazonal da cor da pelagem numa espécie em declínio, a lebre de cauda branca, usando a genómica da adaptação para informar estratégias de conservação. Finalmente, descrevemos os mecanismos regulatórios envolvidos na mudança sazonal da cor da pelagem em lebres, estabelecendo um modelo regulatório para este fenótipo sazonal.

No primeiro manuscrito, estudámos a história evolutiva das lebres, um grupo cosmopolita de 32 espécies que colonizou todos os biomas principais do hemisfério norte e África nos últimos 4-6 milhões de anos. Usámos dados de sequenciação do exoma completo para inferir e datar as relações entre 15 espécies de lebre que representam a distribuição global do género *Lepus*. Estudando os padrões de discordância filogenética e hibridização, revelámos níveis consideráveis de variação genética partilhada entre as espécies, resultante de eventos de hibridização que ocorreram desde o início da divergência do género. Mais ainda, este trabalho infere que a mudança sazonal da cor da pelagem evoluiu em duas linhagens distintas que foram ligadas por um evento de hibridização ancestral. Estudámos o impacto deste evento ancestral de hibridização no genoma, e detetámos introgressão de

regiões com potencial adaptativo em ambientes altamente sazonais, incluindo genes envolvidos na regulação de ritmos circadianos (*ARNTL2*), pigmentação (*TCF4*) e termorregulação (*EBF2*). Os resultados deste trabalho revelam a importância de estudar hibridização no contexto filogenético de um grupo de espécies relacionadas, o que permite separar eventos de hibridização recentes e ancestrais sobrepostos no tempo ao longo da divergência de um grupo de espécies. Por causa desta estratégia comparativa, revelámos uma contribuição significativa e pouco conhecida da hibridização ancestral para níveis atuais de partilha de variação genética entre espécies que podem contribuir para adaptação local.

No segundo manuscrito, estudámos a base genética e história evolutiva da variação contínua da coloração de inverno (gradiente entre branco e castanho) na lebre de cauda branca (*L. townsendii*), uma espécie em declínio na América do Norte. Com um modelo preditivo, demonstrámos que a distribuição da coloração de inverno está correlacionada com a duração da cobertura de neve, o que deverá permitir a camuflagem em habitats com condições de queda neve variáveis. Usando dados de reflectância obtidos com espectrofotometria, dados de genoma completos e genótipos de espectrometria de massa para 74 indivíduos de uma zona de transição na coloração em Colorado, Estados Unidos da América, associámos interações primariamente aditivas entre os genes de pigmentação *EDNRB* e *CORIN* a variações de coloração, com uma possível contribuição epistática do gene *ASIP*. Usámos análises comparativas entre os genomas de 10 espécies de lebres para demonstrar que a variação genética associada ao *EDNRB* e *CORIN* foi mantida durante os 4-6 milhões de anos de divergência do género, enquanto que o alelo castanho do gene *ASIP* tem origem em introgressão com a lebre de cauda negra (*L. californicus*). Estes resultados confirmam a importância da hibridização introgressiva para a evolução da variação da coloração de inverno nas lebres, mas revelam também pela primeira vez a importância de polimorfismos ancestrais balanceados para a adaptação a condições heterogêneas de queda de neve. Previmos que nos próximos 60 anos as condições ambientais irão favorecer um aumento da área ocupada pelo fenótipo castanho em 3.2 vezes, de ~15% da área atual para 49% na década de 2080. Por isso, a variação genética que codifica variações na coloração, que persistiu a pressões seletivas ao longo de milhões de anos e que existe nas atuais populações com coloração castanha no Inverno, é a variação que mais provavelmente irá permitir uma resposta adaptativa às pressões seletivas no futuro que possibilitará a persistência da espécie. Contudo, o sucesso desta resposta depende da variação existente em populações em declínio e que por isso devem ser protegidas para assegurar a sobrevivência a longo prazo da lebre de cauda branca. Este trabalho demonstra como o

conhecimento do processo evolutivo pode ajudar a definir prioridades para a conservação das populações naturais, tendo em vista promover a adaptação às alterações climáticas.

No terceiro manuscrito, estudámos as alterações de expressão génica que ocorrem durante a transição outonal de cor castanha para branca na lebre da montanha dos Alpes. Detetámos a expressão diferencial de 632 genes entre três estágios da muda – “castanho” (início da muda), “intermédio” e “branco” (fim da muda) – com funções conhecidas na regulação do ritmo circadiano, pigmentação e ciclo de crescimento do pelo. Comparando as funções destes genes com alterações de expressão conhecidas que ocorrem durante o ciclo de crescimento do pelo, associámos o início da muda de pelagem à anagénesse e o final da muda à transição para a catagénesse. Estas mudanças de expressão detetadas recapitularam os resultados observados na lebre americana, sugerindo que os mecanismos regulatórios da mudança sazonal da cor do pelo são conservados entre as espécies. Diferenças importantes sugeriram variação sazonal da diferenciação dos melanócitos e integração de sinais nutricionais. Este trabalho contribui para o estabelecimento de um modelo regulatório da mudança sazonal da cor do pelo que é importante para contextualizar a arquitetura funcional e evolução deste fenótipo sazonal.

No geral, os trabalhos desta tese contribuem para um melhor entendimento dos mecanismos evolutivos que influenciam a diversificação fenotípica em grupos de espécies próximas e que divergem rapidamente. Os nossos resultados sublinham a importância do papel de polimorfismos ancestrais e da hibridização introgressiva para os níveis de variação genética partilhados entre espécies. A evolução repetida de polimorfismo na coloração de inverno é um exemplo claro deste conceito, em que a variação ancestral numa espécie e a variação introgridida em três espécies está na origem da adaptação a ambientes com heterogeneidade na cobertura de neve. A divergência recente entre lebres provavelmente contribuiu para a partilha inferida de mecanismos de adaptação, dos mecanismos regulatórios de mudança sazonal de cor semelhantes ao envolvimento de genes da pigmentação em adaptação local, e até ao papel recorrente da hibridização introgressiva na origem de alelos adaptativos. Este trabalho estabelece ainda uma ponte entre a biologia evolutiva e a conservação das espécies, uma vez que a mudança sazonal da cor da pelagem é um carácter crítico para a sobrevivência em habitats boreais e temperados. Mais ainda, ligamos a variação genética pré-existente associada às diferentes colorações de inverno à persistência das espécies em caso de mudanças ambientais rápidas. Assim, o nosso trabalho contribui significativamente para o conhecimento dos mecanismos de evolução adaptativa e mostra a

importância de estudar os mecanismos de adaptação no passado e presente para prever como as espécies se poderão adaptar a futuras mudanças nas pressões seletivas.

Palavras-chave

Polimorfismo ancestral, Hibridização introgressiva, Hibridização ancestral, Mudança sazonal da cor do pelo, Evolução adaptativa, Divergência Rápida, Genómica.

Index

Foreword.....	i
Acknowledgements.....	ii
Summary.....	v
Keywords.....	vii
Sumário.....	viii
Palavras-chave	xi
Index.....	xii
Index of Figures.....	xv
Index of Tables.....	xxxiv
Abbreviations.....	xlili
Chapter I. General Introduction.....	1
1.1. The source of adaptive genetic variation during rapid divergence.....	2
1.2. Maintenance of ancestral polymorphism during rapid divergence.....	4
1.3. Molecular mechanisms of repeated phenotypic evolution during rapid divergence.....	5
1.4. The evolution of seasonal coat color change.....	6
1.5. The evolution of polymorphic seasonal camouflage in hares.....	8
1.6. The continuous winter coat colors of white-tailed jackrabbits.....	11
1.7. Objectives and thesis organization.....	13
1.8. References.....	15
Chapter II. The Legacy of Recurrent Introgression during the Radiation of Hares.....	27
2.1. Abstract and Keywords.....	28
2.2. Introduction.....	29
2.3. Materials and Methods.....	30
2.3.1. Taxon Sampling and Exome Sequencing.....	30
2.3.2. Read Processing and Genotyping.....	32
2.3.3. Species Tree Inference.....	33
2.3.4. Bayesian Divergence Time Inference.....	34
2.3.5. Ancestral Range Reconstruction.....	35
2.3.6. Gene Tree Discordance and Phylogenetic Networks.....	35
2.3.7. Genetic Diversity, Divergence and Admixture.....	36
2.4. Results.....	38
2.4.1. Whole Exome Sequencing Data.....	38

2.4.2. Phylogenetic Relationships among Hares.....	38
2.4.3. Incomplete Lineage Sorting and Introgression.....	39
2.4.4. Genes affected by Ancestral Introgression.....	43
2.5. Discussion.....	44
2.5.1. The Effect of Persistent Gene Flow on Phylogenetic Inference..	44
2.5.2. The Timing and Biogeography of the Lepus Radiation.....	46
2.5.3. The Legacy of Introgression during the Rapid Radiation of Hares	46
2.6. Data Accessibility.....	49
2.7. Funding.....	49
2.8. Acknowledgements.....	50
2.9. References.....	51
2.10. Supplementary Materials.....	61
2.10.1. Supplementary Material and Methods.....	61
2.10.2. Supplementary Figures.....	69
2.10.3. Supplementary Tables.....	82
Chapter III. The Evolution of Seasonal Camouflage in White-Tailed Jackrabbits and its Adaptive Potential to Climate Change.....	98
3.1. Abstract.....	99
3.2. Main Text.....	100
3.3. Acknowledgements.....	110
3.4. References.....	111
3.5. Supporting Information	114
3.5.1. Material and Methods.....	114
3.5.2. Supplementary Text.....	132
3.5.3. Supplementary Figures.....	134
3.5.4. Supplementary Tables.....	168
3.5.5. References.....	191
Chapter IV. Transcriptomic Regulation of Seasonal Coat Color Change in Hares.....	197
4.1. Abstract and Keywords.....	198
4.2. Introduction.....	199
4.3. Materials and Methods.....	201
4.3.1. Sampling, laboratory procedures and sequencing.....	201
4.3.2. Quality control and transcriptome assembly.....	202
4.3.3. Differential expression analysis	203
4.3.4. Functional enrichment analysis.....	204
4.3.5. Quantitative PCR for Agouti gene expression.....	205

4.4. Results.....	206
4.4.1. Assembly, annotation and differential expression.....	206
4.4.2. Gene-ontology term and Reactome pathway analyses.....	209
4.5. Discussion.....	210
4.5.1. The transcriptional landscape of the molt in the context of the hair growth cycle.....	210
4.5.2. Candidate genes associated with circadian rhythm and pigmentation.....	212
4.6. Conclusions and Significance.....	214
4.7. Acknowledgements.....	214
4.8. Competing interests.....	215
4.9. Author Contributions.....	215
4.10. Data Accessibility.....	215
4.11. References.....	217
4.12. Supplementary Materials.....	226
4.12.1. Supplementary Figures.....	226
4.12.2. Supplementary Tables.....	232
Chapter V. General Discussion.....	238
5.1. The sources of adaptive genetic variation during rapid divergence.....	238
5.1.1. The role of hybridization as a source of adaptive variation.....	239
5.1.2. Old polymorphisms, balancing selection and adaptive evolution	241
5.2. Molecular mechanisms of seasonal coat color change in hares.....	242
5.2.1. Regulatory mechanisms underlying seasonal camouflage.....	243
5.2.2. Evolution of polymorphic seasonal camouflage through variation in the pigmentation pathway.....	245
5.2.3. Adaptation to snow cover variation through discrete versus quantitative winter coloration.....	248
5.3. Adaptive evolution and conservation: lessons from seasonal coat color change.....	250
5.4. Conclusions.....	252
5.5. Impact of this work and Future Directions.....	253
5.6. References.....	257
Appendix	267

Index of Figures

Figure 1.1 – The distribution of the six hare species that undergo seasonal coat color change. At the top, and from left to right: Alaskan hare (*Lepus othus*; photo credit Alaska Department of Fish and Wildlife), Arctic hare (*L. arcticus*; photo credit Marie and Alistair Knock at iNaturalist), Mountain hare (*L. timidus*; photo credits habitats.org.uk, José Melo-Ferreira and nadezhdax at iNaturalist). At the bottom, from left to right: Snowshoe hare (*L. americanus*; photo credits Jaco and Lindsey Barnard), White-tailed jackrabbit (*L. townsendii*; photo credits rmbphotography at iNaturalist, and Rick Wicker, Denver Museum of Nature and Science) and Japanese hares (*L. brachyurus*; photo credits Materialschemist at Wikipedia and Mt. Iwaki Conservation Association).10

Figure 1.2 – The distribution of the white-tailed jackrabbit and other native hare species in North America. The location of the polymorphic zone described by Hansen and Bear (1963) where continuous variations in coat colors can be found (inset photo credit Rick Wicker, Denver Museum of Nature and Science) is marked with a star. To provide context, we also display the distribution of seven other North American species (Alaskan hare, *L. othus*; Arctic hare, *L. arcticus*; Snowshoe hare, *L. americanus*; Black-tailed jackrabbit, *L. californicus*; Antelope jackrabbit, *L. alleni*; White-sided jackrabbit, *L. callotis*). Given their small distribution in Mexico, we do not display the distribution of the Black jackrabbit (*L. insularis*) and of the Tehuantepec jackrabbit (*L. flavigularis*). Also, we do not display the distribution of an introduced population of European hare (*L. europaeus*) in the states of Pennsylvania, New York, United States of America, and southern Ontario, Canada.12

Figure 2.1 – Hare (*Lepus* spp.) evolutionary history and biogeography. (a) The distribution of the 15 hare species studied in this work obtained from the IUCN database (<http://www.iucnredlist.org>); (b) Coalescent species tree estimated with *ASTRAL* assigning individuals to species groups ($n > 1$ except for the white-sided jackrabbit); (c) Ancestral range reconstruction under the DIVALIKE+J model implemented in *BioGeoBEARS* on the divergence time tree estimated with *MCMCtree* and calibrated with deep fossil calibration points. Pie charts represent the probability of each potential range, and squares represent the current range of extant taxa. Node labels represent estimated divergence times in millions of years. Confidence intervals,

an alternative biogeographic reconstruction based on *Lepus* fossil calibrations and ancestral ranges for the shoulders of the tree are provided in Supplementary Material.31

Figure 2.2 – The hare (*Lepus* spp.) species tree is underlined by extensive gene tree incongruence. Split network constructed from 8,889 gene trees (5% threshold) shows discordance among the gene tree topologies (cuboid structures represent alternative topologies) in deeper nodes of the species tree. Species are marked in accordance with the continents where they are distributed.40

Figure 2.3 – Admixture events are distributed across the hare (*Lepus* spp.) species tree. The ‘f-branch’ statistic $f_b(C)$ represents excess allele sharing between branches b (y-axis) and C (x-axis) of the species tree in Figure 2.1b. The gradient represents the $f_b(C)$ score, dark gray represents tests not consistent with the species tree (for each branch b , having itself or a sister taxon as donor C) and asterisks denote block jackknifing significance at $P \leq 0.05$ (after Bonferroni correction). Tips of the tree are marked according to their current distribution, and ancestral tips (dashed lines and labeled with letters) are marked according to the ancestral range reconstruction in Figure 2.1c [two colors represent the inference of two ranges with equivalent (~ 0.5) probability]. Ancestral tips are labeled from A to L corresponding to labels in Supplementary Table S2.12 available at the end of the chapter and on Dryad.42

Figure 2.4 – Admixture proportions decrease with genetic divergence between allopatric species. We plot the ‘f-branch’ ($f_b(C)$) values against exome-wide divergence (d_{xy}) for extant species pairs, differentiating species with overlapping (sympatric/parapatric) or non-overlapping (allopatric) distributions. The tendency line represents a linear regression relating d_{xy} and all (significant and non-significant) $f_b(C)$ values calculated with function $lm()$ in R. 43

Figure 2.5 – The impact of ancestral introgression on extant northern latitude species. (a) Events of past and recent admixture inferred in this study involving snowshoe, Alaskan hares, mountain hares and white-tailed jackrabbits. The arrow indicates the direction of ancestral introgression inferred by *PhyloNet* and ‘f-branch’ and dashed lines indicate recent introgression inferred with ‘f-branch’. Values above the lines represent admixture proportion (f) estimated with ‘f-branch’ or inheritance probability (IP) estimated with *PhyloNet*; (b) Genomic distribution of ancestral blocks of

introgression (circles) inferred as shared outlier windows among fraction of admixture (f_d) analysis testing for admixture among snowshoe hares and each one of the three other northern latitude species. Tests were run in 50 kb genomic sliding windows, and outlier windows are in the top 0.5% of the f_d distribution.45

Figure S2.1 – Maximum likelihood tree of 11,949,529 base pair exome alignment (positions with no missing data for any individual), generated using a simultaneous maximum likelihood (ML) search in *RAXML* and rapid bootstrapping run under the GTR+ Γ model of sequence evolution (autoMRE option), and setting the European and pygmy rabbits as outgroups. We show branch supports for branches with support smaller than 100 in bold.69

Figure S2.2 – *ASTRAL* species tree obtained with 8,889 gene trees without a) or with b) assigning individuals to species. Nodes in both trees are numbered and annotated with quartet scores and posterior probabilities. Further information for each node can be found in Supplementary Tables S2.8 and S2.9.70

Figure S2.3 – *SVDquartets* species tree generated with 45,779 unlinked SNPs without a) or with b) assigning individuals to species. For both trees, we show branch supports for branches with support lower than 100% of 1,000 bootstrap rounds.71

Figure S2.4 – Frequencies of the three main topologies around focal branches of the *ASTRAL* species tree (Figure 2.1b). For each internal branch of the tree in a), the frequency of the three possible topologies connecting the four neighboring branches is shown in b). The title of each bar plot in b) corresponds to labeled internal branches in the tree in a). The most frequent topology is shown in red, and the two alternative topologies are shown in blue. On the x-axis of each bar plot, the topology of each quartet is represented using the neighboring branch labels. The branches leading to mountain hares and Alaskan hares, Cape and Ethiopian hares, and Corsican and broom hares were collapsed given the non-monophyly of the individuals of these species (Supplementary Figures S2.2a and S2.3a).72

Figure S2.5 – Divergence time tree of *Lepus*, estimated from alignments of 9,015 protein coding ortholog genes (10,863,822 bp alignment), with three partitions (1st, 2nd and 3rd codon positions) using a) molecular-based dates extrapolated from deep fossil calibrations in the lagomorphs or b) fossil calibrations for the divergence of the genus

Lepus and the *Lepus* species (see Materials and Methods). Node labels in bold black are divergence time estimations in millions of years. Numbers in parenthesis in each node represent node numbers in Supplementary Table S2.5.73

Figure S2.6 – Ancestral range reconstruction for the *Lepus* under the DIVALIKE+J model implemented in *BioGeoBEARS*. We performed ancestral range reconstruction using the two chronograms inferred with *MCMCtree* calibrated with a) deep fossil and b) *Lepus* fossil calibration points. Pie charts show the relative probability of each potential range. Squares at the tips represent the current range of extant species. Node labels represent node numbers in Supplementary Table S2.7.74

Figure S2.7 – Box plots of the normalized Robinson-Foulds distance between chromosome X and autosomes gene trees and the genome-wide species tree (Supplementary Figure S2.2a), and pairwise distance among all gene trees.75

Figure S2.8 – Majority Rule Consensus Tree constructed from 8,889 maximum likelihood gene trees. Tree nodes are annotated with the Internode Certainty and Internode Certainty All.75

Figure S2.9 – Chromosome X species trees. a) *ASTRAL* species tree obtained from 181 chromosome X gene trees and b) *SVDquartets* species obtained from 1,473 unlinked SNPs localized in the X chromosome. Node labels in a) correspond to node labels in Supplementary Table S2.10 and b) 1,000 bootstrap support.76

Figure S2.10 – Minimum D-statistic ($D_{\min}$; Malinsky et al 2018, Nat. Ecol. Evol.) between all possible trios of taxa in the species tree plotted against their Z-score. Significant values are highlighted in orange. The number of significant and non-significant $D_{\min}$ values is given above the graph.77

Figure S2.11 – Rooted pseudo-maximum likelihood networks for *Lepus*, with 0 to 4 reticulations inferred from 8,889 gene trees with *PhyloNet*. Red branches represent reticulations, and blue branches represent variations to the species tree topology in Figure 2.1b. Branch lengths are represented in black and inheritance probabilities are represented in red. Below each network, we report the log likelihood, Bayesian

information criteria (BIC) and Akaike information criteria corrected for small sample sizes (AICc).78

Figure S2.12 – *TreeMix* inference of splits and admixture using 30,709 biallelic SNPs for no migration events ($m=0$) to nine migration events ($m=9$). The white-sided jackrabbit (LCL) was used as outgroup. LAM – Snowshoe hare; LCF – Black-tailed jackrabbit; LGR – Iberian hare; LCR – Corsican hare; LCS – Broom hare; LST – Ethiopian highland hare; LHB – Abyssinian hare; LCP – Cape hare; LFG – Ethiopian hare; LER – European hare; LOT – Alaskan hare; LTM – Mountain hare; LTW – White-tailed jackrabbit; LMS – Manchurian hare.79

Figure S2.13 – Residuals for the *TreeMix* networks of Supplementary Figure S2.12, from 0 to 9 migration events. The white-sided jackrabbit (LCL) was used as outgroup. LAM – Snowshoe hare; LCF – Black-tailed jackrabbit; LGR – Iberian hare; LCR – Corsican hare; LCS – Broom hare; LST – Ethiopian highland hare; LHB – Abyssinian hare; LCP – Cape hare; LFG – Ethiopian hare; LER – European hare; LOT – Alaskan hare; LTM – Mountain hare; LTW – White-tailed jackrabbit; LMS – Manchurian hare.80

Figure S2.14 – Exome-wide observed genetic divergence (gray distribution) and average genetic divergence (colored lines) for fraction of admixture (f_d) outlier windows (Supplementary Table S2.13) between snowshoe hares and a) Alaskan hare, b) Mountain hare and c) white-tailed jackrabbits. The number of outlier windows used to estimate d_{xy} is reported above the graph, and in dark gray we highlight the lower 1% percentile of the distribution.81

Figure 3.1 – Winter coat color polymorphism in white-tailed jackrabbits. (A) Predicted probability of winter brown coats using bioclimatic predictors of winter coloration (snow cover duration, mean diurnal temperature range and isothermality) across the estimated suitable habitat of the white-tailed jackrabbit using 1,312 occurrence records; **(B)** White-tailed jackrabbit specimens from the Denver Museum of Nature and Science representing the extreme ends of the spectrum of winter coat color variation in the species; **(C)** Sampling locations for 74 white-tailed jackrabbit specimens from a transition zone in winter coat color in Colorado, USA, used to study phenotype-to-genotype association in this work. The size of the circles represents sample size in a given location.101

Figure 3.2 – The polygenic genetic basis of winter coat color polymorphism in white-tailed jackrabbits. (A) Genome-wide association ($-\log_{10}$ of P values, assuming an additive inheritance model; 5,557,716 SNPs) with continuous winter coat color (PC1 of a PCA analysis of dorsal reflectance measurements for 61 white-tailed jackrabbits). Points above the dashed line exceed the Bonferroni multiple test corrected threshold of $P=0.05$. **(B and D)** Gene structures of the association regions in chromosome 2 and 8 identified in panel A coincide with the location of *CORIN* **(B)** and *EDNRB* **(D)**, respectively. SNPs with an association P value above the Bonferroni-corrected threshold of $P=0.05$ are highlighted in dark blue. **(C and E)** Boxplots showing the phenotype distribution (PC1 loading) across the three alternative genotypes (BB – homozygous brown allele; BW – heterozygous; WW – homozygous white allele) of the top MassARRAY® associated SNPs in the *CORIN* **(C)** and *EDNRB* **(D)** association regions. **(F)** Mean dorsal reflectance (mean PC1 loading) across the nine possible combinations between *CORIN* and *EDNRB* genotypes. White space represents missing combinations.103

Figure 3.3 – Old alleles contribute to winter coat color polymorphism in white-tailed jackrabbits. (A) Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) between a winter white (DMNS_18801) and one winter brown (DMNS_18808) white-tailed jackrabbit homozygous for opposite alleles at each association region (gray rectangles; Figure S3.17). Orange points represent windows with RND values three standard deviations from the mean ($Z\text{-score} \geq 3$). **(B)** A winter brown (DMNS_18808) white-tailed jackrabbit consistently clusters with black-tailed jackrabbits and is not monophyletic with a conspecific winter white white-tailed jackrabbit (DMNS_18801) in the local topologies of the three association regions of *EDNRB*, *CORIN* and *ASIP*. The local topologies contrast with the multispecies coalescent tree topology (left tree) obtained from 43,430 50 kb genome-wide windows. All represented branches in the species tree have posterior probability higher than 1. Branch labels in the gene trees represent bootstrap values below 80. **(C-D)** Empirical and simulated distributions of d_{xy} genome-wide or for the *EDNRB*, *CORIN* and *ASIP* association regions in this study between winter white or winter brown white-tailed jackrabbits and **(C)** black-tailed jackrabbits or **(D)** Arctic hares. The simulated distributions of d_{xy} were generated with coalescent simulations of the demographic history of the species pairs considered with and without strong selection in the ancestral population.105

Figure 3.4 – Future snow cover conditions will increase the probability of currently endangered winter brown phenotypes. (A) Predicted increase in the probability of brown ($P(\text{brown})_{\text{future}} - P(\text{brown})_{\text{present}}$) in the 2080s, using forecasted predictions of snow residence time (proxy of snow cover; see Figure S3.30 for a comparison of the predictions for $P(\text{brown})$ obtained with the two metrics), mean diurnal temperature range and isothermality in a high-emissions scenario (RCP8.5). The density of probability of brown is predicted to shift in future conditions towards higher probabilities in the southern range (inset). **(B)** Proportion of the current distribution of “brown”, “intermediate” and “white” white-tailed jackrabbits overlapping with areas of the species distribution classified under five conservation categories (Brown et al. 2020). To bin the distribution in three phenotypic categories, we followed the Mills et al. (2018) broad definition of polymorphic zone (“intermediate”), with a polymorphic zone being where $0.2 \leq P(\text{brown}) \leq 0.8$ (Figure. S3.31).108

Figure S3.1 – Comparison of performance of the best species distribution model for five different datasets resulting from applying five thinning options (no thinning, 1 km, 5 km, 10 km or 50 km) to 1,312 white-tailed jackrabbit occurrences. For each thinned dataset, we fitted 48 different maxent models with various combinations of 6 feature classes and 8 regularization multipliers and chose the best model (with the lowest corrected Akaike Information Criteria) to perform a comparison across thinning options (in the panels). Input data (true occurrences and 10,000 random points) were spatially split into 4 groups for cross-validation. Each model was trained on one group (train.AUC), and then tested on each of the other three groups (test.AUC, averaged across 3 test sets). Large differences between training and test AUC (diff.AUC) may be indicative of overfitting. We calculated the omission rate (percent of false negatives) in the test data using a threshold for 0% (MTP, minimum training presence) or 10% omission in the training data (test.orMTP and test.or10pct, respectively), which should be close to 0.10. We also display the number of parameters in each model (parameters).134

Figure S3.2 – Genome-wide association scan (GWAS) with winter coat color of 66 white-tailed jackrabbits from Colorado. Sequence data for 36 white and 30 brown individuals (Table S3.5) was mapped to a white-tailed jackrabbit pseudo-reference in the coordinates of the European rabbit genome; **(A)** The GWAS identified three strong candidate regions in chromosome 2 (*CORIN*), chromosome 4 (*ASIP*) and chromosome 8 (*EDNRB*) as associated with coat color variation [$-\log_{10}(P \text{ value}) \geq 8.61$ after

Bonferroni correction for 20,260,101 tested SNPs]. **(B)** We performed an additional GWAS with 48 individuals (24 brown vs. 24 white) by removing intermediate individuals (Table S3.5), identifying the same association regions [$-\log_{10}(P \text{ value}) \geq 8.56$ after Bonferroni correction for 18,122,190 tested SNPs]. Individual DMNS_18807 was used to generate the white-tailed jackrabbit *de novo* assembled reference genome since its intermediate placement (highlighted in red) in local PCAs with the associated SNPs in **(C)** *CORIN*, **(D)** *ASIP* and **(E)** *EDNRB* suggests this individual is a triple heterozygote.135

Figure S3.3 – Dot plot obtained from mapping the white-tailed jackrabbit *de novo* assembled reference genome (DMNS18807_06042020_pseudohap2.1) to the European rabbit reference genome (OryCun2.0) with *D-Genies*. Dots represent matches with identity higher than 0.5.137

Figure S3.4 – Genome-wide SNP data suggests that white-tailed jackrabbits from Colorado are split into three main clusters. Main principal component axes partitioning genetic variation of white-tailed jackrabbits from Colorado using **(A)** 239,834 independent SNPs segregating among 74 individuals and **(B)** 240,109 independent SNPs segregating among 61 individuals for which we have dorsal reflectance measurements. Colored lines correspond to major geographic regions in Colorado **(C)**.138

Figure S3.5 – Admixture analysis infers that white-tailed jackrabbits from Colorado are split into three main clusters. **(A)** Identification of the best number of ancestral populations (K) for 74 white-tailed jackrabbits by applying the Evanno method (Evanno et al. 2005) to the likelihoods of admixture analyses with 1 to 10 ancestral populations, using the best replicate per K. **(B-D)** Population ancestry plots obtained from admixture analysis of 239,834 independent whole-genome SNPs segregating among 74 white-tailed jackrabbits from Colorado. Labels correspond to population clusters identified in a principal component analysis (Figure S3.4). We present ancestry plots for the best number of ancestral populations (K=3) and K=2 and 4 for context.139

Figure S3.6 – *SplitsTree* network constructed with genetic distances among 74 white-tailed jackrabbit individuals from Colorado. Genetic distances were calculated with *ngsDist* with genome-wide polymorphic sites. For each individual, we indicate its population (colored as in Figure S3.4), its sample label (as in Table S3.3), approximate winter

coat color shade [white, brown or intermediate (light brown or gray) as in Table S3.5] and year of collection.140

Figure S3.7 – Principal component analysis of reflectance measurements from **(A)** 6 dorsal regions of 61 white-tailed jackrabbit specimens from the Denver Museum of Nature and Science collection. **(B)** The two main principal component axes explaining variation in dorsal reflectance measurements reveal no particular clustering of the individuals, concordant with the continuous variation of all components of color. Individuals are labeled as in Table S3.3 and colored according to a discrete winter coat color classification (“white” as light-colored circles or “brown” as dark colored circles). **(C)** Variance explained by the first 10 principal components of the PCA. The first three components explain 77.1% of the data. **(D-F)** Contribution of the several reflectance measurements to the first three principal components. Variables are labeled by body region and reflectance measurement [e.g., DM_B2 corresponds to brightness (B2) of the middle dorsum]. Brightness measurements of all body regions and color components of mid-point of the dorsum load on the first dimension **(D)**, suggesting these are the most important aspects distinguishing individual coat color. Contrast measurements load of PC2 **(E)** and head and rump reflectance measurements load on PC3 **(F)** suggesting that patterning and variation of color between body regions are also important components of winter coat color variation in white-tailed jackrabbits.141

Figure S3.8 – First principal component of dorsal reflectance measurements separates white-tailed jackrabbits with contrasting coat brightness. Next to the plot, we show dorsal photographs of the three individuals with highest and lowest PC1 loadings. Individuals with high loadings are whiter/brighter than individuals with low loadings, which are browner/darker. Photographs were taken from specimens of the Denver Museum of Nature and Science, and the individual codes correspond to voucher codes in Table S3.3.142

Figure S3.9 – Second principal component of dorsal reflectance measurements separates white-tailed jackrabbits with patterned coats from individuals with homogeneous coloration. Next to the plot, we show dorsal photographs of the three individuals with highest and lowest PC2 loadings. Individuals with high loadings show a patterned coat color with lighter and darker patches, while individuals with low loadings tend to be completely white or completely brown. Photographs were taken from specimens of the

Denver Museum of Nature and Science, and the individual codes correspond to voucher codes in Table S3.3.143

Figure S3.10 – Third principal component of dorsal reflectance measurements separates white-tailed jackrabbits with dark head and dorsum and light rump and flank from individuals with homogeneous coloration. Next to the plot, we show dorsal photographs of the three individuals with highest and lowest PC3 loadings. Individuals with low loadings show a patterned coat color with dark head and dorsum and light rump and flanks, while individuals with high loadings tend to be completely white or completely brown. Photographs were taken from specimens of the Denver Museum of Nature and Science, and the individual codes correspond to voucher codes in Table S3.3.144

Figure S3.11 – Genome-wide association scan (GWAS) with winter coat color of white-tailed jackrabbits from Colorado. We performed **(A)** a GWAS with continuous winter coat color using the first principal component of a PCA of dorsal reflectance measurements from 61 white-tailed jackrabbits (Figure S3.7), controlling for population structure (Figure S3.4B) and **(B-C)** a GWAS with discrete winter coat color (“white” or “brown”) (Table S3.5), using genetic variation from 74 white-tailed jackrabbits **(B)** not controlling or **(C)** controlling for population structure (Figure S3.4A).145

Figure S3.12 – *MAUVE* alignment of the two pseudo-haploid 380 scaffolds of the white-tailed jackrabbit genome and chromosome 8 of the European rabbit genome centered on the scaffold 380 association region with winter white and winter brown coat colors. **(A)** Blocks represent conserved homologous segments between haploid genome 1 (used as reference), haploid genome 2 and the European rabbit chromosome 8. Homologous segments are connected by colored lines. Blocks above and below the central line in each track are aligned in the same direction or inverted relatively to the reference, respectively. The scaffold 380 association region **(B)** coincides with the flanking region of two consecutive inversions of the white-tailed jackrabbit genome relatively to the European rabbit genome (marked with two gray arrows in panel A; black arrows indicate blocks aligned in the same direction). **(C)** A local PCA of the associated SNPs in panel B (in dark blue) reveals a separation of the brown Great Plains individuals from other individuals, suggesting a potentially spurious association with coat color.146

Figure S3.13 – Genome location of the 34 SNPs (green, purple or black dots) genotyped with MassARRAY® along association regions in **(A)** scaffold 311 containing *EDNRB*, **(B)** scaffold 342 containing *CORIN* and **(C)** scaffold 245 containing *ASIP*. Genome-wide association *P* values ($-\log_{10} P$ values) with a continuous winter coat color (PC1 of dorsal reflectance measurements for 61 individuals in blue) is shown for *EDNRB* and *CORIN* (from Figure 3.2) or with discrete color (white vs. brown color for 74 individuals in green) is shown for *ASIP* (from Figure S3.11B). Since the genotyped SNPs were selected based on the results of the association with white vs. brown coloration with data mapped to a pseudo-reference based on the European rabbit genome (Figure S3.2), we colored genotypes in green if they were also associated in all four association tests performed with sequence data mapped to the white-tailed jackrabbit genome (Figure 3.2 and Figure S3.11), in purple if they were only significantly associated with white vs. brown color not controlling for population structure (Figure S3.11B), and in black if they were not associated in any test.147

Figure S3.14 – Distribution of discrete winter coat color categories (“white” or “brown”) across *EDNRB*, *CORIN* and *ASIP* genotypes for 81 white-tailed jackrabbit individuals from Colorado. We used the SNPs with highest association with continuous winter coat color (8:79701598, 2:36361155, 4:5424640, Table S3.10) among the MassARRAY® genotyped SNPs to study **(A)** the frequency of two discrete color categories (white or brown) across *EDNRB*, *CORIN* and *ASIP* genotypes; **(B)** the frequency of two discrete color categories (white and brown) across the combined *EDNRB*, *CORIN* and *ASIP* genotypes. The white allele at each gene is highlighted in bold.148

Figure S3.15 – Distribution of continuous winter coat color across *EDNRB*, *CORIN* and *ASIP* genotypes for 59 white-tailed jackrabbit individuals from Colorado. We used the SNPs with highest association with continuous winter coat color (8:79701598, 2:36361155, 4:5424640; Table S3.10) to study the **(A)** phenotype distribution (PC1 loading of a PCA of dorsal reflectance) across the combined *EDNRB* and *CORIN* genotypes; **(B)** Phenotype distribution (PC1 loading) across the three alternative genotypes (BB – homozygous brown allele; BW – heterozygous; WW – homozygous white allele) of the top associated SNP in *ASIP*; **(C)** Phenotype distribution (PC1 loading) across the combined *CORIN* and *ASIP* genotypes; **(D)** Mean phenotype (PC1 loading) across the nine possible combinations between *ASIP* and *CORIN* genotypes of the top associated SNPs; **(E)** Phenotype distribution (PC1 loading) across the combined genotypes of

EDNRB, *CORIN* and *ASIP*. Genotypes are presented in the order *EDNRB*, *CORIN* and *ASIP*. The white allele at each gene is highlighted in bold.

.....149

Figure S3.16 – Tajima’s D (**A**) and nucleotide diversity (**B**) across the *EDNRB* (left panels), *CORIN* (middle panels) and *ASIP* (right panels) association regions (311:3382036-3468296, 342:46856651-47120426 and 245:24188393-24237751, respectively). Statistics were calculated using low-coverage sequence data of 74 white-tailed jackrabbits from Colorado. Tajima’s D and nucleotide diversity were calculated in 20 kb sliding windows (2 kb step size) and windows with Z-score equal or greater than 3 are highlighted in orange. For nucleotide diversity, the dark orange, light orange and black dashed lines represent windows with Z-score above 3, Z-score above 2 and the average of the distribution, respectively.150

Figure S3.17 – High average pairwise genetic divergence among brown and white *EDNRB*, *CORIN* and *ASIP* alleles in white-tailed jackrabbits. Seven white-tailed jackrabbit individuals of various winter coat color shades (**A**) and genotypes (**B**) were selected to produce high-coverage whole-genome sequence data. The complete pelt of DMNS_16612 was not salvageable, but photographs of the specimen upon arrival at the DMNS suggest a brown winter coloration (<http://arctos.database.museum/guid/DMNS:Mamm:16612>), concordant with its genotype (**B**). Average pairwise genetic divergence (**C**) within the association regions between individuals of the same genotype (BB or WW comparisons), among heterozygote individuals (H) and between white or brown homozygote individuals (BW) compared against the average genome-wide divergence among the 21 possible pairwise comparisons with 7 individuals in 1,058,055 20 kb sliding windows (purple). The gray dashed line represents the 99.5% quantile of the genome-wide d_{xy} distribution for reference. (**D**) Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) between a winter white (DMNS_18801) and winter brown (DMNS_18808) white-tailed jackrabbits across the *ASIP* association region (gray rectangle) using high-coverage whole genome data mapped to a white-tailed jackrabbit pseudo-reference in the coordinates of the European rabbit (OryCun 2.0) genome. The pseudo-reference approach allows to map the entire structure of *ASIP*, including the ventral specific exon (black vertical bar outside the association region). Windows with RND values with Z-score ≥ 3 are highlighted in orange. Red and blue dashed lines highlight the position of a white-specific 1,033 bp insertion and of a brown-specific 43 kb deletion, respectively, identified in Jones et al. (2018).151

- Figure S3.18** – Multispecies coalescent tree generated with *ASTRAL-III* with 43,430 gene trees. Node labels correspond to node labels in Table S3.15, containing quartet scores and posterior probabilities for each node. Data used for this analysis was mapped to species specific pseudo-references in the coordinates of the European rabbit genome. Tip labels correspond to sample labels in Table S3.3. Each individual is marked with its respective winter coat color (brown – dark/brown winter coloration; gradient – intermediate coloration; white – white winter coloration).152
- Figure S3.19** – Maximum likelihood tree for 10 hare species obtained with a genome-wide concatenated alignment with 66,166,173 positions with no missing data, from sequence data aligned to species specific pseudo-references in the coordinates of the European rabbit genome. Tip labels correspond to sample labels of Table S3.3. All branches have 100/100 bootstrap support.153
- Figure S3.20** – Maximum likelihood tree of the scaffold 245 association region (245:24188393-24237751) containing *ASIP*. Alignments were generated from high-coverage data mapped to species specific pseudo-references in the coordinates of the white-tailed jackrabbit genome. Node numbers represent bootstrap support lower than 80/100.154
- Figure S3.21** – Maximum likelihood tree of the scaffold 311 association region (311:3382036-3468296) containing *EDNRB*. Alignments were generated from high-coverage data mapped to species specific pseudo-references in the coordinates of the white-tailed jackrabbit genome. Node numbers represent bootstrap support lower than 80/100.155
- Figure S3.22** – Maximum likelihood tree of the scaffold 342 association region (342:46856651-47120426) containing *CORIN*. Alignments were generated from high-coverage data mapped to species specific pseudo-references in the coordinates of the white-tailed jackrabbit genome. Node numbers represent bootstrap support lower than 80/100.156
- Figure S3.23** – Maximum likelihood tree of the scaffold 380 association region (380:35250713-35322089). Alignments were generated from high-coverage data mapped to species specific pseudo-references in the coordinates of the white-tailed

jackrabbit genome. Node numbers represent bootstrap support lower than 80/100.
.....157

Figure S3.24 – Divergence between winter brown white-tailed jackrabbits and black-tailed jackrabbits across the regions associated with winter coat color variation. **(A)** Genome-wide association ($-\log_{10}$ of P values) with continuous color (PC1 of dorsal reflectance) centered at the *EDNRB* (left panel) and *CORIN* (middle panel) association regions and association with discrete color (“white” vs. “brown”) centered at the *ASIP* association region (right panel) (311:3382036-3468296, 342:46856651-47120426 and 245:24188393-24237751, respectively). Points above the dashed line exceed the Bonferroni multiple test corrected threshold of $P = 0.05$ **(B-C)** Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) across the *EDNRB*, *CORIN* and *ASIP* association regions from panel A (light gray rectangles) with high coverage whole-genome data mapped to pseudo-references in the coordinates of **(B)** the white-tailed jackrabbit genome or **(C)** European rabbit genome. Scans were performed with whole-genome data of one white-tailed jackrabbit (DMNS_18808), one black-tailed jackrabbit (Mackay2) and one white-sided jackrabbit as outgroup (LCL_1956). Red and blue dashed lines mark the position of a white-specific 1,033 bp insertion and of a brown-specific 43 kb deletion, respectively, identified in Jones et al. (2018). **(D)** Empirical and simulated distributions of d_{xy} genome-wide or for continuous regions of low divergence between winter white or winter brown white-tailed jackrabbits and black-tailed jackrabbits detected in the genome scans in **(B)**. We plot empirical distributions of d_{xy} for the region of low divergence overlapping with the *ASIP* association region (245:24166001-24224000), the region of low divergence truncated by the start of the *ASIP* association region (245:24188393-24224000), two non-overlapping regions of low divergence inside *CORIN*'s association region (342:46884001-46908000 and 342:46912001-46938000), and one region of low divergence inside *EDNRB*'s association region (311:3398001-3426000). The simulated distributions of d_{xy} were generated with coalescent simulations of the demographic history of the species pairs with and without strong selection in the ancestral population.158

Figure S3.25 – Bayesian inference trees with divergence time estimates (millions of years) for the “white” and “brown” haplotypes of the three regions associated with winter coat color variation in white-tailed jackrabbits. One randomly phased sequence per species

is included to provide context for the divergence of the “brown” and “white” haplotypes within the hare (*Lepus* spp.) radiation.159

Figure S3.26 – Divergence between winter white white-tailed jackrabbits and closely related seasonal coat color changing species across the regions associated with winter coat color variation. Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) across the *EDNRB* (left panel), *CORIN* (middle panel) and *ASIP* (right panel) association regions (light gray rectangles; 311:3382036-3468296, 342:46856651-47120426 and 245:24188393-24237751, respectively). Scans were performed with whole-genome consensus sequences in the coordinates of the white-tailed jackrabbit genome of one white-tailed jackrabbit (DMNS_18801), **(A)** one Arctic hare (UAM_83621), **(B)** one Alaskan hare (UAM_117654) and **(C)** one mountain hare (LTM_CAT_2012). The white-sided jackrabbit (LCL_1956) was used as the outgroup.160

Figure S3.27 – Divergence between winter white white-tailed jackrabbits and snowshoe hare populations across the regions associated with winter coat color variation. Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) across the *EDNRB* (left panel), *CORIN* (middle panel) and *ASIP* (right panel) association regions (light gray rectangles). Scans were performed with whole-genome consensus sequences in the coordinates of the white-tailed jackrabbit genome of one white-tailed jackrabbit (DMNS_18801), **(A)** one Pacific Northwest snowshoe hare (LAM_INE_2013), **(B)** one Rocky Mountains hare (DOlsen1) and **(C)** one Boreal snowshoe hare (Canada_227). The white-sided jackrabbit (LCL_1956) was used as the outgroup. **(D-E)** Empirical and simulated distributions of d_{xy} genome-wide or for the *EDNRB*, *CORIN* and *ASIP* association regions in this study between winter white or winter brown white-tailed jackrabbits and **(D)** Pacific Northwest snowshoe hares or **(E)** Boreal snowshoe hares. **(F-G)** Empirical and simulated distributions of d_{xy} genome-wide or for the regions of low divergence between species within or overlapping with the association regions. In **(F)** we plot empirical distributions of d_{xy} for a region of low divergence overlapping with the *ASIP* association region (245:24188393-24216000), the region of low divergence truncated by the start of the *ASIP* association region (245:24164001-24216000) and two non-overlapping regions of low divergence inside *CORIN*'s association region (342:46884001-46922000 and 342:46928001-46950000). In **(G)** we plot a region of low divergence overlapping with the *ASIP* association region [245:24044001-24198000; truncating this region by the

start of the association region results in an alignment too short (< 20 kb) to generate a distribution] and two regions inside the *CORIN* association region (342:46876001-46926000 and 342:46926001-46950000). In panels **(D-G)** the distributions of d_{xy} were generated with coalescent simulations of the demographic history of the species pairs considered with and without strong selection in the ancestral population.161

Figure S3.28 – Divergence between snowshoe hare populations and other coat color changing species in this study across the *ASIP* association region. Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) across the *ASIP* association region in this work (4:5423743-5474836, dark-gray narrow rectangle) or winter coat color association region from Jones et al. (2018) (4:5340275-5633020, light-gray rectangle). Scans were performed with whole-genome consensus sequences in the coordinates of the European rabbit genome of one **(A)** white-tailed jackrabbit (DMNS_18801), **(B)** one Arctic hare (UAM_83621), **(C)** one Alaskan hare (UAM_117654) and **(D)** one mountain hare (LTM_MAG_1862). The snowshoe hare individuals used were LAM_INE_2013, DOlsen1 and Canada_227, left to right. The white-sided jackrabbit (LCL_1956) was used as an outgroup. Red and blue dashed lines highlight the position of a white-specific 1,033 bp insertion and of a brown-specific 43 kb deletion, respectively, identified in Jones et al. (2018). **(E-F)** Simulated and empirical distributions of d_{xy} between Pacific Northwest snowshoe hares and **(D)** Arctic hares or **(E)** mountain hares, the pairs showing stronger reduction in divergence. For each pair we show empirical distributions of d_{xy} genome-wide, for the *ASIP* association region in this work (245:24188393-24237751), the *ASIP* association region in Jones et al. (2018) and the region of low divergence between species overlapping with the regions of association (4:5442001-5588000 in Arctic hares and 4:5444001-5610000 in mountain hares). The simulated distributions of d_{xy} were generated with coalescent simulations of the demographic history of the species pairs with and without strong selection in the ancestral population.163

Figure S3.29 – Current **(A)** and future **(B)** predicted probability of winter brown coats using current and forecasted (2080s) distributions for mean diurnal temperature range (BIO2), isothermally (BIO3) and snow residence time (proxy of the snow cover duration variable used in the model of Figure 3.1) under a high CO₂ emissions scenario (RCP8.5).164

- Figure S3.30** – Difference in the current distribution of probability of winter brown coats predicted with generalized linear models using current distributions of mean diurnal temperature range (BIO2), isothermally (BIO3) and either snow cover duration or snow residence time across the United States of America.165
- Figure S3.31** – Distribution of winter brown, winter white and polymorphic zones in coat color (mixed or intermediate) across the white-tailed jackrabbit range, defining polymorphic zones as $0.2 \leq P(\text{brown}) \leq 0.8$ following Mills et al. (2018) broad definition of polymorphic zone. Probability of winter brown was predicted using a generalized linear model with current distributions of mean diurnal temperature range (BIO2), isothermally (BIO3) and snow cover duration (Figure 3.1).166
- Figure S3.32** – The predicted range of winter brown white-tailed jackrabbits (WTJR) is always higher under forecasted (green line) than in current (purple line) conditions of mean diurnal temperature range (BIO2), isothermally (BIO3) and snow residence time, no matter the threshold of $P(\text{brown})$ used to classify an area as winter brown.167
- Figure 4.1** – Mountain hare autumn molt and sampling scheme. (A) Three patches of skin were sampled from individuals undergoing autumn molt, classified according to the color of guard hairs: “brown”, “intermediate” (both brown and white hairs) and “white”. (B) Parallel between the molt stages of our sampling scheme and the stages of the hair growth cycle, as established for the spring molt in Ferreira et al., (2017), and hypothesized for the autumn molt (this work).202
- Figure 4.2** – Smear plots showing the number of differentially expressed genes ($\text{FDR} < 0.05$) in three pairwise comparisons between molt stages. Average log counts per million for each gene (dots) are plotted against log fold change. For each comparison, the number of upregulated genes in each stage is shown.207
- Figure 4.3** – A model of the transcriptional landscape of seasonal coat color change. Left panels show the results of this work on the autumn molt of the mountain hare and right panels show the corresponding results on the spring molt of the snowshoe hare, adapted from Ferreira et al., (2017). (A) Patterns of expression of differentially expressed genes in the mountain hare and snowshoe hare datasets are shown as heatmaps of $\log_2$ transformed, mean centered FPKM levels of expression per differentially expressed gene (row) and sample (column; A-C and A-E). Dendrograms

represent the complete linkage hierarchical clustering of Pearson's correlation coefficient between samples. Clusters result from PAM clustering of individual gene expression levels for $k=3$ (B – “brown”; I – “intermediate”; W – “white”). The gray line connects the mean expression value of genes' expression in “brown” (B), “intermediate” (I) and “white” (W). (B) Functional enrichment and candidate gene expression at early and late molt. For clusters in (A), a Gene Ontology and Reactome enrichment analysis was performed and the presence of candidate genes, known to have functions related with molting cycle, circadian rhythms and pigmentation regulation was inspected. Terms and genes specific to mountain hare (this work) and snowshoe hare (Ferreira et al. 2017) are highlighted in brown and green, respectively. Bold black indicates common terms to both species.208

Figure S4.1 – Multidimensional scaling plot with 4 individuals where distance between samples was calculated using the 500 genes with higher dispersion between each sample pair. Distance between samples is the Euclidian distance (average root-mean-square) of the 500 genes' absolute $\log_2$ fold change between samples. Letters identify each individual sample, and colors identify each molting stage.226

Figure S4.2 – Multidimensional scaling plot with 3 individuals where distance between samples was calculated using the 500 genes with higher dispersion between each sample pair. Distance between samples is the Euclidian distance (average root-mean-square) of the 500 genes' absolute $\log_2$ fold change between samples. Letters identify each individual sample, and colors identify each molting stage.227

Figure S4.3 – Clusters are resultant from PAM clustering of individual gene expression levels for $k=9$. The average expression level for the group of genes in each cluster is displayed in black. Letters A, B and C represent mountain hare individuals and letters B, I and W represent the “brown”, “intermediate” and “white” molting stages, respectively.228

Figure S4.4 – Clusters are resultant from PAM clustering of individual gene expression levels for $k=3$. The average expression level for the group of genes in each cluster is displayed in black. Letters A, B and C represent mountain hare individuals and letters B, I and W represent the “brown”, “intermediate” and “white” molting stages, respectively.229

Figure S4.5 – Functional overlap between the mountain hare autumn molt and snowshoe hare spring molt. Overlapping Gene Ontology Biological Process terms and Reactome pathways and the clusters where they are enriched in both species' datasets.230

Figure S4.6 – The expression of candidate agouti signaling peptide (*ASIP*) gene across the autumn molt of (A, C) mountain hares (this work) and (B) spring molt of snowshoe hares (recalculated from Ferreira et al. 2017). For both species (A, B), expression levels are shown in mean centered $\log_2$ FPKM for all individuals in each dataset, derived from RNA-sequencing. For mountain hares, (C) relative expression levels estimated from qPCR ($2^{-\Delta Ct}$) for the *ASIP* hair cycle and ventral isoform are shown for individuals and tissue types, using *ACTB* as a reference gene.231

Figure 5.1 – The phenotypic effects of *EDNRB*, *CORIN* and *ASIP* on winter coat color of white-tailed jackrabbits. **(A)** In mice, shutting down *EDNRB* at different stages of embryogenesis (e, E10.5; f, E11.5; g E12.5; h, E13.5) results in varying degrees of white spotting (see individuals indicated with arrows). Adapted from Shin et al. 1999. We observed similar patches of pigmented areas in intermediate white-tailed jackrabbits; **(B)** The interaction between *CORIN* and *ASIP* genotypes seems to alter the coloration of a mid-section of the hair. All photographs are from white-tailed jackrabbit specimens from the Denver Museum of Nature and Science mammalogy collection and numbers refer to voucher IDs.247

Index of Tables

Table S2.1 – Sampling information, for each species and individual used in this work.	82
Table S2.2 – Technical information for each individual regarding number of raw and clean reads, percentage of missing data, mean target coverage, capture enrichment, number of genotypes and pseudo-reference generation.	84
Table S2.3 – Per species nucleotide diversity.	86
Table S2.4 – Whole-exome genetic divergence (d_{xy}) among species.	86
Table S2.5 – Posteriors for divergence time estimation for each branch in Supplementary Figure S2.5 (rows labeled with numbers). For each branch, we indicate high posterior density (HPD) 95% confidence intervals and corresponding width estimated with Bayesian inference in <i>MCMCtree</i> using divergence time calibrations for the divergence between <i>Lepus</i> and <i>Oryctolagus</i> and for the divergence of the <i>Lepus</i> species obtained from (a) molecular-based dates extrapolated from deep calibrations in the Lagomorphs or (b) <i>Lepus</i> fossil record calibrations (see Materials and Methods). Also, for each partition in the alignment (first, second and third codon of the coding regions), we indicate the posterior of the mean substitution rate (μ or μ) and of the Dirichlet-gamma parameter of substitution rate heterogeneity among branches (σ^2 or σ^2).	87
Table S2.6 – Model comparison for ancestral range reconstruction in <i>BioGeoBEARS</i> using two divergence times estimations obtained from calibrating the <i>Lepus</i> species tree with deep fossil calibration points or with <i>Lepus</i> fossil calibration points. The included models were dispersal-extinction-cladogenesis (DEC), dispersal-vicariance-like (DIVALIKE), and Bayesian inference of historical biogeography for discrete areas (BAYAREALIKE), each with and without founder-event speciation (+J).	88

Table S2.7 – Proportional likelihood values for ancestral range reconstruction under the DIVALIKE+J biogeographic model implemented in <i>BioGeoBEARS</i> using two divergence times estimations obtained from (a) calibrating the <i>Lepus</i> species tree with deep fossil calibration points within Lagomorphs or (b) with <i>Lepus</i> fossil calibration points.	89
Table S2.8 – Branch information for the branches of the coalescent species tree estimated with <i>ASTRAL</i> in Figure 2.1 and Supplementary Figure S2.2b. For each branch, we indicate the quartet score for the first (q1), second (q2) and third (q3) most prevalent topologies, number of quartet trees that support the first (f1), second (f2) and third (f3) most prevalent topologies, the posterior probability for the first (pp1), second (pp2) and third (pp3) most prevalent topologies, the total number of quartets defined around each branch (QC) and the effective number of genes for the branch (EN).	90
Table S2.9 – Branch information for the branches of the coalescent species tree estimated with <i>ASTRAL</i> in Supplementary Figure S2.2a. For each branch, we indicate the quartet score for the first (q1), second (q2) and third (q3) most prevalent topologies, number of quartet trees that support the first (f1), second (f2) and third (f3) most prevalent topologies, the posterior probability for the first (pp1), second (pp2) and third (pp3) most prevalent topologies, the total number of quartets defined around each branch (QC) and the effective number of genes for the branch (EN).	91
Table S2.10 – Branch information for the branches of the coalescent species tree estimated with <i>ASTRAL</i> in Supplementary Figure S2.9a, obtained for X-linked gene trees. For each branch, we indicate the quartet score for the first (q1), second (q2) and third (q3) most prevalent topologies, number of quartet trees that support the first (f1), second (f2) and third (f3) most prevalent topologies, the posterior probability for the first (pp1), second (pp2) and third (pp3) most prevalent topologies, the total number of quartets defined around each branch (QC) and the effective number of genes for the branch (EN).	92
Table S2.11 – Shared heterozygosity in the dataset. For each individual, we indicate the amount of shared and non-shared heterozygous sites with members of other species.	93

Table S2.12 – Admixture proportion as 'f-branch' statistic ($f_b(C)$; from Malinsky et al 2018, Nat Eco Evo) values represented in Figure 2.3 for each branch pair b and C , with Z-score and p-value.	94
--	----

Table S2.13 – Windows of recent and ancient introgression between snowshoe hares (as P3) and mountain hares, Alaskan hares or white-tailed jackrabbits (as P2), detected with fraction of admixture (f_d) scans of introgression with the topology (((Iberian hare,P2),P3),Rabbit). Only significantly introgressed windows (f_d value in the top 0.5% of each distribution) in at least one species are shown. If a window is significant for all three tests it is considered to be a window of ancestral introgression. This table is available in Appendix and on Dryad Digital Repository (doi:10.5061/dryad.bzkh18967).	95
--	----

Table S2.14 – Annotation of the genes in the 119 f_d outlier windows that putatively introgressed from the mountain hare, Alaskan hare and white-tailed jackrabbit common ancestor into snowshoe hares. For each ENSEMBL Gene ID, we provide the chromosome where it is located, the start and end positions of the gene, the external gene name, and Gene Ontology annotation. This table is available in Appendix and on Dryad Digital Repository (doi:10.5061/dryad.bzkh18967).	95
--	----

Table S2.15 – Gene Ontology enrichment analysis obtained with <i>g:Profiler</i> for list of unique gene annotations in the 119 f_d outlier windows that putatively introgressed from the mountain hare, Alaskan hare and white-tailed jackrabbit common ancestor into snowshoe hares.	95
---	----

Table S2.16 – Admixture proportion as f_{hom} (Martin et al., 2015, MBE) between snowshoe hare individuals and one black-tailed jackrabbit, using the topology (((P1,P2),P3),Rabbit) reflecting variation shared recently among snowshoe hare populations and black-tailed jackrabbits. Snowshoe hare samples represent the Pacific Northwest population (LAM_A0604_WA and LAM_A0665_WA), the Boreal population (LAM_Allab3_B) and the Rocky Mountains population (LAM_NBerg67_Rockies).	95
--	----

Table S3.1 – Parameters of two generalized linear models predicting probability of winter brown across the distribution of white-tailed jackrabbits using mean diurnal	
---	--

temperature range (BIO2), isothermally (BIO3) and **(A)** snow cover duration or **(B)** snow residence time (SRT) as proxy of snow cover.168

Table S3.2 – Performance parameters for the two generalized linear models predicting the probability of winter brown across the distribution of white-tailed jackrabbits using mean diurnal temperature range (BIO2), isothermally (BIO3) and **(A)** snow cover duration or **(B)** snow residence time (SRT) as proxy of snow cover.168

Table S3.3 – Sampling information for specimens used to obtain genetic data.169

Table S3.4 – Chromium linked-read data and assembly statistics. Statistics marked with an asterisk (*) were obtained from *Supernova* and statistics marked with a plus (+) sign were obtained from *QUAST*.173

Table 3.5 – Sequencing statistics for low coverage whole-genome data of 74 white-tailed jackrabbit individuals from Colorado. Each sample was mapped to a white-tailed jackrabbit pseudo-reference in the coordinates of the European rabbit genome (66 samples) or to the *de novo* assembled white-tailed jackrabbit genome (74 samples). Individuals marked with an “I” were alternatively classified as having an intermediate coat color. NA, individual not mapped to the white-tailed jackrabbit pseudo-reference.174

Table S3.6 – Sequencing statistics for high coverage whole-genome data from 36 individuals of 10 hare species. Each sample was mapped to a pseudo-reference with the coordinates of the white-tailed jackrabbit (WTJR) genome or European rabbit genome.176

Table S3.7 – Weighted (bellow diagonal) and unweighted (above diagonal) fixation indexes (F_{st}) among the white-tailed jackrabbit population clusters identified with *NGSadmix* under a model with three ancestral populations (Figure S3.5).177

Table S3.8 – Genotypes at selected SNPs located in three association regions with winter coat color detected with genome-wide associations with a quantitative (dorsal reflectance (PC1 loading); -999 is missing data) or discrete (white “W” or brown “B”)

color classification, using samples in bold. Positions are in the European rabbit genome (OryCun2.0) coordinates and 5'-3' direction. Genotypes are colored as black: homozygous brown; dark gray: heterozygous; light gray: homozygous white; and white: missing data (0).178

Table S3.9 – Genotypes of the European rabbit genome reference (haploid), and black-tailed and white-sided jackrabbits, and Arctic, Alaskan and mountain hares. Genotypes are in the European rabbit coordinates and 5'-3' direction. Genotypes are colored as black: homozygous brown; dark gray: heterozygous; light gray: homozygous white. Blue genotypes represent a third allele and N represents missing data.181

Table S3.10 – Association test between genotyped SNPs and the first principal component of a PCA of dorsal reflectance (Figure S3.7). We performed linear regressions in R (function *lm()*) or *PLINK* 1.9 (--linear) of the effect of each SNP on the phenotype assuming an additive model between the alleles at each SNP (0, 1 and 2). All tests controlled for population structure using the main principal component axes partitioning genetic variation of 240,109 independent positions segregating in 61 individuals as covariates (Figure S3.4B). Beta reflects the effect of adding an extra white allele on phenotype.182

Table S3.11 – Linear regression testing association with continuous winter coat color considering the strongest associated SNPs at *EDNRB* and *CORIN* (Table S3.10) as factors. For each *ASIP* SNP, the ADD row gives the association coefficient and *P* value for the *ASIP* SNP while the two other rows give the association parameters and *P* value for the *EDNRB* (8:79701598) and *CORIN* (2:36361155) SNPs. Numbers in bold highlight association where the *P* value is smaller than 0.05. All tests controlled for population structure using the main principal component axes partitioning genetic variation of 240,109 independent positions segregating in 61 individuals as covariates (Figure S3.4B).183

Table S3.12 – Linear regression testing association of additive effects of allele dosage (ADD), dominance deviation from additivity (DOMDEV) and a 2 degree of freedom joint test of both additivity and dominance (GENO_2DF) with continuous winter coat color. All tests controlled for population structure using the main principal component axes

partitioning genetic variation of 240,109 independent positions segregating in 61 individuals as covariates (Figure S3.4B).184

Table S3.13 – Linear regression testing for dominance deviation (DOMDEV) from an Additive model (ADD) using the top associated SNPs at the two other candidate regions as covariates (i.e., Response ~ SNP + top SNP1 + top SNP2 + PC1 + PC2; Table S3.10). The SNPs used as covariates are marked with an asterisk (*). All tests controlled for population structure using the main principal component axes partitioning genetic variation of 240,109 independent positions segregating in 61 individuals as covariates (Figure S3.4B).185

Table S3.14 – Linear regression testing the interaction among all the SNPs genotyped across the *EDNBR*, *CORIN* and *ASIP* association regions. Only interactions with *P* value ≤ 0.05 (not corrected for multiple tests) are shown. For each pair of SNPs, we present the regression coefficient (Beta) and *P* value of the interaction term in the regression analysis in the format Beta/ *P* value.186

Table S3.15 – Information for the nodes in the multispecies coalescent tree estimated with *ASTRAL-III* in Figure 3.3B and S3.18. For each node, we indicate the quartet score for the first (q1), second (q2) and third (q3) most prevalent topologies, number of quartet trees that support the first (f1), second (f2) and third (f3) most prevalent topologies, the posterior probability for the first (pp1), second (pp2) and third (pp3) most prevalent topologies, the total number of quartets defined around each branch (QC) and the effective number of genes for the branch (EN).187

Table S3.16 – Demographic parameters inferred with *G-PhoCs* for the history of divergence between white-tailed jackrabbits and **(A)** black-tailed jackrabbits; **(B)** snowshoe hares from the Boreal population; **(C)** snowshoe hares from Pacific Northwest population and **(D)** Arctic hares, and between Pacific Northwest snowshoe hares and **(E)** Arctic hares and **(F)** mountain hares. Raw estimates of effective population size (θ), population divergence time (τ) and migration rate (M) were converted assuming a mutation rate (μ) of 2.8×10^{-9} and generation time of 2 years. Mean values for each parameter are presented along with 95% highest posterior density (HPD) interval.188

Table S3.17 – Genome-wide tests of admixture with D-statistic among mountain hares, Arctic hares, Alaskan hares, white-tailed jackrabbits, black-tailed jackrabbits and three snowshoe hare populations (Pacific Northwest, Boreal and Rocky Mountains). Pairs of species highlighted in bold show significant (P value $\leq 2.00 \times 10^{-3}$, after Bonferroni multiple test correction) admixture.	189
Table S3.18 – Admixture proportion (f_G ; Martin et al. 2015) between white-tailed jackrabbits and black-tailed jackrabbits.	190
Table S4.1 – Sampling details for the individuals used in this study.	232
Table S4.2 – Sequences, sequencing efficiencies and expected PCR product length of the primers used for qPCR expression analysis of the ventral and hair cycle <i>ASIP</i> isoforms from Jones et al. (2018), Science, and reference genes <i>ACTB</i> (from Fabiana Neves, CIBIO-InBIO, University of Porto) and <i>SDHA</i> (from Giska et al. (2019), PNAS).	232
Table S4.3 – Thermal conditions for quantitative PCR used to amplify <i>ASIP</i> isoforms and reference genes.	232
Table S4.4 – Number of reads for each individual skin sample, before and after filtering for quality.	232
Table S4.5 – <i>Transrate</i> metrics for the raw transcriptome produced with <i>Trinity</i>	233
Table S4.6 – Filtering steps applied to the transcriptome, and the number of <i>Trinity</i> genes retained in each step.	234
Table S4.7 – List of <i>Trinity</i> genes in the transcriptome, their annotation to either Rabbit and/or Mouse ENSEMBL gene database and Associated gene name. NA = Non-Applicable. This table is available in Appendix and online (https://doi.org/10.1002/ece3.5956).	234
Table S4.8 – List of differentially expressed <i>Trinity</i> genes in at least one of the pairwise comparisons performed. For each <i>Trinity</i> gene, log fold change (logFC) and false	

discovery rate (FDR) is indicated, as well as the ENSEMBL annotation to rabbit and/or mouse, associated gene name and Gene Ontology ID. NA=Non-Applicable. This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).234

Table S4.9 – Fisher exact test of overlap between the number of unique ENSEMBL annotations for each group of genes in mountain hare and snowshoe hare molt cycle, considering a background of 9,091 common genes between the skin transcriptomes of both species, calculated with *fisher.test()* in R. This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).234

Table S4.10 – Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 1 resultant from partitioning cluster analysis. This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).234

Table S4.11 – Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 2 resultant from partitioning cluster analysis. This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).234

Table S4.12 – Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 3 resultant from partitioning cluster analysis. This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).234

Table S4.13 – Reactome pathways significantly enriched in the genes of cluster 1 resultant from the partitioning cluster analysis. This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).234

Table S4.14 – Reactome pathways significantly enriched in the genes of cluster 2 resultant from the partitioning cluster analysis. This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).234

Table S4.15 – Reactome pathways significantly enriched in the genes of cluster 3 resultant from the partitioning cluster analysis. This table is available in Appendix and online (https://doi.org/10.1002/ece3.5956).	234
Table S4.16 – Information about the genes annotated to the specific Gene Ontology Biological Process categories in the first and second column. For each gene we show the respective <i>Trinity</i> "gene" code, log fold change and significance value (FDR) in each of the pairwise comparisons in which it is significantly differentially expressed (BSWS – Brown vs White; ISWS – Intermediate vs White; BSIS – Brown vs Intermediate). Also, for each gene the annotation to the ENSEMBL database (ENSEMBL gene code) is given.	235
Table S4.17 – Quantitative PCR results for the amplifications of <i>ASIP</i> (<i>Agouti</i>) hair cycle and ventral isoforms. The raw qPCR results, presented as the threshold cycle (Ct), were used to calculate expression of each <i>ASIP</i> isoform, normalized by the expression of the <i>ACTB</i> or <i>SDHA</i> genes according to the formula $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct^{Agouti} - Ct^{reference\ gene}$. We performed calculations by subtracting the mean Ct of the reference gene from each technical replicate Ct of the <i>ASIP</i> gene, to present the relative expression as normalized individual data points. Results of in the main text and in Figure S4.6 are reported based on <i>ACTB</i> gene.	236
Table S4.18 – Linear regression test results modeling the contribution of "Individual" and "Color" to the expression levels of <i>ASIP</i> , using <i>ACTB</i> as a reference gene. "Brown" and "Individual A" are used as reference levels. Linear regression was performed with <i>pcr_lm()</i> from the R package <i>pcr</i>	237

Abbreviations

AIC – Akaike Information Criterion
AICc – corrected AIC
ACTB – actin beta
A2M – alfa-2-macroglobulin
AMNH – American Museum of Natural History
ARNTL2 – aryl hydrocarbon receptor nuclear translocator-like 2
ASIP – Agouti signaling peptide
BCV – biological coefficient of variation
BIC – Bayesian information criteria
bp – base pairs
CDS – coding sequence
CI – confidence interval
CORIN – Corin, serine peptidase
CPM – counts per million
DEC – Dispersal-Extinction-Cladogenesis
DIVA – Dispersal-Vicariant-Analysis
D_{min} – minimum absolute value of D-statistic
DMNS – Denver Museum of Nature and Science
d_{xy} – genetic distance
EBF2 – early B cell factor 2
EDNRB – Endothelin Receptor type B
ESS – effective sample size
ET-1 – Endothelin-1
f_b(C) – ‘f-branch’ statistic
f_d – fraction of admixture
FDR – false discovery rate
f_G – admixture proportion
f_{hom} – admixture proportion
FPKM – fragments per kilobase per million reads mapped
F_{st} – fixation index
GLM – generalized linear model
GO – Gene Ontology
GPS – global positioning system
GWAS – genome-wide association study

HPD – high posterior density
kb – kilobase pairs
LRT – likelihood ratio test
Ma – million years ago
Mb – megabase pairs
MC1R – Melanocortin 1 Receptor
ML – maximum likelihood
 μ – mutation rate
Myr – millions of years
N_e – effective population size
NMSC – network multispecies coalescent
 π – nucleotide diversity
PAM – partitioning around medoids
PC – principal component
PCA – principal component analysis
PCR – polymerase chain reaction
PDE6H – phosphodiesterase 6H, cGMP-specific, cone, gamma
RND – scaled genetic divergence
RF – Robinson-Foulds distance
SDHA – succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SDM – species distribution model
SNP – single nucleotide polymorphism
SNV – single nucleotide variant
SRT – snow residence time
 τ – population divergence time
TCF4 – transcription factor 4
 θ – population mutation rate
TMRCA – time to the most recent common ancestor
UCM – University of Colorado Museum
UMNH – Utah Museum of Natural History
UMZM – Philip L. Wright Zoological Museum
USA – United States of America
UTRs – Untranslated regions

Chapter I

General Introduction

As we approach a point of irreparable consequences to biodiversity from anthropogenic climate change (Randers and Goluke 2020), it has never been more pressing to understand how organisms adapt to a changing environment (Savolainen et al. 2013; Capblancq et al. 2020). Important lessons can be obtained by studying the evolution and genetic basis of fitness-relevant traits, to understand their origin and how they respond to shifting selective pressures (Bay et al. 2017). For instance, the study of adaptive traits can help us understand if common molecular mechanisms underlie adaptation to the same selective pressures (Manceau et al. 2010; Stern 2013), what sources of genetic variation drive faster adaptation (*de novo* mutation, standing genetic variation or introgressive hybridization; Barrett and Schluter 2008; Hedrick 2013; Marques et al. 2019), and what mutations produce adaptive change (regulatory, coding or structural; Wray 2007; Wellenreuther et al. 2019). Studying adaptive evolution can also reveal how adaptive mutations are established and maintained (interplay of drift, migration and selection; Dittmar et al. 2016; Jamie and Meier 2020), and if variation in fitness-relevant traits is explained by a few genes of large effect or many genes of small effect (Pritchard et al. 2010; Dittmar et al. 2016).

While detailed intraspecific studies can provide fundamental insights on adaptive evolution, comparative studies among closely related groups of species are necessary to broadly assess the evolutionary pathways that underlie the origin of adaptive variation (Berner and Salzburger 2015). For instance, studies focusing on the rapid ecological divergence of many species have brought great insights into the molecular mechanisms that underlie repeated phenotypic evolution (Reed et al. 2011; Lamichhaney et al. 2016; Lewis et al. 2019) or the importance of hybridization during adaptation and speciation (Seehausen 2004; Grant and Grant 2019; Marques et al. 2019). They have also shown that mechanisms promoting adaptation in the present time may have evolved prior to the origin of the species being studied. These findings underscore the importance of ancestral alleles in contemporary local adaptation (Marques et al. 2019) and suggest that ancient hybridization can be at the origin of adaptive genetic variation (Taylor and Larson 2019). Therefore, the study of adaptive

evolution within and across species can contribute to a better understanding of how organisms are able to persist and adapt across multiple evolutionary scales. Ultimately this knowledge can inform management decisions focusing on traits that are vital for adaptation to environmental change and on genetic variation that can quickly seed rapid adaptation (Hoban et al. 2016; Bay et al. 2017).

1.1. The source of adaptive genetic variation during rapid divergence

Theoretical and empirical evidence points to a disproportional role of standing genetic variation as the source of variation for phenotypic diversification during rapid divergence and adaptation (Barrett and Schluter 2008; Hedrick 2013; Marques et al. 2019). There are several explanations for this expectation. First, the waiting times for a beneficial *de novo* mutation to occur are longer ($1/\text{mutation rate}$; Kimura 1984) than for a beneficial allele that is already present in the population (Hedrick 2013), and also longer than the rates of diversification often observed in rapid diverging clades (Marques et al. 2019). Moreover, the selective fixation of new mutations might be counteracted by genetic drift when they are recessive and, therefore, not immediately advantageous (Orr and Betancourt 2001), or when the fitness effects of new mutations are too small (Kopp and Hermisson 2009). On the other hand, standing genetic variation can allow faster adaptation since it typically segregates at higher frequencies in the population (i.e., less likely to be lost due to genetic drift) and has potentially been previously challenged by selection in specific environmental contexts, which increases the probability of its long-term persistence and effective response during repeated selection bouts (Barrett and Schluter 2008; Matuszewski et al. 2015).

Standing genetic variation, either inherited from an ancestral population or introduced via introgression among species, seems therefore more likely to seed adaptation within shorter evolutionary scales (Matuszewski et al. 2015; Marques et al. 2019). Empirical examples that have linked ancestral polymorphism to local adaptation include 15 million year old haplotypes that originated at the split of threespine sticklebacks (*Gasterosteus aculeatus*) from its closest relative (ninespine stickleback, *Pungitius pungitius*), underlying the repeated colonization of freshwater habitats by marine populations 6 million years ago (Nelson and Cresko 2018). Similarly, old haplotypes that display higher divergence within species than the genomic background have been linked to adaptation in geographically distant populations of different organisms. Some notable examples include genetic variation underlying reproductive timing in Atlantic herring (Lamichhaney et al. 2017) and steelhead and Chinook salmon populations (Prince et al. 2017), survival in seaweed fly *Coelopa frigida* (Mérot et al. 2018),

and color polymorphism in stick insects *Timema cristinae* (Lindtke et al. 2017). Introgressive hybridization from a close relative can also contribute to standing genetic variation. Hybridization has been shown to have predominantly negative consequences on fitness (Mayr 1963; Barton 2008), and patterns of introgression tend to be strongly shaped by purifying selection (Schumer et al. 2018; Edelman et al. 2019). Nonetheless, hybridization among diverging lineages may also produce novel allelic combinations that generate increased phenotypic variation, or allow the direct incorporation of adaptive alleles that can seed adaptation when coupled with ecological opportunity (Grant and Grant 2019; Marques et al. 2019). In line with this, population genomic data has shown that introgressive hybridization is pervasive in nature, and has been shown to occur in a great variety of taxonomical groups in connection with the evolution of adaptive polymorphisms (Marques et al. 2019; Taylor and Larson 2019). For example, across animals, introgression has been connected to the evolution of crypsis (Jones et al. 2018; Giska et al. 2019), mimicry patterns (The Heliconius Genome Consortium 2012; Jay et al. 2018), altitude adaptation (Huerta-Sánchez et al. 2014), adaptation to drought (Lamichhaney et al. 2015), pesticide resistance (Song et al. 2011), and pathogen resistance (Bechsgaard et al. 2017; Fijarczyk et al. 2018; Sagonas et al. 2019). Furthermore, the detection of signatures of ancestral introgression among extant species (Wilson and Bernatchez 1998; Alves et al. 2008b; Malinsky et al. 2018; Li et al. 2019; Vanderpool et al. 2020) suggests that the impact of introgression can persist during long evolutionary timescales and across speciation events. This raises the hypothesis that introgression can have much deeper evolutionary impacts on adaptation, with variants introgressed between ancestral lineages being recruited by selection much later in daughter lineages. However, evidence for a lag between introgression and a subsequent increase in frequency in response to selective pressures has only been demonstrated for relatively recent ancestral introgression like among modern humans and Neanderthals or Denisovans (Yair et al. 2020) and allopatric species (Seixas et al. 2018; Giska et al. 2019).

Finally, pre-existent shared variation can have an impact on local adaptation across deeper evolutionary scales, including closely related groups of species, and can thus be in the origin of trans-specific polymorphic traits (Jamie and Meier 2020). For example, shared ancestral polymorphisms have been connected to the evolution of polymorphic social strategies in *Solenopsis* fire ants (Yan et al. 2020), female-limited mimicry in *Papilio* swallowtail butterflies (Zhang et al. 2017), short-wave sensitivity in *Hydrophis* sea snakes (Simões et al. 2020), and crypsis in *Timema* stick insects (Villoutreix et al. 2020). On the other hand, recurrent introgression of the same wing color and patterning genes has been implicated in the evolution of polymorphic wing patterns among *Heliconius* butterflies (Smith and Kronforst 2013; Jay et al. 2018; Kozak et al. 2018). Finally, a combination of both sources

of genetic variation has also been documented, when certain lineages fix one of the alleles but later encounter selective pressures that favor the alternative morphology (Palmer and Kronforst 2020). For example, this seems to have been the case of beak size loci in the radiation of the Darwin's finches, where old haplotypes segregate with beak size across the radiation, but have also been exchanged between species and consequently selected in response to variations in food sources caused by rapid environmental change (Lamichhaney et al. 2015, 2016). Another example is color variation in *Podarcis* wall lizards, where ancient haplotypes responsible for polymorphic coloration are maintained by balancing selection but were also exchanged between species (Andrade et al. 2019).

1.2. Maintenance of ancestral polymorphism during rapid divergence

If ancient standing genetic variation often underlies adaptation across closely related groups of species, what processes may contribute to maintain this shared variation? The probability of survival of an allele in a population depends on the effective population size (N_e) and selective effect size of a mutation, since small populations experience more drift (and thus more rapid loss or fixation of alleles) and large effect alleles tend to be favored or eliminated by selection, depending on the selection coefficient (Clark 1997; Barrett and Schluter 2008; Yeaman and Whitlock 2011). Furthermore, gene flow between populations or species can introduce and maintain variation (Pinho and Hey 2010). If two species maintain shared polymorphisms after divergence, the expected time for the loss of the neutral shared polymorphism is also dependent on population size ($\sim 1.7 N_e$ generations, Clark 1997). Therefore, for closely related species with large effective population sizes, incomplete lineage sorting can be quite prevalent and result in major phylogenetic discordances along the genome and abundant shared polymorphisms that survive across species boundaries long after divergence (Song et al. 2012; Suh et al. 2015; Pease et al. 2016; Edelman et al. 2019; Vanderpool et al. 2020).

Shared polymorphisms can be much older than expected from genetic drift alone. In this case, balancing selection can underlie the maintenance of shared variation across species boundaries, particularly if related species experience similar selective pressures. One common form of balancing selection is frequency dependent selection where the fitness of a phenotype depends on its frequency (Ayala and Campbell 1974). Examples in nature include sexual selection for the 'rare male' morphs (Dijkstra and Border 2018) as observed in guppies (*Poecilia reticulata*) (Hughes et al. 2013), or apostatic selection where predators focus on common forms [e.g., guppies (*P. reticulata*) (Olendorf et al. 2006) and *Plethodon* salamanders (Fitzpatrick et al. 2009)] resulting in the evolution of Batesian mimicry in several systems [e.g.,

Salamander *P. cinereus* (Kraemer and Adams 2014); Papilionidae butterflies (Mallet and Joron 1999; Tsurui-Sato et al. 2019)]. Heterosis, or heterozygote advantage, is another form of balancing selection. Forms of heterosis include antagonist pleiotropy where one allele confers higher survival advantage while the alternative allele increases reproductive success in the seaweed fly (*C. frigida*) (Mérot et al. 2020). A similar example is the proposed trade-off in horn-size in Soay sheep, where large horns in males are selected by females (sexual selection) but smaller horns have increased survival (natural selection), resulting in a net heterozygote advantage (Johnston et al. 2013). Finally, spatially or temporally variable selection is also expected to contribute to maintain polymorphisms in nature (Gillespie 1974; Hedrick 2006; Gulisija and Kim 2015; Wittmann et al. 2017). For example, color polymorphisms evolve in many species in response to heterogeneous selective pressures (Gray and McKinnon 2007) including crypsis across color contrasting environments (Nachman et al. 2003; Mills et al. 2018; Barrett et al. 2019; Villoutreix et al. 2020) or thermoregulatory clines (Rosenblum et al. 2004; Hegna et al. 2013). Other environmental clines created by altitude (Natarajan et al. 2016; Witt and Huerta-Sánchez 2019), or photoperiod and temperature (Kozak et al. 2019) can contribute to create and maintain polymorphisms within and across species.

1.3. Molecular mechanisms of repeated phenotypic evolution during rapid divergence

Similar phenotypes across related species have been the focus of studies wanting to understand if evolution proceeds with similar molecular mechanisms in the face of the same selective challenges (Manceau et al. 2010; Rosenblum et al. 2014). The evolution of identical phenotypes in response to similar selective pressures across species can be underlined by different levels of molecular similarity, from the same pathway, same genes, same source of variation (see section 1.1), or same types of mutations. For example, color polymorphisms often involve changes in the genes of the pigment production (melanogenesis) pathway (Slominski et al. 2004; Hoekstra 2006) and adaptation to high-altitude usually involves changes in the hypoxia-inducible factor oxygen-signaling pathway (Cheviron and Brumfield 2012). Within pathways, changes can occur on the same genes, such as color polymorphism in deer mice (*Peromyscus*) (Steiner et al. 2007; Barrett et al. 2019) or hares (*Lepus*) (Jones et al. 2018; Giska et al. 2019), evolution of patterning across cichlid species (Kratochwil et al. 2018), armor plate patterning in three-spine stickleback populations (Colosimo et al. 2005), beak size in finch species (Lamichhaney et al. 2016) or mimicry in *Heliconius* (Reed et al.

2011) and *Papilio* butterfly species (Zhang et al. 2017). However, even if the same genes are involved, different alleles or mutations may be implicated. For instance, different *doublesex* alleles underlie evolution of female-limited mimicry in *Papilio* butterflies (Palmer and Kronforst 2020) and different *cis*-regulatory mutations underlie patterning across cichlid species (Kratochwil et al. 2018). Mutations can further differ in type, i.e., regulatory, coding or structural mutations. While the literature has described plenty of coding and regulatory mutations affecting the evolution of adaptive evolution (Hoekstra and Coyne 2007; Wray 2007), new long and linked-read technology have allowed a more efficient identification of structural variants connected to local adaptation (Wellenreuther et al. 2019; Meier et al. 2020; Todesco et al. 2020). Therefore, evolution might not proceed with the exact same molecular solutions to the same challenges, but two factors may result in higher predictability. First, evolutionary paths may be limited by genetic constraints, such as pleiotropy or epistasis, or the organization of organisms into developmental networks with specific functions (Feder 2007; Stern 2013; Rosenblum et al. 2014). Finally, predictability might be higher among closely related species given higher similarity in evolutionary pressures and higher levels of shared genetic variation and opportunity for hybridization (Martin and Orgogozo 2013; Stern 2013; Rosenblum et al. 2014). Therefore, in the case of closely related groups of species, adaptive traits may have a single origin as adaptive alleles are often shared or exchanged across species.

1.4. The evolution of seasonal coat color change

Many species have developed forms of seasonal flexibility to adapt to different selective pressures across the year (winter versus summer) (Helm et al. 2013). The cycling of behaviors or physiological traits (e.g., reproduction, molt, migration, hibernation) allow organisms in temperate and boreal habitats to engage in high energetic tasks in periods of higher food abundance or favorable environmental conditions (Bradshaw and Holzapfel 2007; Geyfman et al. 2015). The accurate timing of these cyclic biological processes with seasonal changes depends on physiological mechanisms that perceive changes in the environmental cues (e.g., photoperiod, temperature, snow cover) and trigger the next stage of the life cycle (Schwartz and Andrews 2014). In mammals, variation in day length (photoperiod) perceived by the retina and suprachiasmatic nuclei of the hypothalamus results in the rhythmic production of melatonin in a rate inversely proportional to day length (Goldman 1999). This in turn induces rhythmic hormonal production and gene expression throughout the body (Helm et al. 2013; Schwartz and Andrews 2014).

One intriguing form of seasonal flexibility of at least 22 mammal and bird species inhabiting temperate and boreal environments involves the alternation between a white winter

coat and a brown summer coat tied to their annual molt cycle (Mills et al. 2018; Zimova et al. 2018). The convergent evolution of seasonal coat color change across species of ptarmigan (Jacobsen et al. 1983), foxes (Våge et al. 2005), hamsters (Logan and Weatherhead 1978), weasels (Bissonnette and Bailey 1944), lemmings (Gower et al. 1994) and hares (Flux and Angermann 1990) (see Mills et al. 2018a and Zimova et al. 2018 for comprehensive list of species) is thought to provide camouflage in snow-covered environments (Zimova et al. 2018). Indeed, varying coat colors have a clear adaptive significance since mismatched animals incur higher detection rates by predators resulting in decreased survival (Zimova et al. 2016; Atmeh et al. 2018; Mills et al. 2018; Wilson et al. 2019). Therefore, the fitness value of this trait is intrinsically tied to an accurate synchrony of molt phenology with annual snow cover conditions (Mills et al. 2013). However, molt timing is mostly controlled by photoperiod with little variation across altitude, winter onset or duration (Zimova et al. 2014, 2018, 2020), which should result in an increase in coat color mismatch under forecasted decreases in snow cover duration due to climate change (Mills et al. 2013). In this scenario, adaptation might occur through the evolution of alternative winter coat colors (Mills et al. 2018). Indeed, polymorphism in winter coloration has been documented in several systems correlated with spatial variation in snow seasonality (Mills et al. 2018) including rock ptarmigans (Skoglund and Höglund 2010), arctic foxes (Våge et al. 2005), three species of weasels (*Mustela*) (Bissonnette and Bailey 1944; Rust 1965; Mills et al. 2018) and four hare species (*Lepus*) (Dalquest 1942; Hansen and Bear 1963; Angerbjörn and Flux 1995; Nunome et al. 2014). In the Faroese mountain hare, a shift to winter gray coat colors in response to lower levels of snow cover happened in less than 100 years (Reinert 1982; Giska et al. 2019), suggesting that intraspecific winter coat color polymorphism (white and non-white pelage morphs) could be an important source of rapid adaptation to climate change in seasonal coat color changing systems.

Despite the clear importance of winter coat color polymorphisms for the survival of seasonally color changing systems in the face of environmental change (Mills et al. 2018), there is currently a limited understanding of the potential for an adaptive shift to occur. The genetic basis of color polymorphisms has been evaluated in four species (arctic foxes, Våge et al. 2005; snowshoe hares, Jones et al. 2018; least weasels, Miranda 2018; mountain hares, Giska et al. 2019). Of these, only the studies on hares and weasels have also interpreted genotype-to-phenotype associations along with population history of adaptive alleles, which is necessary to understand the potential for the spread of adaptive variants across populations and how fast an adaptive shift could occur (Miranda 2018; Giska et al. 2019; Jones et al. 2020). Furthermore, only the mountain and snowshoe hare studies partially addressed the evolutionary history of the adaptive alleles in a broader phylogenetic context, which could reveal the potential for the maintenance or exchange of adaptive alleles across seasonal coat

color changing species or resilience of genetic variation to past-selective pressures (Jones et al. 2018; Giska et al. 2019). Finally, none of these works have clearly connected standing genetic variation for alternative winter coat colors to forecasted selective pressures in the future to guide conservation priorities that may promote adaptation to rapid environmental change. However, there is a pressing need to understand the evolutionary history of adaptive traits to inform management strategies looking into preserving adaptive genetic variation that will be important in forecasted environmental change (Savolainen et al. 2013; Hoban et al. 2016; Bay et al. 2017).

Furthermore, given the clear adaptive significance of seasonal coat color change and its evolution across many related species, including phenotypic polymorphisms, this form of seasonal flexibility provides an outstanding opportunity to answer some of the longstanding questions in evolutionary biology (see introductory paragraph and sections 1.1 to 1.3). For instance, the evolution of winter coat color polymorphism within species is suitable to study the genetic basis of local adaptation with genotype-to-phenotype association studies (Crawford and Nielsen 2013; Hoban et al. 2016). Furthermore, cross species comparison of regulatory mechanisms underlying color change or of the genetic basis of color polymorphisms can be used to address questions regarding possible convergent evolution (Manceau et al. 2010; Rosenblum et al. 2014) and the origin and maintenance of adaptive genetic variation (Hedrick 2013; Jamie and Meier 2020).

1.5. The evolution of polymorphic seasonal camouflage in hares

Hares and jackrabbits comprise 32 currently described species (genus *Lepus*) of the family Leporidae that also includes several rabbit genera (Smith et al. 2018). Hares are thought to have originated in North America and dispersed in the last 4-6 million years (Yamada et al. 2002) throughout all major biomes of the Northern Hemisphere and Africa (Smith et al. 2018). Hares are generally associated with open grasslands (Ge et al. 2013) and their widespread distribution suggests physiological and molecular adaptation to contrasting environments, from the Arctic (arctic hare, *L. arcticus*), to temperate (European hare, *L. europaeus*) and desert environments (Cape hare, *L. capensis*), and even high elevation (Ethiopian highland hare, *L. starcki*) (Smith et al. 2018). Hares are thus an appealing system to study the evolution of adaptive traits during rapid divergence, from the types of genetic variation likely to underlie the evolution of polymorphisms to the selective regimes promoting their evolution (Jones et al. 2018; Giska et al. 2019). For instance, hybridization has been described across several hare species pairs (e.g., Alves et al. 2008; Liu et al. 2011; Melo-Ferreira et al. 2012; Tolesa et al. 2017; Jones et al. 2018; Seixas et al. 2018; Kinoshita et al.

2019; Lado et al. 2019) in some cases resulting in adaptive introgression (Jones et al. 2018; Seixas et al. 2018; Giska et al. 2019), suggesting that introgressed variation can be important for adaptive evolution in hares.

Moreover, seasonal coat color change occurs in six hare species inhabiting temperate, boreal, and arctic environments of the Northern Hemisphere: the North American white-tailed jackrabbit (*L. townsendii*; Hansen and Bear 1963), snowshoe hare (*L. americanus*; Dalquest 1942), Arctic hare (*L. arcticus*; Howell 1936) and Alaskan hare (*L. othus*; Merriam 1900) and the Eurasian Japanese hare (*L. brachyurus*; Nunome et al. 2014) and mountain hare (*L. timidus*; Flux 2009) (Figure 1.1). Among these, four species have adapted to varying snowfall conditions through the evolution of discrete (i.e., brown, white or gray in the snowshoe, mountain and Japanese hares; Severaid 1945; Angerbjörn and Flux 1995; Nunome et al. 2014) or continuous winter coat colors (the white-tailed jackrabbit; Hansen and Bear 1963) (Figure 1.1). Therefore, similar selective pressures seem to underlie the maintenance of color variation across these four systems (Mills et al. 2018). The existence of seasonal coat color polymorphisms across closely related species offers outstanding models to study the genetic basis and evolutionary history of adaptive phenotypes across several phylogenetic scales, including if the same or independent evolutionary pathways underlie this adaptive polymorphism within species. It can also contribute to a better understanding of the adaptive potential of seasonal coat color changing species to environmental change.

Recent studies have made important progress regarding the regulatory mechanisms underlying coat color change and the genetic basis of winter coat color polymorphism in hares. Ferreira et al. (2017) looked at gene expression across the spring change from white to brown in snowshoe hares and pointed to the pigmentation, circadian clock or hair growth cycle pathways as likely targets of adaptive change underlying the evolution of alternative winter coat colors. More recently, genome-wide association studies in snowshoe and mountain hares linked *cis*-regulatory variation at the Agouti signaling peptide (*ASIP*) gene in the melanogenesis pathway to the evolution of winter white versus winter brown or winter gray coat colors, respectively (Jones et al. 2018; Giska et al. 2019). In both cases, repeated adaptation to environments with less or no snow cover was seeded by alleles introgressed from other hare species, the black-tailed jackrabbit (*L. californicus*) and the Iberian hare (*L. granatensis*) for snowshoe and mountain hares, respectively. These studies reveal three relevant insights regarding the evolution of winter coat color polymorphism in hares. First, in both cases, a simple genetic basis involving *cis*-regulatory variation in the same gene (*ASIP*) underlies the evolution of winter dark phenotypes. Second, local adaptation evolved through a similar origin of the alleles (introgression) and the same pathway (melanogenesis) and gene (*ASIP*). Third, introgressed variation was important for rapid adaptive shifts in response to

environmental change, from less than 100 years in the Faroese hare and a few thousand generations in the Pacific Northwest snowshoe hare. This suggests that, in the absence of *de novo* mutations or ancestral polymorphisms, hybridization can be an important source of adaptive genetic variation in seasonal coat color changing systems.

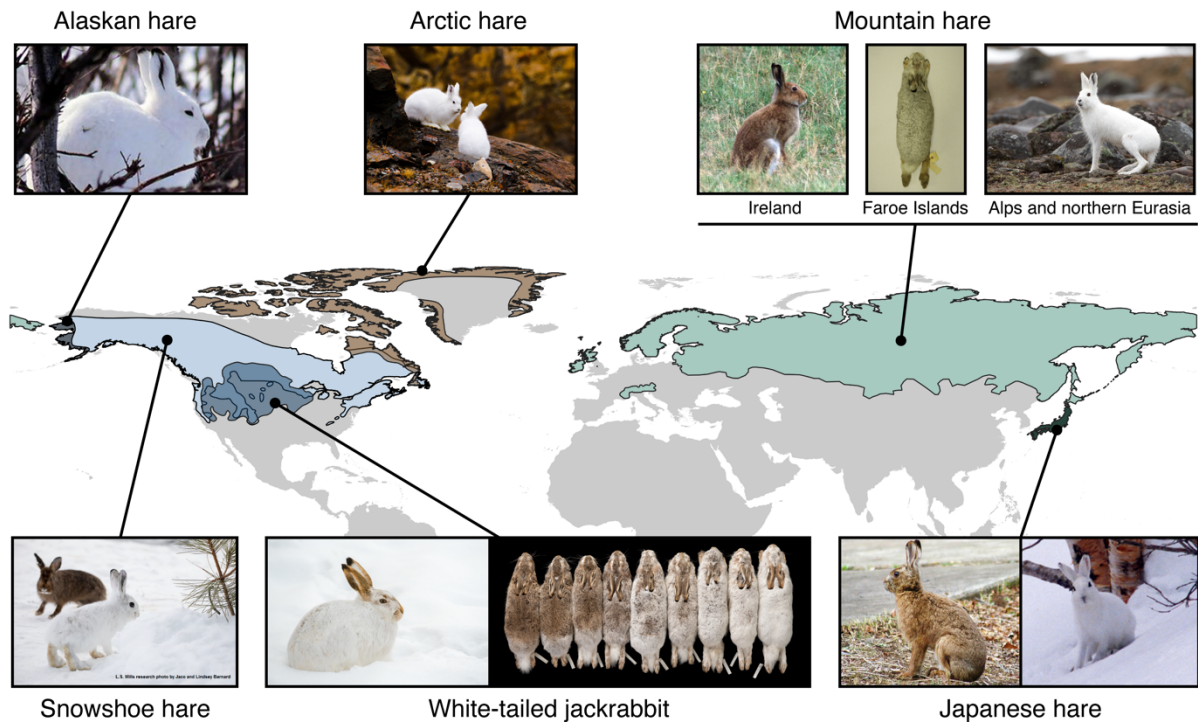


Figure 1.1 – The distribution of the six hare species that undergo seasonal coat color change. At the top, and from left to right: Alaskan hare (*Lepus othus*; photo credit Alaska Department of Fish and Wildlife), Arctic hare (*L. arcticus*; photo credit Marie and Alistair Knock at iNaturalist), Mountain hare (*L. timidus*; photo credits habitats.org.uk, José Melo-Ferreira and nadezhda at iNaturalist). At the bottom, from left to right: Snowshoe hare (*L. americanus*; photo credits Jaco and Lindsey Barnard), White-tailed jackrabbit (*L. townsendii*; photo credits rmbphotography at iNaturalist, and Rick Wicker, Denver Museum of Nature and Science) and Japanese hares (*L. brachyurus*; photo credits MaterialsScientist at Wikipedia and Mt. Iwaki Conservation Association).

Despite this progress, there are still questions left unanswered regarding the evolution of seasonal coat color change in hares and the emergence of intraspecific polymorphism. First, relationships among hare species are currently unresolved, as previous attempts have relied on a few genetic markers resulting in unresolved phylogenies due to the confounding effect of incomplete lineage sorting and hybridization (Halanych et al. 1999; Matthee et al. 2004; Melo-Ferreira et al. 2012; Melo-Ferreira and Alves 2018). Therefore, while mountain and snowshoe hares seem to be distant relatives within *Lepus* (Seixas et al. 2018; Giska et al. 2019), the relationships among the remaining seasonal coat color changing hare species are not clearly resolved. Furthermore, even if mountain and snowshoe hares could potentially

represent independent origins of winter white coats, given the many instances of introgression hybridization in hares, there is the possibility that the ability to alternate color may have been exchanged between species. Also, it is not known if the regulatory basis of seasonal coat color change in snowshoe hares (Ferreira et al. 2017) or the similar genetic basis of winter coat color variation described in snowshoe and mountain hares involving the same gene (Jones et al. 2018; Giska et al. 2019) can be generalized to the other systems. Therefore, a better understanding of the evolution of seasonal coat color change variation in hares would benefit from resolving the evolutionary history of *Lepus* species, understanding the importance of hybridization for the levels of shared variation across the genus and expanding genotype-to-phenotype studies to other coat color changing systems.

1.6. The continuous winter coat colors of white-tailed jackrabbits

Among seasonal coat color changing systems, the white-tailed jackrabbit (*L. townsendii*) stands out as a unique case of continuous variation in winter coat colors (Figure 1.1 and 1.2). The white-tailed jackrabbit is a North American species, historically distributed from the west coast to Lake Michigan in Wisconsin and from the Great Plains in Saskatchewan, Canada to the Rocky Mountains in New Mexico (Lim 1987). They are sympatric with the black-tailed jackrabbit and the snowshoe hare across parts of their range (Figure 1.2). White-tailed jackrabbits molt to winter coats across most of the species range, but classic studies described winter brown populations in British Columbia, Nebraska, Kansas and Colorado (Nelson 1909; Hansen and Bear 1963). Across some populations spanning the continental divide in Colorado, white-tailed jackrabbits display continuous winter coat color variation between white and brown (Hansen and Bear 1963). Thus, unlike the other systems studied so far (Jones et al. 2018; Giska et al. 2019), the genetic architecture underlying this color variation likely involves multiple genes (Hansen and Bear 1963). It could also suggest that white-tailed jackrabbits experience higher variation in snow cover conditions that select for continuous variation in winter coat colors, thus maintaining genetic variants for alternative colorations across the range of this species (Gulisija and Kim 2015; Mills et al. 2018).

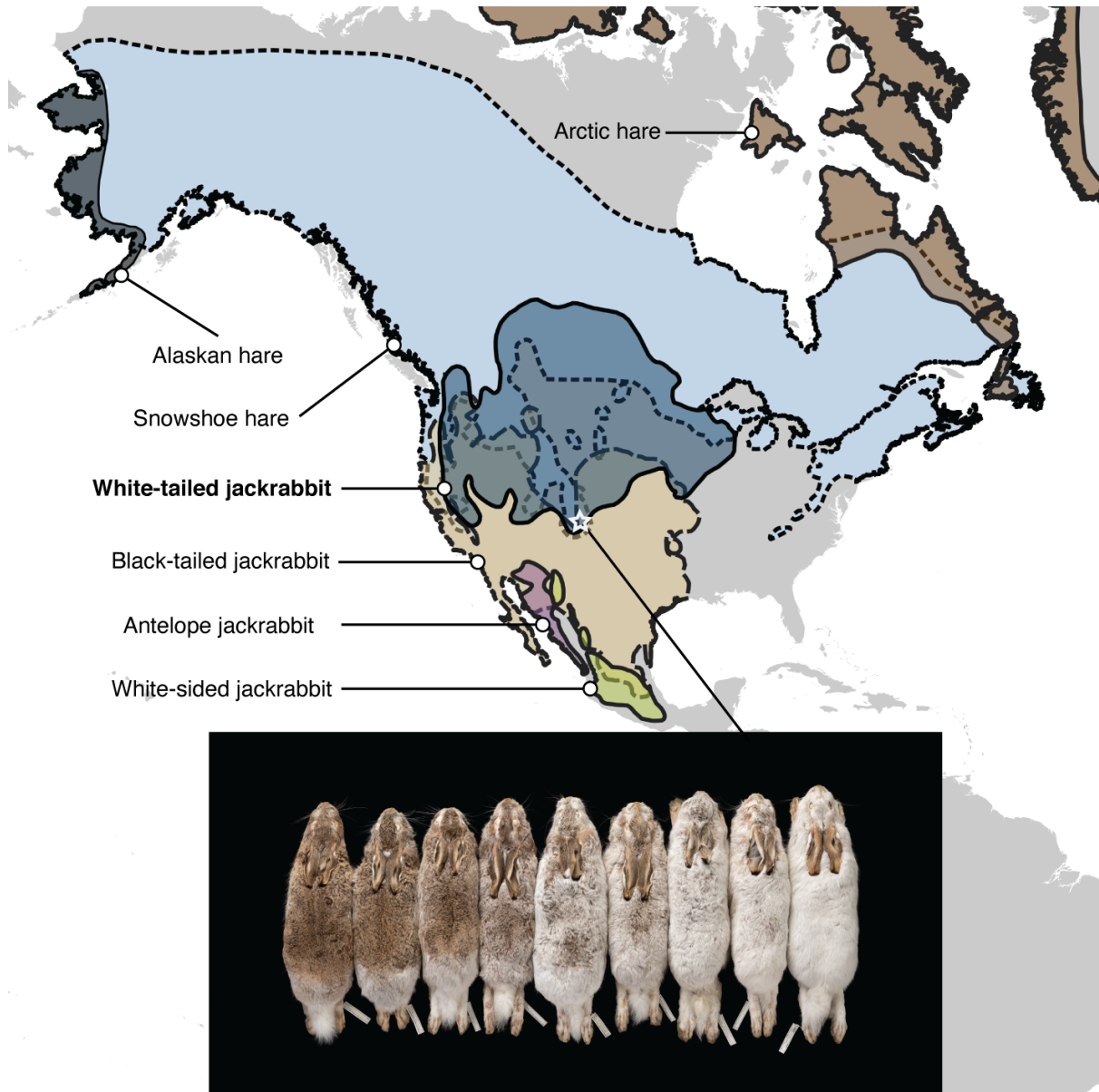


Figure 1.2 – The distribution of the white-tailed jackrabbit and other native hare species in North America. The location of the polymorphic zone described by Hansen and Bear (1963) where continuous variations in coat colors can be found (inset photo credit Rick Wicker, Denver Museum of Nature and Science) is marked with a star. To provide context, we also display the distribution of seven other North American species (Alaskan hare, *L. othus*; Arctic hare, *L. arcticus*; Snowshoe hare, *L. americanus*; Black-tailed jackrabbit, *L. californicus*; Antelope jackrabbit, *L. alleni*; White-sided jackrabbit, *L. callotis*). Given their small distribution in Mexico, we do not display the distribution of the Black jackrabbit (*L. insularis*) and of the Tehuantepec jackrabbit (*L. flavigularis*). Also, we do not display the distribution of an introduced population of European hare (*L. europaeus*) in the states of Pennsylvania, New York, United States of America, and southern Ontario, Canada.

Despite this unique color variation, white-tailed jackrabbits have gained little attention by the scientific community or the general public and seem to have disappeared from parts of its range without notice (Berger et al. 2005; Berger 2008). A recent review of the conservation status of the species revealed that the vast majority of the white-tailed jackrabbit range has experienced severe populations declines, with some historical southern populations now

thought to be extinct or experiencing extirpations (Brown et al. 2020). Brown et al. (2020) did not identify a universal reason for the decline of white-tailed jackrabbits but pointed to a combination of several factors including habitat conversion, climate change and past depredation measurements. Therefore, a clear understanding of the evolution and genetic basis of winter coat color polymorphism in the white-tailed jackrabbit has the potential to contribute to longstanding questions in evolutionary biology while also informing conservation measures directed at promoting species persistence through future climatic shifts.

1.7. Objectives and thesis organization

There is currently a well-established connection between phenotype and fitness of seasonal coat color change (Zimova et al. 2016; Atmeh et al. 2018) and previous work linking genetic variation to color polymorphism in two *Lepus* systems (snowshoe and mountain hares; Ferreira et al. 2017; Jones et al. 2018; Giska et al. 2019; Jones et al. 2020). There are also growing genomic tools available to perform state of the art whole-genome studies in *Lepus* (Ferreira et al. 2017; Jones et al. 2018; Seixas et al. 2018; Giska et al. 2019; Marques et al. 2020). Therefore, the general objective of this thesis is to use the evolution of seasonal camouflage and trans-species winter coat color polymorphism in hares as a model to study the evolutionary mechanisms underlying repeated adaptive evolution. Finally, through the study of the evolutionary basis of a fitness relevant trait that will be key for the future adaptation of seasonal coat color changing species to climate change we aim to inform conservation measurements in the declining white-tailed jackrabbit.

The specific objectives of this work are:

- i. Infer and date the phylogenetic history of hares using exome-wide data for 15 hare species (including four coat color changing species) to produce a better biogeographic context for the evolution of seasonal coat color changing species.
- ii. Infer the contribution of shared polymorphism and introgressed genetic variation during the rapid divergence of hares, and in particular identify shared genetic variation between species that undergo seasonal coat color change.
- iii. Study the genetic basis of continuous winter coloration in white-tailed jackrabbits (*L. townsendii*) across a transition zone in coat color in Colorado, United States of America.

- iv. Study the evolutionary origin of genes associated with winter coloration in white-tailed jackrabbits in the context of the *Lepus* radiation to infer the origin of adaptive variants (*de novo* mutation, standing genetic variation, hybridization).
- v. Study gene expression changes underlying the autumn molt of the Alpine mountain hare (*L. timidus*) and compare it with the transcriptional landscape of seasonal coat color change from a distant relative, the snowshoe hares (*L. americanus*), studied by Ferreira et al. (2017).

This thesis is organized in five chapters. In Chapter I, I provide a general introduction to the thesis. Then, in Chapters II, III and IV I present three scientific manuscripts (published or in preparation). Finally, in Chapter V I discuss the implications of our findings and future directions.

In Chapter II, we use phylogenomic tools to study the reticulate evolutionary history of hares. Using whole-exome data, we estimate and date the relationships and apply state of the art tools to infer hybridization among 15 hare species. Specifically, we study the contribution of ancestral polymorphism and hybridization across rapid divergence of hares to their evolution and adaptation. We infer a paraphyletic relationship among snowshoe hares and three other monophyletic seasonal coat color changing species (mountain and arctic hare, and the white-tailed jackrabbit). However, we detect hybridization among the common ancestors of these two lineages and we further explore the functional consequences of this ancestral hybridization to the adaptation of these species. This work has been published at the SCI indexed journal *Systematic Biology* (<https://doi.org/10.1093/sysbio/syaa088>).

In Chapter III, we study the genetic basis and evolutionary history of the continuous winter coat color in white-tailed jackrabbits (*L. townsendii*) in the context of the rapid divergence of hares. We find a multigenic basis of the trait involving at least three genes, *EDNRB*, *CORIN* and *ASIP*, revealing a more complex genetic architecture than that previously inferred in the snowshoe and mountain hares. We determine that ancestral polymorphism, either maintained by balancing selection throughout the hare radiation or shared from black-tailed jackrabbits through introgression, underlies the evolution of this polymorphic quantitative phenotype in white-tailed jackrabbits. Finally, we determine that winter brown phenotypes are predicted to expand in forecasted scenarios of snow cover conditions pointing to the importance of preserving endangered winter brown populations of white-tailed jackrabbits. This paper is currently under preparation for submission to a peer reviewed journal.

Finally, in Chapter IV, we infer gene expression changes across the Autumn molt from brown to white of Alpine mountain hares (*L. timidus*). We find differential gene expression of

genes involved in the pigmentation, circadian clock and hair growth cycle pathways. These results reveal clear parallels with gene expression changes studied in the Spring molt from white to brown in snowshoe hares, suggesting that the mechanisms of molt regulation are largely conserved across species and molts. This work is published in the SCI-indexed journal *Ecology and Evolution* (<https://doi.org/10.1002/ece3.5956>).

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Chapter II

The Legacy of Recurrent Introgression during the Radiation of Hares

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2.1. Abstract

Hybridization may often be an important source of adaptive variation, but the extent and long-term impacts of introgression have seldom been evaluated in the phylogenetic context of a radiation. Hares (*Lepus*) represent a widespread mammalian radiation of 32 extant species characterized by striking ecological adaptations and recurrent admixture. To understand the relevance of introgressive hybridization during the diversification of *Lepus*, we analyzed whole exome sequences (61.7 Mb) from 15 species of hares (1 – 4 individuals per species), spanning the global distribution of the genus, and two outgroups. We used a coalescent framework to infer species relationships and divergence times, despite extensive genealogical discordance. We found high levels of allele sharing among species and show that this reflects extensive incomplete lineage sorting and temporally layered hybridization. Our results revealed recurrent introgression at all stages along the *Lepus* radiation, including recent gene flow between extant species since the last glacial maximum, but also pervasive ancient introgression occurring since near the origin of the hare lineages. We show that ancient hybridization between northern hemisphere species has resulted in shared variation of potential adaptive relevance to highly seasonal environments, including genes involved in circadian rhythm regulation, pigmentation, and thermoregulation. Our results illustrate how the genetic legacy of ancestral hybridization may persist across a radiation, leaving a long-lasting signature of shared genetic variation that may contribute to adaptation.

Keywords

Ancient introgression; Hybridization; Adaptation; Phylogenomics; *Lepus*

2.2. Introduction

Species radiations are often accompanied by extensive gene flow between nascent lineages (e.g., Lamichhaney et al. 2015; Árnason et al. 2018; Malinsky et al. 2018; Li et al. 2019; Barth et al. 2020). Genetic signatures of hybridization between several closely related species could either represent recent or ongoing introgressive hybridization (Eaton et al. 2015), or the remnants of hybridization among ancestral populations that remain shared among contemporary species (Malinsky et al. 2018; Li et al. 2019). Although these alternatives can be difficult to differentiate in large radiations (Eaton et al. 2015; Malinsky et al. 2018; Vanderpool et al. 2020), both ancient and contemporary introgression has been linked to local adaptation in several systems (e.g., Liu et al. 2015; Gittelman et al. 2016; Meier et al. 2017; Barlow et al. 2018; Giska et al. 2019; Svoldal et al. 2020). Thus, unraveling the tempo and contribution of introgression to standing genetic variation within and among species remains a critical step in understanding the overall importance of introgression to evolution.

Reconstructing the history of hybridization between several closely related species requires inferring evolutionary relationships among species while considering the two primary processes – incomplete lineage sorting and gene flow – that may cause sharing of genetic variation among populations (Malinsky et al. 2018). The network multispecies coalescent (NMSC) model (Than et al. 2011; Solís-Lemus et al. 2017; Degnan 2018) offers one promising framework that appears to resolve species relationships in the face of multiple reticulation events and rapid speciation (Kozak et al. 2018; Edelman et al. 2019). However, the NMSC is still prohibitive for large datasets and relies on the user to choose the number of migration events based on *a priori* hypotheses (Yu and Nakhleh 2015). Alternatively, site-based summary statistics based on tree asymmetries (e.g., Green et al. 2010; Pease and Hahn 2015), or admixture proportions (e.g., Reich et al. 2009; Martin et al. 2015; Malinsky et al. 2018) are simpler to implement, but offer less power for localizing the timing and number of introgression events when recurrent hybridization is layered across a phylogeny (Malinsky et al. 2018). A combination of methods is thus most appropriate to infer a species tree that may have layered events of hybridization throughout time (e.g., Kozak et al. 2018; Malinsky et al. 2018; Edelman et al. 2019; Li et al. 2019).

Hares and jackrabbits comprise a group of 32 currently classified species (genus *Lepus*; Smith et al. 2018) whose common ancestor likely originated in North America and spread throughout most of the Northern Hemisphere and Africa presumably in the last 4-6 millions of years (myr) (Yamada et al. 2002; Matthee et al. 2004; Melo-Ferreira et al. 2012). Hares are primarily associated with open grasslands, but can be found across a broad range of biomes (e.g., desert, forest, or arctic) and elevations (e.g., from sea level to the Himalayan

or Ethiopian plateau; Smith et al., 2018). The *Lepus* radiation also provides multiple case studies of hybridization and introgression, with admixture detected among several extant pairs of species (e.g., Liu et al. 2011; Melo-Ferreira et al. 2012; Tolesa et al. 2017; Jones et al. 2018; Seixas et al. 2018; Lado et al. 2019; Kinoshita et al. 2019). Selection on introgressed variation has been hypothesized to have aided the range expansion of the Iberian hare (Seixas et al. 2018), and has been directly linked to convergent adaptive evolution of non-white winter coats in populations of two species that change the color of their pelage seasonally (Jones et al. 2018; Giska et al. 2019; Jones et al. 2020). These studies suggest that the relatively recent exchange of genetic variation among extant *Lepus* species has provided an important source of adaptive variation. However, the phylogenetic relationships among *Lepus* species remain poorly resolved (Halanych et al. 1999; Matthee et al. 2004; Melo-Ferreira et al. 2012; Melo-Ferreira and Alves 2018), and the contribution of ancient gene flow to the *Lepus* evolutionary history remains unknown.

Here, we use exome-wide data to infer the evolutionary history of 15 *Lepus* species and show that hybridization between lineages has likely occurred since the origin of the radiation. The combination of incomplete lineage sorting and these temporally layered events of hybridization have resulted in extremely high levels of shared genetic variation among extant species, including species that currently occur on different continents. We then use the case of ancient admixture among northern latitude species that occupy highly seasonal environments to investigate the gene content and possible functional relevance of introgressed genomic regions. Our work demonstrates that recurrent introgression throughout evolutionary history has made a substantial contribution to genetic variation within and among species of this widespread mammalian radiation.

2.3. Materials and Methods

2.3.1. Taxon Sampling and Exome Sequencing

We generated new genome-wide resequencing data targeting 207,691 exonic and non-coding regions [totaling 61.7 Megabases (Mb)] from 14 hare species (30 individuals) and the outgroup pygmy rabbit (*Brachylagus idahoensis*; 2 individuals). We combined these data with published whole exomes from four snowshoe hares [*Lepus americanus*; NCBI Sequence Read Archive BioProject PRJNA420081 from Jones et al. (2018, 2020b)] and extracted data from the reference genome of the European rabbit (*Oryctolagus cuniculus*; OryCun2.0; Carneiro et al., 2014) to use as a second outgroup. Our total sample of 15 hare species (34

individuals, 1 to 4 individuals per species) and 2 outgroup species (3 individuals) included species from all major regions of the *Lepus* native distribution: Africa (3 species), Africa and Eurasia (1 species), Eurasia (6 species) and North America (5 species, see Figure 2.1 and Supplementary Table S2.1 available at the end of this chapter and at Dryad; doi:10.5061/dryad.bzkh18967).

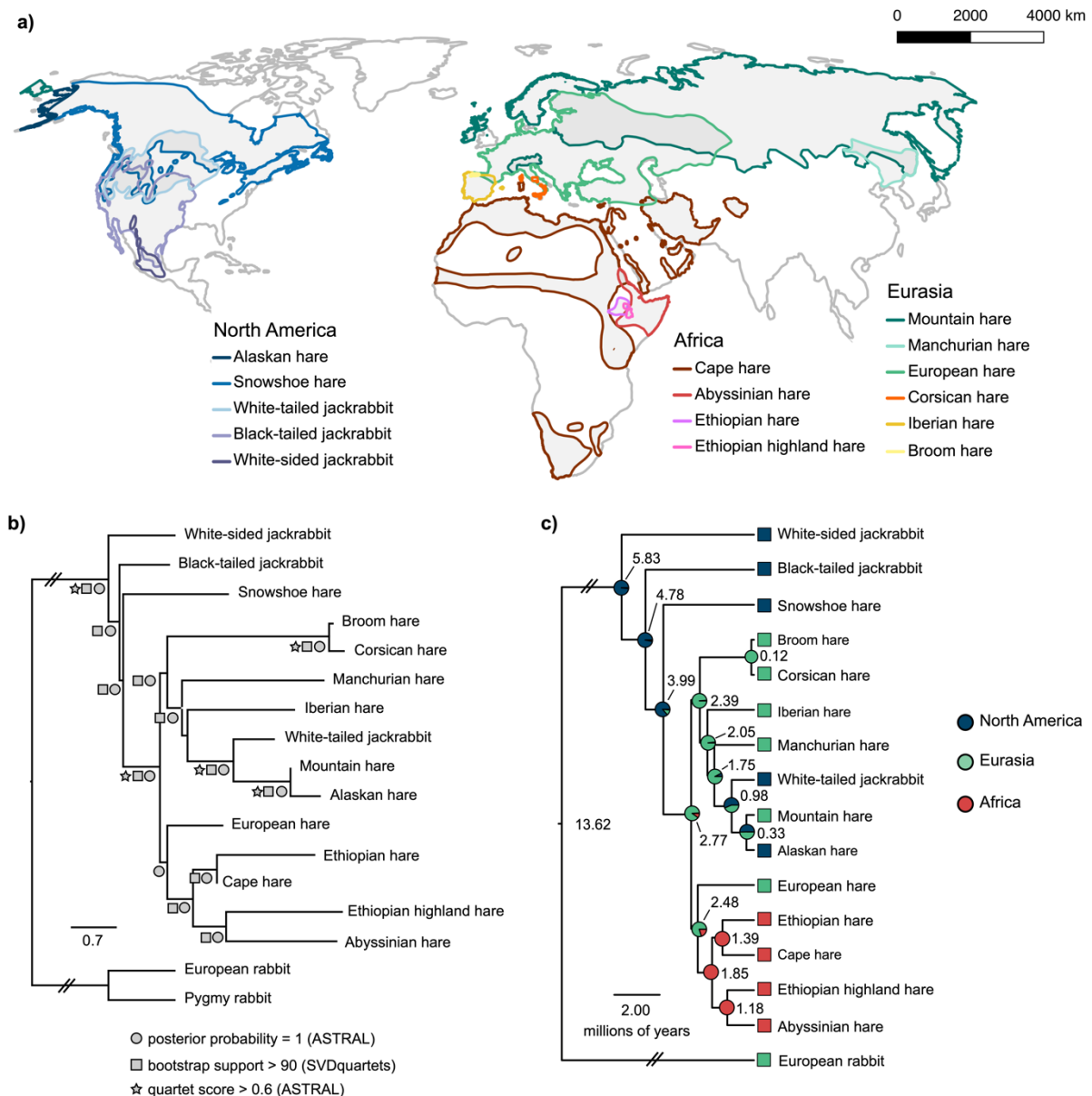


Figure 2.1 – Hare (*Lepus* spp.) evolutionary history and biogeography. (a) The distribution of the 15 hare species studied in this work obtained from the IUCN database (<http://www.iucnredlist.org>); (b) Coalescent species tree estimated with ASTRAL assigning individuals to species groups ($n > 1$ except for the white-sided jackrabbit); (c) Ancestral range reconstruction under the DIVALIKE+J model implemented in BioGeoBEARS on the divergence time tree estimated with MCMCtree and calibrated with deep fossil calibration points. Pie charts represent the probability of each potential range, and squares represent the current range of extant taxa. Node labels represent estimated divergence times in millions of years. Confidence intervals, an alternative biogeographic reconstruction based on *Lepus* fossil calibrations and ancestral ranges for the shoulders of the tree are provided in Supplementary Material.

Exome capture experiments were performed following the procedures outlined in Jones et al. (2018) and in the Supplementary Material and Methods. Briefly, we obtained samples as tissue or extracted DNA including samples from previous studies or through loans from collaborators (Supplementary Table S2.1). Depending on the sample, genomic DNA was isolated using a saline extraction method (Sambrook et al. 1989) or DNeasy Blood & Tissue Kit (QIAGEN) (Supplementary Table S2.1 and Supplementary Materials and Methods). We prepared Illumina sequencing libraries for each sample following Meyer and Kircher (2010) with minor modifications [see Supplementary Material and Methods and Jones et al. (2018)]. Sequencing libraries were then enriched using NimbleGen SeqCap EZ v.4.3 protocol and a custom capture design consisting of 213,164 probes targeting ~25 Mb of protein-coding exons, ~28 Mb of untranslated regions, and ~9 Mb of intron/intergenic regions (Jones et al. 2018). Hybridization reactions were performed in two separate equimolar pools of indexed libraries (31 and 29 libraries, Supplementary Table S2.1), together with samples used for other studies. The target enriched pools were each sequenced across two lanes of an Illumina HiSeq1500 sequencer [125 base pairs (bp) paired-end reads] at CIBIO-InBIO's New-Gen sequencing platform, Portugal.

2.3.2. Read Processing and Genotyping

We trimmed adapters, low-quality bases, merged overlapping reads, and removed PCR duplicates from raw reads using the *expHTS* pipeline (v.0.Mar112016; <https://github.com/msettles/expHTS>). We then used *pseudo-it* (v1; Sarver et al. 2017) to generate pseudo-reference exomes for each species by iteratively mapping (four iterations and allowing ambiguity codes) cleaned single and paired-end reads from one individual per species (Supplementary Table S2.1) to the European rabbit reference genome (OryCun2.0) (Carneiro et al. 2014). For snowshoe hares and black-tailed jackrabbits (*L. californicus*), we used pseudo-references generated by Jones et al. (2018). The genomes of hares and European rabbits are broadly syntenic, despite two known large scale fusions/fissions (Robinson et al. 2002), and sufficiently closely related that iterative mapping to the rabbit reference is not expected to bias genotyping (Sarver et al. 2017; Marques et al. 2020).

We then mapped data from all individuals to each species-specific pseudo-reference using *bwa-mem* (v.0.7.12-r1039; Li 2013) with default options. Mapped reads were sorted with *samtools* (v1.4; Li et al. 2009), assigned to read groups, filtered for duplicates (*Picard* v1.140; <http://broadinstitute.github.io/picard/>), and realigned for insertion-deletion length variation using *GATK* (v3.4.46; Van der Auwera et al. 2013). We calculated coverage statistics and capture efficiency using *CalculateHSMetrics* from *Picard*. For each individual, we called and

filtered genotypes using the *bcftools* (v1.4; Li 2011) mpileup, call and filter pipeline. We excluded indels and filtered single nucleotide variant genotypes with low mapping (MQ < 20) or phred-scaled quality (QUAL < 20), low sequencing depth (DP < 6), excess sequencing depth (> 3 x individual coverage, see Supplementary Table S2.2), sites less than 10 bases from an indel (--SnpGap 10), and low-quality non-reference homozygous or heterozygous genotypes (GQ ≤ 20). We used the filtered genotypes to construct consensus exome fasta sequences in the OryCun2.0 coordinate system.

2.3.3. Species Tree Inference

We used a concatenated alignment without missing data to estimate a single bifurcating phylogeny using a maximum likelihood (ML) search and rapid bootstrapping run under the GTR+ Γ model of sequence evolution (autoMRE option) in *RAxML* (v8.2.10; Stamatakis 2018). We then used two complementary methods to infer multispecies-coalescent trees while accounting for local variation in phylogenetic histories along the genome. First, we extracted 50 kilobase (kb) alignments from the exome capture targeted regions plus 200 bp of flanking sequences using *msa_split* (*phast* 1.4; <http://compugen.cshl.edu/phast/>) and *bedtools* (1.9; Quinlan and Hall 2010), considering a balance between the expected extent of linkage disequilibrium in hares (10-20 kb; Jones et al. 2018) and the retention of information for phylogenetic analysis (alignment length > 1 kb). For each window, we filtered positions with missing data for > 30% of individuals using *TriSeq* (*TriFusion* 1.0.0; <http://odiogosilva.github.io/TriFusion/>) excluded windows smaller than 1 kb, and used *RAxML* to estimate local maximum likelihood gene trees (GTR+ Γ , 100 bootstraps). For each gene tree, we used the corresponding bootstraps to calculate a tree certainty score based on the sum of certainty scores for all internodes of a tree (-L MRE option in *RAxML*; Salichos and Rokas 2013; Salichos et al. 2014; Kobert et al. 2016). The internode certainty score weighs the support of the bipartition represented by a given internode in the gene tree against the support of the second most prevalent conflicting bipartition (Salichos et al. 2014) present in the bootstrap trees. In our case, the maximum theoretical value of the tree certainty score is 31, or $k - 3$ with k equal to the number of taxa (Salichos et al. 2014). Only trees with certainty score above 5 were used in the species tree inference. We unrooted the gene trees using *R* package *ape* (Paradis et al. 2004) and estimated a consensus species tree using *ASTRAL-III* (5.6.3; Zhang et al. 2018).

We also estimated coalescent species trees using only variable sites with *SVDquartets* (Chifman and Kubatko 2014) implemented in *PAUP** (4a163; Swofford 2003). For the analyses based on variable sites, we recovered single nucleotide variants (SNVs) distanced

10 kb along the genome (within targeted regions and 200 bp flanking regions) using *snp-sites* (v2.3.3; Page et al. 2016), a custom script and *bedtools intersect*, and excluded sites with missing information for > 30% of the individuals using a custom script (available at <https://github.com/evochange>). For both *SVDquartets* and *ASTRAL* analyses, species trees were estimated with and without assigning species identities and using sites/intervals genome-wide or only from the X chromosome. The European and/or pygmy rabbits were included for all analyses requiring outgroups. Additional details on the phylogenetic analyses are provided in the Supplementary Materials and Methods.

2.3.4. Bayesian Divergence Time Inference

We performed Bayesian inference of divergence times in the inferred species tree using an approximate maximum likelihood method and assuming an autocorrelated relaxed molecular clock, implemented in *MCMCtree* (*PAML* v.4.9; Yang 2007) and described in dos Reis and Yang (2011, 2019). For one individual per species (Supplementary Table S2.1), we extracted the coding sequence for all genes included in our capture design (18,798 genes in the OryCun2.0 ENSEMBLE 94 database) with *bedtools getfasta*, after selecting the longest transcript per gene using R package *biomaRt* (v2.34.2; Durinck et al. 2005, 2009). We excluded alignments with more than 20% missing data using *AMAS* (Borowiec 2016) (see Supplementary Materials and Methods for details). With these, we constructed a concatenated alignment with three partitions, corresponding to the three codon positions. We assumed GTR+ Γ for the model of sequence evolution and we used the prior of 3.33 for the average substitution rate per site per 100 million years, following Matthee et al. (2004). *Lepus* is poorly represented in the fossil record. The earliest hare record dates to the early Pleistocene [2.5 million years ago (Ma); White 1991; Lopez-Martinez 2008], which is much more recent than molecular estimates for the genus extrapolated from deeper fossil calibrations (4-6 Ma; Yamada et al. 2002). Therefore, we used two different calibrations for the species tree. We either (1) used molecular estimates of 4-6 myr for the time of the most recent common ancestor (TMRCA) of *Lepus* extrapolated from deep fossil record calibrations of the order Lagomorpha (Yamada et al. 2002) and 9.7-14.5 myr for the TMRCA of *Oryctolagus-Lepus* divergence (Matthee et al. 2004) or (2) used fossil estimates of 2.5 myr for the lower bound of *Lepus* diversification and constrained the root to be no older than 4.8 myr, which is when the fossil record suggests that the common ancestor of rabbits and hares existed (Hibbard 1963; White 1991). We discarded the first 1,000,000 samples as burn-in and ran the program until we gathered 1,000,000 samples from the posterior, sampling every 10 iterations, and repeated the analysis twice to ensure convergence. We checked for lack of convergence between the

two runs by confirming a linear correlation between posterior times, trendless trace plots, and high effective sample size values ($ESS > 200$) following dos Reis and Yang (2019). Finally, we also checked for a linear relationship between posterior times and confidence interval widths in infinite sites plots (Inoue et al. 2010).

2.3.5. Ancestral Range Reconstruction

We used the R package *BioGeoBEARS* (Matzke 2013, 2014a, 2014b) to estimate ancestral ranges for the species in our dated phylogenies. For this analysis, we assigned each species to North America, Eurasia or Africa. Note that the Cape hare *sensu lato* is distributed in Africa and Eurasia, but Lado et al. (2019) showed deep divergence and non-monophyly of African and Eurasian lineages. Because our Cape hare samples represent the African lineages, we assigned the Cape hare distribution to Africa. In *BioGeoBEARS*, we implemented a maximum likelihood framework to estimate ancestral ranges under three biogeographical models: DEC (dispersal-extinction-cladogenesis; Ree and Smith 2008), DIVA (dispersal-vicariance analysis; Ronquist 1997) and BayArea (Bayesian inference of historical biogeography for discrete areas; Landis et al. 2013). For each method, we tested each model with and without founder-event speciation (j parameter; Matzke 2014b), resulting in six models in total: DEC, DEC+J, DIVALIKE, DIVALIKE+J, BAYAREALIKE and BAYAREALIKE+J. The best fitting model was assessed with the likelihood ratio test (LRT), the Akaike Information criterion (AIC) and corrected AIC (AICc).

2.3.6. Gene Tree Discordance and Phylogenetic Networks

We performed a series of analyses to explore the amount and effect of gene tree discordance in our dataset. To minimize misinterpreting poor phylogenetic resolution as discordance, we retained only the filtered gene trees used as input for *ASTRAL*. These trees were used to estimate a *SplitsTree4* network (v4.14.6; Huson and Bryant 2006) with the option “Consensus Network with distances as means” and a 5% weight threshold to draw a split, and a majority rule consensus tree with *RAxML* (-L MRE option), including internode certainty scores (Salichos et al. 2014). We then used *DiscoVista* (Sayyari et al. 2018) to plot *ASTRAL* quartet frequencies around nodes of interest. Finally, we calculated Robinson-Foulds normalized distances between gene trees and the *ASTRAL* species tree where individuals are not assigned to species, and among gene trees using the function *RF.dist()* from the R package *phangorn* (2.4.0; Schliep 2011). This metric varies between 0 (no discordance between trees) and 1 (complete discordance).

We used *PhyloNet* (v3.6.6; Yu and Nakhleh 2015) to model species relationships under the network multispecies coalescent model, using all local genealogies (tree certainty scores > 5). Given our extensive dataset, we applied the pseudo-maximum likelihood inference of species networks (Yu and Nakhleh 2015). We ran *InferNetwork_MPL* (Yu and Nakhleh 2015) with 0 up to 4 migration events (due to computational limitations), associating individuals to species (option -a), and optimizing branch lengths and inheritance probabilities to compute likelihoods for each proposed network (option -o). We used the best likelihoods per run to calculate BIC and AICc following Yu et al. (2012, 2014) to compare the resulting networks. Networks were visualized with *IcyTree* (<https://icytree.org>; last accessed July 2019).

Given that the pseudo-maximum likelihood network inference is computationally intensive (Yu and Nakhleh 2015), network support could not be inferred with bootstraps. Therefore, we additionally used *Treemix* and ‘f-branch’ statistics (see below) to determine if ancestral reticulations were consistently inferred across methods. We reconstructed ancestral population graphs with *TreeMix* (v1.13, options -global, -noss and -se; Pickrell and Pritchard 2012) following the steps used to generate an SNV dataset for *SVDQuartets* but only including *Lepus* species. We randomly phased the SNV alignments with a custom script (available at <https://github.com/evochange>) and created the *TreeMix* input using the script *TreeMix_from_nex.py* (<https://github.com/mgharvey/>). We allowed 0 to 9 migration events and used the white-sided jackrabbit (*L. callotis*) as the outgroup relative to all other *Lepus* species (see *Results*).

2.3.7. Genetic Diversity, Divergence and Admixture

We used the *genomics general* toolkit (<https://github.com/simonhmartin/>; last accessed January 14, 2019) to estimate pairwise genetic distances (d_{xy}) between species and nucleotide diversity (π) within species, and a custom script (available at <https://github.com/evochange>) to calculate the number of heterozygous sites per individual and the subset shared between at least one individual of each pair of species (used as proxy for shared variation). All diversity estimates were based on a genome-wide concatenated alignment, where we excluded sites with missing information for > 30% of the individuals.

We then used *genomics general* and custom scripts (available at <https://github.com/evochange>) to calculate several variants of the D-statistics (Green et al. 2010) from the informative sites in the same filtered alignment, treating the European rabbit sequence as the ancestral state (additional details are provided in the Supplementary Materials and Methods). Briefly, we calculated the minimum absolute value of D ($D_{\min}$) (Malinsky et al. 2018) for all possible species trios. We calculated Z-scores for each D value

using a 1 Mb block jackknife approach. After finding the minimum D per trio, D values with Bonferroni-corrected $P \leq 0.05$ were considered significantly different from zero. We then calculated the ‘f-branch’ statistic ($f_b(C)$) (Malinsky et al. 2018). The ‘f-branch’ statistic measures admixture proportion between species C and branch b by calculating admixture proportion among all possible $f(A,B,C,O)$ combinations where A are all descendants of branch a (sister to b), B are all descendants of branch b , and C is the donor taxa. $f_b(C)$ is the minimum f value across all possible B and the median across all possible A . A significant $f_b(C)$ value means that all descendants B of branch b share alleles with C , which is more parsimoniously explained by an event of ancestral introgression from C to b (Malinsky et al. 2018). Using the inferred species tree, we determined all conformations ($A,B;C,O$) needed to calculate $f_b(C)$ for all pairs of C species and b branches following Malinsky et al. (2018) with custom scripts (available at <https://github.com/evochange>) and R package *treeman* (1.1.3; Bennett et al. 2017). For each conformation, we calculated ‘admixture proportion’ (f_G) as defined in Martin et al. (2015) and Malinsky et al. (2018) and Z-scores with 1 Mb block jackknife approach following Malinsky et al. (2018). ‘f-branch’ values with Bonferroni-corrected $P \leq 0.05$ were considered significantly different from zero. We also calculated f_{hom} (Martin et al. 2015) between black-tailed jackrabbits (P3) and several snowshoe hare populations (as P1 and P2) to evaluate levels of admixture estimated with $f_b(C)$ for this species pair (see *Results and Discussion*).

We also used *ABBABABAwindows.py* from *genomics general* to estimate the fraction of admixture (f_d) (Martin et al. 2015) across 50 kb genomic sliding windows (≥ 100 sites, 5 kb steps), to localize tracts of introgression in the genomes of northern latitude species. We performed three scans testing introgression between snowshoe hares (*L. americanus*) as P3 and Alaskan hares (*L. othus*), mountain hares (*L. timidus*) or white-tailed jackrabbits (*L. townsendii*) as alternative P2, using the Iberian hare (*L. granatensis*) as P1. Windows of top 0.5% f_d were considered significant. Following Liu et al. (2015), we considered that significant windows in all three tests reflected introgression between snowshoe hares and the ancestral lineage of white-tailed jackrabbits/mountain hares/Alaskan hares, while significant windows in only one test result from recent introgression between the focal extant species. We obtained the annotation of genes in these windows from the European rabbit reference using *biomaRt* and used unique annotations to perform an enrichment analysis in *g:Profiler* (accessed September 2019; Raudvere et al. 2019) using default parameters. We also calculated d_{xy} for f_d outlier windows and the exome-wide d_{xy} distribution between the focal pair of P2-P3 species using *popgenWindows.py* from *genomics general* to calculate d_{xy} in windows of 50 kb (> 2000 sites, 5 kb steps).

2.4 Results

2.4.1. Whole Exome Sequencing Data

Custom DNA captures showed high efficiency (32.3 average fold-enrichment) and specificity (average 10% of sequenced bases off-target; Supplementary Table S2.2). Mapping cleaned reads onto species-specific pseudo-references resulted in an average target sequencing coverage of 16× (5-35× on average per sample; Supplementary Table S2.2) with 57.8 million genotyped sites per individual (Supplementary Table S2.2). Two lower coverage individuals (one hare individual and one pygmy rabbit individual) and one locality duplicate (one hare) were removed from the final dataset to maximize data quality and avoid geographic redundancy (Supplementary Table S2.1). Combining new and published data, all analyses were performed on a dataset of 15 hare species (32 individuals), one pygmy rabbit, and the European rabbit reference genome, unless otherwise noted (see Supplementary Table S2.1).

2.4.2. Phylogenetic Relationships among Hares

The overall topologies of the concatenated ML phylogeny (11,949,529 positions with no missing data) and of the multispecies-coalescent species trees of *ASTRAL* (8,889 gene trees estimated from 50 kb genomic intervals; alignment lengths between 1 kb and 29 kb) and *SVDquartets* (45,779 unlinked SNVs) were largely concordant (Supplementary Figures S2.1, S2.2 and S2.3). Most branching relationships were highly supported in general (*ASTRAL* posterior probabilities = 1, *SVDquartets* bootstraps > 90; Figure 2.1 and Supplementary Figures S2.1, S2.2, S2.3 and S2.4) with most species recovered as monophyletic (Supplementary Figures S2.1, S2.2a and S2.3a). The only exceptions involved very closely related species with disputed taxonomy, such as paraphyly of the mountain hare with the Alaskan hare, and the broom hare (*L. castroviejo*) with the Corsican hare (*L. corsicanus*) (Alves et al. 2008a; Melo-Ferreira et al. 2012), or paraphyly of a species with known deep intraspecific divergence, the Cape hare (*L. capensis*), with the Ethiopian hare (*L. fagani*) (Lado et al. 2019; Supplementary Figures S2.1, S2.2 and S2.3 and Supplementary Tables S2.3 and S2.4).

Our two calibration strategies recovered overlapping 95% high posterior density (HPD) intervals of divergence times for relatively recent splits (e.g., diversification of Eurasian and African species; Supplementary Table S2.5), but the *Lepus*-based fossil calibrations suggested more recent ages for deeper nodes (Supplementary Table S2.5 and Figure S2.5).

For instance, the hare radiation was estimated at ~5.83 million year ago (95% HPD 6.17-5.34 Ma) using fossil calibrations outside *Lepus*, while *Lepus* fossil calibration sets it at ~4.05 Ma (95% HPD 5.00-3.18 Ma). All species tree analyses were consistent in showing that the deepest branching events involved North American species: white-sided jackrabbit, black-tailed jackrabbit, and snowshoe hare (Figure 2.1b). In accordance, ancestral reconstruction of biogeographic distributions based on the best fitting DIVALIKE+J model supported a North America origin, with subsequent colonization of Eurasia (2.85 and 1.99 Ma for deep and *Lepus*-fossil calibration, respectively) and Africa (1.85 and 1.33 Ma) (Figure 2.1c and Supplementary Table S2.6). We also found support for one or more re-colonization events of North America from Eurasia, represented by white-tailed jackrabbits and Alaskan hares (Figure 2.1 and Supplementary Figure S2.6 and Table S2.7).

2.4.3. Incomplete Lineage Sorting and Introgression

We recovered a highly supported species tree across phylogenetic methods, albeit with considerable phylogenetic discordance among sequenced regions [average Robinson-Foulds (RF) pairwise distance between local trees was 0.73]. No local tree completely recovered the species tree topology (minimum RF distance between gene and species tree was 0.13; Supplementary Figure S2.7) and the majority rule consensus tree showed low internode certainty (Supplementary Figure S2.8). Phylogenetic discordance was also apparent in the *ASTRAL* species tree with all but five branches showing quartet scores below 0.6 (Figure 2.1b, Supplementary Figures S2.2, S2.4 and Tables S2.8 and S2.9). The inference of a concordant species tree across *ASTRAL* and *SVDquartets* suggests that the low quartet scores are not highly impacted by gene tree estimation error (Molloy and Warnow 2018). The splits network analysis of individual gene trees also supported many alternative relationships (represented by cuboid structures connecting alternative topologies) particularly involving deeper branches (Figure 2.2). We estimated similar levels of discordance between X-linked and autosomal genealogies and the inferred species trees (Supplementary Figure S2.7). Furthermore, a species tree inferred with X-linked data differed from the genome-wide species tree and showed lower overall branch support (Supplementary Figure S2.9 and Table S2.10). Nucleotide diversity was relatively low within species (0.13-0.63%) and overlapped with estimates of absolute genetic divergence between species ($d_{xy} = 0.17$ -1.11%; Supplementary Tables S2.3 and S2.4). However, these ranges reflect some taxonomic uncertainties. For example, some instances of low interspecific divergence concern species with debated species-level status (e.g., the Corsican-broom hare and mountain-Alaskan hare complexes; Alves et al. 2008; Melo-Ferreira et al. 2012), while high diversity may reflect intraspecific

cryptic divergence (e.g., snowshoe hares, Cheng et al. 2014; Melo-Ferreira et al. 2014; Cape hares, Lado et al. 2019).

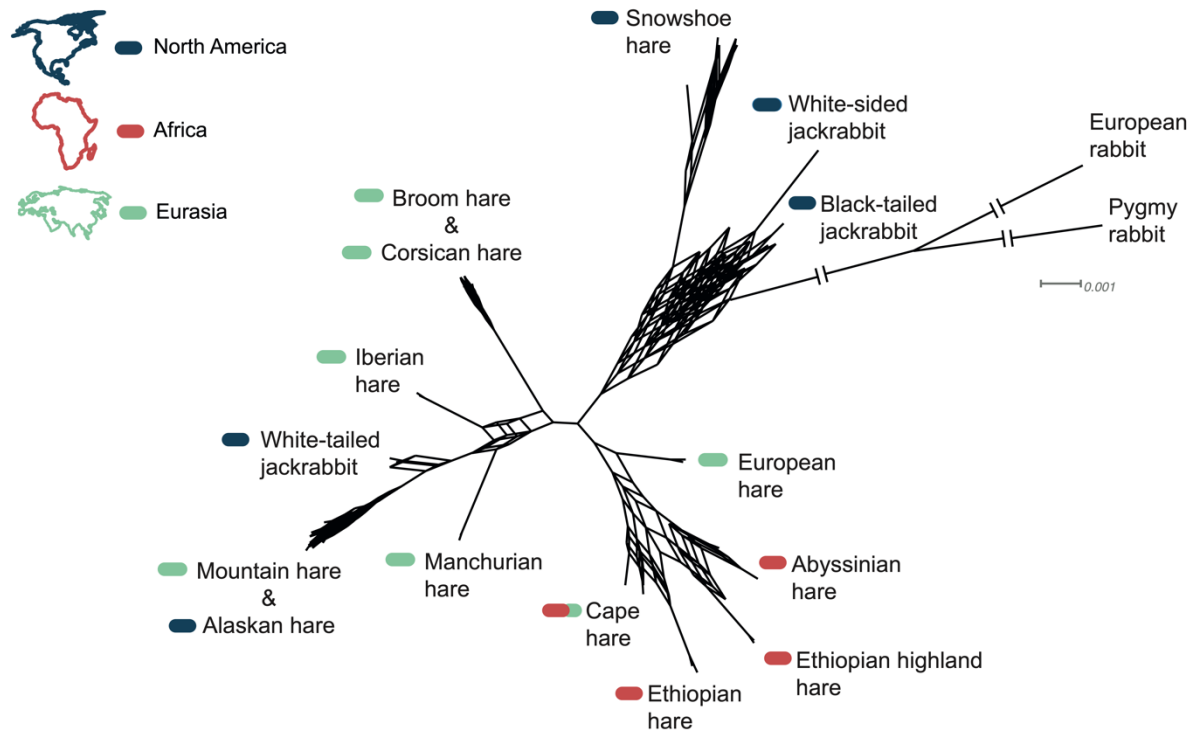


Figure 2.2 – The hare (*Lepus* spp.) species tree is underlined by extensive gene tree incongruence. Split network constructed from 8,889 gene trees (5% threshold) shows discordance among the gene tree topologies (cuboid structures represent alternative topologies) in deeper nodes of the species tree. Species are marked in accordance with the continents where they are distributed.

On average, 49% of heterozygous sites were shared between species (Supplementary Table S2.11). Considerable phylogenetic discordance among gene trees combined with such high levels of shared polymorphism between species could be explained by incomplete lineage sorting, secondary introgression, or a combination of both processes. We found that 88% of the minimum absolute D-statistics ($D_{\min}$) (Malinsky et al. 2018) for all trios of species in our dataset were significantly different from zero (Bonferroni-corrected $P \leq 0.05$; Supplementary Figure S2.10), providing overwhelming support for gene flow either between extant species pairs or between ancestral lineages (Malinsky et al. 2018). Our inferences with the multispecies network coalescent model (Supplementary Figure S2.11), ancestral population graph reconstruction (based on 30,709 biallelic SNVs; Supplementary Figures S2.12 and S2.13), and estimates of admixture proportions among species based on the ‘f-branch’ metric ($f_b(C)$) (Figure 2.3) were all consistent with recurrent gene flow between species layered across the diversification of *Lepus*. With $f_b(C)$ (Figure 2.3) and network analysis (Supplementary Figures S2.11 and S2.12), we detected introgression among extant

species pairs within all of the major geographic regions that are currently sympatric, suggesting ongoing or recent hybridization. For example, strong gene flow was found between black-tailed jackrabbits and snowshoe hares in North America ($f_b(C) = 19\%$; $P = 2.04 \times 10^{-169}$), or between European brown hares and mountain hares from Eurasia ($f_b(C) = 18\%$; $P = 1.73 \times 10^{-233}$; Figure 2.3 and Supplementary Table S2.12). In general, we found decreased admixture proportions with increased genetic divergence between species, although this correlation was only significant when considering species with non-overlapping distributions (Figure 2.4). Several species pairs with current contact zones showed admixture even when genetic divergence was relatively high, such as snowshoe hares and black-tailed jackrabbits ($d_{xy} = 0.97\%$; TMRCA ~ 4.8 Ma, Figure 2.1c, Supplementary Figure S2.5 and Tables S2.4 and S2.5).

We also found evidence for introgression between ancestral populations, which has likely affected deeper branches of the species tree. These ancestral events were detected as network reticulations, as significant $f_b(C)$ values among ancestral branches (y-axis in Figure 2.3) or similar admixture levels shared among species within clades (x-axis in Figure 2.3). We detected ancestral events of introgression connecting major clades within Eurasia (e.g., European hares and the ancestor of the mountain hare/Alaskan hare/white-tailed jackrabbit clade), African and Eurasian lineages (e.g., ancestral of all African species and the Corsican and broom hare ancestral), and Eurasian and North American lineages (e.g., snowshoe hares and the ancestral lineage of the mountain hare/Alaskan hare/white-tailed jackrabbit; Figure 2.3, 2.4, Supplementary Figures S2.11 and S2.12, and Table S2.12). Finally, we found significant introgression among species from different continents, such as North American hares and species from Africa and Western Europe (Figure 2.3 and 2.4), which was also suggested by a network with two reticulations and by ancestral population graph reconstruction (Supplementary Figures S2.11c and S2.12). These results suggest that introgression affected the very early branches of the *Lepus* radiation, and that the genetic legacy of these gene flow events persists in the gene pool of descendant species today (Figure 2.3 and 2.4).

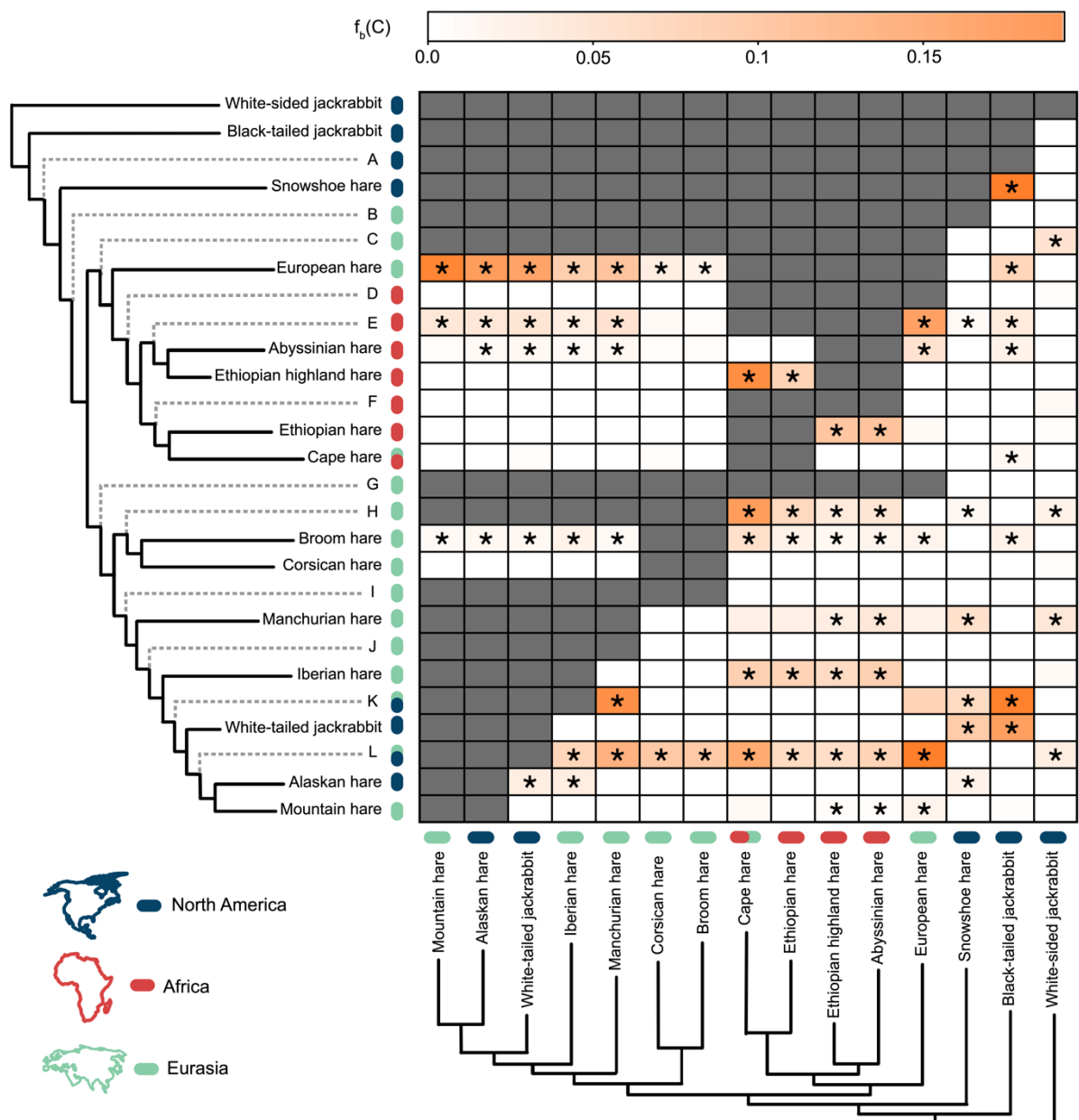


Figure 2.3 – Admixture events are distributed across the hare (*Lepus* spp.) species tree. The ‘f-branch’ statistic $f_b(C)$ represents excess allele sharing between branches b (y-axis) and C (x-axis) of the species tree in Figure 2.1b. The gradient represents the $f_b(C)$ score, dark gray represents tests not consistent with the species tree (for each branch b , having itself or a sister taxon as donor C) and asterisks denote block jackknifing significance at $P \leq 0.05$ (after Bonferroni correction). Tips of the tree are marked according to their current distribution, and ancestral tips (dashed lines and labeled with letters) are marked according to the ancestral range reconstruction in Figure 2.1c [two colors represent the inference of two ranges with equivalent (~ 0.5) probability]. Ancestral tips are labeled from A to L corresponding to labels in Supplementary Table S2.12 available at the end of the chapter and on Dryad.

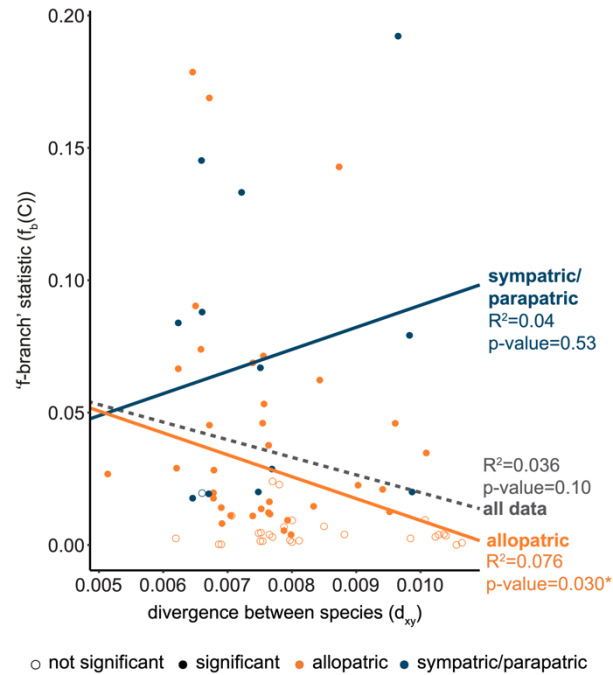


Figure 2.4 – Admixture proportions decrease with genetic divergence between allopatric species. We plot the 'f-branch' ($f_b(C)$) values against exome-wide divergence (d_{xy}) for extant species pairs, differentiating species with overlapping (sympatric/parapatric) or non-overlapping (allopatric) distributions. The tendency line represents a linear regression relating d_{xy} and all (significant and non-significant) $f_b(C)$ values calculated with function $lm()$ in R.

2.4.4. Genes affected by Ancestral Introgression

We consistently detected ancestral introgression between the ancestors of coat color changing species in our dataset: white-tailed jackrabbits, mountain hares, Alaskan hares, and snowshoe hares (Figure 2.3 and Supplementary Figure S2.11). Motivated by recent work showing that introgressive hybridization has shaped local adaptation in seasonal coat color changing species (Jones et al. 2018, 2020a; Giska et al. 2019), we examined the contribution of gene flow to standing variation and local adaptation in these species. We detected 119 putative windows of ancient introgression across all major chromosomes, highlighting the genome-wide contribution of ancestral introgression (Figure 2.5; Supplementary Table S2.13). The f_d outlier windows of ancestral introgression contained 54 annotated genes (Supplementary Table S2.14). This set of genes was enriched for the gene ontology term "E-Box binding" (3 of the 54 genes; Supplementary Table S2.15), a DNA motif found in the promoters of many genes, suggesting that genomic regions affected by ancient introgression may be enriched for transcription factors involved in *trans*-regulation of gene expression. Among these transcription factors we found the circadian clock related gene *ARNTL2* (Sasaki et al. 2009) and pigmentation related gene *TCF4* (Furumura et al. 2001; Le Pape et al. 2009) (Figure 2.5). In addition, the list of 54 genes includes a gene involved in brown fat

differentiation (*EBF2*; Rajakumari et al. 2013), and a photoreceptor-related gene (*PDE6H*; Kohl et al. 2012).

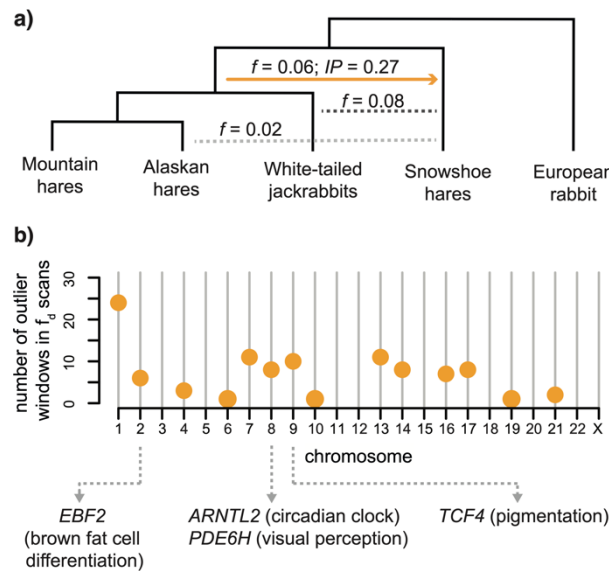


Figure 2.5 – The impact of ancestral introgression on extant northern latitude species. (a) Events of past and recent admixture inferred in this study involving snowshoe, Alaskan hares, mountain hares and white-tailed jackrabbits. The arrow indicates the direction of ancestral introgression inferred by *PhyloNet* and ‘f-branch’ and dashed lines indicate recent introgression inferred with ‘f-branch’. Values above the lines represent admixture proportion (f) estimated with ‘f-branch’ or inheritance probability (IP) estimated with *PhyloNet*; (b) Genomic distribution of admixture blocks of introgression (circles) inferred as shared outlier windows among fraction of admixture (f_d) analysis testing for admixture among snowshoe hares and each one of the three other northern latitude species. Tests were run in 50 kb genomic sliding windows, and outlier windows are in the top 0.5% of the f_d distribution.

2.5. Discussion

We used phylogenetic analyses of whole exome data to tease apart signatures of stochastic lineage sorting and admixture across the evolutionary history of the *Lepus* radiation. By accounting for these sources of phylogenetic discordance, we were able to detect pervasive introgression across the evolution of this recent and rapid mammalian radiation. Below, we discuss the biogeographic and evolutionary implications of our analyses, focusing on the long-term impacts of temporally layered hybridization in shaping patterns of shared genetic variation within and among extant species.

2.5.1. The Effect of Persistent Gene Flow on Phylogenetic Inference

We present a resolved genome-wide phylogeny for the genus *Lepus* despite extensive incomplete lineage sorting and pervasive gene flow. Our genome-wide analysis covered all

major lineages across the worldwide range of hares, substantially extending previous analyses based on more limited genetic sampling (Halanych et al. 1999; Melo-Ferreira et al. 2012; Ge et al. 2013; Tolesa et al. 2017). A systematic evaluation of species limits and hare taxonomy is beyond the scope of our work, as it would require genome-wide data from an expanded inter and intraspecific sample. However, our sampling should not bias the estimated phylogenetic relationships and may even underestimate species diversity. Thus, our inferences of shared variation across species are likely to be conservative.

To estimate species relationships, we combined species tree inferences that do not account for gene flow with network-based inferences that explicitly consider introgression. The resulting topologies were generally consistent between methods when considering up to three reticulations (Figure 2.1 and Supplementary Figure S2.11). However, there were limits to this approach. For example, the alternative placement of European, Corsican, and broom hares, as closer to the Eurasian or African *Lepus* clades depended on the number of reticulations considered (Supplementary Figure S2.11). This uncertainty likely reflects long-term admixture among Eurasian and African lineages, including the European hare (Figure 2.3 and Supplementary Figure S2.11) whose range overlaps with species from both continents (Figure 2.1a). Another example is the alternative sister relationship of snowshoe hares and black-tailed jackrabbits in the network with no reticulation (Supplementary Figure S2.11), which could result from ancestral introgression between these species (see below). While an increased number of reticulation events could better represent the widespread gene flow uncovered in our work, allowing for more than three reticulations resulted in increased branch compression and therefore did not help resolve gene-to-gene incongruences (Supplementary Figure S2.11; Yu and Nakhleh 2015; Wen et al. 2016).

We found that gene trees constructed from X-linked loci or autosome-linked loci showed similar levels of discordance with the exome-wide species tree (Supplementary Figure S2.7). In general, the X chromosome could be expected to show less phylogenetic discordance due to its smaller effective population size and faster lineage sorting, and a tendency to accumulate hybrid incompatibilities (i.e., the large X-effect; Fontaine et al. 2015; Edelman et al. 2019; Li et al. 2019). Although there is evidence for reduced X-linked gene flow between some hybridizing European lineages (Seixas et al. 2018), very little is known about the genetic architecture of reproductive isolation between hare species. Moreover, less phylogenetic discordance of loci involved in reproductive isolation may not be always expected, particularly when speciation events are clustered in time (Wang and Hahn 2018) as is the case in *Lepus* (Figure 2.1c and Supplementary Figure S2.5).

2.5.2. The Timing and Biogeography of the *Lepus* Radiation

Our results suggest that the *Lepus* diversification followed major climatic shifts that occurred during the late Miocene, Pliocene and Pleistocene, similar to other terrestrial mammals (Simpson 1947; Ge et al. 2013). We inferred a North American origin between 5.83 Ma and 4.05 Ma [deep or *Lepus* fossil calibrations, respectively, in agreement with Hibbard (1963), Halanych et al. (1999), Melo-Ferreira et al. (2012) and Ge et al. (2013)], coinciding with a global cold and dry period of the late Miocene that favored the expansion of grasslands worldwide (Osborne and Beerling 2006; Ge et al. 2013). The establishment of the Bering land bridges during late Pliocene and Pleistocene glacial periods (Simpson 1947; Hopkins 1959; Cook et al. 2016) may have allowed the subsequent colonization of Eurasia (~2.77-1.99 Ma). Finally, we inferred secondary colonization of North America from Eurasia within the last 1 million years (Figure 2.1 and Supplementary Figures S2.5 and S2.6; Halanych et al. 1999), in agreement with the Eurasia-North America exchange of cold-adapted fauna during Pleistocene glacial periods (Simpson 1947; Hoberg et al. 2012).

We note that our finding of pervasive introgression may distort aspects of these biogeographic inferences (Leaché et al. 2014; Solís-Lemus et al. 2016; Long and Kubatko 2018; Li et al. 2019; Jiao et al. 2020). If the inferred topology of the *Lepus* phylogeny is correct, as discussed above, then the general biogeographic reconstructions are probably robust. However, the timing of these events remains tentative given potential estimation bias caused by ancestral gene flow (Leaché et al. 2014; Li et al. 2019) and a lack of varied, independent, and reliable calibration points.

2.5.3. The Legacy of Introgression during the Rapid Radiation of Hares

Our inferences indicate that lineages often hybridized when they came into contact during the worldwide expansion of *Lepus*. Some of the inferred reticulation events overlap with known recent introgression, such as between mountain hares and European hares (Levänen et al. 2018) or between snowshoe hares and black-tailed jackrabbits (Jones et al. 2018, 2020a). However, our analysis also revealed prevailing signatures of deeper hybridization between ancestral populations, suggesting a persistent contribution of secondary introgression during the diversification of hares. These past hybridization events have resulted in extensive shared polymorphism among extant species (Figure 2.3 and Supplementary Tables S2.11, S2.12 and Figures S2.10, S2.11 and S2.12), with significant admixture still

detected among species with non-overlapping distributions, even from different continents (Figure 2.3, 2.4 and Supplementary Table S2.12).

Our analyses highlight how ancient gene flow can obscure accurate detection of contemporary hybridization. Similar to other systems (Malinsky et al. 2018; Edelman et al. 2019; Li et al. 2019), we estimated high levels of introgression based on D-statistics (e.g., 88% of $D_{\min}$ values across all possible species trios were significant), but less reticulation after taking phylogeny into account (e.g., 33% of 'f-branch' statistics were significant). These findings suggest that phylogenetic correlation causes non-independence of summary-statistics and can thus lead to false pairwise inferences of gene flow between species (Eaton et al. 2015; Malinsky et al. 2018; Li et al. 2019). Given these results, signatures of hybridization among closely related species should be interpreted in the context of broader phylogenetic relationships whenever possible.

We also detected some discrepancies between the magnitude of gene flow inferred here and in previous works, which underscores the challenges of quantifying introgression across a reticulating radiation. For instance, we inferred substantial admixture proportions between black-tailed jackrabbits and snowshoe hares ($f_b(C) = 19\%$, Figure 2.3 and Supplementary Table S2.12). These estimates are one order of magnitude higher than recent studies suggesting that ~2-3% of genomic variation in the Pacific Northwest snowshoe hare populations descends from hybridization with black-tailed jackrabbits in the last ~10,000 generations (Jones et al. 2018, 2020a). Ancient introgression persisting in all snowshoe hare populations could reconcile this discrepancy. Indeed, we recovered consistent results ($f_{\text{hom}} \sim 3\%$) when we infer admixture proportions among black-tailed jackrabbits and different snowshoe hare populations (Supplementary Table S2.16), which likely reflects recent and geographically localized introgression. However, our estimates of overall admixture proportions between these species should be taken with caution as they depend on the accurate reconstruction of a short internal branch not fully supported across methods, and a species (snowshoe hare) that has been involved in multiple instances of introgression with different hare lineages (Figure 2.3, 2.5 and Supplementary Table S2.12 and Figure S2.11).

Introgression between species is often limited by purifying selection against hybrid incompatibilities (Schumer et al. 2018; Edelman et al. 2019), which agrees with the predominantly negative consequences of hybridization (Mayr 1963). Nonetheless, hybridization is also expected to produce novel allelic combinations that increase phenotypic variation (Grant and Grant 2019; Marques et al. 2019). If coincident with ecological opportunity, introgressed variation could broadly facilitate adaptation (Grant and Grant 2019; Taylor and Larson 2019). Recent work has shown at least two instances where introgression between *Lepus* species has driven local adaptation (Jones et al. 2018; Giska et al. 2019), and

standing introgressed variation may have contributed even more generally to adaptation during the radiation. We found that genetic variation introduced by ancient hybridization can persist through several speciation events (Figure 2.3 and 2.4).

While much of large reservoir of shared variation may reflect stochastic sorting of neutral variation, some may have been maintained by selection (Guerrero and Hahn 2017; Jamie and Meier 2020) and helped facilitate colonization of the diverse habitats currently inhabited by hare species, from desert to arctic environments (Ge et al. 2013; Smith et al. 2018). In this respect, introgression between the ancestor of mountain hares/Alaskan hares/white-tailed jackrabbits and snowshoe hares (or an ancestral lineage) is particularly intriguing. These four species have adapted to highly seasonal environments through striking forms of phenotypic plasticity (e.g., seasonal coat color change; Mills et al. 2013; Zimova et al. 2018) that have been at least partially shaped by adaptive introgression at the *Agouti* pigmentation gene from non-color changing species (*ASIP*; Jones et al. 2018; Giska et al. 2019). Here we estimated that a pulse of ancient introgression occurred at least 0.71 Ma (*Lepus* fossil calibration; Supplementary Figure S2.5) and affected genomic regions containing genes associated with circadian rhythm regulation (*ARNTL2*; Sasaki et al. 2009), pigmentation (*TCF4*; Furumura et al. 2001; Le Pape et al. 2009), thermoregulation (*EBF2*; Rajakumari et al. 2013) and visual perception (*PDE6H*; Kohl et al. 2012). While *ASIP* would also be a likely candidate for adaptive introgression between these lineages (Jones et al. 2018), our exome sequencing coverage of this region was too sparse for detailed window-based analysis.

The functions of these introgressed genes overlap with common physiological adaptations of northern latitude animals to seasonal conditions, such as higher metabolic rates, regulation of body temperature and non-shivering thermogenesis (Hart et al. 1965; Feist and Rosenmann 1975; Rogowitz 1990; Pyörnilä et al. 2008; Sheriff et al. 2009), seasonal camouflage (Grange 1932; Hewson 1958; Hansen and Bear 1963; Mills et al. 2018; Zimova et al. 2018), and visual acuity in response to dim winter light in northern latitudes (Stokkan et al. 2013). Furthermore, the functions of two of these genes, pigmentation and circadian rhythms, are linked to pathways activated during seasonal coat color changing molts (Ferreira et al. 2017, 2020a). The functional relevance of these candidates to local adaptation must await further testing. Nonetheless, substantial introgression along the rapid diversification of a group of organisms, as we describe here, may bolster genetic variation within species and have a greater role in local adaptation than previously anticipated (Grant and Grant 2019; Taylor and Larson 2019). However, we also cannot exclude that some of these shared variants have been maintained by long-term balancing selection rather than secondary introgression (Supplementary Figure S2.14; Smith and Kronforst 2013; Liu et al. 2015; Guerrero and Hahn

2017). Regardless of origin, the *Lepus* radiation provides an intriguing system by which to test the long-term evolutionary importance of shared genetic variation across a rapid radiation.

2.6. Data Accessibility

Raw sequence reads used in this article are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession numbers SRR12020579 to SRR12020510 and BioProject number PRJNA639005. Supplementary materials, including data files and/or online-only appendices, can be found at the end of this chapter and in the Dryad Digital Repository (<https://doi.org/10.5061/dryad.bzkh18967>). The pipeline and custom scripts used for this study are available at <https://github.com/evochange>.

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2.10. Supplementary Materials

2.10.1. Supplementary Material and Methods

Exome Capture Design and Laboratory Work

Genomic DNA was extracted using either a saline extraction method (Sambrook et al. 1989) or the DNeasy Blood & Tissue Kit (QIAGEN) (Supplementary Table S2.1) and quantified using a Qubit fluorometer (Invitrogen). For exome capture, we used the capture design and followed the exome library generation protocol described in Jones et al. (2018). The capture probe set was designed to target all protein-coding and untranslated regions (UTRs) annotated in the European rabbit (*Oryctolagus cuniculus*) reference genome (OryCun2.0) and novel UTRs inferred from previously published snowshoe hares (*Lepus americanus*) transcriptomes (Ferreira et al. 2017). The capture probe set consisted of 213,164 probes covering 61.73 Megabases (Mb), of which ~25 Mb are protein-coding regions, ~28 Mb are untranslated regions and ~9 Mb are intron/intergenic regions. Library preparation steps followed a modified version of Meyer and Kircher (2010). Genomic DNA was sheared to ~300 base pairs (bp) with a Covaris E220evolution ultrasonicator, followed by cleaning using homemade 1.2X carboxyl-coated magnetic beads (Rohland and Reich 2012). Blunt-end repair, adapter ligation and adapter fill-in were done according to Meyer and Kircher (2010). To avoid over-amplification, we determined the number of cycles for dual-indexing PCR by performing quantitative PCR using a DyNamo Flash SYBR Green qPCR kit (Thermo Scientific) and a Stratagene Mx3000P thermocycler (Applied Biosystems). Dual-indexing PCR was performed in 50 µl Herculanase II fusion polymerase and 3.5µl library template. The PCR thermal conditions consisted of an initial denaturation at 98°C for 2 minutes, followed by 10-16 cycles of 98°C for 20 seconds, and 60°C for 20 seconds, and 72°C for 20 seconds, with a final elongation step at 72°C for 5 minutes. We quantified individually indexed libraries using an Implen P330 NanoPhotometer or Qubit (Invitrogen Qubit Quantitation system LTI) and visualized on an Agilent 2230 TapeStation. Then, we performed hybridization reactions following NimbleGen SeqCap EZ v.4.3 protocol in two separate sets of equimolarly pooled indexed libraries (Supplementary Table S2.1) together with samples not included in this work, for a total of 31 and 29 libraries in each pool. The 72-hour hybridization reaction included 5 µg of C0t-1 DNA isolated from snowshoe hare liver to prevent capture of repetitive regions. After hybridization, we washed and recovered hybridized DNA fragments following the NimbleGen protocol (Roche). We performed a post-capture PCR for each captured library pool with 1X Herculanase II reaction buffer, 200 µM each dNTP, 2µM each primer, 1µl Herculanase II fusion

polymerase and 20µl library template. The thermal profile of the PCR consisted of a 45 second denaturation step at 95°C, 16 cycles of 98°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final elongation step at 72°C for 5 minutes. The amplified libraries were cleaned with 1.8X AMPure XP beads (Agencourt). Enrichment of the capture reaction was evaluated with qPCR using a DyNAmo FLASH SYBR Green qPCR kit with both on-target and off-target primer sets. The two pools were each sequenced in two lanes of an Illumina HiSeq1500 sequencer (125 bp paired-end reads) at CIBIO-InBIO's New-Gen sequencing platform, Portugal.

Genotype Calling and Filtering

We mapped the data of all individuals (including those used to generate a pseudo-reference) back to each species-specific pseudo-reference using *bwa mem* (v.0.7.12-r1039; Li 2013) with default options. We created and sorted resulting bam files using *samtools* (1.4; Li et al. 2009). We then added read groups (*AddOrReplaceReadGroups*), removed duplicates (*MarkDuplicates*) and performed indel realignment (*RealignerTargetCreator* and *IndelRealigner*) using *Picard* (v1.140; <http://broadinstitute.github.io/picard/>). We calculated coverage statistics and capture efficiency using *Picard's CalculateHSMetrics* and called variants individually using the *bcftools* (1.4; Li 2011) mpileup, call and filter pipeline. We excluded indels and filtered genotypes with mapping quality smaller than 20 (MQ < 20), with phred based quality smaller than 20 (QUAL < 20), depth smaller than 6 (DP < 6), with depth more than 3 times higher than the average coverage for that individual (Supplementary Table S2.2), less than 10 bases from an indel (--Snpgap 10), and with genotype quality smaller than 20 if homozygous derived or heterozygous (GQ ≤ 20). With a custom script (available at <https://github.com/evochange>), we constructed consensus genome sequences for each individual, where we included all genotype positions passing filter and all other genomic positions were hard masked. We opted to call genome-wide sequences to facilitate the generation of input files for the various analysis. Furthermore, given all genomes were in the same coordinate system as the European rabbit reference genome (OryCun2.0), we could quickly access annotations for any given region.

Generating Alignments for Species Tree Inference

To generate a maximum likelihood tree for the concatenated whole exome, we retained positions with information for all individuals (no missing data allowed) from whole-

genome alignments using *TriSeq* (1.0.1rc2), from *TriFusion* (<http://odiogosilva.github.io/TriFusion/>).

For the coalescent species tree analysis using *ASTRAL*, we generated 50 kilobase (kb) windows with no overlap from the individual genome consensus sequences using *msa_split* (--windows 50000,0) from *phast* (v1.4; <http://compugen.cshl.edu/phast/>). Then, to determine which windows contained regions included in the exome capture, we used *bedtools* (1.9; Quinlan and Hall 2010) *makewindows* to produce a bed file with the genomic windows' coordinates and we excluded windows not included in targeted exome regions extended by 200 bp. Furthermore, for each alignment, we filtered positions with missing data for more than 30% of the individuals using *TriSeq* and, finally, excluded alignments shorter than 1000 bp. Then, we estimated a tree for each window alignment with *RAXML* (v8.2.10; Stamatakis 2018) using a simultaneous maximum likelihood (ML) search and 100 rapid bootstraps, under the GTR+ Γ model of sequence evolution (-f a option), and setting the European rabbit and pygmy rabbit as outgroups. Prior to the *ASTRAL* analysis, we unrooted the gene trees using *R* package *ape* (Paradis et al. 2004). For each resultant gene tree, we used the bootstrap trees generated by *RAXML* to calculate a majority rule consensus tree and calculate tree certainty scores using *RAXML* (-L MRE option) (Salichos et al. 2014).

We also estimated a coalescent species tree with single nucleotide variants (SNVs) with *SVDquartets* (Chifman and Kubatko 2014). Using genome-wide sequences, we produced an alignment containing only *Lepus* species, and used *snp-sites* (v2.3.3; Page et al. 2016) to extract a vcf file containing SNVs in the alignment. We filtered positions in this vcf with missing data for more than 30% of the individuals using a custom script (*filter_missing_vcf.py*; available at <https://github.com/evochange>). Furthermore, we filtered out positions not included in the targeted regions extended by 200 bp, using *bedtools* intersect. We recovered SNVs distanced 10 kb along the genome using a custom script (*filter_bed.py*; available at <https://github.com/evochange>) to respect the assumption of independence among sites. With the resulting filtered SNV bed file, we produced a SNV fasta alignment containing 45,779 SNVs for all *Lepus* individuals, European rabbit and pygmy rabbit using *bedtools getfasta*. This multi species alignment was converted to nexus with *AMAS* (option convert) (Borowiec 2016).

Generating Alignments for Bayesian Time Inference

To extract the coding sequence (CDS) of all genes included in our capture (18,798 genes) we selected the longest transcript per gene using *biomaRt* (v2.34.2; Durinck et al. 2005, 2009) package in *R*. Then, we extracted the coding exons using *bedtools getfasta* along

with a gff3 file for each gene from the rabbit ENSEMBL 94 database (OryCun2.0). We concatenated the coding exons using custom scripts (available at <https://github.com/evochange>). We filtered alignments with more than 20% missing data as calculated by *AMAS summary*, which resulted in 9015 CDS alignments. With these, we constructed a concatenated alignment in phylip format with three partitions corresponding to the three codon positions using a custom script (available at <https://github.com/evochange>), *AMAS concat* and *AMAS convert*.

Generating SNV alignments for TreeMix

To perform ancestral population graph reconstruction with *TreeMix* (Pickrell and Pritchard 2012), our pipeline was similar to the *SVDquartets* analysis, except that we only included *Lepus* species and used the white-sided jackrabbit (*L. callotis*) as outgroup. We used a custom script (*fake_phase_fasta_algn.py* available at <https://github.com/evochange>) to randomly phase the SNV alignment for each chromosome. Then, we used the script *TreeMix_from_nex.py* (<https://github.com/mgharvey>) to convert phased fasta alignments to the *TreeMix* input, assigning all alleles from a species to a single population to calculate allele counts. This script excluded all multi-allelic sites.

Nucleotide Diversity, Genetic Divergence and Admixture Proportion

To calculate several variants of the D-statistics (D_{min} , $f_b(C)$ and f_d) in our dataset, genetic divergence (d_{xy}) and nucleotide diversity (π), we used or based our analysis in the genomics general collection of scripts and tutorials by Simon Martin (<https://github.com/simonhmartin/>; last accessed January 14, 2019). All scripts mentioned in this section are from genomics general, unless otherwise stated. We started by converting an alignment of the genomic consensus sequences for all *Lepus* individuals and the European rabbit reference to a geno file (we included all genomic positions to preserve genomic coordinates) using the *seq2Geno.py* script. Using *filterGenotypes.py*, we filtered genotypes with missing data for more than 30% of the individuals.

Genetic Divergence and Nucleotide Diversity

With the script *popgenWindows.py*, we calculated π per species and pairwise d_{xy} in non-overlapping sliding windows of 1 Mb with more than 100 sites and averaged across all windows to obtain an exome wide estimate per species.

The Minimum D-statistics (D_{min})

For D_{min} , we calculated derived allele frequencies for all species using *freq.py*, grouping all individuals of a species using a population file (--popFile) and fixing the European rabbit as the outgroup. With a custom *R* script (*D-stat.R* available at <https://github.com/evochange>), we calculated *D* (ABBA-BABA; Green et al. 2010) for all combinations of possible trios of species in our dataset (1,365 calculations). To calculate Z-scores, we performed block jackknifing using *jackknife.R*, establishing blocks of 1 Mb and removing blocks with missing data. *D* values with Bonferroni-corrected $P \leq 0.05$ were considered significantly different from zero.

The ‘f-branch’ Statistic

The ‘f-branch’ statistic ($f_b(C)$) requires the calculation of ‘admixture proportion’ (f_G) among all species pairs (Martin et al. 2015; Malinsky et al. 2018). Therefore, in addition to calculating allele frequencies for all species as described for D_{min} , and to produce P3a and P3b frequencies, we 1) calculated allele frequencies for all individuals per species with two samples, 2) calculated allele frequencies splitting species sampling in two populations for species with more than two samples or; 3) split the geno file entry for the single white-sided jackrabbit in two haploid chromosomes with a custom script (*make_haploid_geno.py* available at <https://github.com/evochange>), and used the two resulting entries to calculate two sets of allele frequencies, following Martin et al. (2015). With a custom script (*fd_tree_calculations.R* available at <https://github.com/evochange>) that implements the *R* package *treeman* (1.1.3; (Bennett et al. 2017), we calculated the necessary taxa combinations for $fb(C)$ based on the *ASTRAL* species tree (Figure 2.1), following Malinsky et al. (2018). Then, we calculated f_G for each combination of taxa (using *f_G.R* available at <https://github.com/evochange>), calculating Z-scores for all combinations following what is described for D_{min} . The exception was the white-sided jackrabbit, for which we randomly shuffled the alleles in each position for every jackknife iteration to avoid biases in phasing. We finally calculated $f_b(C)$ for each branch *b* and species *C* as described by Malinsky et al., (2018) (with *fb_C.R* available at <https://github.com/evochange>)

The Fraction of Admixture (f_d)

We performed three f_d scans with the conformation ((Iberian hare, P2), Snowshoe hare), where P2 is either Alaskan hare (*L. othus*), mountain hare (*L. timidus*) or white-tailed jackrabbit (*L. townsendii*). We used *ABBABABAWindows.py* to calculate f_d in sliding windows

of 50 kb (5 kb step and minimum of 100 sites) and defined outliers as the top 0.5% f_d values in each test. We inspected the functions of genes contained in the outlier windows, by annotating them using *biomaRt* package in *R* and the OryCun2.0 reference, and performing an enrichment analysis in *g:Profiler* (accessed September 2019; Raudvere et al. 2019) with the default parameters. We also compared the average d_{xy} for f_d outlier windows against the exome-wide d_{xy} distribution between each pair of P2 and P3 species calculation d_{xy} in windows of 50 kb, with 5 kb step for windows with more than 2000 sites, using *popgenWindows.py*.

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2.10.2. Supplementary Figures

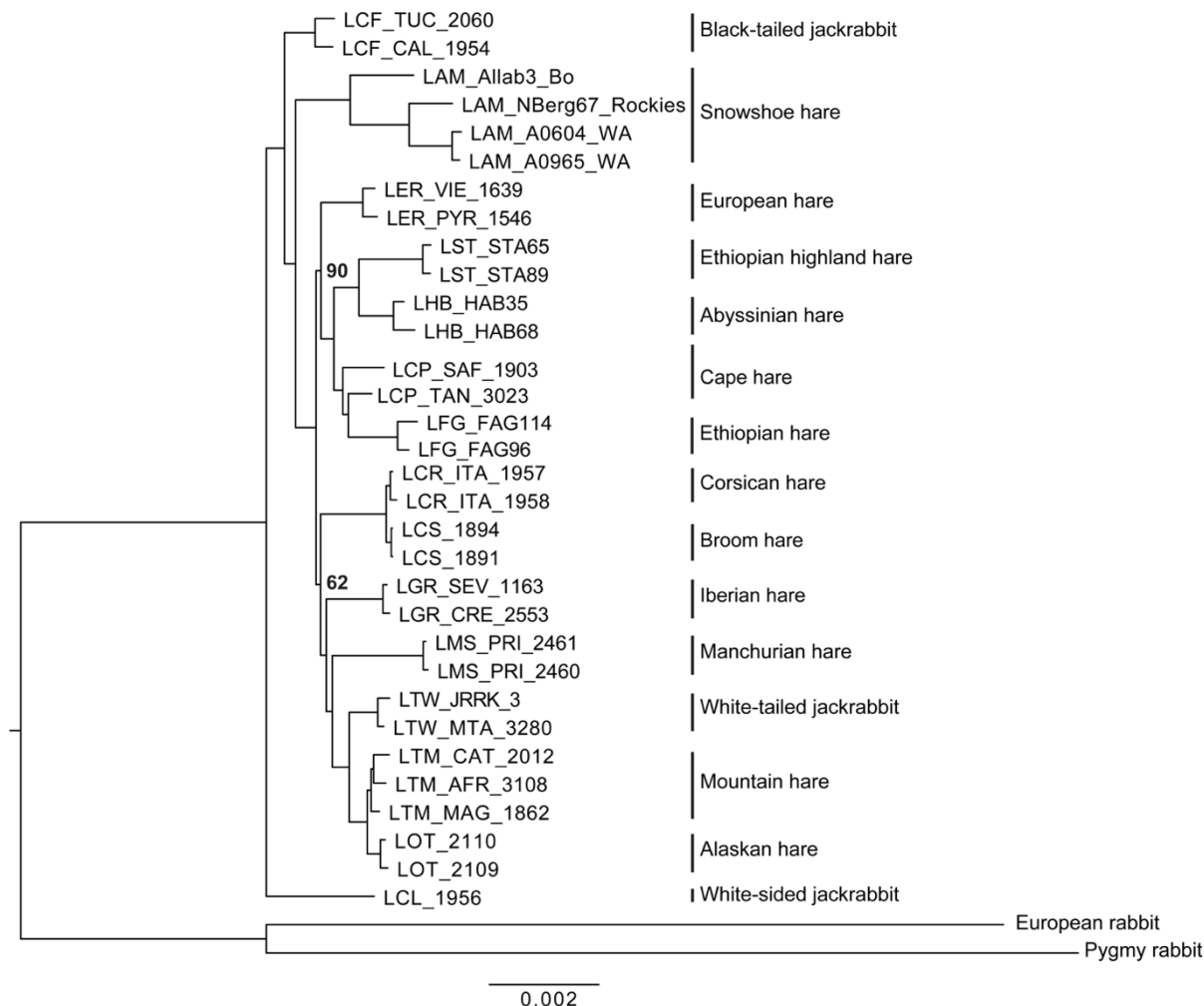


Figure S2.1 – Maximum likelihood tree of 11,949,529 base pair exome alignment (positions with no missing data for any individual), generated using a simultaneous maximum likelihood (ML) search in *RAxML* and rapid bootstrapping run under the GTR+ Γ model of sequence evolution (autoMRE option), and setting the European and pygmy rabbits as outgroups. We show branch supports for branches with support smaller than 100 in bold.

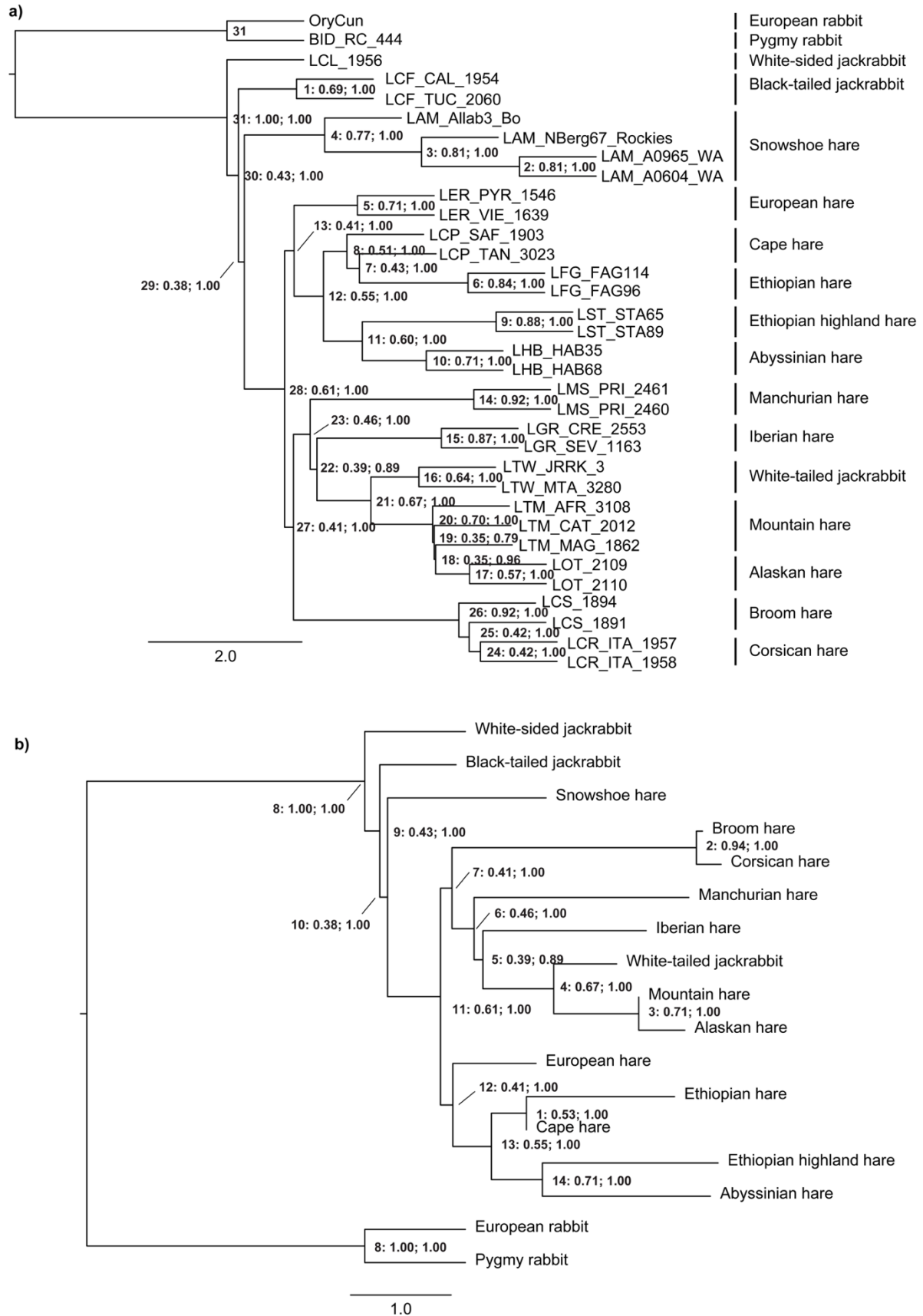


Figure S2.2 – ASTRAL species tree obtained with 8,889 gene trees without a) or with b) assigning individuals to species. Nodes in both trees are numbered and annotated with quartet scores and posterior probabilities. Further information for each node can be found in Supplementary Tables S2.8 and S2.9.

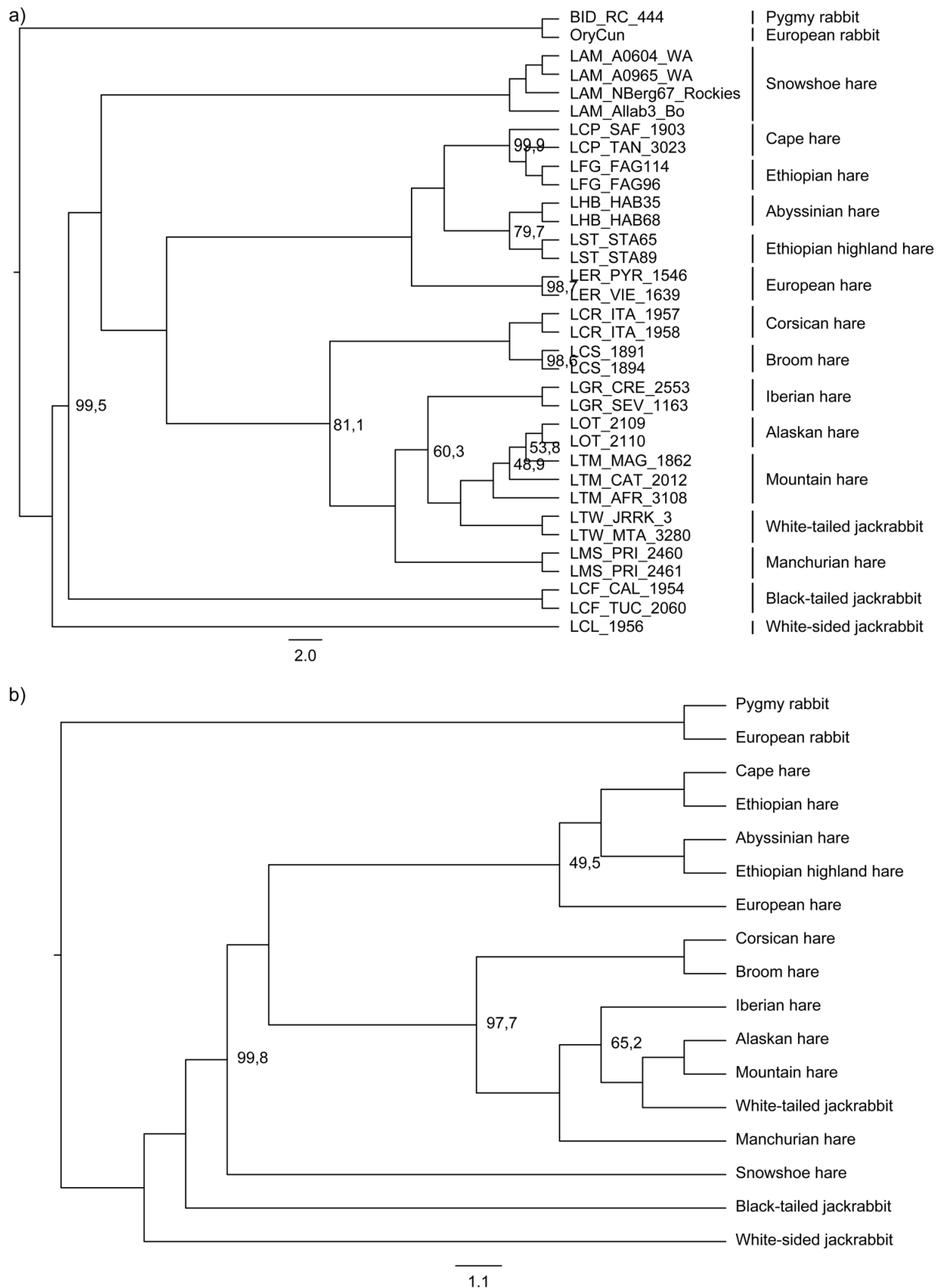
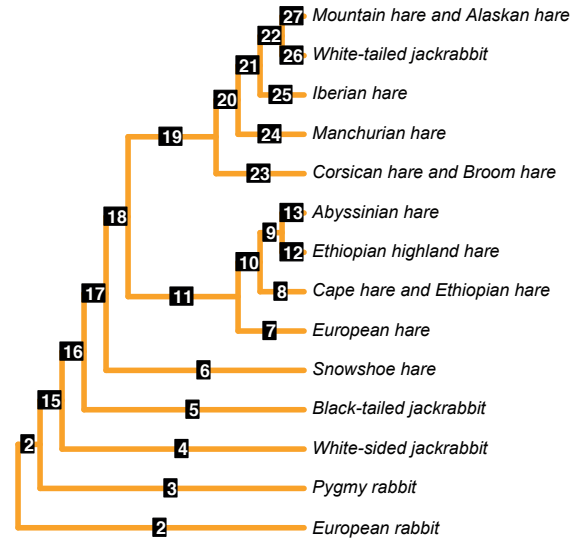


Figure S2.3 – *SVDquartets* species tree generated with 45,779 unlinked SNVs without a) or with b) assigning individuals to species. For both trees, we show branch supports for branches with support lower than 100% of 1,000 bootstrap rounds.

a)



b)

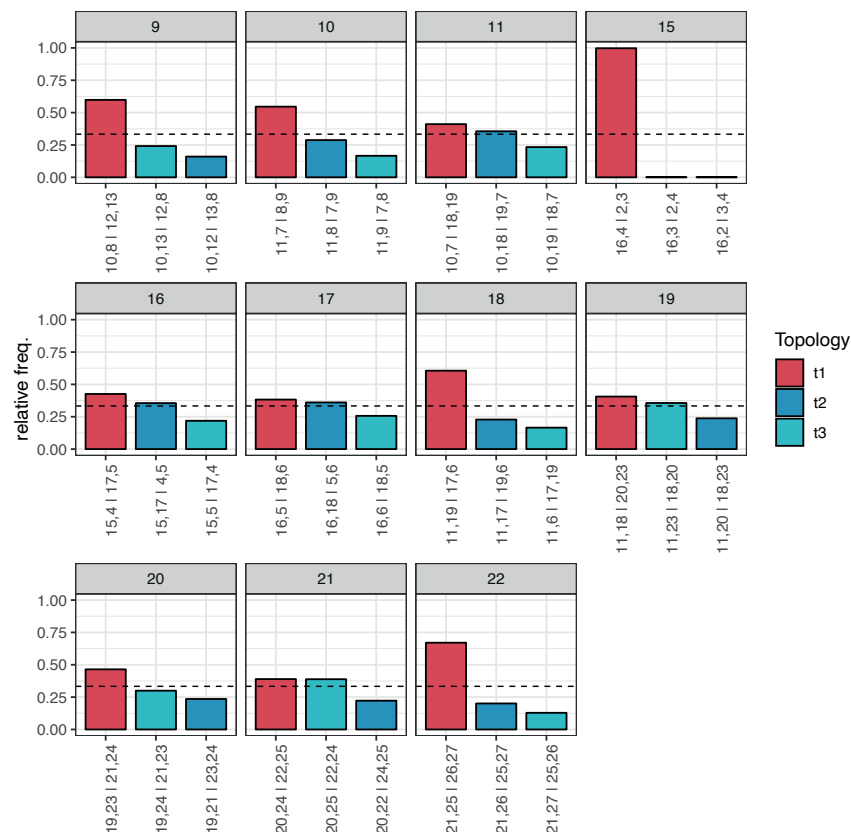


Figure S2.4 – Frequencies of the three main topologies around focal branches of the *ASTRAL* species tree (Figure 2.1b). For each internal branch of the tree in a), the frequency of the three possible topologies connecting the four neighboring branches is shown in b). The title of each bar plot in b) corresponds to labeled internal branches in the tree in a). The most frequent topology is shown in red, and the two alternative topologies are shown in blue. On the x-axis of each bar plot, the topology of each quartet is represented using the neighboring branch labels. The branches leading to mountain hares and Alaskan hares, Cape and Ethiopian hares, and Corsican and broom hares were collapsed given the non-monophyly of the individuals of these species (Supplementary Figures S2.2a and S2.3a).

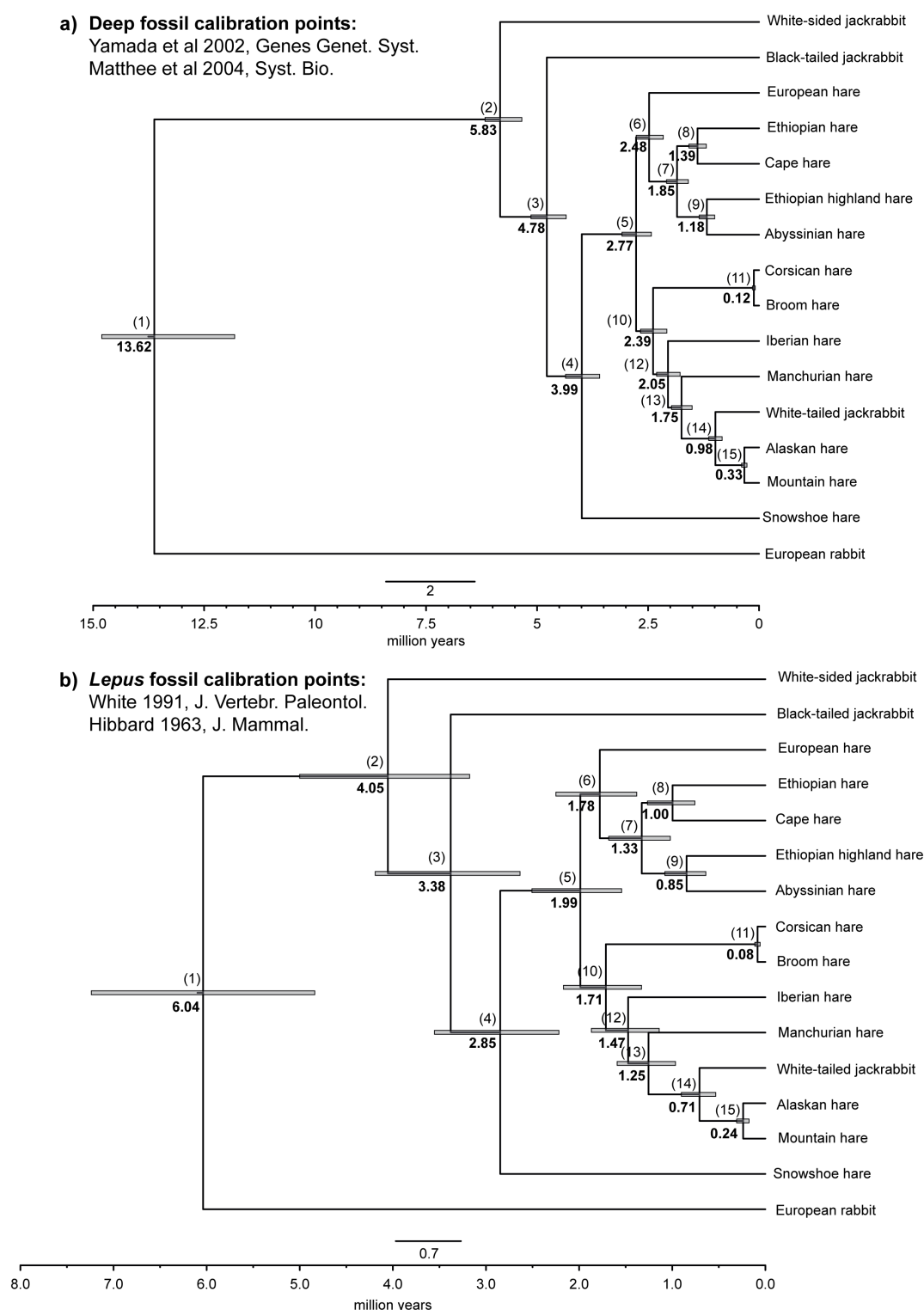


Figure S2.5 - Divergence time tree of *Lepus*, estimated from alignments of 9,015 protein coding ortholog genes (10,863,822 bp alignment), with three partitions (1st, 2nd and 3rd codon positions) using a) molecular-based dates extrapolated from deep fossil calibrations in the lagomorphs or b) fossil calibrations for the divergence of the genus *Lepus* and the *Lepus* species (see Materials and Methods). Node labels in bold black are divergence time estimations in millions of years. Numbers in parenthesis in each node represent node numbers in Supplementary Table S2.5.

The evolution and molecular bases of seasonal coat color variation in hares:
gene expression and the shared contribution of ancestral polymorphism and introgression

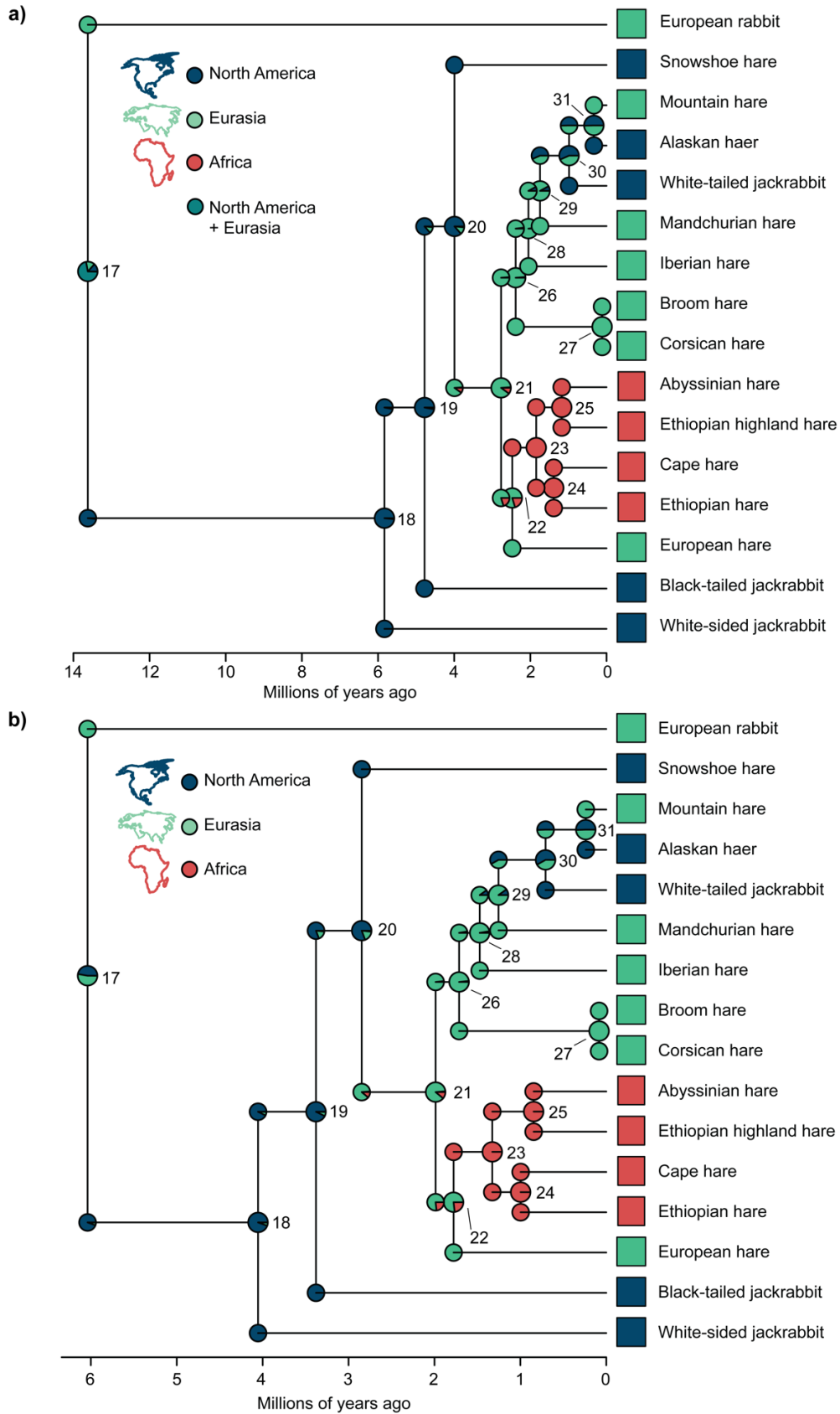


Figure S2.6 – Ancestral range reconstruction for the *Lepus* under the DIVALIKE+J model implemented in *BioGeoBEARS*. We performed ancestral range reconstruction using the two chronograms inferred with *MCMCtree* calibrated with a) deep fossil and b) *Lepus* fossil calibration points. Pie charts show the relative probability of each potential range. Squares at the tips represent the current range of extant species. Node labels represent node numbers in Supplementary Table S2.7.

The evolution and molecular bases of seasonal coat color variation in hares: gene expression and the shared contribution of ancestral polymorphism and introgression

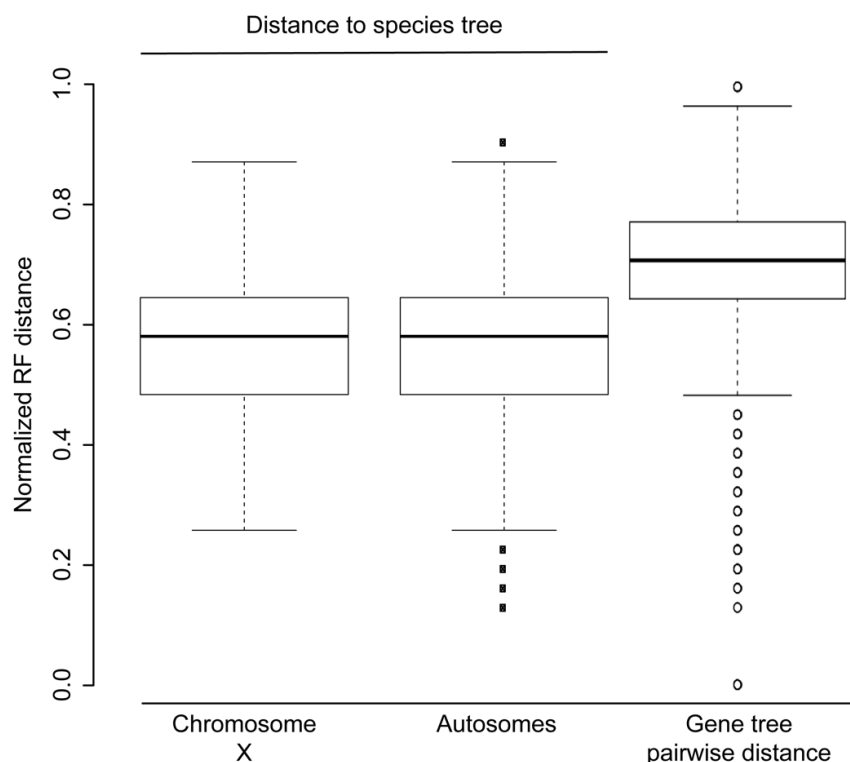


Figure S2.7 – Box plots of the normalized Robinson-Foulds distance between chromosome X and autosomes gene trees and the genome-wide species tree (Supplementary Figure S2a), and pairwise distance among all gene trees.

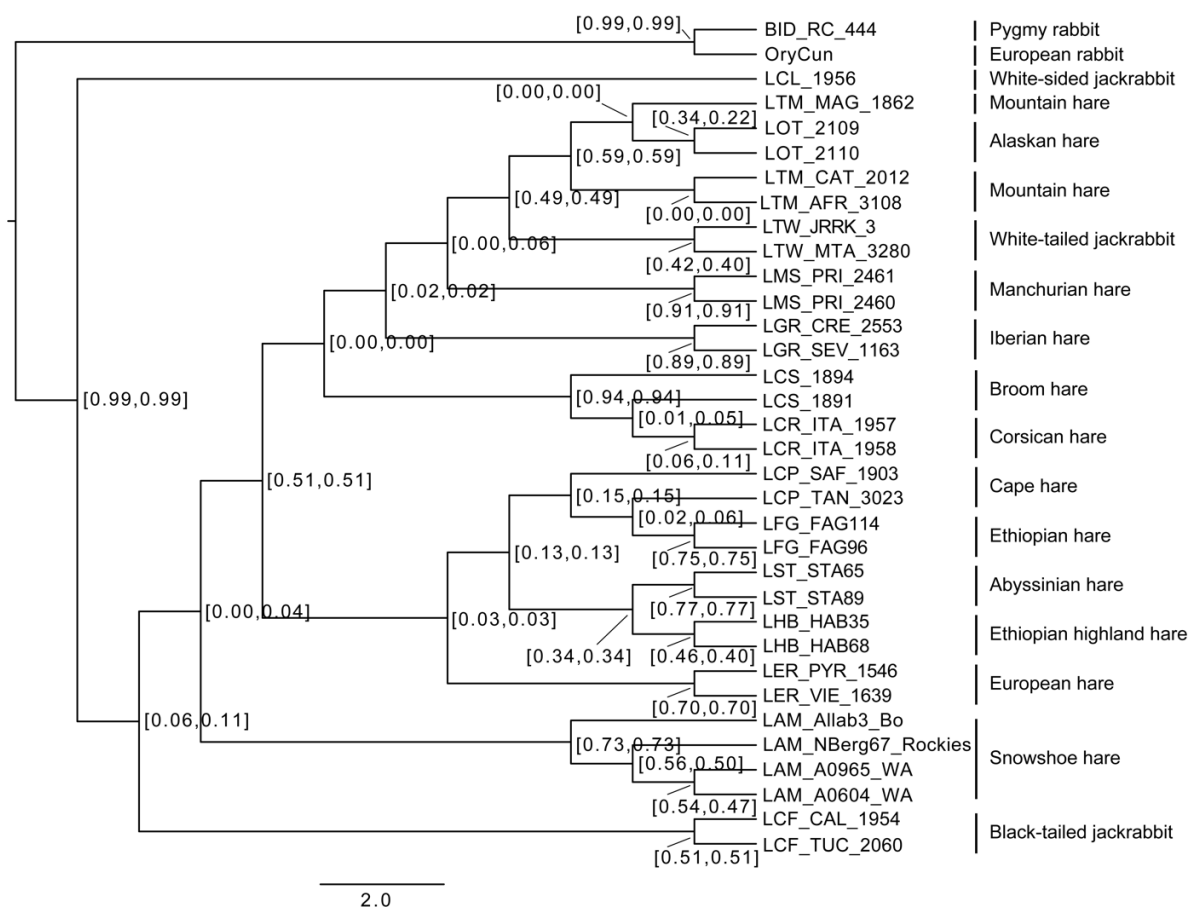


Figure S2.8 – Majority Rule Consensus Tree constructed from 8,889 maximum likelihood gene trees. Tree nodes are annotated with the Internode Certainty and Internode Certainty All.

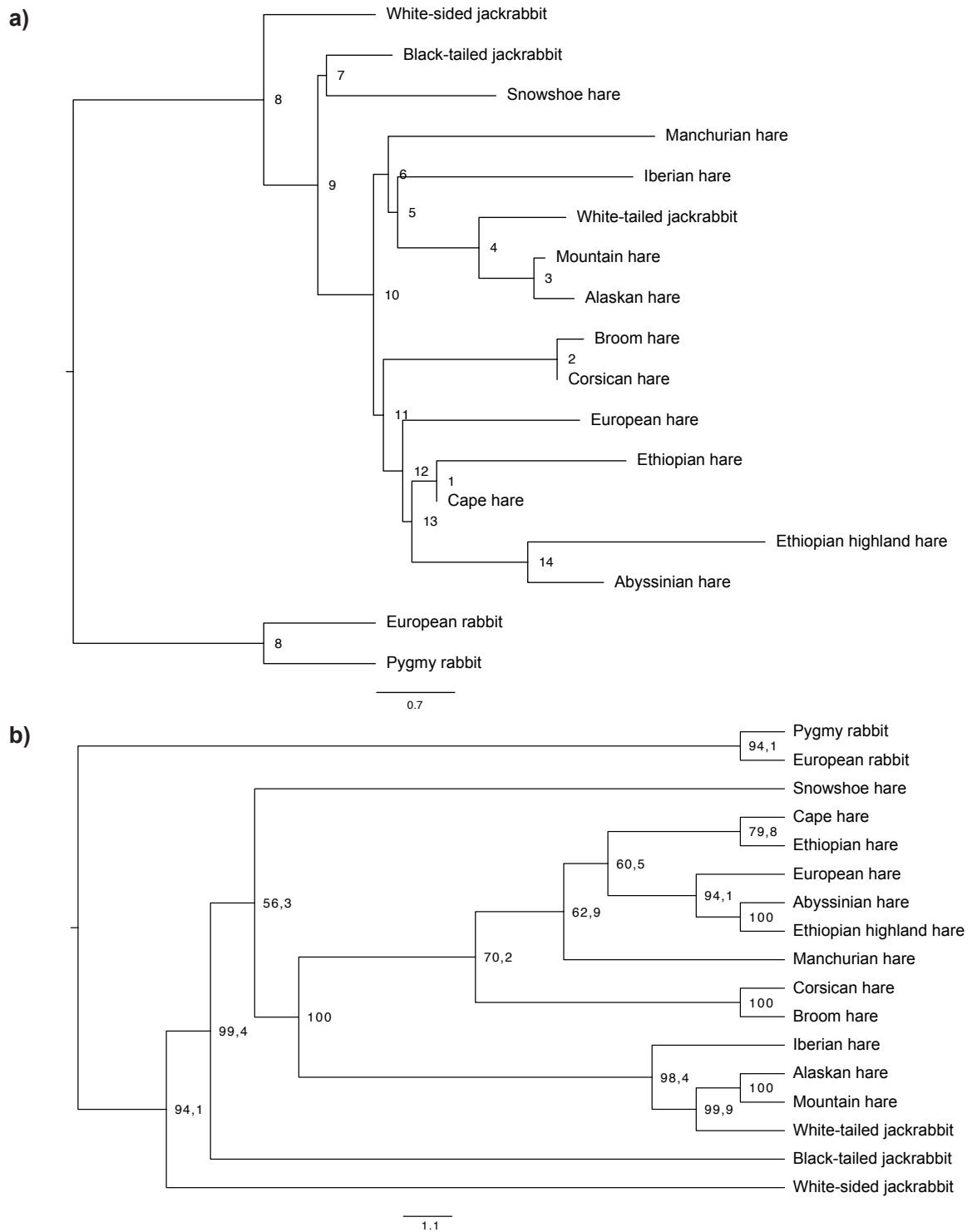


Figure S2.9 – Chromosome X species trees. a) *ASTRAL* species tree obtained from 181 chromosome X gene trees and b) *SVDquartets* species obtained from 1,473 unlinked SNVs localized in the X chromosome. Node labels in a) correspond to node labels in Supplementary Table S2.10 and b) 1,000 bootstrap support.

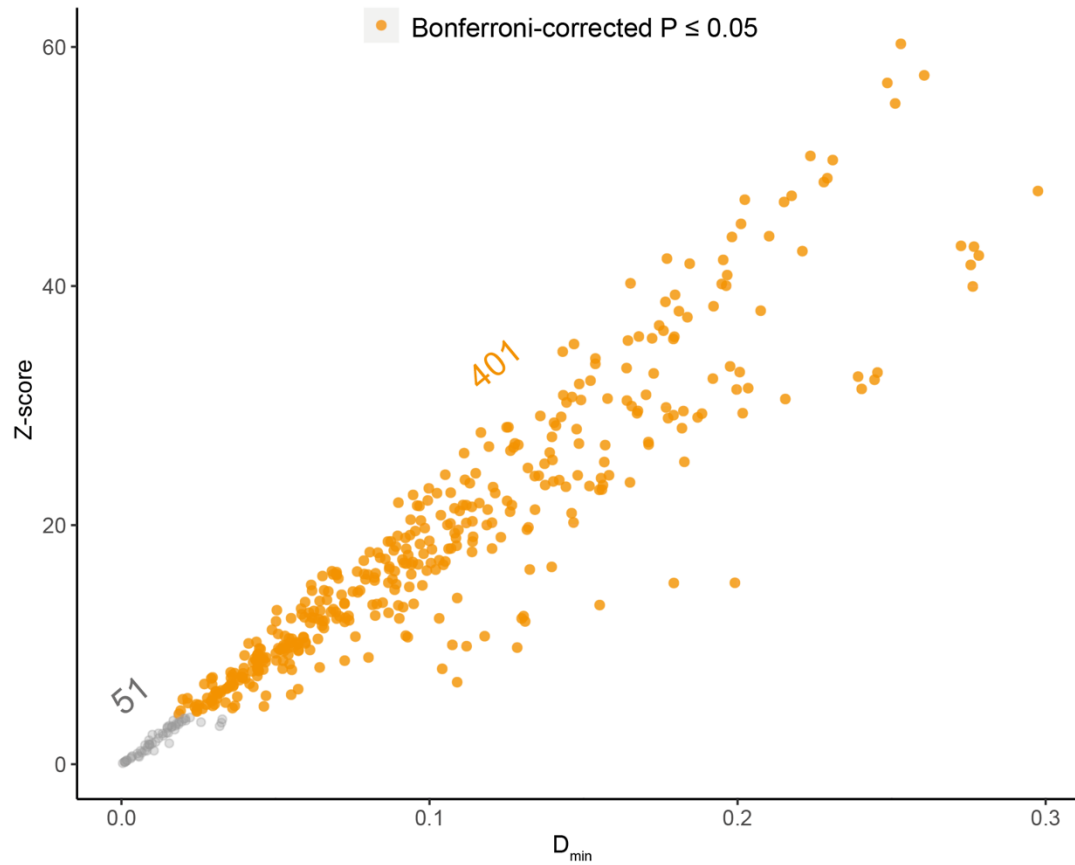


Figure S2.10 – Minimum D-statistic ($D_{\min}$; Malinsky et al 2018, Nat. Ecol. Evol.) between all possible trios of taxa in the species tree plotted against their Z-score. Significant values are highlighted in orange. The number of significant and non-significant $D_{\min}$ values is given above the graph.

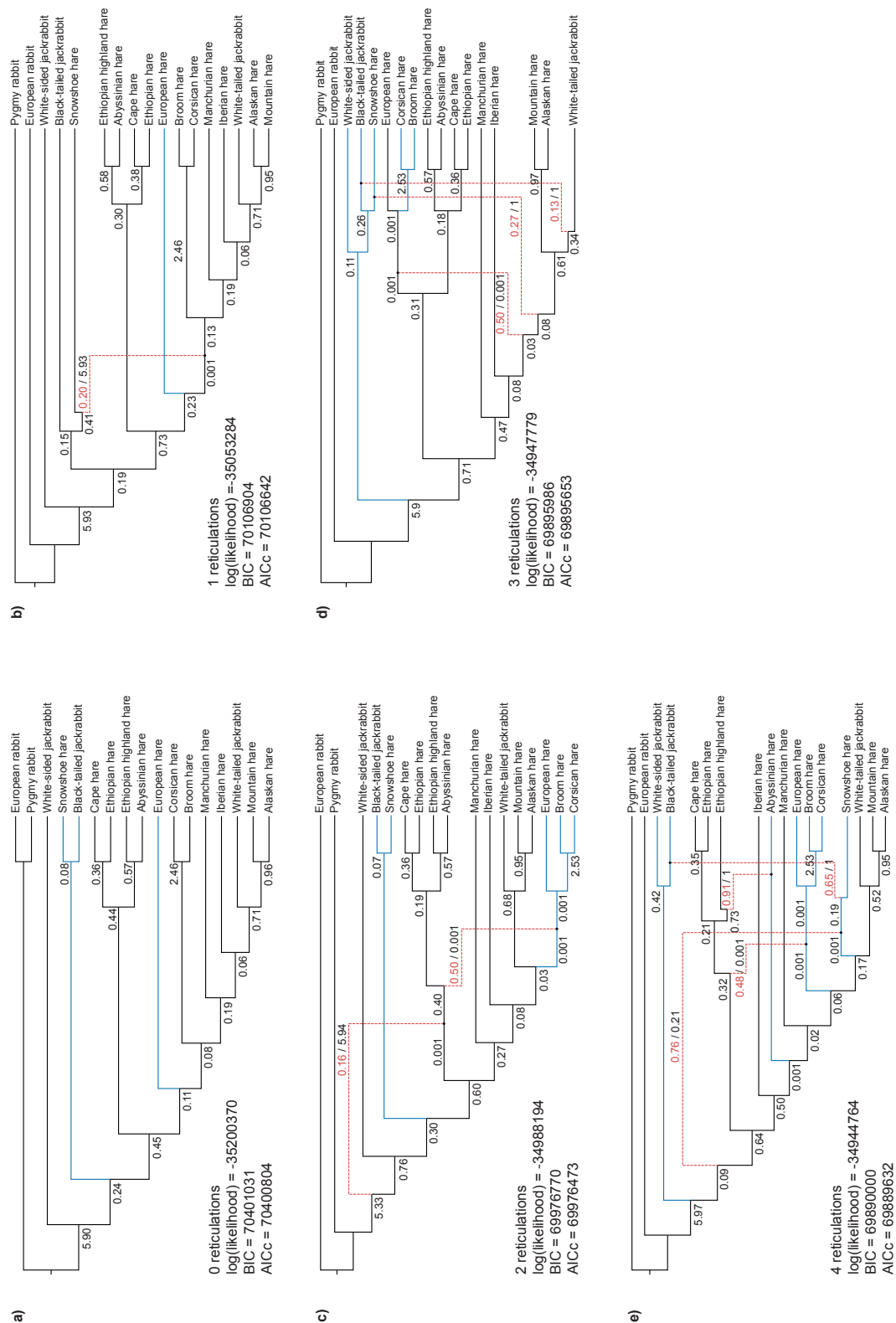


Figure S2.11 – Rooted pseudo-maximum likelihood networks for *Lepus*, with 0 to 4 reticulations inferred from 8,889 gene trees with *PhyloNet*. Red branches represent reticulations, and blue branches represent variations to the species tree topology in Figure 2.1b. Branch lengths are represented in black and inheritance probabilities are represented in red. Below each network, we report the log likelihood, Bayesian information criteria (BIC) and Akaike information criteria corrected for small sample sizes (AICc).

The evolution and molecular bases of seasonal coat color variation in hares: gene expression and the shared contribution of ancestral polymorphism and introgression

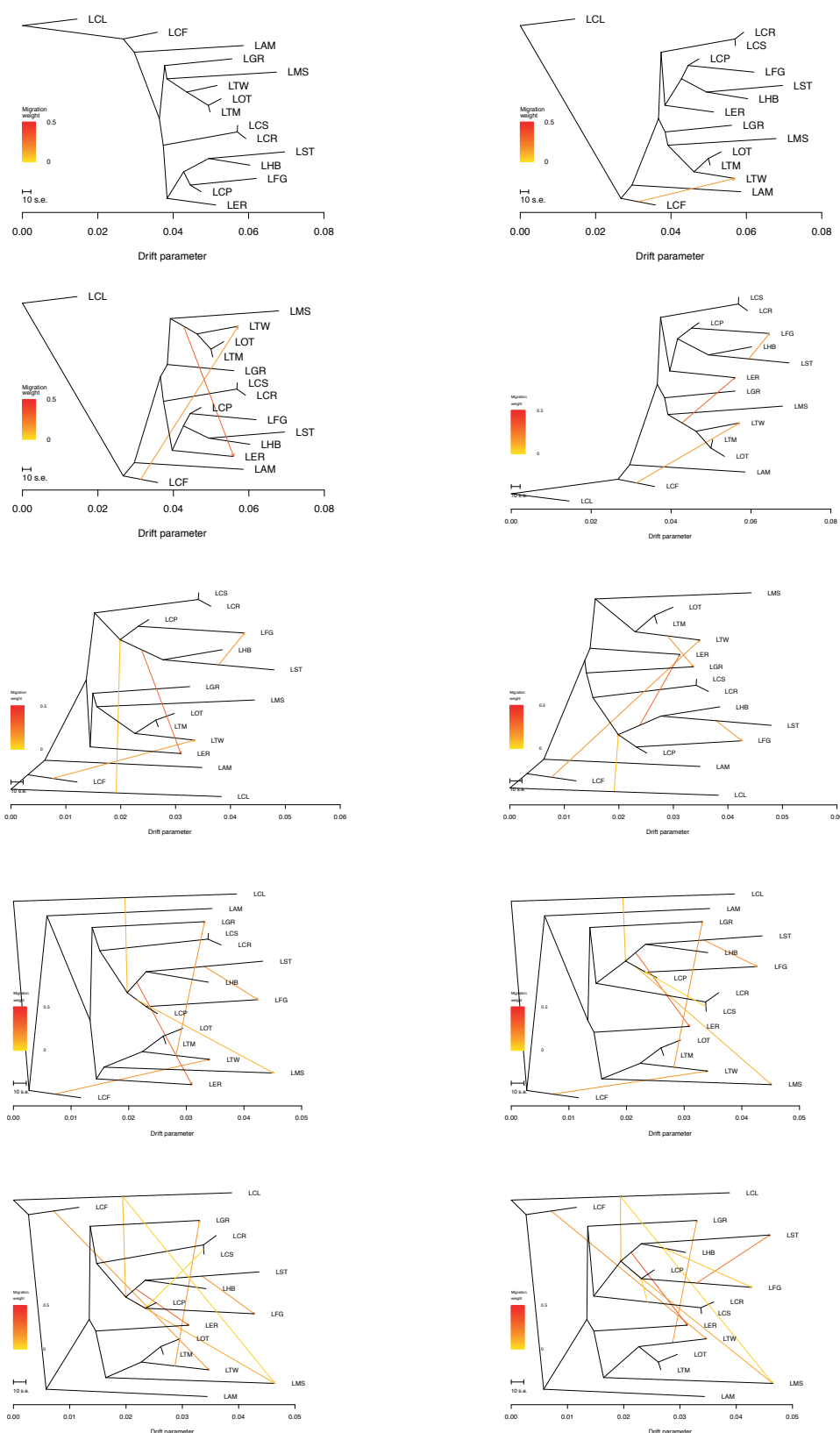


Figure S2.12 – *TreeMix* inference of splits and admixture using 30,709 biallelic SNVs for no migration events ($m=0$) to nine migration events ($m=9$). The white-sided jackrabbit (LCL) was used as outgroup. LAM – Snowshoe hare; LCF – Black-tailed jackrabbit; LGR – Iberian hare; LCR – Corsican hare; LCS – Broom hare; LST – Ethiopian highland hare; LHB – Abyssinian hare; LCP – Cape hare; LFG – Ethiopian hare; LER – European hare; LOT – Alaskan hare; LTM – Mountain hare; LTW – White-tailed jackrabbit; LMS – Manchurian hare.

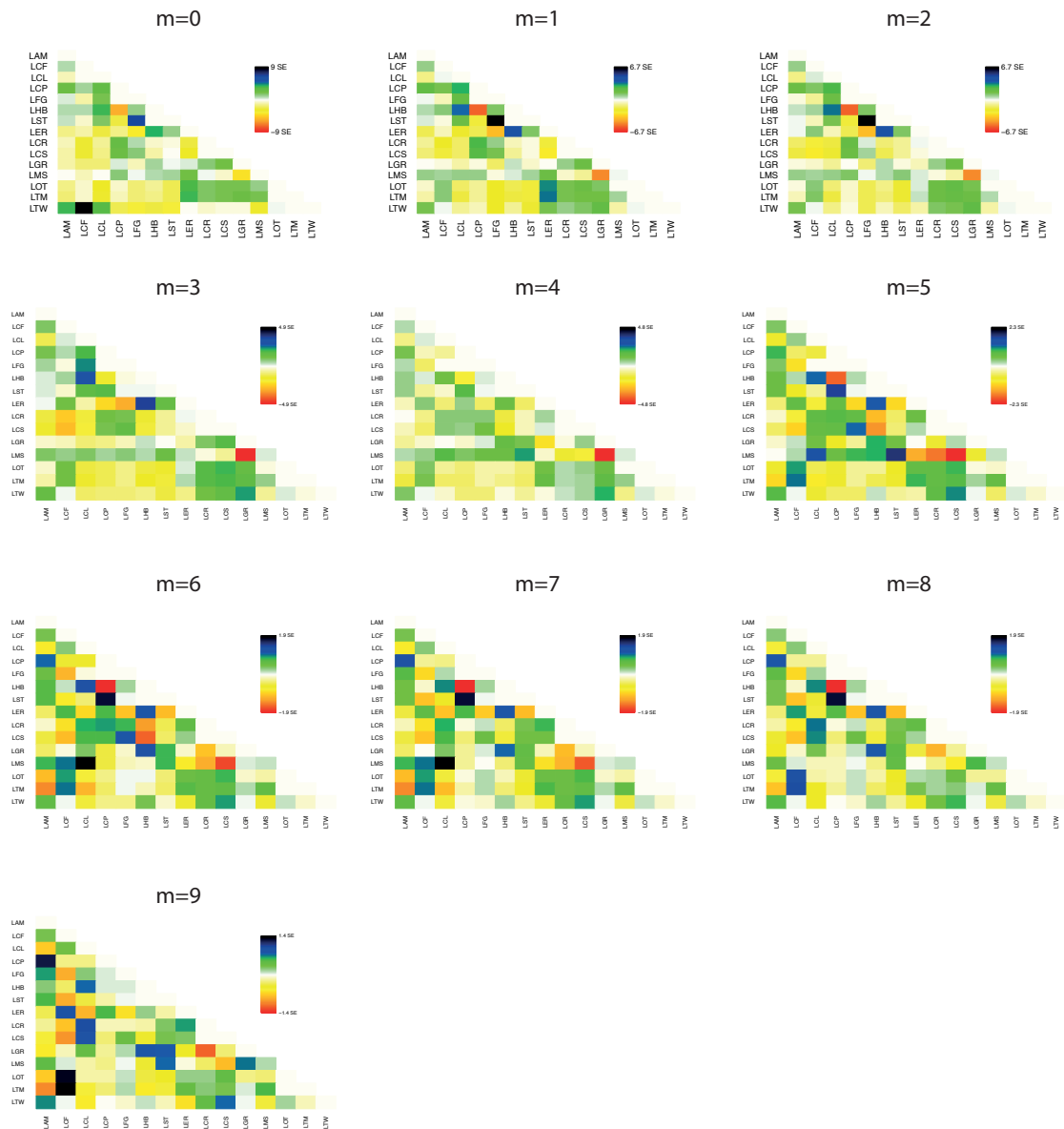


Figure S2.13 – Residuals for the *TreeMix* networks of Supplementary Figure S2.12, from 0 to 9 migration events. The white-sided jackrabbit (LCL) was used as outgroup. LAM – Snowshoe hare; LCF – Black-tailed jackrabbit; LGR – Iberian hare; LCR – Corsican hare; LCS – Broom hare; LST – Ethiopian highland hare; LHB – Abyssinian hare; LCP – Cape hare; LFG – Ethiopian hare; LER – European hare; LOT – Alaskan hare; LTM – Mountain hare; LTW – White-tailed jackrabbit; LMS – Manchurian hare.

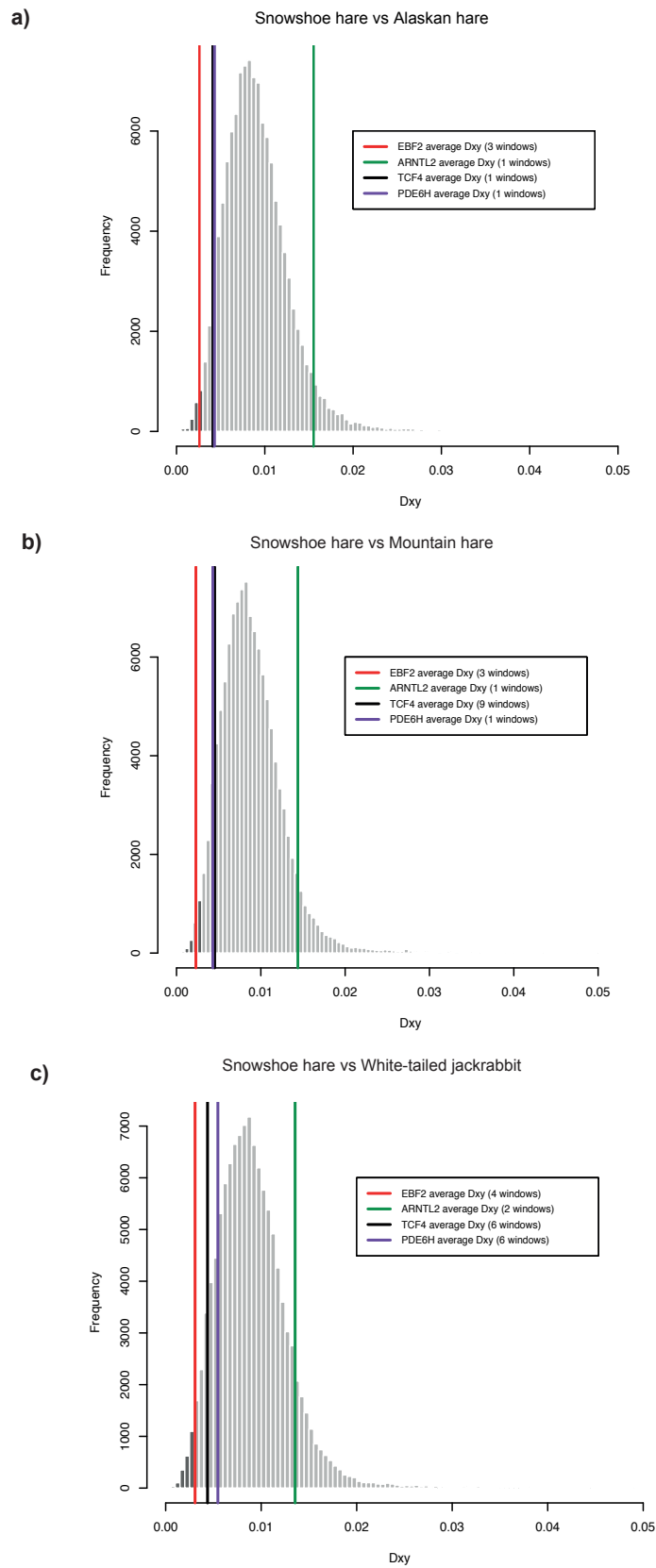


Figure S2.14 – Exome-wide observed genetic divergence (gray distribution) and average genetic divergence (colored lines) for fraction of admixture (f_d) outlier windows (Supplementary Table S2.13) between snowshoe hares and a) Alaskan hare, b) Mountain hare and c) white-tailed jackrabbits. The number of outlier windows used to estimate d_{xy} is reported above the graph, and in dark gray we highlight the lower 1% percentile of the distribution.

2.10.3. Supplementary Tables

Table S2.1 - Sampling information, for each species and individual used in this work.

Species	Common name	Sample ID	Sample origin	Location	Data origin	Extraction method	Capture effort	Sequencing effort	Used as pseudo-reference	Included in all major analysis	Included in divergence time estimation
<i>Brachylagus idahoensis</i>	Pygmy rabbit	BID_CG461	CIBIO-InBIO	Wyoming, United States of America	This work	Sent as DNA	1	20160128_hiseq		Yes	
<i>Brachylagus idahoensis</i>	Pygmy rabbit	BID_RC_444	CIBIO-InBIO	Wyoming, United States of America	This work	Sent as DNA	2	20160812_hiseq	Yes	Ex.	
<i>Lepus americanus</i>	Snowshoe hare	LAM_A0604_WA	Ref. 1	Washington, United States of America	Ref. 1	Ref. 1	Ref.1	Ref.1		Yes	
<i>Lepus americanus</i>	Snowshoe hare	LAM_A0965_WA	Ref. 1	Washington, United States of America	Ref. 1	Ref. 1	Ref.1	Ref.1		Yes	
<i>Lepus americanus</i>	Snowshoe hare	LAM_Allab3_Boreal	Ref. 1	Pennsylvania, United States of America	Ref. 1	Ref. 1	Ref.1	Ref.1		Yes	Yes
<i>Lepus americanus</i>	Snowshoe hare	LAM_NBerg67	Ref. 2	Wyoming, United States of America	Ref. 2	Ref. 2	Ref. 2	Ref. 2		Yes	
<i>Lepus californicus</i>	Black-tailed jackrabbit	LCF_CAL_1954	CIBIO-InBIO	Mexico	This work	Saline	2	20160812_hiseq		Yes	Yes
<i>Lepus californicus</i>	Black-tailed jackrabbit	LCF_TUC_2060	CIBIO-InBIO	Tucson, Arizona, United States of America	This work	DNeasy	2	20160812_hiseq		Yes	
<i>Lepus callotis</i>	White-sided jackrabbit	LCL_1956	CIBIO-InBIO	Mexico	This work	DNeasy	1	20160128_hiseq	Yes	Yes	Yes
<i>Lepus capensis</i>	Cape hare	LCP_SAF_1903	CIBIO-InBIO	South Africa	This work	Saline	1	20160128_hiseq		Yes	Yes
<i>Lepus capensis</i>	Cape hare	LCP_TAN_3023	CIBIO-InBIO	Tan-Tan, Morocco	This work	DNeasy	1	20160128_hiseq	Yes	Yes	
<i>Lepus corsicanus</i>	Apennine hare	LCR_ITA_1957	CIBIO-InBIO	Castello Porzianno, Italy	This work	DNeasy	1	20160128_hiseq		Yes	Yes
<i>Lepus corsicanus</i>	Apennine hare	LCR_ITA_1958	CIBIO-InBIO	Corsica, France	This work	DNeasy	1	20160128_hiseq	Yes	Yes	
<i>Lepus castroviejo</i>	Broom hare	LCS_1891	CIBIO-InBIO	Argovejo, Spain	This work	Saline	1	20160128_hiseq		Yes	
<i>Lepus castroviejo</i>	Broom hare	LCS_1894	CIBIO-InBIO	Cantabria, Spain	This work	Saline	1	20160128_hiseq	Yes	Yes	Yes
<i>Lepus europaeus</i>	European hare	LER_PYR_1546	CIBIO-InBIO	Jujols, Oriental Pyrenees, France	This work	DNeasy	2	20160812_hiseq		Yes	
<i>Lepus europaeus</i>	European hare	LER_ALM_1580	CIBIO-InBIO	Germany	This work	DNeasy	1	20160128_hiseq	Yes	Ex.	

<i>Lepus europaeus</i>	European hare	LER_VIE_1639	CIBIO-InBIO	Vienna, Austria	This work	DNeasy	2	20160812_hiseq		Yes	Yes
<i>Lepus fagani</i>	Ethiopian hare	LFG_FAG114	Zelalem Tolesa	West Shewa, Ethiopia	This work	Sent as DNA	1	20160128_hiseq	Yes	Yes	
<i>Lepus fagani</i>	Ethiopian hare	LFG_FAG96	Zelalem Tolesa	Adolla/Guji, Ethiopia	This work	Sent as DNA	1	20160128_hiseq		Yes	Yes
<i>Lepus granatensis</i>	Iberian hare	LGR_CRE_2553	CIBIO-InBIO	Ciudad Real, Spain	This work	DNeasy	1	20160128_hiseq	Yes	Yes	Yes
<i>Lepus granatensis</i>	Iberian hare	LGR_SEV_1163	CIBIO-InBIO	Sevilla, Spain	This work	DNeasy	1	20160128_hiseq		Yes	
<i>Lepus habessinicus</i>	Abyssinian hare	LHB_HAB35	Zelalem Tolesa	Fedis, eastern Ethiopia	This work	Sent as DNA	1	20160128_hiseq		Yes	Yes
<i>Lepus habessinicus</i>	Abyssinian hare	LHB_HAB68	Zelalem Tolesa	Wolaita, southern Ethiopia	This work	Sent as DNA	1	20160128_hiseq	Yes	Yes	
<i>Lepus mandshuricus</i>	Manchurian hare	LMS_PRI_2460	CIBIO-InBIO	Primorski territory, river Malinovka, Russia	This work	DNeasy	1	20160128_hiseq	Yes	Yes	
<i>Lepus mandshuricus</i>	Manchurian hare	LMS_PRI_2461	CIBIO-InBIO	Primorski territory, river Orekhovka, Russia	This work	DNeasy	2	20160812_hiseq		Yes	Yes
<i>Lepus othus</i>	Alaskan hare	LOT_2109	UAM (UAM42143)	Frosty road, Alaska, United States of America	This work	Ref. 3	1	20160128_hiseq	Yes	Yes	
<i>Lepus othus</i>	Alaskan hare	LOT_2110	UAM (UAM45545)	Nimiuk Point Baldwin Peninsula, Alaska, United States of America	This work	Ref. 3	1 and 2	20160812_hiseq		Yes	Yes
<i>Lepus starcki</i>	Ethiopian highland hare	LST_STA65	Zelalem Tolesa	North Showa/Oromia, Ethiopia	This work	Sent as DNA	1	20160128_hiseq	Yes	Yes	Yes
<i>Lepus starcki</i>	Ethiopian highland hare	LST_STA89	Zelalem Tolesa	Guraghe, central Ethiopia	This work	Sent as DNA	1	20160128_hiseq		Yes	
<i>Lepus timidus</i>	Mountain hare	LTM_AFR_3108	CIBIO-InBIO	Commune Araches, Arbaron, France	This work	DNeasy	2	20160812_hiseq		Yes	
<i>Lepus timidus</i>	Mountain hare	LTM_FIN_2191	CIBIO-InBIO	Jyvaskyla, Keski Suomi, Finland	This work	DNeasy	1	20160128_hiseq	Yes	Ex.	
<i>Lepus timidus</i>	Mountain hare	LTM_CAT_2012	CIBIO-InBIO	From captivity, originally from Finland.	This work	DNeasy	2	20160812_hiseq		Yes	Yes
<i>Lepus timidus</i>	Mountain hare	LTM_MAG_1862	CIBIO-InBIO	Magadan, Russia	This work	DNeasy	2	20160812_hiseq		Yes	
<i>Lepus townsendii</i>	White-tailed jackrabbit	LTW_JRRK_3	CIBIO-InBIO	Sublette County, Wyoming, United States of America	This work	DNeasy	1	20160128_hiseq	Yes	Yes	Yes
<i>Lepus townsendii</i>	White-tailed jackrabbit	LTW_MTA_3280	CIBIO-InBIO	Whitlash, Montana, United States of America	This work	DNeasy	2	20160812_hiseq		Yes	

Ref 1, Jones et al 2018, Science; Ref 2, Jones et al 2020, Evolution; Ref 3, Melo-Ferreira, 2012; DNeasy, DNeasy Blood & Tissue Kit (QIAGEN).

Note: This table was modified from its original published version for brevity.

Table S2.2 - Technical information for each individual regarding number of raw and clean reads, percentage of missing data, mean target coverage, capture enrichment, number of genotypes and pseudo-reference generation.

Species	Sample	Capture effort	No. raw paired reads	1) Remove contaminants, duplicates and low-quality trimming			2) Combine overlapping reads				3) Discard short reads				% missing data	Mean target coverage	% target bases at 10X	Capture fold enrichment	% bases off bait	No. genotypes called
				No. paired end reads	No. single reads	% of original reads	No. combined pairs	No. uncombined pairs	% combined pairs	% of original reads	Short discarded (< 50bp)	No. cleaned pair reads	No. cleaned single reads	% of original reads						
Pygmy rabbit	BID_RC_444	2	4133089	3778011	29151	92%	2694421	1083590	71%	59%	30468	1057324	2719370	58%	50%	9.0	34%	33.01	9%	36469826
Pygmy rabbit	BID_CG461	1	5650465	4604884	124829	83%	2715959	1888925	59%	59%	59159	1835756	2834798	58%	39%	10.6	44%	31.8	10%	46884276
Snowshoe hare	LAM_A0604_WA	Ref.1	20903389	16910103	488526	82%	5760536	11149567	34%	68%	542157	10810473	6045999	66%	14%	32.6	81%	31.5	9%	84057109
Snowshoe hare	LAM_A0965_WA	Ref. 1	16225762	13346516	354600	83%	5487534	7858982	41%	66%	408787	7613042	5679287	64%	18%	25.8	76%	32.0	9%	75023166
Snowshoe hare	LAM_Allab3_Boreal	Ref. 1	40211456	25674528	1989518	66%	18375906	7298622	72%	43%	2315266	6165557	19183223	39%	16%	34.2	79%	30.0	17%	77601897
Snowshoe hare	LAM_NBerg67_Rockies	Ref. 1	11858946	8965838	827395	79%	2627854	6337984	29%	68%	957307	5823815	3012111	62%	31%	17.4	60%	30.9	11%	60613260
Black-tailed jackrabbit	LCF_CAL_1954	2	14458717	10816369	264476	76%	9224262	1592107	85%	44%	100052	1507908	9472885	43%	17%	20.1	75%	33.4	10%	66642092
Black-tailed jackrabbit	LCF_TUC_2060	2	9555072	7148093	205249	76%	5016225	2131868	70%	50%	85342	2056101	5211899	49%	22%	14.9	65%	32.3	10%	62085858
White-sided jackrabbit	LCL_1956	1	20433818	17600879	176491	87%	13392972	4207907	76%	76%	173817	4061118	13542435	53%	16%	35.5	80%	32.4	10%	78258218
Cape hare	LCP_SAF_1903	1	12570426	11237068	100358	90%	9485556	1751512	84%	52%	104753	1662062	9570611	51%	19%	22.0	73%	33.4	9%	66233115
Cape hare	LCP_TAN_3023	1	7720636	6650941	75856	87%	3848955	2801986	58%	62%	84712	2728760	3913325	61%	24%	15.2	63%	31.5	9%	62893927
Apennine hare	LCR_ITA_1957	1	10938227	8285103	76222	76%	7603366	681737	92%	41%	66887	633254	7661184	41%	31%	14.3	57%	33.5	10%	52279511
Apennine hare	LCR_ITA_1958	1	6633498	5657638	72776	86%	3366237	2291401	60%	60%	81226	2221962	3427226	59%	26%	12.7	55%	31.9	9%	56623225
Broom hare	LCS_1891	1	9126117	5730961	86846	63%	3994322	1736639	70%	41%	70804	1679279	4067724	41%	26%	11.9	55%	31.3	11%	56749489
Broom hare	LCS_1894	1	12730787	7403613	111507	59%	5512108	1891505	74%	37%	91264	1822057	5601799	36%	29%	14.0	57%	30.9	13%	55913200

European hare	LER_ALM_1580	1	4752913	2364716	47491	50%	1294614	1070102	55%	37%	35213	1040836	1336158	36%	28%	4.9	7%	28.39	13%	22105101
European hare	LER_PYR_1546	2	8856514	6050102	162167	69%	5080518	969584	84%	41%	59513	920512	5232244	40%	68%	11.4	52%	33.6	10%	51418466
European hare	LER_VIE_1639	2	8108051	6901720	170216	86%	5055815	1845905	73%	55%	81721	1773717	5216498	54%	24%	14.1	61%	32.8	10%	57572770
Ethiopian hare	LFG_FAG114	1	4220285	3882475	41767	92%	3036961	845514	78%	57%	38007	813002	3073233	56%	48%	8.8	34%	33.5	8%	37559219
Ethiopian hare	LFG_FAG96	1	5944519	5416664	50283	92%	4261666	1154998	79%	56%	47776	1113648	4305523	55%	34%	11.4	49%	33.4	9%	48438244
Iberian hare	LGR_CRE_2553	1	6375769	5895279	68941	93%	3582174	2313105	61%	65%	70936	2251959	3641325	64%	32%	14.3	55%	32.5	8%	55164602
Iberian hare	LGR_SEV_1163	1	9343607	7298788	73723	79%	5116372	2182416	70%	51%	77345	2117442	5177724	50%	24%	15.7	64%	31.9	10%	62433081
Abyssinian hare	LHB_HAB35	1	5310602	4883789	57972	93%	3171710	1712079	65%	63%	57749	1661292	3222720	62%	34%	11.0	47%	32.4	8%	50053634
Abyssinian hare	LHB_HAB68	1	6875762	6338415	78265	93%	3924277	2414138	62%	64%	76745	2346495	3993440	63%	26%	14.2	60%	31.9	9%	59877656
Manchurian hare	LMS_PRI_2460	1	6181531	5412108	35109	88%	4400525	1011583	81%	52%	35578	982937	4428702	52%	35%	11.5	49%	33.7	8%	48119739
Manchurian hare	LMS_PRI_2461	2	5118486	4340720	95984	86%	3386416	954304	78%	53%	41814	917596	3477294	52%	38%	9.1	39%	33.8	8%	43767644
Alaskan hare	LOT_2109	1	8676332	7596917	91541	88%	4645864	2951053	61%	61%	93857	2870393	4724208	60%	23%	17.4	66%	31.8	9%	65003323
Alaskan hare	LOT_2110	1 and 2	6506564	13138771	224350	83%	8787473	4351298	67%	69%	153086	4218769	8991266	55%	30%	13.7	56%	32.0	8%	57147905
Ethiopian highland hare	LST_STA65	1	7463375	6817750	75363	92%	4470584	2347166	66%	62%	78227	2279232	4535654	61%	25%	14.7	62%	32.1	9%	60365506
Ethiopian highland hare	LST_STA89	1	4987064	4619986	56279	93%	2847158	1772828	62%	65%	54517	1725079	2896669	64%	35%	10.8	46%	32.3	8%	49047045
Mountain hare	LTM_AFR_3108	2	7940427	6945437	172994	89%	4554376	2391061	66%	60%	87435	2312612	4718384	59%	18%	15.8	64%	32.7	8%	62213553
Mountain hare	LTM_CAT_2012	2	6638031	5839983	150627	89%	3671101	2168882	63%	61%	75741	2101327	3813542	60%	23%	13.7	56%	32.5	8%	55921540
Mountain hare	LTM_FIN_2191	1	11452993	8351427	111730	73%	4054095	4297332	49%	56%	117504	4196164	4149489	55%	30%	18.8	72%	30.14	10%	74637308
Mountain hare	LTM_MAG_1862	2	6641522	5611544	148105	86%	4294948	1316596	77%	53%	67548	1256954	4435147	52%	28%	11.7	53%	33.5	8%	52655645
White-tailed jackrabbit	LTW_JRRK_3	1	7789293	6662734	75158	86%	2884684	3778050	43%	68%	82911	3705912	2949069	67%	23%	16.0	64%	30.9	9%	66705053
White-tailed jackrabbit	LTW_MTA_3280	2	7801644	6732958	174780	87%	4484722	2248236	67%	59%	87149	2170450	4650139	58%	27%	14.9	60%	32.9	9%	57746815

Ref 1, Jones et al 2018, Science

Table S2.3 – Per species nucleotide diversity.

Species	Nucleotide diversity (π)
Snowshoe hare (<i>Lepus americanus</i>)	0.51%
Black-tailed jackrabbit (<i>L. californicus</i>)	0.63%
Cape hare (<i>L. capensis</i>)	0.54%
Broom hare (<i>L. castroviejo</i>)	0.14%
Corsican hare (<i>L. corsicanus</i>)	0.13%
European hare (<i>L. europaeus</i>)	0.37%
Ethiopian hare (<i>L. fagani</i>)	0.30%
Iberian hare (<i>L. granatensis</i>)	0.26%
Abyssinian hare (<i>L. habessinicus</i>)	0.33%
Manchurian hare (<i>L. mandschuricus</i>)	0.18%
Alaskan hare (<i>L. othus</i>)	0.23%
Ethiopian highland har (<i>L. starcki</i>)	0.20%
Mountain hare (<i>L. timidus</i>)	0.29%
White-tailed jackrabbit (<i>L. townsendii</i>)	0.38%
White-sided jackrabbit (<i>L. callotis</i>)	0.44%

Table S2.4 - Whole-exome genetic divergence (d_{xy}) among species.

[illegible]

Table S2.5 – Posteriors for divergence time estimation for each branch in Supplementary Figure S2.5 (rows labeled with numbers). For each branch, we indicate high posterior density (HPD) 95% confidence intervals and corresponding width estimated with Bayesian inference in *MCMCtree* using divergence time calibrations for the divergence between *Lepus* and *Oryctolagus* and for the divergence of the *Lepus* species obtained from **(a)** molecular-based dates extrapolated from deep calibrations in the Lagomorphs or **(b)** *Lepus* fossil record calibrations (see Materials and Methods). Also, for each partition in the alignment (first, second and third codon of the coding regions), we indicate the posterior of the mean substitution rate (μ or μ) and of the Dirichlet-gamma parameter of substitution rate heterogeneity among branches (σ^2 or σ^2).

		a) Deep fossil calibration points			b) <i>Lepus</i> fossil calibration points		
		Posterior mean	95% HPD CI	HPD CI width	Posterior mean	95% HPD CI	HPD CI width
Branch label in Figure S5	1	0.1362	(0.1181, 0.1481)	0.0299	0.0604	(0.0484, 0.0724)	0.024
	2	0.0583	(0.0534, 0.0617)	0.0083	0.0405	(0.0318, 0.05)	0.0183
	3	0.0478	(0.0435, 0.0514)	0.0079	0.0338	(0.0263, 0.0419)	0.0156
	4	0.0399	(0.0359, 0.0436)	0.0077	0.0285	(0.0222, 0.0355)	0.0134
	5	0.0277	(0.0243, 0.0309)	0.0066	0.0199	(0.0154, 0.0251)	0.0096
	6	0.0248	(0.0216, 0.0277)	0.0061	0.0178	(0.0138, 0.0225)	0.0087
	7	0.0185	(0.0159, 0.0208)	0.0049	0.0133	(0.0102, 0.0168)	0.0066
	8	0.0139	(0.0119, 0.0158)	0.0039	0.0100	(0.0076, 0.0127)	0.0051
	9	0.0118	(0.01, 0.0135)	0.0035	0.0085	(0.0064, 0.0108)	0.0044
	10	0.0239	(0.0208, 0.0267)	0.0059	0.0171	(0.0133, 0.0217)	0.0084
	11	0.0012	(0.0009, 0.0015)	0.0006	0.0008	(0.0006, 0.0011)	0.0006
	12	0.0205	(0.0178, 0.0231)	0.0053	0.0147	(0.0114, 0.0187)	0.0073
	13	0.0175	(0.0151, 0.0197)	0.0047	0.0125	(0.0097, 0.0159)	0.0063
	14	0.0098	(0.0083, 0.0113)	0.0030	0.0071	(0.0053, 0.009)	0.0037
	15	0.0033	(0.0027, 0.0039)	0.0012	0.0024	(0.0018, 0.0031)	0.0013
mu (1st codon)		0.0458	(0.02, 0.0762)	0.0562	0.0709	(0.0367, 0.1104)	0.0737
mu (2nd codon)		0.0345	(0.015, 0.058)	0.0429	0.0537	(0.0274, 0.0836)	0.0562
mu (3rd codon)		0.1586	(0.0688, 0.2636)	0.1947	0.2637	(0.1376, 0.4071)	0.2695
Sigma ² (1st codon)		3.3819	(2.3756, 4.5785)	2.2857	4.7525	(3.2943, 6.3445)	3.0502
Sigma ² (2nd codon)		3.4956	(2.4617, 4.7318)	2.3441	4.8661	(3.3687, 6.4676)	3.0988
Sigma ² (3rd codon)		3.5286	(2.497, 4.7363)	2.3329	4.9017	(3.4015, 6.5167)	3.1152
Log likelihood		-42.274	(-54.98, -30.14)	24.84	-42.2936	(-55.013, -30.163)	24.85

Table S2.6 – Model comparison for ancestral range reconstruction in *BioGeoBEARS* using two divergence times estimations obtained from calibrating the *Lepus* species tree with deep fossil calibration points or with *Lepus* fossil calibration points. The included models were dispersal-extinction-cladogenesis (DEC), dispersal-vicariance-like (DIVALIKE), and Bayesian inference of historical biogeography for discrete areas (BAYAREALIKE), each with and without founder-event speciation (+J).

Models	Ln likelihood	Number of parameters	d	e	j	AIC	AICc
a) Deep fossil calibration points							
DEC	-21.26	2	0.021	1.00E-12	0.00	46.53	47.45
DEC+J	-13.81	3	1.00E-12	1.00E-12	0.13	33.62	35.62
DIVALIKE	-22.76	2	0.035	1.00E-12	0.00	49.52	50.44
DIVALIKE+J	-13.28	3	1.00E-12	1.00E-12	0.12	32.55	34.55
BAYAREALIKE	-32.06	2	0.049	0.16	0.00	68.12	69.05
BAYAREALIKE+J	-14.49	3	1.00E-07	1.00E-07	0.13	34.99	36.99
b) <i>Lepus</i> fossil calibration points							
DEC	-21.34	2	0.033	1.00E-12	0.000	46.69	47.61
DEC+J	-13.81	3	1.00E-12	1.00E-12	0.130	33.62	35.62
DIVALIKE	-22.02	2	0.059	1.00E-12	0.000	48.04	48.96
DIVALIKE+J	-13.28	3	1.00E-12	1.00E-12	0.120	32.55	34.55
BAYAREALIKE	-30.89	2	0.059	0.27	0.000	65.77	66.69
BAYAREALIKE+J	-14.49	3	1.00E-07	1.00E-07	0.130	34.99	36.99

d, rate of dispersal; e, rate of extinction; j, likelihood of founder event speciation at cladogenesis; AIC, Akaike's information criterion; AICc, Akaike's information criterion, with correction for sample size.

Table S2.7 – Proportional likelihood values for ancestral range reconstruction under the DIVALIKE+J biogeographic model implemented in *BioGeoBEARS* using two divergence times estimations obtained from **(a)** calibrating the *Lepus* species tree with deep fossil calibration points within Lagomorphs or **(b)** with *Lepus* fossil calibration points.

a) Deep fossil calibration points								
Tip/ Node	None	NA	EU	AF	NAEU	NAAF	EUAF	NAEUAF
White-sided jackrabbit	0	1	0	0	0	0	0	0
Black-tailed jackrabbit	0	1	0	0	0	0	0	0
European rabbit	0	0	1	0	0	0	0	0
Ethiopian hare	0	0	0	1	0	0	0	0
Cape hare	0	0	0	1	0	0	0	0
Ethiopian highland hare	0	0	0	1	0	0	0	0
Abyssinian hare	0	0	0	1	0	0	0	0
Corsican hare	0	0	1	0	0	0	0	0
Broom hare	0	0	1	0	0	0	0	0
Iberian hare	0	0	1	0	0	0	0	0
Manchurian hare	0	0	1	0	0	0	0	0
White-tailed jackrabbit	0	1	0	0	0	0	0	0
Alaskan hare	0	1	0	0	0	0	0	0
Mountain hare	0	0	1	0	0	0	0	0
Snowshoe hare	0	1	0	0	0	0	0	0
European rabbit	0	0	1	0	0	0	0	0
17	0	0.133	0.146	1.74E-04	0.718	6.23E-04	9.38E-04	1.90E-03
18	0	0.983	0.013	1.29E-03	1.03E-12	2.52E-03	3.32E-14	3.49E-14
19	0	0.970	0.024	5.09E-03	6.77E-13	8.83E-14	8.76E-14	7.29E-25
20	0	0.865	0.119	0.016	3.78E-12	4.27E-13	1.68E-13	1.95E-24
21	0	0.010	0.889	0.100	6.42E-14	3.87E-14	1.48E-12	4.43E-25
22	0	2.76E-24	0.797	0.203	7.82E-25	3.93E-25	1.59E-12	4.32E-36
23	0	1.47E-25	5.88E-25	1.000	6.44E-49	1.11E-36	2.31E-36	4.71E-60
24	0	7.63E-26	2.18E-25	1.000	4.31E-49	7.77E-37	1.13E-36	3.53E-60
25	0	5.49E-26	1.57E-25	1.000	3.32E-49	8.15E-37	1.18E-36	4.17E-60
26	0	0.012	0.988	4.17E-25	6.51E-14	7.29E-27	8.60E-27	9.68E-38
27	0	6.80E-28	1.000	4.39E-28	2.88E-38	4.27E-52	2.59E-38	2.80E-62
28	0	0.019	0.981	1.35E-25	1.43E-13	1.11E-26	3.42E-26	1.56E-37
29	0	0.079	0.921	1.73E-25	9.31E-13	3.31E-26	1.99E-25	6.61E-37
30	0	0.566	0.434	1.10E-25	2.04E-12	6.45E-26	2.19E-25	2.46E-36
31	0	0.504	0.496	1.37E-26	3.52E-12	9.03E-26	1.45E-25	1.72E-36
b) <i>Lepus</i> fossil calibration points								
Tip/ Node	None	NA	EU	AF	NAEU	NAAF	EUAF	NAEUAF
White-sided jackrabbit	0	1	0	0	0	0	0	0
Black-tailed jackrabbit	0	1	0	0	0	0	0	0
European rabbit	0	0	1	0	0	0	0	0
Ethiopian hare	0	0	0	1	0	0	0	0
Cape hare	0	0	0	1	0	0	0	0
Ethiopian highland hare	0	0	0	1	0	0	0	0
Abyssinian hare	0	0	0	1	0	0	0	0
Corsican hare	0	0	1	0	0	0	0	0
Broom hare	0	0	1	0	0	0	0	0
Iberian hare	0	0	1	0	0	0	0	0
Manchurian hare	0	0	1	0	0	0	0	0
White-tailed jackrabbit	0	1	0	0	0	0	0	0
Alaskan hare	0	1	0	0	0	0	0	0
Mountain hare	0	0	1	0	0	0	0	0
Snowshoe hare	0	1	0	0	0	0	0	0
European rabbit	0	0	1	0	0	0	0	0
17	0	0.133	0.146	0.000	0.718	0.001	0.001	0.002
18	0	0.983	0.013	0.001	2.62E-13	0.003	8.45E-15	8.90E-15
19	0	0.970	0.024	0.005	4.34E-13	5.66E-14	2.98E-14	1.91E-25
20	0	0.865	0.119	0.016	2.56E-12	2.89E-13	8.45E-14	7.97E-25

21	0	0.010	0.889	0.100	4.51E-14	2.72E-14	1.00E-12	2.17E-25
22	0	1.42E-24	0.797	0.203	4.00E-25	1.99E-25	1.13E-12	1.57E-36
23	0	7.59E-26	3.03E-25	1	1.71E-49	4.10E-37	8.52E-37	8.94E-61
24	0	3.94E-26	1.12E-25	1	1.15E-49	2.88E-37	4.19E-37	6.76E-61
25	0	2.84E-26	8.09E-26	1	8.86E-50	3.02E-37	4.39E-37	7.98E-61
26	0	0.012	0.988	2.15E-25	4.62E-14	3.71E-27	4.41E-27	3.52E-38
27	0	3.51E-28	1.000	2.27E-28	1.07E-38	1.14E-52	9.59E-39	5.36E-63
28	0	0.019	0.981	6.95E-26	1.02E-13	5.68E-27	1.76E-26	5.72E-38
29	0	0.079	0.921	8.94E-26	6.68E-13	1.71E-26	1.02E-25	2.45E-37
30	0	0.566	0.434	5.69E-26	1.47E-12	3.33E-26	1.13E-25	9.12E-37
31	0	0.504	0.496	7.04E-27	2.53E-12	4.66E-26	7.46E-26	6.38E-37

NA, North America; EU, Eurasia; AF, Africa; NAEU, North America + Eurasia; NAAF, North America + Africa; EUAF, Eurasia + Africa; NAEUAF, North America + Eurasia + Africa.

Table S2.8 – Branch information for the branches of the coalescent species tree estimated with *ASTRAL* in Figure 2.1 and Supplementary Figure S2.2b. For each branch, we indicate the quartet score for the first (q1), second (q2) and third (q3) most prevalent topologies, number of quartet trees that support the first (f1), second (f2) and third (f3) most prevalent topologies, the posterior probability for the first (pp1), second (pp2) and third (pp3) most prevalent topologies, the total number of quartets defined around each branch (QC) and the effective number of genes for the branch (EN).

Node	q1	q2	q3	f1	f2	f3	pp1	pp2	pp3	QC	EN
1	0.53	0.29	0.18	4702.8	2588.3	1597.9	1.00	0.00	0.00	416	8889
2	0.94	0.03	0.03	8368.2	243.3	277.5	1.00	0.00	0.00	836	8889
3	0.71	0.15	0.13	6344.7	1364.3	1180.0	1.00	0.00	0.00	324	8889
4	0.67	0.20	0.13	5959.2	1786.0	1143.8	1.00	0.00	0.00	500	8889
5	0.39	0.39	0.22	3460.2	3451.4	1977.5	0.89	0.11	0.00	644	8889
6	0.46	0.30	0.24	4129.8	2665.0	2094.2	1.00	0.00	0.00	1368	8889
7	0.41	0.36	0.24	3608.0	3166.7	2114.4	1.00	0.00	0.00	3960	8889
8	1.00	0.00	0.00	8866.4	12.2	10.4	1.00	0.00	0.00	31	8889
9	0.43	0.22	0.36	3787.2	1944.8	3157.1	1.00	0.00	0.00	116	8889
10	0.38	0.26	0.36	3402.1	2282.1	3204.7	1.00	0.00	0.00	600	8889
11	0.61	0.17	0.23	5386.2	1473.7	2029.1	1.00	0.00	0.00	3000	8889
12	0.41	0.23	0.36	3652.5	2074.9	3161.6	1.00	0.00	0.00	2160	8889
13	0.55	0.29	0.17	4853.5	2557.2	1478.3	1.00	0.00	0.00	768	8889
14	0.60	0.16	0.24	5318.4	1422.7	2147.9	1.00	0.00	0.00	416	8889

Table S2.9 – Branch information for the branches of the coalescent species tree estimated with *ASTRAL* in Supplementary Figure S2.2a. For each branch, we indicate the quartet score for the first (q1), second (q2) and third (q3) most prevalent topologies, number of quartet trees that support the first (f1), second (f2) and third (f3) most prevalent topologies, the posterior probability for the first (pp1), second (pp2) and third (pp3) most prevalent topologies, the total number of quartets defined around each branch (QC) and the effective number of genes for the branch (EN).

Node	q1	q2	q3	f1	f2	f3	pp1	pp2	pp3	QC	E N
1	0.69	0.16	0.15	6113.06	1431.06	1344.89	1.00	0.00	0.00	87	8889
2	0.81	0.09	0.10	7240.26	801.26	847.48	1.00	0.00	0.00	31	8889
3	0.81	0.09	0.10	7216.02	795.27	877.72	1.00	0.00	0.00	60	8889
4	0.77	0.12	0.12	6813.30	1048.39	1027.31	1.00	0.00	0.00	375	8889
5	0.71	0.15	0.14	6298.01	1302.48	1288.51	1.00	0.00	0.00	192	8889
6	0.84	0.09	0.07	7452.48	790.87	645.65	1.00	0.00	0.00	31	8889
7	0.43	0.25	0.32	3854.10	2233.70	2801.20	1.00	0.00	0.00	60	8889
8	0.51	0.30	0.19	4525.58	2638.41	1725.00	1.00	0.00	0.00	312	8889
9	0.88	0.06	0.06	7856.15	504.72	528.13	1.00	0.00	0.00	60	8889
10	0.71	0.14	0.15	6317.92	1237.08	1334.00	1.00	0.00	0.00	60	8889
11	0.60	0.16	0.24	5318.45	1422.69	2147.87	1.00	0.00	0.00	416	8889
12	0.55	0.29	0.17	4853.51	2557.19	1478.30	1.00	0.00	0.00	768	8889
13	0.41	0.23	0.36	3652.46	2074.95	3161.59	1.00	0.00	0.00	2160	8889
14	0.92	0.04	0.04	8189.29	358.55	341.15	1.00	0.00	0.00	207	8889
15	0.87	0.06	0.07	7721.88	537.94	629.18	1.00	0.00	0.00	175	8889
16	0.64	0.16	0.20	5720.56	1414.15	1754.30	1.00	0.00	0.00	135	8889
17	0.57	0.20	0.22	5085.48	1820.65	1982.87	1.00	0.00	0.00	31	8889
18	0.35	0.34	0.32	3078.08	2991.55	2819.37	0.96	0.03	0.00	60	8889
19	0.35	0.31	0.34	3074.24	2767.78	3046.98	0.79	0.00	0.20	87	8889
20	0.70	0.16	0.14	6245.00	1430.34	1213.67	1.00	0.00	0.00	216	8889
21	0.67	0.20	0.13	5959.20	1786.05	1143.75	1.00	0.00	0.00	500	8889
22	0.39	0.39	0.22	3460.16	3451.39	1977.45	0.89	0.11	0.00	644	8889
23	0.46	0.30	0.24	4129.79	2665.02	2094.19	1.00	0.00	0.00	1368	8889
24	0.42	0.25	0.33	3771.61	2191.84	2925.55	1.00	0.00	0.00	31	8889
25	0.42	0.21	0.37	3708.67	1872.07	3308.27	1.00	0.00	0.00	60	8889
26	0.92	0.04	0.04	8206.03	327.90	355.07	1.00	0.00	0.00	627	8889
27	0.41	0.36	0.24	3607.97	3166.67	2114.36	1.00	0.00	0.00	3960	8889
28	0.61	0.23	0.17	5386.19	2029.09	1473.73	1.00	0.00	0.00	3000	8889
29	0.38	0.26	0.36	3402.12	2282.14	3204.74	1.00	0.00	0.00	600	8889
30	0.43	0.22	0.36	3787.16	1944.78	3157.05	1.00	0.00	0.00	116	8889
31	1.00	0.00	0.00	8866.42	10.42	12.16	1.00	0.00	0.00	31	8889

Table S2.10 – Branch information for the branches of the coalescent species tree estimated with *ASTRAL* in Supplementary Figure S2.9a, obtained for X-linked gene trees. For each branch, we indicate the quartet score for the first (q1), second (q2) and third (q3) most prevalent topologies, number of quartet trees that support the first (f1), second (f2) and third (f3) most prevalent topologies, the posterior probability for the first (pp1), second (pp2) and third (pp3) most prevalent topologies, the total number of quartets defined around each branch (QC) and the effective number of genes for the branch (EN).

Node	q1	q2	q3	f1	f2	f3	pp1	pp2	pp3	QC	EN
1	0.47	0.30	0.23	84.82	54.31	41.87	1.00	0.00	0.00	416	181
2	0.86	0.07	0.07	156.41	12.08	12.51	1.00	0.00	0.00	800	181
3	0.60	0.20	0.20	107.86	36.13	37.01	1.00	0.00	0.00	324	181
4	0.68	0.18	0.14	123.41	32.76	24.83	1.00	0.00	0.00	500	181
5	0.39	0.36	0.25	70.43	65.82	44.75	0.71	0.25	0.03	644	181
6	0.42	0.31	0.27	75.70	56.79	48.51	0.97	0.02	0.01	2268	181
7	0.38	0.26	0.35	69.65	47.34	64.01	0.73	0.04	0.23	600	181
8	0.98	0.01	0.01	178.00	1.97	1.03	1.00	0.00	0.00	31	181
9	0.59	0.22	0.19	107.28	39.36	34.36	1.00	0.00	0.00	300	181
10	0.60	0.18	0.22	108.32	32.77	39.91	1.00	0.00	0.00	2772	181
11	0.39	0.28	0.33	70.90	50.24	59.85	0.85	0.04	0.11	3960	181
12	0.44	0.28	0.28	79.95	51.06	49.99	1.00	0.00	0.00	1280	181
13	0.39	0.39	0.23	70.25	70.02	40.73	0.50	0.48	0.02	768	181
14	0.77	0.11	0.12	139.02	20.04	21.94	1.00	0.00	0.00	416	181
15	0.67	0.15	0.18	120.47	27.73	32.80	1.00	0.00	0.00	60	181

Table S2.11 – Shared heterozygosity in the dataset. For each individual, we indicate the amount of shared and non-shared heterozygous sites with members of other species.

Species	Samples	Total length of alignment	No. heterozygous sites	No. missing sites	% of missing sites	Effective length	Percentage of heterozygous sites	No. non-shared heterozygous sites	No. shared heterozygous sites	% of shared heterozygous sites
Snowshoe hare	LAM_A0604_WA	70630551	183349	1991169	3%	68639382	0.27%	120569	62780	34%
Snowshoe hare	LAM_A0965_WA	70630551	174646	4502237	6%	66128314	0.26%	113906	60740	35%
Snowshoe hare	LAM_Allab3_Bo	70630551	283570	3968806	6%	66661745	0.43%	194412	89158	31%
Snowshoe hare	LAM_NBerg67_Rockies	70630551	91188	14646293	21%	55984258	0.16%	57250	33938	37%
Black-tailed jackrabbit	LCF_CAL_1954	70630551	411863	7647257	11%	62983294	0.65%	258026	153837	37%
Black-tailed jackrabbit	LCF_TUC_2060	70630551	352888	11169502	16%	59461049	0.59%	218563	134325	38%
White-sided jackrabbit	LCL_1956	70630551	264573	3315852	5%	67314699	0.39%	156807	107766	41%
Cape hare	LCP_SAF_1903	70630551	244436	7730802	11%	62899749	0.39%	185692	58744	24%
Cape hare	LCP_TAN_3023	70630551	242011	10985956	16%	59644595	0.41%	168564	73447	30%
Apennine hare	LCR_ITA_1957	70630551	56580	19385423	27%	51245128	0.11%	20975	35605	63%
Apennine hare	LCR_ITA_1958	70630551	47039	18725348	27%	51905203	0.09%	14751	32288	69%
Broom hare	LCS_1891	70630551	62780	16018035	23%	54612516	0.11%	19203	43577	69%
Broom hare	LCS_1894	70630551	60490	21784228	31%	48846323	0.12%	21354	39136	65%
European hare	LER_PYR_1546	70630551	163861	21889330	31%	48741221	0.34%	84881	78980	48%
European hare	LER_VIE_1639	70630551	220046	15166071	21%	55464480	0.40%	121172	98874	45%
Ethiopian hare	LFG_FAG114	70630551	80462	33296089	47%	37334462	0.22%	44879	35583	44%
Ethiopian hare	LFG_FAG96	70630551	142078	22715887	32%	47914664	0.30%	86863	55215	39%
Iberian hare	LGR_CRE_2553	70630551	127463	17173349	24%	53457202	0.24%	67095	60368	47%
Iberian hare	LGR_SEV_1163	70630551	151704	10819549	15%	59811002	0.25%	79371	72333	48%
Abyssinian hare	LHB_HAB35	70630551	147372	21457468	30%	49173083	0.30%	84669	62703	43%
Abyssinian hare	LHB_HAB68	70630551	155491	13024827	18%	57605724	0.27%	86537	68954	44%
Manchurian hare	LMS_PRI_2460	70630551	76687	22991702	33%	47638849	0.16%	37351	39336	51%
Manchurian hare	LMS_PRI_2461	70630551	78383	27328699	39%	43301852	0.18%	38384	39999	51%
Alaskan hare	LOT_2109	70630551	129938	9383535	13%	61247016	0.21%	42365	87573	67%
Alaskan hare	LOT_2110	70630551	117974	15415743	22%	55214808	0.21%	37266	80708	68%
Ethiopian highland hare	LST_STA65	70630551	102991	12646720	18%	57983831	0.18%	51459	51532	50%
Ethiopian highland hare	LST_STA89	70630551	77920	22376286	32%	48254265	0.16%	37947	39973	51%
Mountain hare	LTM_AFR_3108	70630551	154779	10838299	15%	59792252	0.26%	59482	95297	62%
Mountain hare	LTM_CAT_2012	70630551	108986	16219984	23%	54410567	0.20%	40600	68386	63%
Mountain hare	LTM_MAG_1862	70630551	129172	18944734	27%	51685817	0.25%	48103	81069	63%
White-tailed jackrabbit	LTW_JRRK_3	70630551	208801	9607582	14%	61022969	0.34%	105569	103232	49%
White-tailed jackrabbit	LTW_MTA_3280	70630551	211186	15960312	23%	54670239	0.39%	104337	106849	51%
Average							0.28%	Average		49%

Table S2.12 – Admixture proportion as 'f-branch' statistic ($f_b(C)$; from Malinsky et al 2018, Nat Eco Evo) values represented in Figure 2.3 for each branch pair b and C , with Z-score and P-value.

Branch b	Branch C	'f-branch'	Z-score	P-value
L	European hare	0.19	23.04	1.8E-117
Snowshoe hare	Black-tailed jackrabbit	0.19	27.74	2.0E-169
K	Black-tailed jackrabbit	0.19	24.44	5.9E-132
European hare	Mountain hare	0.18	32.63	1.7E-233
Ethiopian highland hare	Cape hare	0.17	15.97	1.9E-57
K	Manchurian hare	0.16	15.12	1.1E-51
H	Cape hare	0.15	19.14	1.3E-81
European hare	Alaskan hare	0.15	28.66	1.4E-180
White-tailed jackrabbit	Black-tailed jackrabbit	0.14	22.94	2.0E-116
E	European hare	0.14	31.85	1.4E-222
European hare	White-tailed jackrabbit	0.13	29.10	3.8E-186
L	Cape hare	0.12	28.34	1.2E-176
L	Manchurian hare	0.11	29.36	1.8E-189
Ethiopian hare	Abyssinian hare	0.09	20.60	2.6E-94
European hare	Manchurian hare	0.09	21.85	7.8E-106
Ethiopian hare	Ethiopian highland hare	0.08	25.72	6.9E-146
L	Broom hare	0.08	21.09	1.1E-98
L	Corsican hare	0.08	20.68	5.4E-95
White-tailed jackrabbit	Snowshoe hare	0.08	34.79	3.8E-265
European hare	Iberian hare	0.07	15.11	1.4E-51
Iberian hare	Cape hare	0.07	7.81	5.9E-15
Iberian hare	Abyssinian hare	0.07	8.79	1.5E-18
Iberian hare	Ethiopian hare	0.07	8.34	7.7E-17
K	European hare	0.07	3.67	2.4E-04
Ethiopian highland hare	Ethiopian hare	0.07	12.42	2.0E-35
L	Abyssinian hare	0.07	27.61	9.6E-168
L	Iberian hare	0.06	13.06	5.6E-39
European hare	Black-tailed jackrabbit	0.06	11.78	4.9E-32
H	Ethiopian hare	0.06	16.85	9.9E-64
L	Ethiopian hare	0.06	23.86	8.7E-126
K	Snowshoe hare	0.06	25.97	1.2E-148
Iberian hare	Ethiopian highland hare	0.05	7.84	4.5E-15
L	Ethiopian highland hare	0.05	27.28	7.6E-164
Broom hare	Cape hare	0.05	14.78	2.0E-49
Manchurian hare	Snowshoe hare	0.05	23.27	9.5E-120
Abyssinian hare	European hare	0.05	11.89	1.3E-32
C	White-sided jackrabbit	0.04	3.25	1.1E-03
E	Manchurian hare	0.04	18.43	7.5E-76
E	White-tailed jackrabbit	0.04	15.56	1.3E-54
H	Abyssinian hare	0.04	10.90	1.1E-27
Manchurian hare	Abyssinian hare	0.04	6.20	5.5E-10
E	Alaskan hare	0.04	15.19	3.9E-52
E	Mountain hare	0.03	15.39	2.0E-53
Manchurian hare	White-sided jackrabbit	0.03	3.18	1.5E-03
E	Black-tailed jackrabbit	0.03	8.87	7.4E-19
H	Ethiopian highland hare	0.03	11.31	1.1E-29
Alaskan hare	Iberian hare	0.03	14.56	5.4E-48
Manchurian hare	Ethiopian highland hare	0.03	5.45	5.0E-08
European hare	Corsican hare	0.03	5.85	4.8E-09
L	White-sided jackrabbit	0.03	4.01	6.2E-05
E	Iberian hare	0.03	11.62	3.3E-31
Alaskan hare	White-tailed jackrabbit	0.03	4.65	3.3E-06
Manchurian hare	Ethiopian hare	0.02	3.48	5.1E-04
Manchurian hare	Cape hare	0.02	2.27	2.3E-02
Broom hare	Black-tailed jackrabbit	0.02	11.92	9.1E-33
Abyssinian hare	Black-tailed jackrabbit	0.02	7.12	1.1E-12
Broom hare	Ethiopian hare	0.02	16.82	1.8E-63
Alaskan hare	Snowshoe hare	0.02	20.32	7.9E-92
H	White-sided jackrabbit	0.02	3.26	1.1E-03
European hare	Broom hare	0.02	4.04	5.3E-05
Manchurian hare	European hare	0.02	2.88	4.0E-03
Broom hare	Iberian hare	0.02	11.09	1.4E-28
Mountain hare	European hare	0.02	6.29	3.1E-10
Broom hare	European hare	0.02	10.03	1.1E-23
Broom hare	Abyssinian hare	0.02	12.05	2.0E-33
Abyssinian hare	White-tailed jackrabbit	0.01	6.92	4.6E-12
H	Snowshoe hare	0.01	6.18	6.6E-10

Broom hare	Alaskan hare	0.01	10.14	3.8E-24
Broom hare	White-tailed jackrabbit	0.01	8.85	9.0E-19
Cape hare	Black-tailed jackrabbit	0.01	4.39	1.1E-05
Abyssinian hare	Manchurian hare	0.01	6.86	6.8E-12
Broom hare	Ethiopian highland hare	0.01	12.54	4.2E-36
Broom hare	Manchurian hare	0.01	9.14	6.1E-20
Ethiopian hare	European hare	0.01	3.55	3.9E-04
Abyssinian hare	Iberian hare	0.01	5.62	2.0E-08
F	White-sided jackrabbit	0.01	2.72	6.6E-03
Iberian hare	White-sided jackrabbit	0.01	1.56	1.2E-01
Abyssinian hare	Alaskan hare	0.01	5.17	2.3E-07
Mountain hare	Cape hare	0.01	3.67	2.4E-04
Broom hare	Mountain hare	0.01	6.00	1.9E-09
E	Corsican hare	0.01	3.22	1.3E-03
E	Snowshoe hare	0.01	4.93	8.4E-07
Cape hare	White-tailed jackrabbit	0.01	3.72	2.0E-04
Abyssinian hare	Mountain hare	0.01	3.84	1.2E-04
E	Broom hare	0.01	2.80	5.1E-03
Mountain hare	Abyssinian hare	0.01	4.24	2.2E-05
Abyssinian hare	Corsican hare	0.00	2.57	1.0E-02
Cape hare	Corsican hare	0.00	2.47	1.4E-02
D	White-sided jackrabbit	0.00	0.98	3.3E-01
Ethiopian hare	White-sided jackrabbit	0.00	1.36	1.7E-01
Mountain hare	Black-tailed jackrabbit	0.00	1.71	8.7E-02
Mountain hare	Ethiopian highland hare	0.00	4.09	4.4E-05
Abyssinian hare	Broom hare	0.00	2.16	3.1E-02
Corsican hare	White-sided jackrabbit	0.00	2.03	4.2E-02
Cape hare	Snowshoe hare	0.00	2.85	4.4E-03
Abyssinian hare	Snowshoe hare	0.00	2.41	1.6E-02
Ethiopian hare	Manchurian hare	0.00	1.80	7.2E-02
Alaskan hare	Manchurian hare	0.00	1.65	9.9E-02
Broom hare	Snowshoe hare	0.00	3.15	1.6E-03
Mountain hare	Ethiopian hare	0.00	1.50	1.3E-01
Cape hare	Alaskan hare	0.00	0.98	3.3E-01
Ethiopian hare	Iberian hare	0.00	0.84	4.0E-01
Cape hare	Broom hare	0.00	0.85	3.9E-01
Cape hare	Mountain hare	0.00	0.71	4.8E-01
Ethiopian highland hare	White-sided jackrabbit	0.00	0.33	7.4E-01
Alaskan hare	Corsican hare	0.00	0.22	8.3E-01
Alaskan hare	Broom hare	0.00	0.17	8.7E-01
Alaskan hare	White-sided jackrabbit	0.00	0.01	9.9E-01
A	White-sided jackrabbit	0.00	0.00	1.0E+00
Abyssinian hare	Cape hare	0.00	0.00	1.0E+00
Abyssinian hare	Ethiopian hare	0.00	0.00	1.0E+00
Abyssinian hare	White-sided jackrabbit	0.00	0.00	1.0E+00
Alaskan hare	Abyssinian hare	0.00	0.00	1.0E+00
Alaskan hare	Black-tailed jackrabbit	0.00	0.00	1.0E+00
Alaskan hare	Cape hare	0.00	0.00	1.0E+00
Alaskan hare	Ethiopian hare	0.00	0.00	1.0E+00
Alaskan hare	Ethiopian highland hare	0.00	0.00	1.0E+00
Alaskan hare	European hare	0.00	0.00	1.0E+00
B	Black-tailed jackrabbit	0.00	0.00	1.0E+00
B	White-sided jackrabbit	0.00	0.00	1.0E+00
Black-tailed jackrabbit	White-sided jackrabbit	0.00	0.00	1.0E+00
Broom hare	White-sided jackrabbit	0.00	0.00	1.0E+00
C	Black-tailed jackrabbit	0.00	0.00	1.0E+00
C	Snowshoe hare	0.00	0.00	1.0E+00
Cape hare	Abyssinian hare	0.00	0.00	1.0E+00
Cape hare	Ethiopian highland hare	0.00	0.00	1.0E+00
Cape hare	European hare	0.00	0.00	1.0E+00
Cape hare	Iberian hare	0.00	0.00	1.0E+00
Cape hare	Manchurian hare	0.00	0.00	1.0E+00
Cape hare	White-sided jackrabbit	0.00	0.00	1.0E+00
Corsican hare	Abyssinian hare	0.00	0.00	1.0E+00
Corsican hare	Alaskan hare	0.00	0.00	1.0E+00
Corsican hare	Black-tailed jackrabbit	0.00	0.00	1.0E+00
Corsican hare	Cape hare	0.00	0.00	1.0E+00
Corsican hare	Ethiopian hare	0.00	0.00	1.0E+00
Corsican hare	Ethiopian highland hare	0.00	0.00	1.0E+00
Corsican hare	European hare	0.00	0.00	1.0E+00
Corsican hare	Iberian hare	0.00	0.00	1.0E+00

The evolution and molecular bases of seasonal coat color variation in hares:
gene expression and the shared contribution of ancestral polymorphism and introgression

Corsican hare	Manchurian hare	0.00	0.00	1.0E+00
Corsican hare	Mountain hare	0.00	0.00	1.0E+00
Corsican hare	Snowshoe hare	0.00	0.00	1.0E+00
Corsican hare	White-tailed jackrabbit	0.00	0.00	1.0E+00
D	Alaskan hare	0.00	0.00	1.0E+00
D	Black-tailed jackrabbit	0.00	0.00	1.0E+00
D	Broom hare	0.00	0.00	1.0E+00
D	Corsican hare	0.00	0.00	1.0E+00
D	Iberian hare	0.00	0.00	1.0E+00
D	Manchurian hare	0.00	0.00	1.0E+00
D	Mountain hare	0.00	0.00	1.0E+00
D	Snowshoe hare	0.00	0.00	1.0E+00
D	White-tailed jackrabbit	0.00	0.00	1.0E+00
E	White-sided jackrabbit	0.00	0.00	1.0E+00
Ethiopian hare	Alaskan hare	0.00	0.00	1.0E+00
Ethiopian hare	Black-tailed jackrabbit	0.00	0.00	1.0E+00
Ethiopian hare	Broom hare	0.00	0.00	1.0E+00
Ethiopian hare	Corsican hare	0.00	0.00	1.0E+00
Ethiopian hare	Mountain hare	0.00	0.00	1.0E+00
Ethiopian hare	Snowshoe hare	0.00	0.00	1.0E+00
Ethiopian hare	White-tailed jackrabbit	0.00	0.00	1.0E+00
Ethiopian highland hare	Alaskan hare	0.00	0.00	1.0E+00
Ethiopian highland hare	Black-tailed jackrabbit	0.00	0.00	1.0E+00
Ethiopian highland hare	Broom hare	0.00	0.00	1.0E+00
Ethiopian highland hare	Corsican hare	0.00	0.00	1.0E+00
Ethiopian highland hare	European hare	0.00	0.00	1.0E+00
Ethiopian highland hare	Iberian hare	0.00	0.00	1.0E+00
Ethiopian highland hare	Manchurian hare	0.00	0.00	1.0E+00
Ethiopian highland hare	Mountain hare	0.00	0.00	1.0E+00
Ethiopian highland hare	Snowshoe hare	0.00	0.00	1.0E+00
Ethiopian highland hare	White-tailed jackrabbit	0.00	0.00	1.0E+00
European hare	Snowshoe hare	0.00	0.00	1.0E+00
European hare	White-sided jackrabbit	0.00	0.00	1.0E+00
F	Alaskan hare	0.00	0.00	1.0E+00
F	Black-tailed jackrabbit	0.00	0.00	1.0E+00
F	Broom hare	0.00	0.00	1.0E+00
F	Corsican hare	0.00	0.00	1.0E+00
F	European hare	0.00	0.00	1.0E+00
F	Iberian hare	0.00	0.00	1.0E+00
F	Manchurian hare	0.00	0.00	1.0E+00
F	Mountain hare	0.00	0.00	1.0E+00
F	Snowshoe hare	0.00	0.00	1.0E+00
F	White-tailed jackrabbit	0.00	0.00	1.0E+00
G	Black-tailed jackrabbit	0.00	0.00	1.0E+00
G	Snowshoe hare	0.00	0.00	1.0E+00
G	White-sided jackrabbit	0.00	0.00	1.0E+00
H	Black-tailed jackrabbit	0.00	0.00	1.0E+00
H	European hare	0.00	0.00	1.0E+00
I	Abyssinian hare	0.00	0.00	1.0E+00
I	Black-tailed jackrabbit	0.00	0.00	1.0E+00
I	Cape hare	0.00	0.00	1.0E+00
I	Ethiopian hare	0.00	0.00	1.0E+00
I	Ethiopian highland hare	0.00	0.00	1.0E+00
I	European hare	0.00	0.00	1.0E+00
I	Snowshoe hare	0.00	0.00	1.0E+00
I	White-sided jackrabbit	0.00	0.00	1.0E+00
Iberian hare	Black-tailed jackrabbit	0.00	0.00	1.0E+00
Iberian hare	Broom hare	0.00	0.00	1.0E+00
Iberian hare	Corsican hare	0.00	0.00	1.0E+00
Iberian hare	European hare	0.00	0.00	1.0E+00
Iberian hare	Manchurian hare	0.00	0.00	1.0E+00
Iberian hare	Snowshoe hare	0.00	0.00	1.0E+00
J	Abyssinian hare	0.00	0.00	1.0E+00
J	Black-tailed jackrabbit	0.00	0.00	1.0E+00
J	Broom hare	0.00	0.00	1.0E+00
J	Cape hare	0.00	0.00	1.0E+00
J	Corsican hare	0.00	0.00	1.0E+00
J	Ethiopian hare	0.00	0.00	1.0E+00
J	Ethiopian highland hare	0.00	0.00	1.0E+00
J	European hare	0.00	0.00	1.0E+00

J	Snowshoe hare	0.00	0.00	1.0E+00
J	White-sided jackrabbit	0.00	0.00	1.0E+00
K	Abyssinian hare	0.00	0.00	1.0E+00
K	Broom hare	0.00	0.00	1.0E+00
K	Cape hare	0.00	0.00	1.0E+00
K	Corsican hare	0.00	0.00	1.0E+00
K	Ethiopian hare	0.00	0.00	1.0E+00
K	Ethiopian highland hare	0.00	0.00	1.0E+00
K	White-sided jackrabbit	0.00	0.00	1.0E+00
L	Black-tailed jackrabbit	0.00	0.00	1.0E+00
L	Snowshoe hare	0.00	0.00	1.0E+00
Manchurian hare	Black-tailed jackrabbit	0.00	0.00	1.0E+00
Manchurian hare	Broom hare	0.00	0.00	1.0E+00
Manchurian hare	Corsican hare	0.00	0.00	1.0E+00
Mountain hare	Broom hare	0.00	0.00	1.0E+00
Mountain hare	Corsican hare	0.00	0.00	1.0E+00
Mountain hare	Iberian hare	0.00	0.00	1.0E+00
Mountain hare	Manchurian hare	0.00	0.00	1.0E+00
Mountain hare	Snowshoe hare	0.00	0.00	1.0E+00
Mountain hare	White-sided jackrabbit	0.00	0.00	1.0E+00
Mountain hare	White-tailed jackrabbit	0.00	0.00	1.0E+00
Snowshoe hare	White-sided jackrabbit	0.00	0.00	1.0E+00
White-tailed jackrabbit	Abyssinian hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	Broom hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	Cape hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	Corsican hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	Ethiopian hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	Ethiopian highland hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	European hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	Iberian hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	Manchurian hare	0.00	0.00	1.0E+00
White-tailed jackrabbit	White-sided jackrabbit	0.00	0.00	1.0E+00

Table S2.13 – Windows of recent and ancient introgression between snowshoe hares (as P3) and mountain hares, Alaskan hares or white-tailed jackrabbits (as P2), detected with fraction of admixture (f_d) scans of introgression with the topology (((Iberian hare,P2),P3),Rabbit). Only significantly introgressed windows (f_d value in the top 0.5% of each distribution) in at least one species are shown. If a window is significant for all three tests it is considered to be a window of ancestral introgression. This table is available in Appendix and on Dryad Digital Repository (doi:10.5061/dryad.bzkh18967).

Table S2.14 – Annotation of the genes in the 119 f_d outlier windows that putatively introgressed from the mountain hare, Alaskan hare and white-tailed jackrabbit common ancestor into snowshoe hares. For each ENSEMBL Gene ID, we provide the chromosome where it is located, the start and end positions of the gene, the external gene name, and Gene Ontology annotation. This table is available in Appendix and on Dryad Digital Repository (doi:10.5061/dryad.bzkh18967).

Table S2.15 – Gene Ontology enrichment analysis obtained with *g:Profiler* for list of unique gene annotations in the 119 f_d outlier windows that putatively introgressed from the mountain hare, Alaskan hare and white-tailed jackrabbit common ancestor into snowshoe hares.

Source	GO Term	GO Term ID	Adjusted P-value	Annotated genes
GO:MF	E-box binding	GO:0070888	0.049967888	ENSOCUG000000017812, ENSOCUG000000007097, ENSOCUG000000002669

Table S2.16 - Admixture proportion as f_{hom} (Martin et al., 2015, MBE) between snowshoe hare individuals and one black-tailed jackrabbit, using the topology (((P1,P2),P3),Rabbit) reflecting variation shared recently among snowshoe hare populations and black-tailed jackrabbits. Snowshoe hare samples represent the Pacific Northwest population (LAM_A0604_WA and LAM_A0665_WA), the Boreal population (LAM_Allab3_B) and the Rocky Mountains population (LAM_NBerg67_Rockies).

P1	P2	P3	Admixture proportion
LAM_Allab3_Bo	LAM_A0604_WA	LCF_CAL_1954	2.74%
LAM_Allab3_Bo	LAM_A0965_WA	LCF_CAL_1954	3.02%
LAM_Allab3_Bo	LAM_NBerg67_Rockies	LCF_CAL_1954	2.84%
LAM_A0965_WA	LAM_A0604_WA	LCF_CAL_1954	-0.21%
LAM_NBerg67_Rockies	LAM_A0604_WA	LCF_CAL_1954	-0.24%
LAM_NBerg67_Rockies	LAM_A0965_WA	LCF_CAL_1954	0.12%

Chapter III

The Evolution of Seasonal Camouflage in White-Tailed Jackrabbits and its Adaptive Potential to Climate Change

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3.1. Abstract

Preserving genetic diversity is fundamental for conservation since standing adaptive variation is a critical component of evolutionary responses to rapid environmental changes. Here, we study the distribution and evolutionary history of winter coat color variation in the white-tailed jackrabbit (*Lepus townsendii*), a seasonal coat color changing species in decline, to understand the future adaptive potential of this species to changes in snow cover duration. With a predictive model, we show that the probability of white versus brown winter coat color is strongly correlated with snow cover. Using whole-genome data, we show that additive interactions among pigmentation genes *EDNRB* and *CORIN*, with possible epistatic contribution of *ASIP*, underlie continuous brown to white winter coat color variation. Comparative genomics across 10 *Lepus* species showed that the associated alleles diverged early in the radiation of the genus ~4-6 million years ago and were maintained within white-tailed jackrabbit populations through a combination of recent secondary introgression and long-term balancing selection. Using forecasted declines in snow cover, we predict that natural selection will favor a 3.2-fold increase in the range of winter brown phenotypes, which will occupy nearly half of the southern portion of the range by 2080. An adaptive response to rapid environmental change is thus critically dependent on the ancient standing genetic variation for winter brown coloration that has been pre-tested by selection for millions of years. However, the winter brown morph is most prevalent across severely declining white-tailed jackrabbit populations, which must be preserved to ensure an effective and rapid response to the predict change in selective pressures. The detailed genotype-to-phenotype-to-fitness map uncovered by our study provides new insights into the evolution of seasonal phenotypic plasticity which can be used to guide conservation efforts in the face of climate change.

Preservation of genetic diversity is a primary goal of conservation biology, reflecting the critical role that standing genetic variation is thought to play in promoting rapid adaptation to changing environments (Barrett and Schluter 2008; Bay et al. 2017). While there has been tremendous progress understanding the genetic basis of adaptive trait variation in some species, rarely has such information been used to guide the conservation of declining populations (Kardos and Shafer 2018). These translational shortcomings reflect the difficulties of identifying ecologically relevant traits in declining species and mapping their genetic basis, connecting such variation to fitness in nature, and then applying genotype-to-phenotype-to-fitness maps to guide conservation efforts that facilitate future adaptive responses (Barrett and Hoekstra 2011; Hoban et al. 2016; Kardos and Shafer 2018).

Flexible shifts in morphology, physiology, and behavior cued by circannual changes in photoperiod allow many species to buffer the challenges of strongly seasonal environments (Helm et al. 2013). Among these forms of phenotypic plasticity, seasonal molts to white winter pelage have evolved in five groups of mammals (at least 18 species) to maintain crypsis in snow covered environments (Mills et al. 2018; Zimova et al. 2018). Flexible winter camouflage has been shown to be closely tied to survival in snowshoe hares (Zimova et al. 2016; Wilson et al. 2019) and in several species appears to be vulnerable to environmental mismatch caused by global declines in snow cover duration (Mills et al. 2013; Atmeh et al. 2018). We examined how snow cover variation has shaped the long-term evolution and future adaptive potential of winter coat color in white-tailed jackrabbits (*Lepus townsendii*), a North American prairie species experiencing population declines across large portions of its range (Brown et al. 2020). Winter pelage color in white-tailed jackrabbits varies from brown to white (Hansen and Bear 1963) and tracks local snow cover duration (Mills et al. 2018), thus providing a compelling fitness-relevant trait for understanding evolutionary responses to climate change.

To determine how geographic variation in winter coat color is structured by local climate variables, we used a range-wide collection of 1,312 georeferenced specimens derived from museum archives (collection years 1800-2019) to estimate a high-resolution species distribution model from 19 bioclimatic variables (Figures 3.1A and S3.1). We then used climate covariates and a subset of 196 specimens where winter coat color could be categorized as mostly white or brown to build a predictive model of winter phenotype across the inferred species distribution (Figure 3.1A, Tables S3.1-S3.2). Consistent with previous work (Mills et al. 2018), the probability of winter white pelage increased positively with snow cover duration and snow seasonality (mean diurnal temperature range, BIO2) and negatively with snow transience (isothermality, BIO3) (Tables S3.1 and S3.2). Our probabilistic map predicted a mosaic of winter white or brown populations separated by extensive intervening regions of intermediate color probabilities (Figure 3.1). Previous research has described continuous

winter coat color variation in some jackrabbit populations, suggestive of a multigenic basis (Hansen and Bear 1963; Figure 3.1). Notably, the mosaic and continuous winter coat color variation across the range of white-tailed jackrabbits differs from other hare species, where color variation is more discrete (Jones et al. 2018; Giska et al. 2019) and winter white coats are predominant.

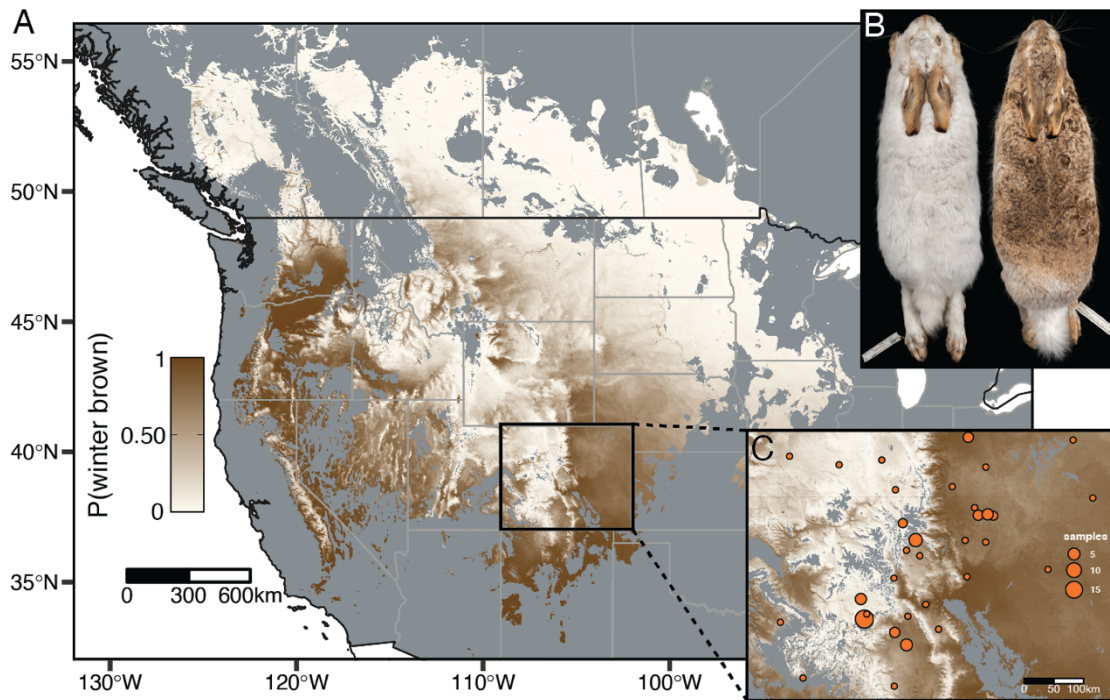


Figure 3.1 – Winter coat color polymorphism in white-tailed jackrabbits. (A) Predicted probability of winter brown coats using bioclimatic predictors of winter coloration (snow cover duration, mean diurnal temperature range and isothermality) across the suitable habitat of the white-tailed jackrabbit estimated using 1,312 occurrence records; (B) White-tailed jackrabbit specimens from the Denver Museum of Nature and Science representing the extreme ends of the spectrum of winter coat color variation in the species; (C) Sampling locations for 74 white-tailed jackrabbit specimens from a transition zone in winter coat color in Colorado, United States of America, used to study phenotype-to-genotype association in this work. The size of the circles represents sample size in a given location.

To dissect the genetic basis of winter coat color in white-tailed jackrabbits, we used linked-read technology to sequence (62.5× coverage; Table S3.3 and S3.4) and assemble a reference white-tailed jackrabbit genome (48.03 Mb scaffold N50; Figures S3.2 and S3.3; Table S3.4), which we annotated using gene models and chromosome linkage from the European rabbit reference genome (Carneiro et al. 2014). We also sampled DNA from 74 jackrabbit specimens collected along a broad transition in snow cover across the Rocky Mountains and Great Plains of Colorado, United States of America (USA) (Figures 3.1 and S3.4, Tables S3.3) and sequenced their genomes to ~1.8× individual coverage (0.6-4.9×; Table S3.5). Seven of these jackrabbits, representing a range of white (N=1), intermediate (N=3), and brown (N=3) winter coats, were also re-sequenced to higher individual coverage (~12.2×; Table S3.3 and S3.6). Analysis of 239,834 unlinked, single-nucleotide

polymorphisms (SNPs) revealed weak population genetic structure (Figures S3.4-3.6) characterized by low genetic differentiation between geographic regions (weighted fixation index, $F_{st} = 0.031-0.050$; Table S3.7). All sequenced individuals were initially categorized as having mostly white or brown winter coats in our range-wide phenotypic model (Figure 3.1, Table S3.5). However, spectrophotometric analysis of 61 individuals (across 6 dorsal regions; Figure S3.7) indicated more continuous coat colors (Figure 3.2A) caused primarily by variation in brightness, hue, and contrast of the dorsum of the body (51% of variation explained by PC1; Figures S3.7 and S3.8), but also by patterning (14.6% of variation explained by PC2; Figures S3.7 and S3.9) and color variation across body regions (7.5 % of variation explained by PC3; Figures S3.7 and S3.10).

Genome-wide association tests between 5,557,716 SNPs and PC1 of the spectrophotometric data revealed significant associations in two scaffolds each containing a single gene involved in melanogenesis (Figure 3.2). The association was robust to the inclusion of the top two principal components (PC1 and PC2) of population genetic variation as covariates (Figure S3.11). One association was on chromosome 2 (scaffold 342; $P = 2.73 \times 10^{-11}$; Figure S3.11A) and centered on *CORIN* (Figure 3.2B), a serine protease gene expressed in dermal papilla hair follicles that modifies melanogenesis in mice as a suppressor of the Agouti signaling protein (*ASIP*) (Enshell-Seijffers et al. 2007). Loss-of-function mutations in *CORIN* have been associated with an enlarged pheomelanin band and overall lighter pelage color in tigers (Xu et al. 2017) and mice (Avigad Laron et al. 2019). The other association (scaffold 311; $P = 7.17 \times 10^{-14}$; Figure S3.11A) was centered on the Endothelin receptor B (*EDNRB*, Figure 3.2D), a G-protein coupled receptor on chromosome 8 essential to the migration, proliferation, and differentiation of melanocyte precursors during embryonic development and post-natal hair cycling (Takeo et al. 2016; Li et al. 2017). *EDNRB* mutations cause white spotting or coats due to the absence of melanocytes (i.e., “piebald” phenotypes; Hosoda et al. 1994; Takeo et al. 2016). For a deeper exploration of our genomic data, we performed additional association tests treating coat color as a binary trait (white or brown) for each of the 74 sequenced jackrabbits (Table S3.5). This qualitative scan suggested two additional associations, including *ASIP* on chromosome 4 (scaffold 245; $P = 1.99 \times 10^{-9}$ for association; Figure S3.11B) and a non-coding region on chromosome 8 near a gene (alpha-2-macroglobulin, *A2M*; scaffold 380; $P = 2.15 \times 10^{-9}$; Figure S3.11B) not known to be involved in melanogenesis. The association on scaffold 380 coincides with the breakpoint of a structural re-arrangement relative to the European rabbit and a local PCA of the region separates the Great Plains population from other individuals, suggesting a spurious correlation with coat color (Figure S3.12). Given a potentially spurious association and unclear connection with pigmentation, we excluded this region from subsequent analysis. *ASIP* antagonizes the

melanocortin 1 receptor (*MC1R*) shifting melanogenesis to lighter phaeomelanin production or inhibiting the production of pigment (Le Pape et al. 2009). *Cis*-regulatory variation of the gene determines winter white versus brown coats in snowshoe hares (*L. americanus*; Jones et al. 2018) and mountain hares (*L. timidus*; Giska et al. 2019).

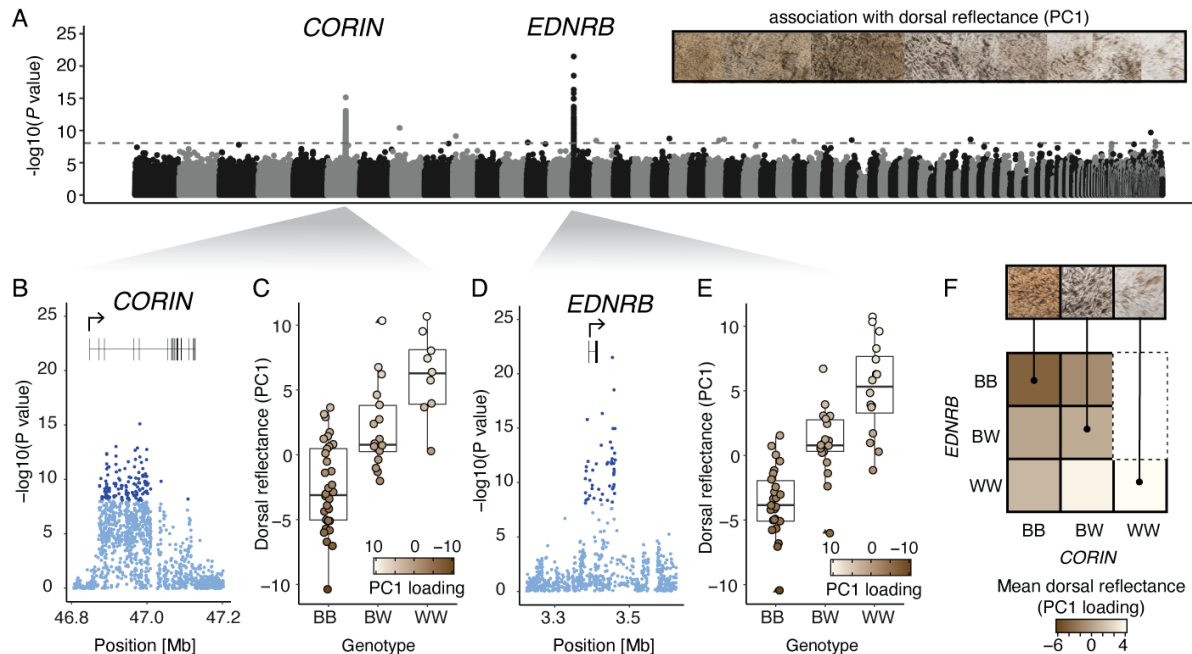


Figure 3.2 – The polygenic genetic basis of winter coat color polymorphism in white-tailed jackrabbits. (A) Genome-wide association ($-\log_{10}$ of P values, assuming an additive inheritance model; 5,557,716 SNPs) with continuous winter coat color (PC1 of a PCA analysis of dorsal reflectance measurements for 61 white-tailed jackrabbits). Points above the dashed line exceed the Bonferroni multiple test corrected threshold of $P=0.05$. (B and D) Gene structures of the association regions in chromosome 2 and 8 identified in panel A coincide with the location of *CORIN* (B) and *EDNRB* (D), respectively. SNPs with an association P value above the Bonferroni-corrected threshold of $P=0.05$ are highlighted in dark blue. (C and E) Boxplots showing the phenotype distribution (PC1 loading) across the three alternative genotypes (BB – homozygous brown allele; BW – heterozygous; WW – homozygous white allele) of the top MassARRAY® associated SNPs in the *CORIN* (C) and *EDNRB* (D) association regions. (F) Mean dorsal reflectance (mean PC1 loading) across the nine possible combinations between *CORIN* and *EDNRB* genotypes. White space represents missing combinations.

Next, we sought to validate low-coverage color associations and examine dominance and epistatic relationships. We used mass spectrometry to generate high confidence diploid genotypes for 59 of the sequenced jackrabbits with spectrophotometric data for 34 SNPs across the associated regions of *CORIN* ($n=13$), *EDNRB* ($n=9$), and *ASIP* ($n=12$) (Figure S3.13, Tables S3.3, S3.8, S3.9). *CORIN* and *EDNRB* remained strongly associated (PC1 linear regression of top associated SNPs; *CORIN*, $P = 6.82 \times 10^{-9}$; *EDNRB*, $P = 7.73 \times 10^{-12}$; Tables S3.10 and S3.11, Supplementary Text 1), showing largely additive (Figure 3.2C, E and S3.15; all $P > 0.05$, dominance deviation test; Tables S3.12 and S3.13, Supplementary Text 1) and independent effects on winter coat color (linear regression; $P > 0.05$ for all pairwise comparisons; Figures 3.2F, S3.15; Table S3.14, Supplementary Text 1). *ASIP* was only associated with color variation when tested independently from the other genes (Tables S3.10

and S3.11). However, all genotyped SNPs at *ASIP* showed epistatic interactions with at least one *CORIN* variant ($P \leq 0.05$, but non-significant after multiple test correction; Table S3.14, Supplementary Text 1), consistent with known functional interactions between these genes (Enshell-Seijffers et al. 2007). *CORIN* acts downstream and suppresses the effect of *ASIP* (Enshell-Seijffers et al. 2007), which likely contributes to the weaker association found at *ASIP* (Figure S3.15). Thus, continuous winter coat color in white-tailed jackrabbits appears primarily determined by additive genetic variation at *CORIN* and *EDNRB*, with a possible epistatic contribution of *ASIP*.

Genome-wide scans within white-tailed jackrabbits revealed local shifts in allele frequencies towards common variants (Tajima's D values above 99.7% of the Z -score distribution in low coverage population samples; Figure S3.16), combined with increased absolute genetic divergence between white and brown associated alleles of *CORIN*, *EDNRB*, and *ASIP* (Z -score ≥ 3 ; Figures 3.3A and S3.17). Elevated divergence between alternative alleles suggests that the variation underlying locally adaptive seasonal coat colors is old and did not arise from *de novo* mutations in the species. To further examine the evolutionary history of these genes, we combined new ($n=10$) and previously published ($n=18$) whole genome sequences ($\sim 7.5 - 33.5 \times$) from 29 individuals and 9 other *Lepus* species, including four other species showing seasonal coat color change and five non-color changing species (Table S3.3 and S3.6). Overall species relationships placed white-tailed jackrabbits as closely related to three other Eurasian (mountain hares) or North America [Arctic hares (*L. arcticus*) and Alaskan hares (*L. othus*)] species showing seasonal coat color change, consistent with previous phylogenomic analyses (Figures 3.3B, S3.18, S3.19 and Table S3.15; Ferreira et al. 2020). However, local genealogies of *CORIN*, *EDNRB*, and *ASIP* showed strikingly different topologies, with brown alleles from white-tailed jackrabbits consistently clustering with black-tailed jackrabbits and white alleles grouping with other winter white species (Figures 3.3B, S3.20 to S3.22).

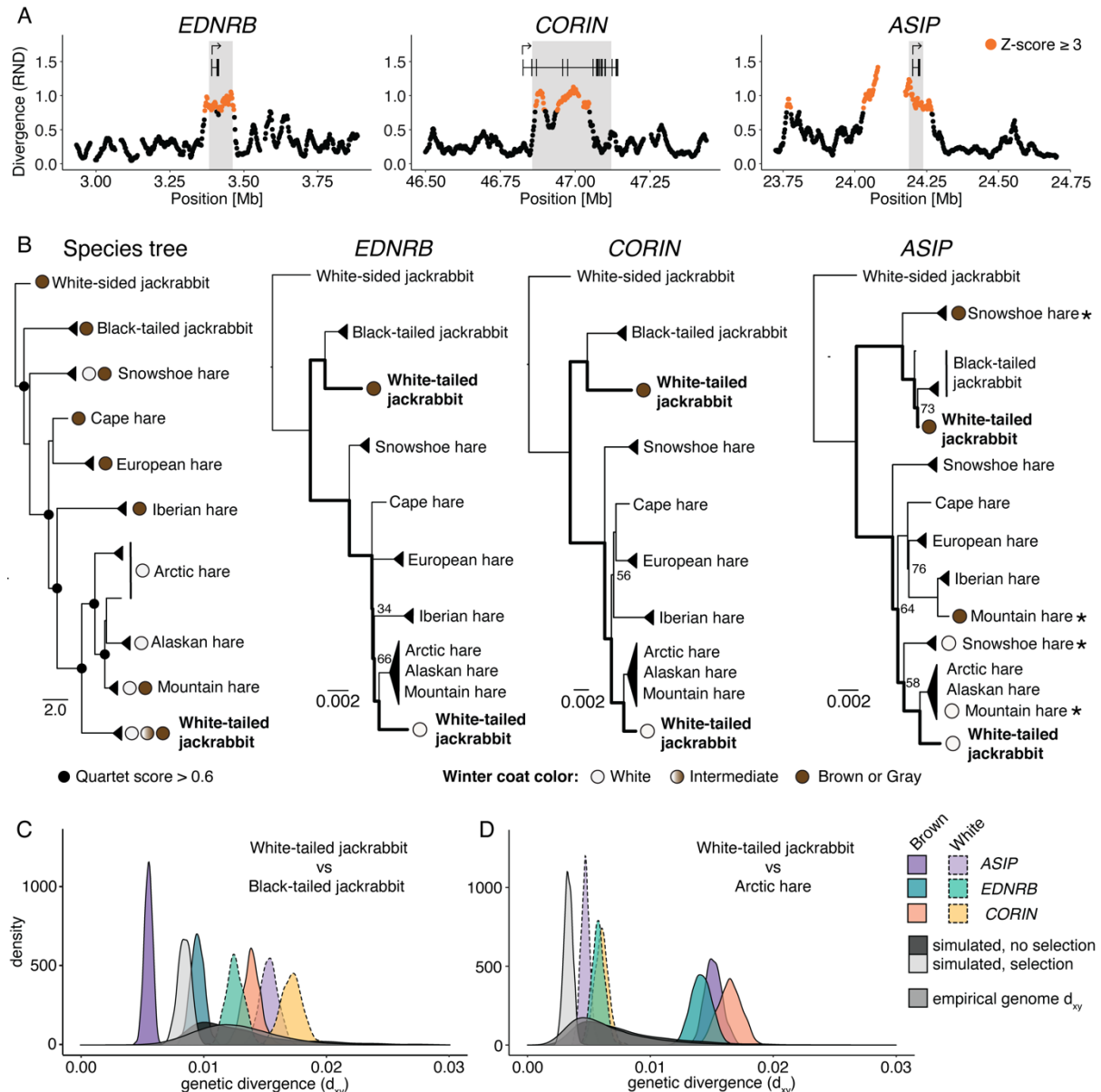


Figure 3.3 - Old alleles contribute to winter coat color polymorphism in white-tailed jackrabbits. (A) Scaled absolute divergence (RND) in 20-kb sliding windows (2-kb step size) between a winter white (DMNS_18801) and one winter brown (DMNS_18808) white-tailed jackrabbit homozygous for opposite alleles at each association region (gray rectangles; Figure S3.17). Orange windows have RND values three standard deviations from the mean ($Z\text{-score} \geq 3$). (B) A winter brown (DMNS_18808) white-tailed jackrabbit consistently clusters with black-tailed jackrabbits and is not monophyletic with a conspecific winter white white-tailed jackrabbit (DMNS_18801) in the local topologies of the three association regions of *EDNRB*, *CORIN* and *ASIP*. Asterisks in the *ASIP* tree highlight the mountain and snowshoe hare lineages where evolution of alternative winter coloration has been linked to *ASIP* (Jones et al. 2018; Giska et al. 2019). The local topologies contrast with the multispecies coalescent tree topology (left tree) obtained from 43,430 50 kb genome-wide windows. All represented branches in the species tree have posterior probability higher than 1. Branch labels in the gene trees represent bootstrap values below 80. (C-D) Empirical and simulated distributions of d_{xy} genome-wide or for the *EDNRB*, *CORIN* and *ASIP* association regions in this study between winter white or winter brown white-tailed jackrabbits and (C) black-tailed jackrabbits or (D) Arctic hares. The simulated distributions of d_{xy} were generated with coalescent simulations of the demographic history of the species pairs considered with and without strong selection in the ancestral population.

The gene-species tree discordance caused primarily by brown alleles at each gene tree could either reflect the long-term maintenance of an ancient color polymorphism in white-tailed jackrabbits, or more recent hybridization between species (Hedrick 2013). To disentangle these alternatives, we tested if absolute genetic divergences between winter brown white-tailed jackrabbits and black-tailed jackrabbits across each gene were significantly lower than expected. Consistent with a model of ancient polymorphism, levels of divergence across *CORIN* and *EDNRB* both overlapped with the 99.5% confidence interval of the empirical genome-wide distribution and simulated expectations under various models that do not incorporate gene flow (Figures 3.3C and S3.24, Table S3.16). We could not completely rule out a scenario of ancient hybridization at *EDNRB* given the observation of unexpectedly low divergence across a narrow (28 kb) interval of the association region. However, the significant high divergence between the alternative *EDNRB* color alleles (88 kb region) and phylogenetic discordance (79.8 kb association region) extended much beyond this narrow interval, suggesting that processes other than recent gene flow were at play in the origin of the deep polymorphism (Figure 3.3A-B and S3.24). Local genealogies for both genes show that the color-associated alleles split near the origin of the *Lepus* radiation (Figure S3.25A-B), with molecular clock-estimated times to a common ancestor at both genes exceeding three million years (myr) [*EDNRB* = 4.2 myr (95% HPD 3.3-5.0 myr); *CORIN* = 3.3 myr (95% HPD 2.9-4.3 myr)]. These results show that ancestral balanced polymorphisms, likely maintained by selection imposed by spatially varying environments, play a key role in the evolution of critical seasonal adaptations.

In contrast, the *ASIP* brown allele segregating in white-tailed jackrabbits appears to have been acquired through more recent gene flow from black-tailed jackrabbits. We observed significantly reduced divergence between the brown allele and black-tailed jackrabbits across the entire *ASIP* interval (~49 kb; Z-score ≤ -3 ; Figures 3.3C and S3.24, Table S3.16), even relative to a simulated model of strong selection reducing ancestral variation (and therefore d_{xy}) prior to the species split (Figures 3.3C and S3.24D). We also found a strong genome-wide signature of gene flow between these ecologically similar and geographically overlapping species, further supporting a model of introgressive hybridization at *ASIP* (D-statistic = 0.19, P value = 4.4×10^{-143} ; admixture proportion = 4%, P value = 1.20×10^{-80} ; Table S3.17-3.18; Ferreira et al. 2020). Introgression of genetic variation at *ASIP* has now been linked to the evolution of polymorphic seasonal camouflage in three hare species – snowshoe hares (Jones et al. 2018), mountain hares (Giska et al. 2019), and white-tailed-jackrabbits (Figure 3.3C). Intriguingly, hybridization with black-tailed jackrabbits appears to have contributed to the independent evolution of locally adaptive brown winter coats in populations of snowshoe hares and white-tailed jackrabbits. These results underscore the broader potential of hybridization

to contribute to local adaptation (Taylor and Larson 2019) and suggest that certain genes may be evolutionary hotspots for introgressive hybridization. However, adaptive introgression at *ASIP* appears to have played a secondary and comparatively minor phenotypic role to the maintenance of variation at *CORIN* and *EDNRB* in the evolution of locally adaptive winter camouflage in white-tailed jackrabbits.

We also tested if introgression has contributed to the evolution of white-associated variation across *Lepus* species. Genealogies across the *EDNRB* and *CORIN* association regions show that relatedness among winter white alleles generally follows species tree relationships, with levels of divergence between species falling within neutral expectations without gene flow (Figures 3.3, S3.26 and S3.27, Table S3.16). Contrastingly, the *ASIP* gene tree showed non-monophyletic species relationships, weakly grouping closely related color changing species (i.e., mountain hares, arctic hares, Alaskan hares, white-tailed jackrabbits) with two snowshoe hare lineages (Figure 3.3B). We also detected long regions of low divergence between all the winter white species at *ASIP* (~90 to 166 kb regions with Z-score ≤ -3 ; Figures S3.27 and S3.28), which could reflect ancient gene flow between winter white lineages as hypothesized by two previous studies (Jones et al. 2018; Ferreira et al. 2020b). However, our simulations show that the low estimates of d_{xy} could also be compatible with a strong selective sweep at *ASIP* in the shared ancestor of these species (Figures S3.27 and S3.28; Table S3.16).

Our work indicates the long-term evolutionary maintenance of a seasonal coat color polymorphism in white-tailed jackrabbits. Seasonal snow cover is predicted to decline dramatically across the northern hemisphere over the next century (Pederson et al. 2011), suggesting that ancestral genetic variation underlying seasonal camouflage may play a critical role in fostering rapid evolutionary responses to climate change (Matuszewski et al. 2015; Mills et al. 2018). To explore the magnitude to which changes in snow cover conditions may shift local selective pressures for camouflage in white-tailed jackrabbits, we used our predictive framework (Figure 3.1A) to project the probability of winter brown camouflage in 2080. Based on forecasted distributions for snow seasonality (BIO2), snow transience (BIO3), and snow residence time (a proxy of snow cover), we predict a massive shift towards winter brown coats across much of the southern range of the white-tailed jackrabbits (Figures 3.4, S3.29 to S3.32 and Tables S3.1-S3.2). Under this model, winter brown morphs are expected to occupy nearly half (~49%) of the southern (USA) range of white-tailed jackrabbits. This represents a 3.2-fold increase relatively to the current distribution (current proportion of winter brown [$P(\text{brown}) > 0.8$] area is ~15%; Figure 3.4A), and in no portion of the range did the model predict a decrease in winter brown probability ($\Delta P_{\text{brown}} \geq 0$; Figure 3.4A). These projections were for a relatively high CO₂ emissions scenario (RCP8.5) and comparable

environmental forecasts were not available for the northern portion of the range where winter white coats are common. Nonetheless, it appears that future rapid environmental shifts will strongly favor winter brown morphs across much of the range of white-tailed jackrabbits (Figure 3.4A).

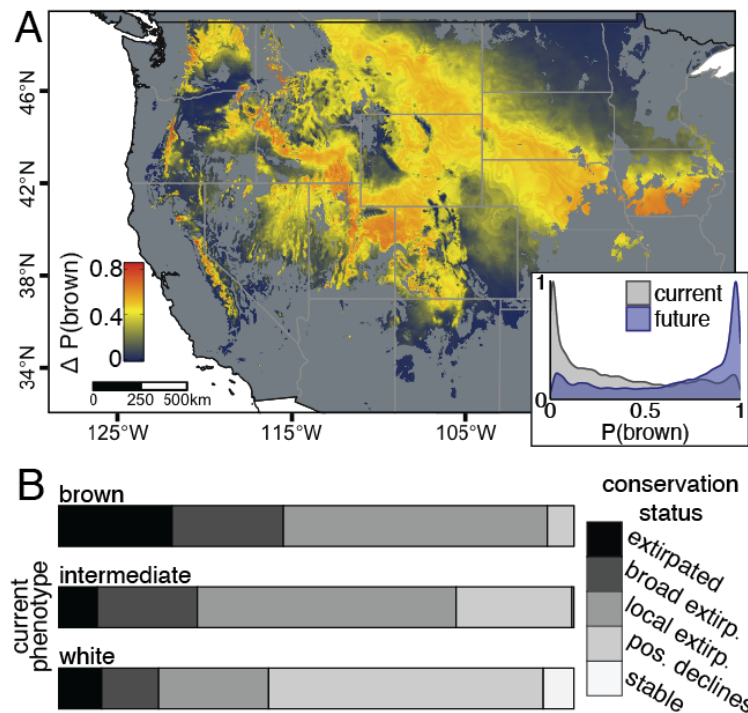


Figure 3.4 – Future snow cover conditions will increase the probability of currently endangered winter brown phenotypes. (A) Predicted increase in the probability of brown [$P(\text{brown})_{\text{future}} - P(\text{brown})_{\text{present}}$] in the 2080s, using forecasted predictions of snow residence time (proxy of snow cover; see Figure S3.30 for a comparison of the predictions for $P(\text{brown})$ obtained with the two metrics), mean diurnal temperature range and isothermality in a high-emissions scenario (RCP8.5). The density of probability of brown is predicted to shift in future conditions towards higher probabilities in the southern range (inset). (B) Proportion of the current distribution of “brown”, “intermediate” and “white” white-tailed jackrabbits overlapping with areas of the species distribution classified under five conservation categories (Brown et al. 2020). To bin the distribution in three phenotypic categories, we followed the Mills et al. (2018) broad definition of polymorphic zone (“intermediate”), with a polymorphic zone being where $0.2 \leq P(\text{brown}) \leq 0.8$ (Figure. S3.31).

The adaptive capacity of the white-tailed jackrabbit to future climate change is likely strongly dependent on standing variation for darker winter phenotypes (Bourne et al. 2014; Kopp and Matuszewski 2014). However, a recent review of the white-tailed jackrabbit conservation status across USA and Canada (Brown et al. 2020) suggests that current populations with a high probability of harboring winter brown variation are disproportionately experiencing local declines or extirpation (Pearson’s χ^2 test P value = 0.0; Cramer’s V = 0.32; Figure 3.4B and S3.31). Given the high fitness cost of camouflage mismatch compromising long-term species survival (Zimova et al. 2016), the potential loss of winter brown variation could jeopardize adaptive responses to climate change thereby threatening the persistence of white-tailed jackrabbits over much of its current range. We suggest that actions prioritizing

the persistence of and connectivity between populations that harbor critical winter brown variation will be crucial to the conservation of white-tailed jackrabbits in the face of ongoing environmental changes.

Safeguarding the adaptive potential of populations is a canonical principle of conservation biology. However, direct incorporation of evolutionary genetics into applied conservation efforts remains relatively rare, in part due to the difficulty of identifying traits under direct selection by rapid environmental change (Barrett and Hoekstra 2011; Hoban et al. 2016; Kardos and Shafer 2018). Our results illustrate how dissecting the genetic basis and evolutionary history of traits underlying environmental adaptation may be used to guide the conservation of threatened populations (Figure 3.4). While identifying conspicuous large-effect adaptive traits may not be feasible in many species, landscape-level analysis of genomic data can provide an effect proxy for discovering alleles that have been critical to fitness during past selective challenges (Wang and Bradburd 2014; Hoban et al. 2016). Preservation of such variation could offer a powerful tool to facilitate evolutionary rescue through adaption in populations threatened by anthropogenic climate change.

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3.5. Supporting Information

3.5.1. Material and Methods

Spatial Distribution Modeling

We compiled occurrence records of white-tailed jackrabbits (*Lepus townsendii*) for our species distribution models (SDMs) from three publicly available databases (VertNet, GBIF, and Arctos) and Natural History collections. In VertNet, we searched for all occurrence records with genus = "lepus" and specific epithet = "townsendii" (accessed April 2, 2020 at <http://portal.vertnet.org/search?q=specific epithet:townsendii+genus:lepus>). In GBIF, we searched for all occurrence records with the scientific name *Lepus townsendii* (accessed April 1, 2020 at <https://www.gbif.org>; DOI for this search is <https://doi.org/10.15468/dl.5rne5a>). In Arctos, we searched for all occurrence records of "lepus townsendii" (accessed April 2, 2020 at <https://arctosdb.org>). We combined this information with records we collected from specimens from the American Museum of Natural History (AMNH), the Denver Museum of Nature and Science (DMNS), University of Colorado Boulder Museum of Natural History (UCM), Utah Museum of Natural History (UMNH) and the Philip L. Wright Zoological Museum (UMZM), University of Montana. After extensive manual curation to remove duplicates and errors, we retained 1,312 occurrences records with unique GPS coordinates for further analysis. The full code and dataset used for this analysis will be made available at https://github.com/tjthurman/wtjr_sdm.

Many species distribution models assume that occurrence data are free of sampling bias, but this is rarely the case, particularly for museum specimens (Aiello-Lammens et al. 2015). When sampling effort is unknown, a possible method to reduce bias is to spatially thin the input occurrence data, retaining the maximum number of records that are a certain minimum distance apart. We used the *thin* function in the R package *spThin* to spatially thin the occurrence dataset at 1 km, 5 km, 10 km, and 50 km distances (Aiello-Lammens et al. 2015). Thus, together with the non-thinned data (i.e., a thinning distance of 0 km), we analyzed 5 separate datasets of occurrence data for our SDMs.

Additionally, we used the 19 bioclimatic variables from version 2 of the WorldClim database (Fick and Hijmans 2017) as the environmental predictors for our SDM. We downloaded the data for our study area at 30 arc-second resolution using the *getData* function in the *raster* R package (Hijmans 2020).

We used the popular Maxent method to fit our SDMs (Phillips et al. 2006). Maxent models have a variety of parameters, so we used the *ENMevaluate* function in the R package

ENMeval (Muscarella et al. 2014) to run multiple models and evaluate their performance to pick the best combination of parameters. For each dataset, we performed SDMs using 6 sets of feature classes and 8 regularization multipliers, for a total of 48 models per dataset. For all models, we used 10,000 randomly sampled points from our study area, generated with the *randomPoints* function in the R package *dismo* (Hijmans et al. 2017), as background points for model fitting and cross validation (Phillips and Dudík 2008). We used the “checkerboard2” setting in *ENMevaluate* to spatially stratify input data for cross validation. For each model, we calculated a variety of performance metrics: the area under the receiver-operator curve (AUC) for the training data, the average AUC for the test data, the corrected Akaike Information Criteria (AICc), and the omission rate (percent of false negatives) in the test data using the threshold for 10% omission in the training data (or10pct).

To choose a single model for further analysis, we first selected the best model for each dataset (each thinning distance), choosing the model with the lowest AICc. We then compared models across datasets, evaluating the average test AUC (larger is better), the average difference between the training AUC and the test AUC (smaller is better), and the omission rate in the test data (or10pct, closer to 0.10 is better). The overall best model came from the unthinned dataset and used all possible feature classes with a regularization multiplier of 1 (Figure S3.1). Finally, we converted the predictions from this best fitting SDM into a binary range map of predicted presence and absence. We used the *threshold* function in the *dismo* R package to apply a threshold to these predictions such that the sensitivity (true positive rate) of our final presence/absence model is 95%.

Phenotype Modeling under Current and Future Environmental Conditions

We predicted winter phenotype for white-tailed jackrabbits following the method of Mills et al. (2018). We classified 196 white-tailed jackrabbits as either winter white or winter brown, then fit a binomial GLM using three environmental predictors: the number of days of snow cover [as calculated by Mills et al. (2018)], and Bioclim variables 2 and 3 from version 2 of the WorldClim database (Fick and Hijmans 2017)]. From this fitted model, we predicted the probability of winter brown [P(brown)] across the white-tailed jackrabbit SDM-based range map.

Brown et al. (2020) surveyed museum records, primary literature, wildlife surveys, and agency reports to determine the conservation status of white-tailed jackrabbits at the state and provincial level in the United States of America and Canada. They distinguished five categories, in order of decreasing conservation concern: extirpated, broad extirpations, local extirpations, possible declines, and presumed stable. We sought to understand how white-

tailed jackrabbits' phenotype was distributed across these conservation statuses. We first converted the continuous prediction of winter phenotype, $P(\text{brown})$, into discrete phenotypic classes. We used the "broad" criteria of Mills et al. (2018) for defining polymorphic/mixed areas, such that phenotypes were classified as brown when $P(\text{brown}) > 0.8$, as white when $P(\text{brown}) < 0.2$, and as mixed/polymorphic when $0.2 \leq P(\text{brown}) \leq 0.8$. We then used the *mask* and *area* functions in the *raster* R package to calculate the area of overlap, in km^2 , between each of the three phenotypic categories and each of the 5 conservation categories. We used a χ^2 -contingency test to determine whether phenotypes were non-randomly distributed across conservation categories.

To predict the white-tailed jackrabbit winter phenotype in the future, we first compiled future projections of our environmental predictor variables. We were unable to find a future projection of snow cover equivalent to the snow cover metric from Mills et al. (2018) that we used for predicting current winter phenotypes. Instead, we used projections of a related variable, snow residence time (SRT), as calculated by the United States National Forest Climate Change Maps project (United States Forest Service 2019, available at <https://www.fs.fed.us/rm/boise/AWAE/projects/national-forest-climate-change-maps.html>).

They calculated historical SRT for 1975-2005, and also projected SRT for the 2080s under the RCP8.5 pathway from an average of five of the top ranked global models (NCAR-CCSM4, GFDL-CM3, GISS-E2-R, IPSL-CM5A-LR, MRI-CGCM3) from the CMIP5 climate modelling project. Their data covered only the continental United States and were available at a resolution of 2.5 arc minutes. We used the *disaggregate* function in the *raster* R package to upscale the historical and future SRT data to the same 30 arc second resolution as our other data.

We obtained future projections of Bioclim variables 2 and 3 from the GCM downscaled data portal of the CGIAR Research Program on Climate Change, Agriculture and Food Security (CAAFS, 2014 at http://ccafs-climate.org/data_spatial_downscaling/). We downloaded projections of each Bioclim variable at 30 arc-second resolution for the 2080s under the RCP8.5 pathway from the same 5 top ranked models, then averaged across the models to obtain our final projection for each variable. We re-fit our binomial GLM of current winter color phenotype, replacing the snow cover variable with the estimate of historical SRT. This required us to remove 1 phenotyped individual from Canada for which historical SRT was not estimated. We then used this second GLM based on SRT to predict both current winter color phenotype and future winter color phenotype in the 2080s. Finally, we estimated the increase in the probability of winter brown phenotype is $P(\text{brown})_{\text{future}} - P(\text{brown})_{\text{current}}$.

Phenotypic Analyses in Museum Specimens

Winter coat color variation in white-tailed jackrabbits varies between a pure white coat to completely brown similar to the summer coloration (Figure 3.1). This variation had been described by Hansen and Bear (1963) and contrasts with the winter coat color polymorphism in other seasonal coat color varying hares, which usually display discrete distribution of coat colors (i.e., brown, gray or white; Nunome et al. 2014; Jones et al. 2018; Giska et al. 2019). We characterized winter coat color variation in 61 white-tailed jackrabbits deposited at the Mammalogy collection of the Denver Museum of Nature and Science (Table S3.3). We used a USB4000 Miniature Fiber Optic Spectrophotometer (Ocean Optics) with a PX-2 pulsed xenon light source and the *OCEANVIEW-U* software (Ocean Insight, version 1.6.7). We measured reflectance on 45-degree angle directly on the specimen across 6 points of the body in triplicate: 3 dorsal points (1-head, 2-middle dorsum and 3-lower dorsum or rump), 3 points in the flank (1-upper flank or shoulder, 2-middle flank, 3-lower flank or rump). We processed the output of *OCEANVIEW-U* using the software *RCLR* (v0.9.28; <http://post.queensu.ca/~mont/color/analyze.html>) and extracted 8 color measurements reflecting brightness (B2 and B3), hue (H1, H3, H4c) and contrast (S3, S6 and S5c). We performed principal component analysis on the scaled and centralized measurements with function *prcomp()* from the *stats* R package and functions of the *factoextra* R package (<https://CRAN.R-project.org/package=factoextra>).

Genome Assembly and Annotation

To generate a reference for the white-tailed jackrabbit, we chose an individual (DMNS_18807) from Colorado that was heterozygous for variation at *EDNRB*, *CORIN* and *ASIP* association regions (Figure S3.2), based on initial analyses using pseudo-reference mappings and genome-wide association with discrete coat color differences (individuals classified as brown or white) of 66 white-tailed jackrabbits (see “*Association mapping*”). The *de novo* genome assembly was generated using Chromium linked-read data (10 X Genomics, San Francisco, USA). A single Chromium library generated from heart tissue was sequenced on an Illumina NovaSeq lane using 2 X 150 bp paired-end reads. The generated reads were used to assemble a reference genome with *Supernova* (v2.1.1) (Weisenfeld et al. 2017), using `--maxreads=1265935139`.

We assessed assembly quality using *Supernova* and *QUAST* (v5.0.2; Gurevich et al. 2013). We aligned the two pseudo-haploid references obtained from *Supernova* to the European rabbit ENSEMBL reference genome (OryCun2.0; Carneiro et al. 2014) using *D-GENIES* (accessed April 2020; Cabanettes and Klopp 2018) for an initial assessment of completeness and synteny. We also used *LIFTOFF* (Shumate and Salzberg 2020) to map the

white-tailed jackrabbit genome and OryCun2.0 and obtain a preliminary annotation from de European rabbit reference genome. We also used *MAUVE* (2015-02-25 build 0; Darling et al. 2004) to align scaffolds 245, 311, 342 and 380 against the European rabbit genome chromosomes 4, 8, 2 and 8, respectively, and inspect for the existence of structural variants.

Tissue Collection for Genomic DNA

We obtained tissue samples from 98 individual white-tailed jackrabbits from specimens in museum collections (N=79) that were originally collected in Colorado (N=65), Montana (N=8), North Dakota (N=3), Minnesota (N=1) and Oregon (N=2), or from specimens collected in Colorado in 2018 (N=19). Museum specimens were obtained from the AMNH, DMNS, UCM, UMNH, and UMZM collections (Table S3.3). Using appropriate sterile techniques (nitrile gloves, face mask and clean instruments), we used a fresh scalpel blade to extract a piece of tissue approximately 0.7-1.5 cm² from ventral skin or dried skull muscle of each specimen. We also took ventral and dorsal pictures from each specimen to record their winter phenotype. Tissues from field collected specimens (N=19) were obtained in January 2018 in Cochetopa Dome and adjacent areas in Colorado, United States of America (Table S3.3). Animals were humanely euthanized under an approved Colorado Parks and Wildlife permit and associated Animal Use Protocols approved through the University of Montana Institutional Animal Care and Use Committee (IACUC). From each specimen, we collected GPS coordinates, biometric information and photographs. We also collected tissue from the liver, heart, lung, kidney and spleen that was immediately frozen in liquid nitrogen until use. The collected specimens and tissues were deposited at the DMNS Mammal collection (Table S3.3).

We extracted DNA from historical tissues under a UV hood in a separate lab facility using DNeasy Blood & Tissue kit (QIAGEN) reagents and a dedicated protocol. Briefly, we rehydrated a small piece of skin (3 mm²) in a Lo-Bind Eppendorf tube with 1 x STE buffer (100mM NaCl, 10 mM Tris-HCl pH 7.5, 1mM EDTA) for 30 minutes and vortexed after 30 minutes for 3 to 5 minutes (Bi et al. 2013). In a sterile surface, we cut the skin in small pieces using a sterilized blade. For muscle tissue, we placed the same amount of tissue in a sterile surface, hydrated it with a small amount of STE buffer (~50 µL) and similarly cut it in small pieces using a blade and tweezers. We placed the tissue in a new tube with 180 µL of Buffer ATL (QIAGEN), 40 µL of proteinase K, 20 µL 1M DTT, and 10 µL 0.5M EDTA (Shapiro and Hofreiter 2012). We placed samples in a rotisserie at 50 °C for 24 hours, added another 20 µL of proteinase K and incubated for an additional 24 hours. Then, we added 200 µL of Buffer AL (QIAGEN) and 200 µL of 100% Ethanol, homogenizing the samples after each step. We transferred the samples to a QiaQuick column (QIAGEN) and centrifuged at 8000 rpm for 1

minute. We discard the flow-through and added 500 μ L of Buffer AW1 (QIAGEN). After centrifuging at 8000 rpm for 1 minute, we replaced the collection tube and added 500 μ L of Buffer AW2 (QIAGEN). After centrifuging at 14,000 rpm for 3 minutes, we replaced the collection tube with a new 1.5 mL labeled LoBind tube (QIAGEN). We added 100 μ L of preheated (70 °C) 1:10 Buffer AE (QIAGEN) to the center of the spin column's membrane and let sit for 5-15 minutes. We centrifuged at 8000 rpm for 1 minute and repeated the previous step, adding another 100 μ L of 1:10 Buffer AE (QIAGEN) to the membrane. After centrifuging again at 8000 rpm for 1 minute, we disposed of the spin columns and froze the extracted DNA at -20°C until further reactions.

We extracted DNA from modern specimens using the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN).

Whole-Genome Library Preparation

For population genetic and phenotype-genotype association analyses of white-tailed jackrabbits, we prepared whole-genome libraries following two different protocols for historical and modern samples both adapted from Meyer and Kircher (2010) protocols with modifications described in Kircher et al. (2012). For historical white-tailed jackrabbit samples from museum collections (Table S3.3 and S3.5), we prepared libraries in a dedicated room with positive hair pressure and UV irradiated before experimental procedures. Starting with 200 ng of DNA, we performed an initial step of blunt-end repair that included USER enzyme (New England BioLabs, Inc.) to remove uracil bases resulting from cytosine deamination (Briggs et al. 2010). We performed an adapter ligation step to add Illumina adapters to both ends of each DNA fragment, followed by an adapter fill-in step. We determined the number of cycles necessary for indexing PCR by performing a quantitative PCR (Maxima SYBR Green qPCR, ThermoFisher), and then performed an indexing PCR to add sample-specific dual indexes following Kircher et al. (2012). We modified the indexing PCR step relatively to Kircher et al. (2012) by using the AccuPrime™ Pfx DNA polymerase for its proofreading activity and by performing 4 PCR replicates per sample to increase library complexity. Sample quality and quantity was evaluated using a High Sensitivity TapeStation Screentape (Agilent Technologies) and KAPA Library Quantification Kit for Illumina libraries (KAPA Biosystems). The Select-a-Size DNA Clean & Concentrator Kit (Zymo Research) was used to remove adapter dimer and library fragments smaller than 100 bp prior to sequencing. We pooled all libraries in equimolar quantities and performed a test run in 1 lane of Illumina HiSeqX at Novogene (Novogene Corporation Ltd., Davis, California, USA). After evaluating the output of this initial sequencing run (number of reads per library, insert sizes and percentage of mapped

reads) we adjusted our pooling strategy and pooled libraries with similar qualities in four different equimolar pools. Three pools were sequenced across two lanes each and one pool was sequenced in one lane of an Illumina HiSeqX at Novogene (Novogene Corporation Ltd., Davis, California, USA), for a total of 7 lanes. The outputs of all lanes were used in subsequent analyses.

To prepare whole-genome libraries for 20 white-tailed jackrabbit samples collected between 2016 (one museum voucher) and 2018 (19 field specimens) we fragmented DNA using a Bioruptor® Pico (Diagenode). We purified fragmented DNA using a 1.6 X suspension of Sera-Mag beads (Rohland and Reich 2012) and performed size selection as in the Illumina's TruSeq DNA PCR-free Sample Preparation protocol using AMPure XP beads (Beckman Coulter Life Sciences). This resulted in DNA with about 350 base pairs in length. We then followed the Meyer & Kircher (2010) protocol to obtain dual-indexed libraries for each sample. Starting with 50 µL of fragmented DNA, we performed an initial blunt-end repair step, followed by adapter ligation and fill-in, where we inactivated the enzyme with a heatkill step following Kircher et al. (2012). Each library was indexed with a unique combination of two barcodes using an indexing PCR step. In all steps, libraries were purified using Sera-Mag bead suspensions. We evaluated the quality of each library by running them on a D1000 Tapestation Screentape (Agilent Technologies) and quantifying them using the KAPA Library Quantification Kit for Illumina libraries (KAPA Biosystems). We generated one equimolar pool that was sequenced in one lane of an Illumina HiSeq X at Novogene (Novogene Corporation Ltd., Davis, California, USA). This same protocol was used to generate high-coverage whole genome data for five white-tailed jackrabbit specimens that were pooled in two equimolar pools of two and three samples and sequenced across one lane of an Illumina HiSeqX each at Novogene.

The remaining high-coverage whole genome data for 21 individuals was prepared with the KAPA HyperPrep kit (KAPA Biosystems). DNA was fragmented using a Covaris E220evolution ultrasonicator and size-selected to 300-1000 base pairs using 0.6X and 0.85X ratio of Sera-Mag beads (Rohland and Reich 2012). During library preparation, we used quantitative PCR (DyNAmo Flash SYBR Green qPCR kit, ThermoFisher) to determine the number of PCR cycles for indexing. Final libraries were quantified with Qubit™ dsDNA BR Assay kit and pooled across nine pools containing one to three libraries, or with libraries for other publications (Bauer6). Select-a-Size DNA Clean & Concentrator Kit (Zymo Research) was used to remove any remaining adapter dimer. The nine pools were sequenced in one lane of HiSeq4000 each at Novogene (Novogene Corporation Ltd., Davis, California, USA). Part of this sequencing data was used for other publications (Table S3.3).

High-coverage whole-genome sequencing data for certain European, Iberian and mountain hare individuals was obtained from previously publications (Table S3.3; Seixas 2017; Seixas et al. 2018; Giska et al. 2019).

Pseudo-Reference Generation

We repeated most analyses either using read data mapped to species specific pseudo-references generated from iterative mapping to the European rabbit reference genome (OryCun2.0) or to the *de novo* assembled white-tailed jackrabbit reference genome. Mapping to rabbit-based pseudo-references allowed us to confirm the annotation of the associated regions for the population level analyses (Figure S3.2), use the European rabbit as the outgroup for species tree analyses (Figure 3.3B, S3.18 and S3.19), use the detailed annotation of the European rabbit to select neutral genomic regions for demographic analyses (Figure 3.3C-D, S3.24, S3.27, S3.28; Table S3.16) and study inter-specific divergence across the *ASIP* association region in comparison with results from Jones et al. (2018) (Figure S3.17, S3.24 and S3.28). Results are consistent across both references and except for the analyses indicated above we only report results based on the white-tailed jackrabbit reference genome. To generate pseudo-references based on OryCun2.0 or the white-tailed jackrabbit genome we used *pseudo-it* (Sarver et al. 2017) which performed iterative mapping of paired and single-read data of one individual per species (Table S3.3) for four iterations allowing ambiguity codes.

Processing, Mapping and Genotyping with Whole-Genome Data

We performed quality control on whole-genome low-coverage read data from 74 white-tailed jackrabbit individuals using *Trimmomatic* (version 0.38; Bolger et al. 2014) with options ILLUMINACLIP 3:20:7:2, TRAILING:15, SLIDINGWINDOW:5:15 and MINLEN:25. We merged overlapping reads using *pear* (version 0.9.11; Zhang et al. 2014) with option *-n 25*. We then used *bwa mem* (v.0.7.12-r1039; Li 2013) to align cleaned reads to the *de novo* assembled white-tailed jackrabbit genome or a white-tailed jackrabbit pseudo-reference in the European rabbit reference coordinates, attributing read groups with option *-R*. Then, we used *samtools* (version 1.9; Li et al. 2009) to sort or merge reads from different lanes. We removed duplicates with *Picard* (version 2.22.3; <http://broadinstitute.github.io/picard/>) and performed indel realignment with *GATK* (3.8-1-0-gf15c1c3ef; Van der Auwera et al. 2013). Finally, we determined alignment quality and depth using *qualimap* (v.2.2.2; Okonechnikov et al. 2016).

To process whole-genome high-coverage read data for 36 *Lepus* specimens for phylogenetic analyses, we used *Trimmomatic* (version 0.35; Bolger et al. 2014) with options ILLUMINACLIP:2:30:10:1:true, SLIDINGWINDOW:4:15, and MINLEN:50. We merged overlapping reads using *Flash2* (v2.2.00; Magoč and Salzberg 2011) with options -m10 -x 0.2 -r 150 -z. We then used *bwa mem* to align cleaned reads to 1) species specific pseudo-references with European rabbit genome coordinates or 2) the *de novo* assembled white-tailed jackrabbit genome for the white-tailed jackrabbit individuals and species-specific pseudo-references in the white-tailed jackrabbit genome coordinates for all remaining *Lepus* specimens. We added read groups, sorted, merged reads, removed duplicates, performed indel realignment and determined alignment quality and depth as described before for low-coverage libraries.

We also performed variant discovery and genotyping for whole-genome high-coverage data from 10 hare species (36 individuals). We used *bcftools* (version 1.9; Li 2011) mpileup, call and filter to call genotypes for each individual, excluding reads with mapping quality below 20 (-q 20), excluding bases with quality below 20 (-Q 20), using the multiallelic caller option (-m), excluding indels, and keeping sites with format depth higher or equal to 6 (FMT/DP ≥ 6), info depth smaller than three times the average coverage (INFO/DP) (Table S3.6) and genotype quality below 30 (FMT/GQ ≥ 30). We used *bcftools view* to produce a vcf file only containing genotypes that passed the filters.

Population Genetic Analyses

To perform population genetic analyses for the white-tailed jackrabbit Coloradan population, we used low-coverage data for 74 individuals mapped to the white-tailed jackrabbit reference genome as input for *ANGSD* (0.928; Korneliussen et al. 2014). Given that we used historical DNA, we first identified positions in the genome that could potentially have suffered from *post-mortem* deamination in the historical samples (Briggs et al. 2010). We used all the historical samples (N=54) to identify C to T or G to A substitutions relatively to the reference (which is a contemporaneous individual), calculating allele frequencies based on genotype likelihoods with *ANGSD* (Kim et al. 2011) fixing the reference allele as the major allele (-doMajorMinor 4, -doMaf 1 and -GL1), removing reads with multiple best hits (-uniqueOnly 1) and flags above 255 (-remove_bads), and only considering bases and mappings with minimum quality score of 20 (-minQ 20 and -minMapQ 20). Every position in which the reference allele was C or G, and the minor allele was T or A (respectively) with allele frequency of at least 0.02 (at least 2 alleles in 54 individuals) was removed from further analyses.

To better understand the population history of Coloradan white-tailed jackrabbits, we used the low-coverage dataset to perform principal component analysis (PCA) with *ANGSD*, using the single-read sampling approach (IBS). Genotype likelihoods were calculated using the *samtools* method (Li 2011), as implemented in *ANGSD* (-GL 1), and for all *ANGSD* analyses in this study we used the following filtering criteria unless otherwise noted: -uniqueOnly 1, -remove_bads 1, -minMapQ 20, -minQ 20, -SNP_pval 1e-6 and -minMaf 0.05. We also restricted analyses to sites present in at least 55 individuals with (-minInd and -setMinDepth set to 55) and with depth not higher than three times the total depth (-setMaxDepth 390). We used *PLINK* (v1.90b6.18; Purcell et al. 2007) to select a SNP every 10 kb (--make-bed and --bp-space) resulting in a dataset of 239,834 SNPs. To correct for population structure in the association mapping, we also performed a PCA for the subset of 61 individuals that were phenotyped with spectrophotometry techniques (Table S3.3). In this case, we adjusted the filtering parameters that rely on sample size (-minInd 45, -setMinDepth 45, -setMaxDepth 312), resulting in a dataset of 240,109 independent SNPs.

We used the same set of SNPs as input for *NGSadmix* (from *ANGSD* 0.928) to estimate individual admixture proportions from 1 to 10 ancestral populations (Skotte et al. 2013). We used *ANGSD* to produce input files in the Beagle format (-doGlf 2), and then ran *NGSadmix* specifying 1 to 10 ancestral populations (K), generating 50 replicate runs per K. We used the Evanno method (Evanno et al. 2005) as implemented in *CLUMPAK* (accessed July 2020; <http://clumpak.tau.ac.il/bestK.html>) to estimate the best number of ancestral populations (K).

We also estimated a neighbor-net network based on pairwise genetic distances between individuals using *ngsDist* (v.1.0.9; Vieira et al. 2015). We used *ANGSD* to estimate genetic distances from genotype posterior probabilities (-doGeno 8 and -doPost 1) based on 22,862,441 genome-wide polymorphic sites. We used *splitsTree4* (Huson and Bryant 2006) to construct a neighbor-net network from the resulting pairwise genetic distances.

Finally, we calculated weighted and unweighted F_{st} among the populations defined by *NGSadmix* assuming 3 ancestral populations (best K under the Evanno method; Figure S3.5). We used *ANGSD* to estimate per site allele frequencies (-dosaf 1, -gl 1) for the same set of 239,834 unlinked SNPs used for the PCA analysis. We also used the consensus genome sequence for the white-sided jackrabbit (*L. callotis*) as the ancestral state (see “Phylogenetic Analysis” for how the consensus genome was generated) given its ancestral position relatively to all other *Lepus* species [see Figure 3.3B, S3.18, S3.19 and Ferreira et al. (2020)]. We then estimated two-dimensional site allele frequency spectrums per scaffold among three population pairs using *realSFS* from *ANGSD* (Nielsen et al. 2012). For computational

efficiency, we restricted this analysis to the 50 longest scaffolds, that comprise 81% (2.28 Gb out of 2.80 total Gb) of the white-tailed reference genome.

Association Mapping

We first used low-coverage whole genome data from 66 white-tailed jackrabbits (Table S3.5) from Colorado mapped to a white-tailed jackrabbit pseudo-reference (in the European rabbit genome coordinates) to detect association of allele frequency differences to winter coat color classified discretely as “white” (N=36) or “brown” (N=30) in *ANGSD* (-yBin, -doAsso 1, -doCounts 1, -doMajorMinor 1, -doMaf 1) (Kim et al. 2011). Filtering criteria dependent on sample size were also adjusted (-minInd 50, -setMinDepth 50 and -setMaxDepth 300). As described before, we restricted analysis to non-C to T or G to A substitutions. Additionally, we restricted analysis to positions with information for at least 9 brown and 9 white individuals. The associated SNPs from this analysis were used to construct a multiplex for *MassARRAY*® genotyping (see “*Design and association analysis with MassARRAY*® genotypes”).

Then, we generated the white-tailed jackrabbit reference genome and increased our dataset of Coloradan individuals to 74 individuals, of which 61 were phenotyped using spectrophotometric techniques (Table S3.3 and S3.5). We used the first principal component of a PCA of reflectance measurements taken across the dorsal region of 61 museum specimens as a response variable (-yQuant) in a linear regression analysis for allele frequency association as implemented in *ANGSD* assuming an additive genotype [-doAsso 2 -model 1 -doPost 1 -GL 1 -doCounts 1 -doMajorMinor 1 -doMaf 1; Skotte et al. (2012)]. We used the previously described filtering criteria, adjusting filters that are based on sample size (-minInd 45 -setMinDepth 45 -setMaxDepth 312) and adding score test specific filters (-minCount 5 and -minHigh 5). We repeated the analysis correcting for population structure (option -cov) using the first two principal components of a PCA performed with polymorphism data of 240,109 independent SNPs for the 61 individuals. We determined that SNPs were associated with the phenotype if their association $-\log_{10}(P \text{ value})$ was above ~8.05, corresponding to a $P \leq 0.05$ after Bonferroni multiple test correction considering 5,557,716 tested SNPs.

To take advantage of the entire dataset of 74 white-tailed jackrabbit individuals from Colorado, we also performed a case-control association (-yBin) in *ANGSD* (-doAsso 1, -doCounts 1, -doMajorMinor 1, -doMaf where we classified individuals in two winter coat color categories (“white” or “brown”) based on photographs taken of all museum specimens (Table S3.5). We used the filtering criteria specified before except for -minMaf and adjusted filters that are based on sample size (-minInd 55, -setMinDepth 55 and -setMaxDepth 390). We repeated the analysis correcting for population structure using the first two principal

components of a PCA performed with polymorphism data of 239,834 independent SNPs for the 74 individuals as described above. For the analysis without correction for population structure, we determined that SNPs were associated with the phenotype if their association - $\log_{10}(P \text{ value})$ was above ~ 8.66 , corresponding to a $P \leq 0.05$ after Bonferroni multiple test correction considering 22,862,441 tested SNPs. When correcting for population structure, the multiple-test corrected $-\log_{10}(P \text{ value})$ threshold was ~ 8.23 , corresponding to the test of 8,509,882 genome-wide SNPs.

Design and Association Analysis with MassARRAY® Genotypes

To confirm the genotype-phenotype associations within the candidate regions containing *EDNRB* (chromosome 8 in the European rabbit genome or scaffold 311 in the white-tailed jackrabbit genome), *CORIN* (chromosome 2/ scaffold 342) and *ASIP* (chromosome 4/scaffold 245) we genotyped 34 SNPs in 95 white-tailed jackrabbits from the transition zone in winter coat color in Colorado (N=81 individuals) and from other states including Oregon (N=2), Montana (N=8), Minnesota (N=1) and North Dakota (N=3) (Table S3.3). Due to low amounts of extracted DNA for some museum-sampled individuals, we were not able to genotype all individuals for which we performed spectrophotometric analyses (genotyped 59 out of 61 individuals) nor for all individuals for which we generated low coverage whole-genome sequencing data (genotyped 71 out of 74 individuals; Table S3.3). All extra samples were collected from museum specimens and DNA extraction for all samples was performed as described in “*Tissue collection for genomic DNA*”. Genotyped SNPs were chosen before we generated the white-tailed jackrabbit reference genome and performed spectrophotometric measurements of the DMNS white-tailed jackrabbit collection. Therefore, we chose SNPs associated with winter coat color classified as “white” (N=36) or “brown” (N=30) in a dataset of 66 white-tailed jackrabbits using data mapped to a white-tailed jackrabbit pseudo-reference in the European rabbit genome reference coordinates (Table S3.3 and S3.5). To perform this GWAS analysis we did the same quality filters described before (see “*Population genetic analyses*” and “*Association mapping*”) and performed the GWAS as described in “*Association mapping*”. Among the 20,260,101 tested SNPs, we detected SNPs that were in association with $-\log_{10}(P \text{ value})$ higher than 8.61, corresponding to a $P \text{ value} \leq 0.05$ after Bonferroni multiple test correction. From this GWAS, 26 SNPs were compatible in a MassARRAY® plex. In order to complete the MassARRAY® plex, we performed an additional GWAS removing potential intermediate individuals (total N=48; “brown” N=24 and “white” N=24; Figure S3.2 and Table S3.5). Among 18,122,190 tested SNPs, we selected SNPs with an association $-\log_{10}(P \text{ value})$ higher than 8.56, corresponding

to a P value ≤ 0.05 after Bonferroni multiple test correction (Figure S3.2). Six SNPs were compatible with the 26 SNPs selected above and were included in the MassARRAY® plex. We also selected two potential non-synonymous amino-acid substitutions in *ASIP* (4:5439658) and *CORIN* (2:36370674) to be genotyped. The set of 34 SNPs was genotyped using the MassARRAY® technology at the Genome Transcriptome Facility in the University of Bordeaux, France.

After genotyping, we confirmed the association of the 34 SNPs to the quantitative phenotype (PC1 of the PCA of reflectance measurements taken across the dorsal region of 61 museum specimens; Figure S3.7) using linear regressions in R (function *lm()*) and in PLINK 1.9 (--option linear) under an additive model of inheritance (i.e., testing for the effect of adding an extra “white” allele on phenotype). To correct for population structure, we used the first two PCs of the PCA with 240,109 independent genome-wide SNPs from 61 individuals as covariates in the regression (option --covar in PLINK). To control for the effect of all genes on the phenotypic response and determine possible interactions among genes, we took the top associated SNPs in the *EDNRB* (8:79701598) and *CORIN* (2:36361155) association regions, and repeated the linear regression using these two SNPs as additional factors in the regression (option --condition-list) in PLINK. We also tested for a dominance deviation from additivity in PLINK using the genotypic model (option --linear genotypic) to understand if there was any statistic support for a deviation from additivity in any of the association regions. We did this either testing each SNP independently or using the highest associated region in each region as covariate, to rule out any potential interaction between SNPs. Finally, we tested for a significant interaction among all pairs of SNPs in PLINK (option --epistasis) to test for possible epistatic interactions considering interactions with a P value smaller than 0.05 as significant after multiple test correction (1,004 tests).

Phylogenetic Analyses

We used high-coverage whole-genome data from 10 hare species either mapped to European rabbit-based or white-tailed jackrabbit-based pseudo-references as input for various phylogenetic analyses. Regardless of reference, the method used to produce genome-wide consensus fasta sequences for each individual was the same. From filtered vcf files, we produced a consensus fasta file for each individual. We used *samtools faidx* and *bcftools consensus* to inject called genotypes into the reference fasta sequence used to map each individual’s read data. Then, we used a custom script to produce a bed file containing coordinates for all called positions and used *bedtools* (v2.27.1-65-gc2af1e7-dirty; Quinlan and Hall 2010) *complement* to produce a bed file with coordinates of non-called positions. We

used this bed file and *bedtools maskfasta* to hard-mask non-called positions in consensus fasta sequences.

To generate a maximum likelihood tree from genome-wide sequences in the coordinates of the European rabbit genome, we used *AMAS* (Borowiec 2016) *trim* to remove positions with missing data from whole-genome alignments. The resulting concatenated alignment was used to estimate a single bifurcating phylogeny using a maximum likelihood search and rapid bootstrapping run under the GTR+ Γ model of sequence evolution (autoMRE option) *RAXML* (8.2.12; Stamatakis 2018), using the European rabbit as outgroup.

To infer a multispecies-coalescent tree using whole-genome consensus fasta sequences in the coordinates of the European rabbit genome, we partitioned whole-genome consensus sequences in 50 kb non-overlapping windows using *msa-split* (option --windows 50000,0) from *phast* (1.4) (<http://compugen.cshl.edu/phast/>). We used *AMAS trim* to remove positions with missing data for more than 30% of the individuals. Finally, we used alignments longer than 10 kb to estimate maximum likelihood trees using *IQ-TREE* (2.0.3; Nguyen et al. 2015), using *ModelFinder* to determine the best substitution model (-m MFP; Kalyaanamoorthy et al. 2017), assessing branch support with 1,000 ultrafast bootstrap approximation replicates (-bb 1000; Thi Hoang et al. 2017) and SH-like approximate likelihood ratio test (-alrt 1000; Guindon et al. 2010). We used the resulting maximum-likelihood gene trees to generate a species tree with *ASTRAL-III* (5.7.3; Zhang et al. 2018).

To generate maximum-likelihood gene trees for all association regions, we extracted the alignments from consensus fasta sequences in the coordinates of the white-tailed jackrabbit genome using *samtools faidx*. We estimated phylogenies using a maximum likelihood search and rapid bootstrapping run under the GTR+ Γ model of sequence evolution (autoMRE option) *RAXML* (8.2.12; Stamatakis 2018), using the white-sided jackrabbit as outgroup.

To estimate divergence times among the two alleles in each association region, we took advantage of the phased diploid *de novo* white-tailed jackrabbit genome. We randomly phased the consensus sequence for scaffold 245 (localization of *ASIP*), 311 (localization of *EDNRB*) and 342 (localization of *CORIN*) for one individual per *Lepus* species or snowshoe hare population [snowshoe hares (DOlsen1, Lam_ine_2013, 8897, Canada_227), European hare (LER_VIE_1639), Iberian hare (LGR_CRE_2553), Arctic hare (UAM_83621), Alaskan hare (UAM_117654), mountain hare (LTM_MAG_1862), white-sided jackrabbit (LCL_1956), Cape hare (LCP_SAF_1905) and black-tailed jackrabbit (MacKay2)]. We aligned these sequences against the two haploid sequences of scaffold 245, 311 and 342 of the white-tailed jackrabbit genome and chromosomes 2, 4 and 8 of the European rabbit reference genome, respectively, with *MAUVE* (2015-02-25 build 0; Darling et al. 2004). We extracted an alignment

of each association region and used Bayesian Inference to estimate gene trees and divergence times in *BEAST2* (v.2.6.3; Bouckaert et al. 2019). We assumed a strict molecular clock and HKY substitution model using empirical base frequencies. We used a Calibrated Yule Model for the tree prior, used a gamma distribution as a prior for the birth rate and clock rate parameter, an exponential distribution for the gamma shape and a log normal distribution for the kappa parameter. We used a log-normal distribution as a prior for the *Oryctolagus-Lepus* node age with a median of 11.8 million years (Matthee et al. 2004). We ran the MCMC chain for 5 million steps, discarding the first 50,000 runs as burn-in and sampling every 100 steps. We evaluated convergence with *Tracer* (v1.7.1; Rambaut et al. 2018) and estimated trees using *TreeAnnotator* provided with *BEAST*.

Window-based Divergence and Diversity Analysis within White-tailed Jackrabbits

We used high-coverage whole genome sequence data from three winter brown (DMNS_16612, DMNS_19793, DMNS_18808), three intermediate (DMNS_18791, DMNS_18800, DMNS_18803) and one winter white (DMNS_18801) white-tailed jackrabbits (~12X coverage) from the Colorado transition-zone to study divergence across the association regions. We also included the white-sided jackrabbit as an outgroup (LCL_1956). We use filtered vcf files as input for *Genomics General* (download October 2019; https://github.com/simonhmartin/genomics_general), converting them to a geno file using the *parseVCFs.py* script. Then, we performed scans of genetic divergence among all possible pairs of individuals, including the white-sided jackrabbit, in 20 kb overlapping windows (2 kb step, 5,000 sites each) with *popgenWindows.py*. We calculated scaled genetic divergence (RND; Rosenzweig et al. 2016) among each individual, using the white-sided jackrabbit as outgroup, and defined outlier windows as the ones distancing three standard deviations from the mean of the d_{xy} distribution (i.e., Z-score ≥ 3).

We also used *PoPoolation* (v1.2.2; Kofler et al. 2011) to estimate Tajima's D and nucleotide diversity (π) for low-coverage WGS data for the 74 white-tailed jackrabbits from Colorado. We merged individual bam files with *samtools merge* and created a mpileup file using *samtools mpileup*, filtering reads with low mapping quality (-q 20) and positions with excess coverage (-d 390). We first masked positions in the proximity of indels (using *PoPoolation* scripts *identify-genomic-indel-regions.pl* with option --indel-window 5 and *filter-pileup-by-gtf.pl*) and uniformized the coverage to half the maximum number of individuals following authors' recommendations (*subsample-pileup.pl* with option --target-coverage 37 and --method withoutreplace). We then calculated corrected Tajima's D and π in 20 kb sliding windows with 2 kb step using the parameters --min-count 2 --min-coverage 4 --max-coverage

--min-qual 20 and --min-covered-fraction 0.25. For Tajima's D and π distributions, outlier windows were defined as the ones distancing three standard deviations from the mean (i.e., $Z\text{-score} \geq 3$).

Window-based Divergence among Hare Species

We performed window-based divergence analyses among hare species using high-coverage whole genome data following the same pipeline described above for divergence within white-tailed jackrabbits (see "*Window-based divergence and diversity analysis within white-tailed jackrabbits*"). We calculated divergence among specific pairs using one individual per species or population (see figure legends) and between all species and the white-sided jackrabbit individual in 20 kb overlapping windows (2 kb step, 5,000 sites each) with *popgenWindows.py*. We calculated scaled genetic divergence (RND) among each species, using the white-sided jackrabbit as outgroup, and defined outlier windows as the ones distancing three standard deviations from the mean of the distribution (i.e., $Z\text{-score} \leq -3$).

Topology Weighting

To study the discordance of phylogenetic signals within the association regions and better understand the evolutionary history of each variant associated with winter coloration in white-tailed jackrabbits, we performed topology weighting analysis within scaffolds 245, 311 and 342 using *Twisst* (Martin and Van Belleghem 2017). We used high coverage whole genome data for one black-tailed jackrabbit (Mackay2), one winter brown (DMNS_18808) and one winter white (DMNS_18801) white-tailed jackrabbit, and the white-sided jackrabbit (LCL_1956) as outgroup. We used biallelic sites to estimate neighbor-joining trees in windows of 50 SNPs using *phyml_sliding_windows.py* from *genomics_generals*. We then used *Twisst* (<https://github.com/simonhmartin/twisst>) to performed topology weighting using the generated trees for each scaffold.

Admixture Summary Statistics

We used *genomics general* to calculate derived allele frequencies for all white-tailed jackrabbits, black-tailed jackrabbits, snowshoe hare populations, Arctic hares, Alaskan hares and mountain hares using *freq.py*, grouping all individuals of a species using a population file (--popFile) and using the white-sided jackrabbit as outgroup. With a custom *R* script (*D-stat.R* available at <https://github.com/evochange>), we calculated the D -statistic (or ABBA-BABA;

Green et al. 2010). To calculate Z-scores, we performed block jackknifing using *jackknife.R* (from *genomics general*), establishing blocks of 1 Mb and removing blocks with missing data. D-values with Bonferroni-corrected $P \leq 0.05$ were considered significantly different from zero. We used the same method and a custom *R* script (*f_G.R* available at <https://github.com/evochange>) to calculate admixture proportion (f_G) among white-tailed and black-tailed jackrabbits.

Demography and Coalescent Simulations of Genetic Divergence

To test if introgression among white-tailed jackrabbits and black-tailed jackrabbits or among seasonal coat color changing species (snowshoe, mountain, Alaskan and Arctic hares) could have contributed for the evolution of winter white or winter brown alleles, we compared empirical d_{xy} distributions across putative introgressed regions with expected divergence under simulated expectations with and without gene flow. We used *G-PhoCs* (Gronau et al. 2011) to obtain demographic parameters for the coalescent simulations: ancestral and current effective population sizes, population divergence times and migration rates. Since this analysis relies of neutral non-repetitive sequencing data, we opted to use consensus sequences in the coordinates of the European rabbit genome where intergenic non-repetitive genomic regions have been annotated. For the various comparisons, we used consensus whole-genome fasta sequences for two white-tailed jackrabbits (DMNS_18801 and DMNS_18808), two black-tailed jackrabbit individuals (Burke4, MacKay2), two Arctic hares (UAM_66285 and UAM_83621), two mountain hares (LTM_MAG_1862 and LTM_CAT_2012), two snowshoe hares from de Boreal population (Canada2 and Allab3) and two snowshoe hares from the Pacific Northwest population (8897 and LAM_INE_2013). We modelled the snowshoe hare populations separately given the deep split between them and possible difference in genetic basis of winter coat color polymorphism (Melo-Ferreira et al. 2014; Jones et al. 2020a, 2020b). We extracted sequence fragments of 1 kb from intergenic regions, distancing at least 50 kb from each other and at least 20 kb from the nearest gene using gene coordinates from ENSEMBL (accessed 18 April 2020). We masked repeats identified in the European rabbit reference genome with *RepeatMasker* and downloaded as a bed file from UCSC Genome Browser (<https://genome-euro.ucsc.edu>). After masking, alignments with more than 40% missing data were removed. This resulted in pairwise datasets with various numbers of fragments. For divergences between with white-tailed jackrabbits and black-tailed jackrabbits, Arctic hares, Boreal snowshoe hares or Pacific Northwest snowshoe hares, we generated datasets with 6712, 3735, 5297 or 7190 fragments, respectively. For divergence between Pacific Northwest snowshoe hares and Arctic hares or mountain hares,

we generated datasets with 4,195 or 8,505 fragments, respectively. In *G-PhoCs*, we modeled parameters inferring bidirectional migration. We run each model three times and checked for convergence with *Tracer* (v1.7.1; Rambaut et al. 2018). We adjusted the number of interactions and scaling factors for each model by inspecting effective sample sizes (ESS) in *Tracer*. To model the divergence between white-tailed jackrabbits and Arctic hares, we ran *G-PhoCs* for three million iterations, sampling every 5, and used a scaling factor of 1000 for theta and 10 for the migration rate. For every other model we ran *G-PhoCs* for one million iterations, sampling every 10, used a scaling factor of 10,000 for theta and 0.1 for the migration rate. We converted the scaled demographic parameters obtained from *G-PhoCs* assuming $\theta=4N_e\mu$, $\tau=T\mu/g$ and $M=m/\mu$, where N_e is the effective population size (in numbers of individuals), g is the average generation time in years, T is the absolute population divergence time in years, and m is the probability of migration in a single generation. We assumed a mutation rate of 2.8×10^{-9} (Seixas et al. 2018) and a generation time of 2 years.

We then used the estimated parameters from each model (Table S3.16) in *msms* (Ewing et al. 2010) to simulate 1,000 20 kb fragments under four demographic models: 1) the full demographic model, to determine the reliability of the simulations to recover the genome-wide empirical divergence distribution; 2) the full demographic model without migration, which represents divergence with incomplete lineage sorting only; 3) the full demographic model with a strong selective sweep in the ancestral population, to understand if low divergence could be explained by fixation of an allele prior to species divergence (Cruickshank and Hahn 2014); 4) the full demographic model with a strong selective sweep in the ancestral population with migration. For model 3 and 4, we introduced a beneficial allele 25,000 generations before the split of the two lineages and with selection coefficient of $s=0.1$. For simulations with white-tailed jackrabbits, the introduced allele had an additive effect. For simulations of the divergence between snowshoe hares and Arctic or mountain hares, the introduced allele was dominant, as the winter white allele in these species is dominant (Jones et al. 2018; Giska et al. 2019). The initial frequency of this allele was $\frac{1}{2} N_e$ and we retained simulations where the beneficial allele was not lost (flag -SFC). We then calculated genetic divergence (d_{xy}) among the simulated sequences using a custom script, and we compared these distributions with empirical distribution of d_{xy} for each of the candidate association regions or the longest continuous region of significant low RND among species (see Figures S3.24, S3.27 and S3.28 for the coordinates of these regions). To generate an empirical distribution, we generated 1,000 alignments by sampling 20,000 sites with replacement from each region and calculated d_{xy} for each replicate (Giska et al. 2019). To infer significant introgression, we determined if the 99.5% quantile of the empirical d_{xy} distribution was smaller than the 0.5% quantile of the simulated d_{xy} distribution without migration and with selection.

3.5.2. Supplementary Text

Supplementary Text 1 - Phenotype-Genotype Associations with MassARRAY® Genotypes

We used the top associated SNPs in the association tests of 34 genotyped SNPs with the major principal component axis explaining variation in dorsal reflectance measurements of 61 white-tailed jackrabbit individuals (2:36361155, 4:5424640 and 8:79701598; Table S3.10) to understand the interaction between alleles at each candidate region. We first analyzed how genotypes correlated with a discrete classification of the phenotype (individuals classified as “white” or “brown”). Classifying each individual in two discrete binary white/brown categories suggests that the white allele of each gene is dominant over the brown allele, as most heterozygotes are classified as winter “white” (using all genotyped individuals from Colorado, N=81; Figure S3.14A). However, this association between genotype and phenotype is not perfect in particular for *CORIN* and *ASIP* genotypes, since several homozygote “brown” individuals are classified as winter “white”. A deviation from perfect association may be related with the predominant effect of *EDNRB* and the interaction among the genes. For instance, when the genotypes of all genes are considered in combination, most winter “brown” individuals are triple homozygote “brown” (Figure S3.14B), suggesting that underlying *EDNRB* genotype influences the perceived phenotype of *CORIN* and *ASIP* in Figure S3.14A. The nearly perfect association of winter “brown” phenotypes with the triple heterozygote genotype, including *ASIP* genotypes, likely explains why *ASIP* is strongly associated in the case-control GWAS (Figure S3.2 and S3.11).

A discrete classification of white-tailed jackrabbit individuals in two color categories is, however, a simplification of the continuous winter coat color variation in the species (Figure S3.7B). When we use the first axis of a PCA of dorsal reflectance measurements from 61 individuals as proxy for winter coat color variation, the phenotype of heterozygote individuals at each locus tends to be intermediate between the two homozygote categories, rather than resembling one of them (Figure 3.2C and E, Figure S3.15B). This is more in line with an additive interaction among the alleles of each locus instead of a clear dominant/recessive inheritance pattern. Indeed, the deviation from additivity is not significant (Table S3.12), even when accounting for the effect of all genes in the linear regression (Table S3.13).

Furthermore, individuals with genotypes with higher number of *EDNRB* and *CORIN* white alleles show increased PC1 loadings (Figure 3.2F, Figure S3.15A), suggesting that the action of the two genes of larger effect is independent and additive. Given these results and the known functions of both genes (see main text), we hypothesize that the *EDNRB* genotype

influences melanocyte development in each molt, with the winter white allele potentially being associated with low numbers of differentiated melanocytes during anagen (Takeo et al. 2016; Li et al. 2017). Then, the *CORIN* genotype would contribute to determine the color produced by each fully developed melanocyte through its interaction with *ASIP*, with the white *CORIN* allele determining the production of lighter pigments (Enshell-Seijffers et al. 2007; Xu et al. 2017; Avigad Laron et al. 2019).

The effect of *ASIP* is less strong and disappears when considering the effect of the other genes (Tables S3.10-S3.11). As in the other genes, a higher number of *ASIP* white alleles results in lighter coloration (Figure S3.15B-E), though this effect appears to be dependent on *CORIN* genotype. For instance, for each *ASIP* genotype, the number of *CORIN* white allele contributes towards higher PC1 loadings (Figure S3.15C-D), in line with the increase in the pulse of *ASIP* expression controlled by *CORIN* genotypes in tigers (Xu et al. 2017) and mice (Avigad Laron et al. 2019). Therefore, it is possible that *ASIP*'s association is closely tied to its interaction with *CORIN* (Enshell-Seijffers et al. 2007; Avigad Laron et al. 2019). In fact, we detect an epistatic interaction between all *ASIP*s SNPs with *CORIN* SNPs, though non-significant after multiple test correction (Table S3.14).

3.5.3. Supplementary Figures

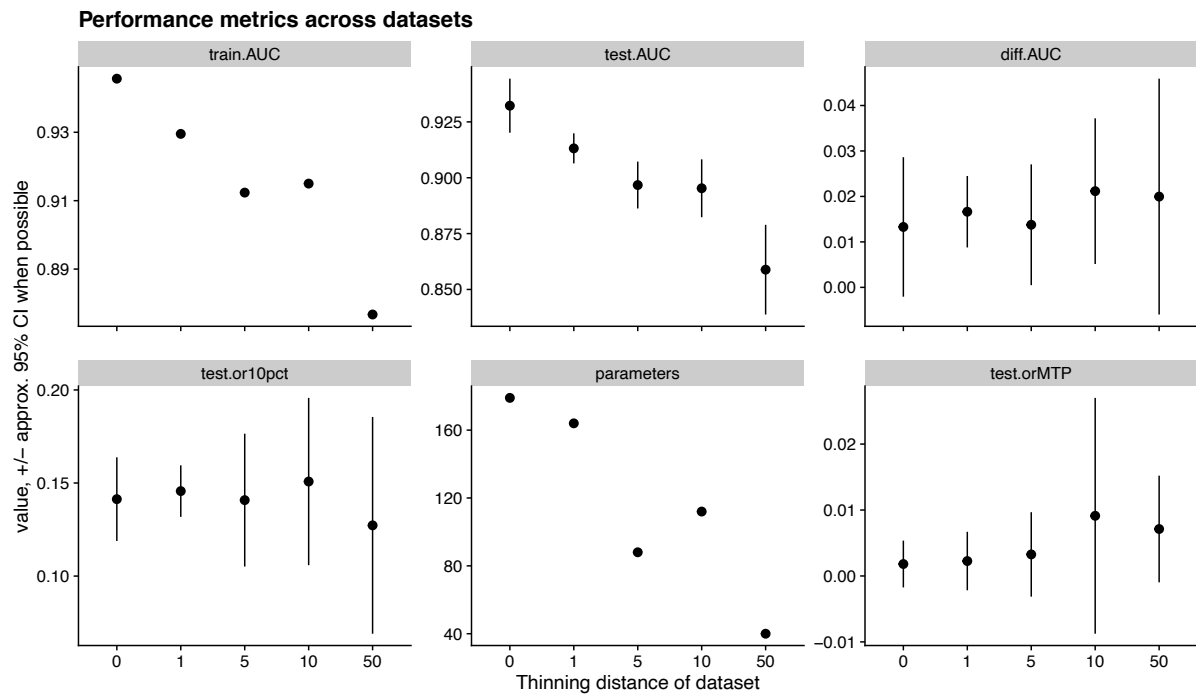


Figure S3.1 – Comparison of performance of the best species distribution model for five different datasets resulting from applying five thinning options (no thinning, 1 km, 5 km, 10 km or 50 km) to 1,312 white-tailed jackrabbit occurrences. For each thinned dataset, we fitted 48 different maxent models with various combinations of 6 feature classes and 8 regularization multipliers and chose the best model (with the lowest corrected Akaike Information Criteria) to perform a comparison across thinning options (in the panels). Input data (true occurrences and 10,000 random points) were spatially split into 4 groups for cross-validation. Each model was trained on one group (train.AUC), and then tested on each of the other three groups (test.AUC, averaged across 3 test sets). Large differences between training and test AUC (diff.AUC) may be indicative of overfitting. We calculated the omission rate (percent of false negatives) in the test data using a threshold for 0% (MTP, minimum training presence) or 10% omission in the training data (test.orMTP and test.or10pct, respectively), which should be close to 0.10. We also display the number of parameters in each model (parameters).

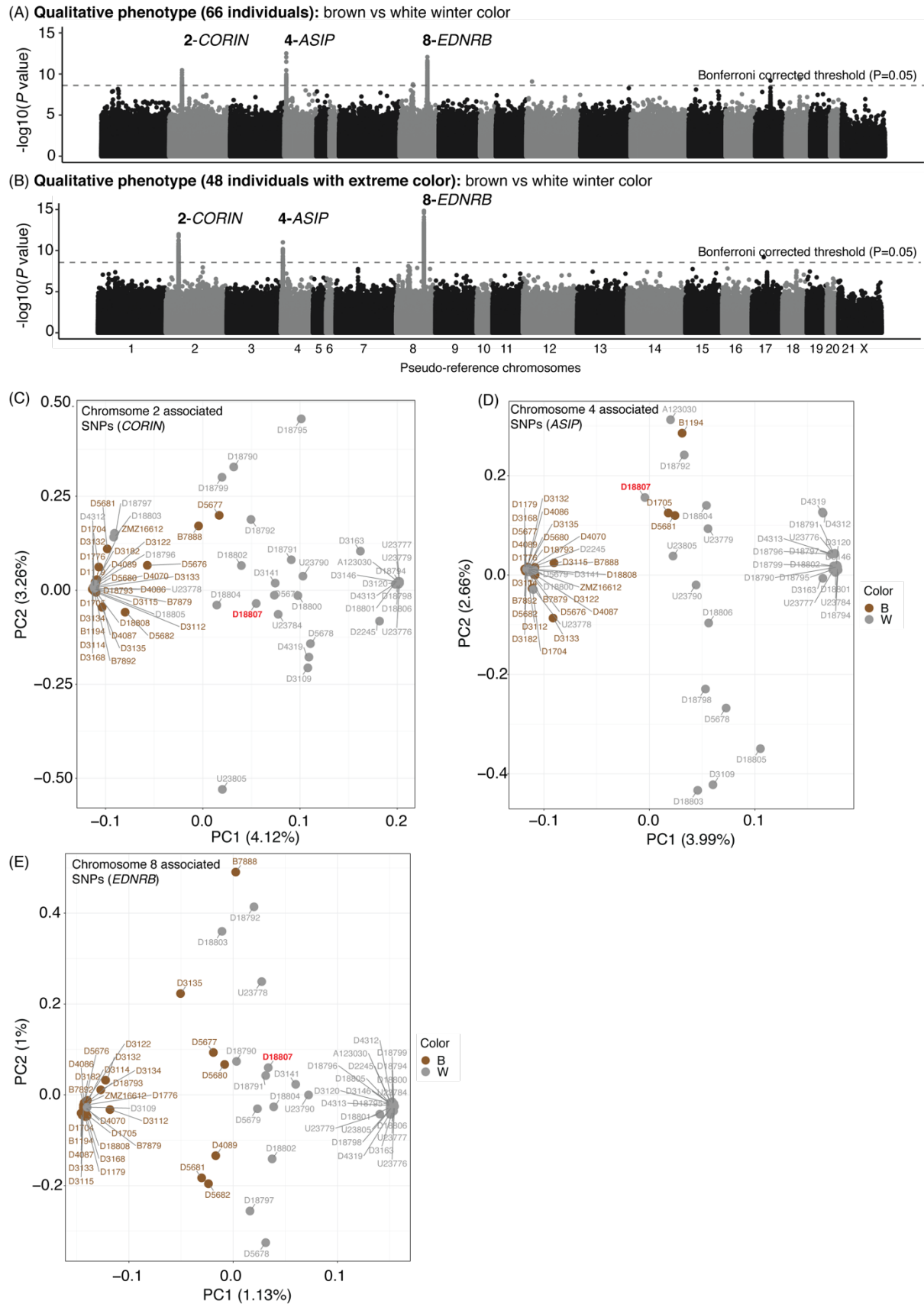


Figure S3.2 – Genome-wide association scan (GWAS) with winter coat color of 66 white-tailed jackrabbits from Colorado. Sequence data for 36 white and 30 brown individuals (Table S3.5) was mapped to a white-tailed jackrabbit pseudo-reference in the coordinates of the European rabbit genome; (A) The GWAS identified three strong candidate regions in chromosome 2 (*CORIN*), chromosome 4 (*ASIP*) and chromosome 8 (*EDNRB*) as associated with coat color variation [$-\log_{10}(P \text{ value}) \geq 8.61$]

The evolution and molecular bases of seasonal coat color variation in hares:
gene expression and the shared contribution of ancestral polymorphism and introgression

after Bonferroni correction for 20,260,101 tested SNPs]. **(B)** We performed an additional GWAS with 48 individuals (24 brown vs. 24 white) by removing intermediate individuals (Table S3.5), identifying the same association regions [$-\log_{10}(P \text{ value}) \geq 8.56$ after Bonferroni correction for 18,122,190 tested SNPs]. Individual DMNS_18807 was used to generate the white-tailed jackrabbit *de novo* assembled reference genome since its intermediate placement (highlighted in red) in local PCAs with the associated SNPs in **(C)** *CORIN*, **(D)** *ASIP* and **(E)** *EDNRB* suggests this individual is a triple heterozygote.

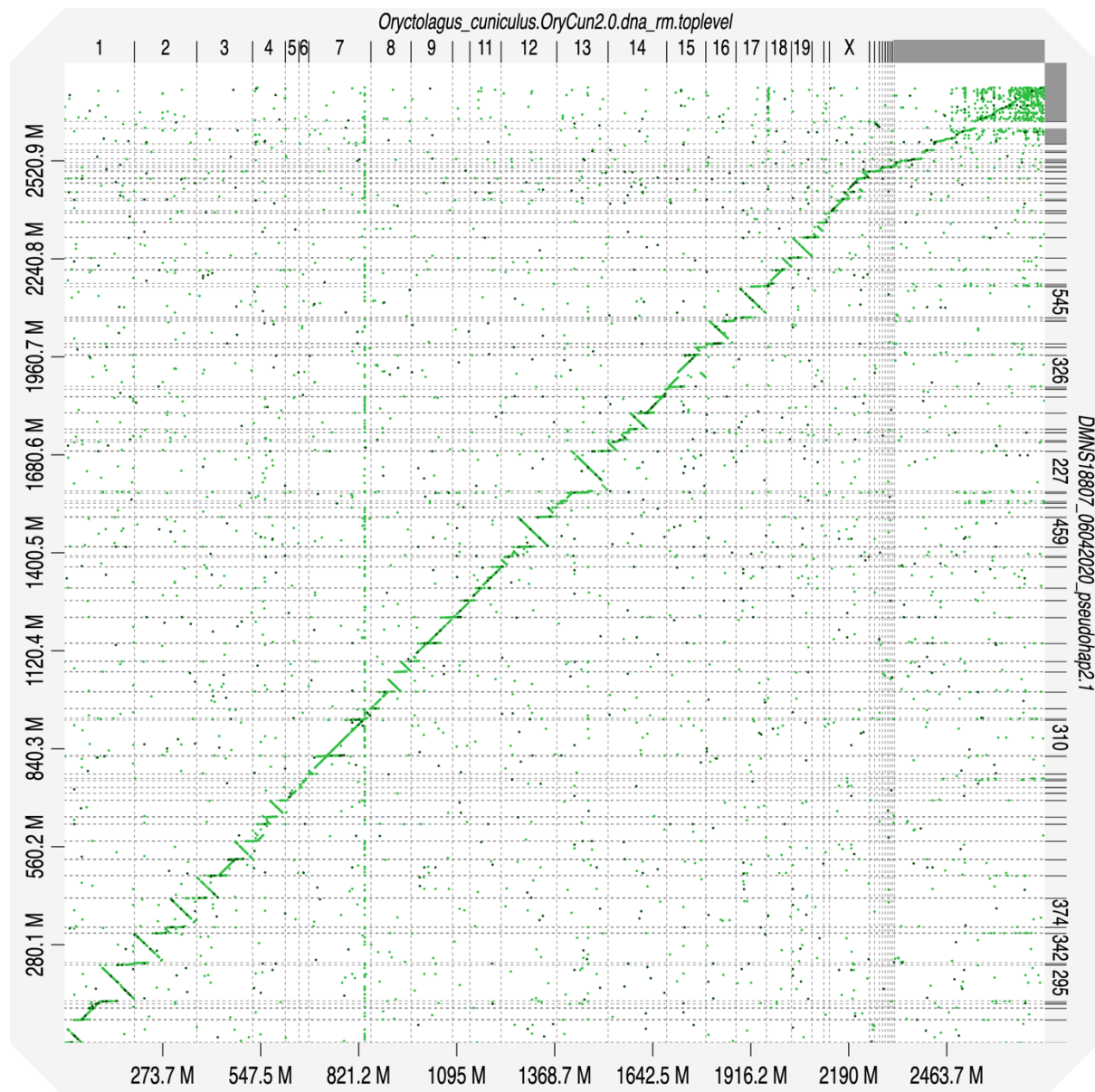
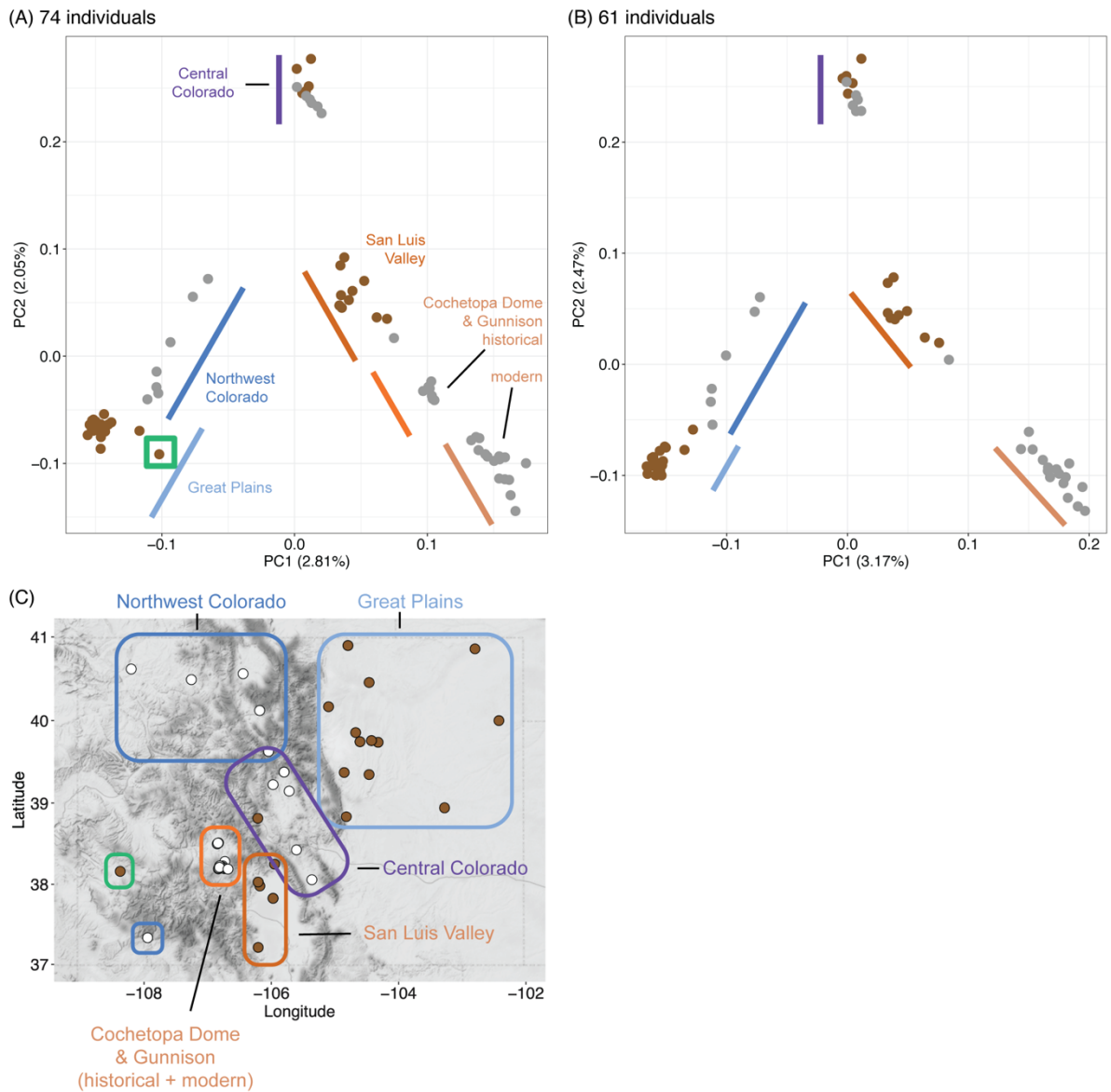


Figure S3.3 – Dot plot obtained from mapping the white-tailed jackrabbit *de novo* assembled reference genome (DMNS18807_06042020_pseudohap2.1) to the European rabbit reference genome (OryCun2.0) with *D-Genies*. Dots represent matches with identity higher than 0.5.



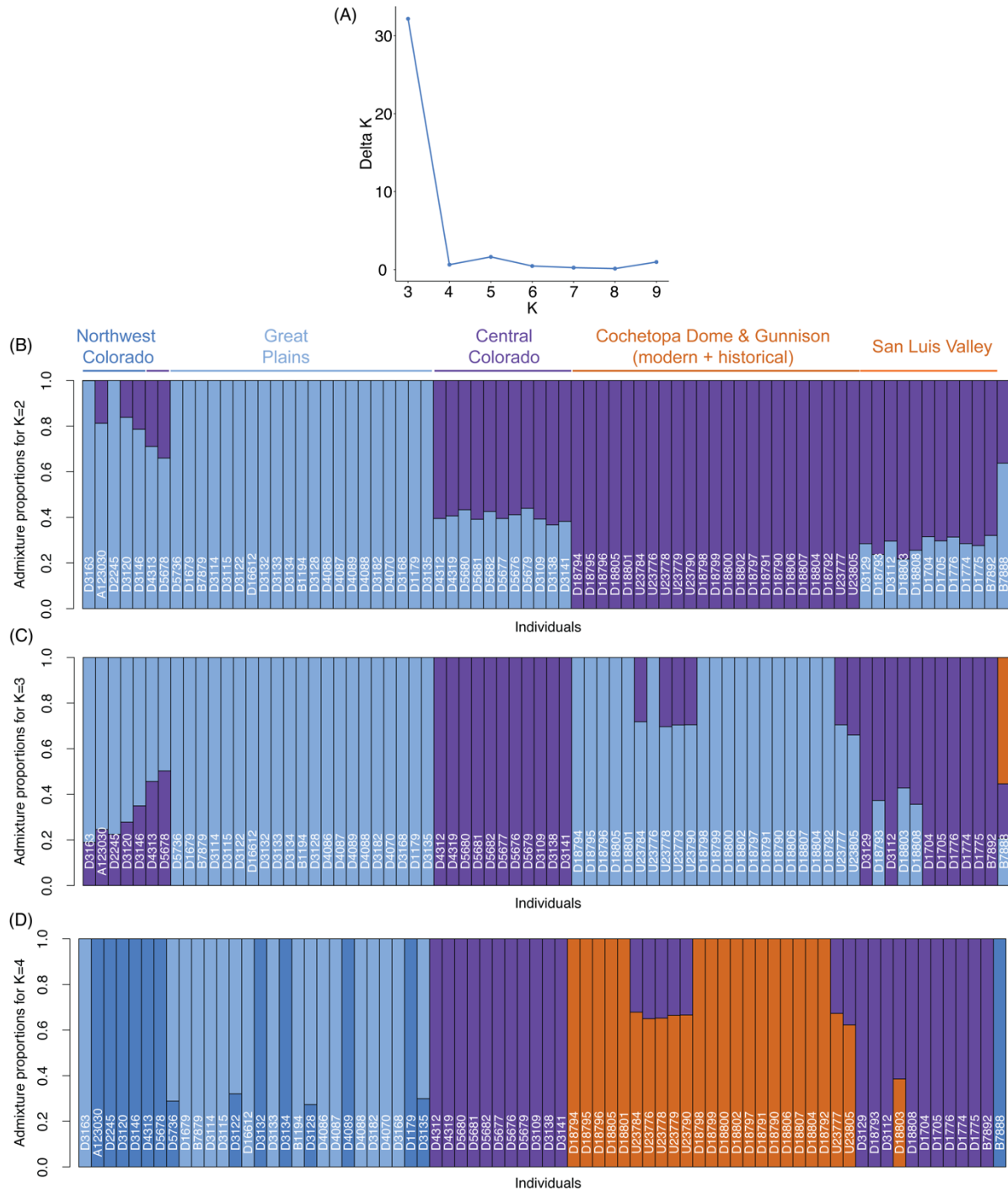


Figure S3.5 – Admixture analysis infers that white-tailed jackrabbits from Colorado are split into three major clusters. **(A)** Identification of the best number of ancestral populations (K) for 74 white-tailed jackrabbits by applying the Evanno method (Evanno et al. 2005) to the likelihoods of admixture analyses with 1 to 10 ancestral populations, using the best replicate per K. **(B-D)** Population ancestry plots obtained from admixture analysis of 239,834 independent whole-genome SNPs segregating among 74 white-tailed jackrabbits from Colorado. Labels correspond to population clusters identified in a principal component analysis (Figure S3.4). We present ancestry plots for the best number of ancestral populations (K=3) and K=2 and 4 for context.

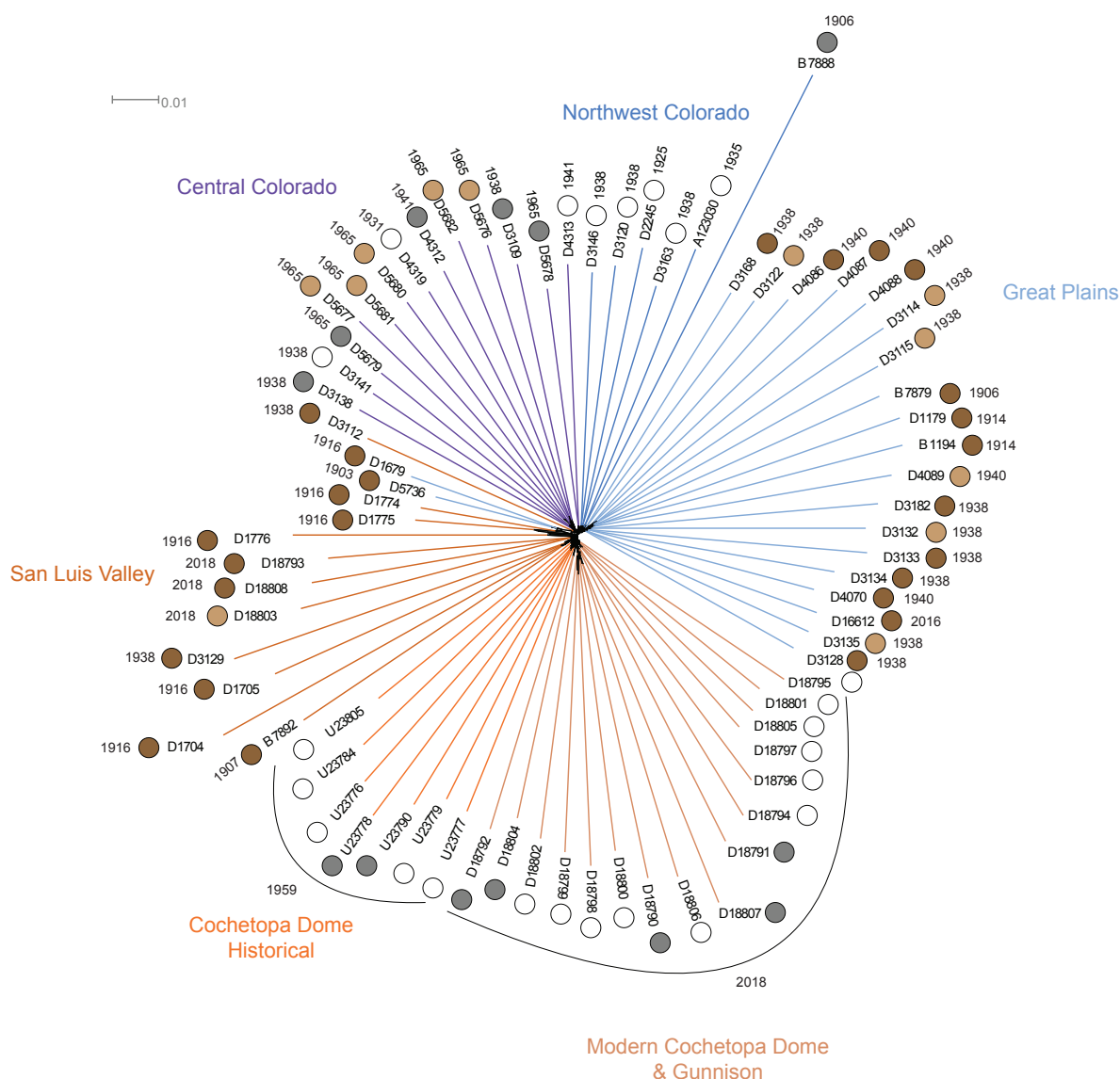


Figure S3.6 – *SplitsTree* network constructed with genetic distances among 74 white-tailed jackrabbit individuals from Colorado. Genetic distances were calculated with *ngsDist* with genome-wide polymorphic sites. For each individual, we indicate its population (colored as in Figure S3.4), its sample label (as in Table S3.3), approximate winter coat color shade [white, brown or intermediate (light brown or gray) as in Table S3.5] and year of collection.

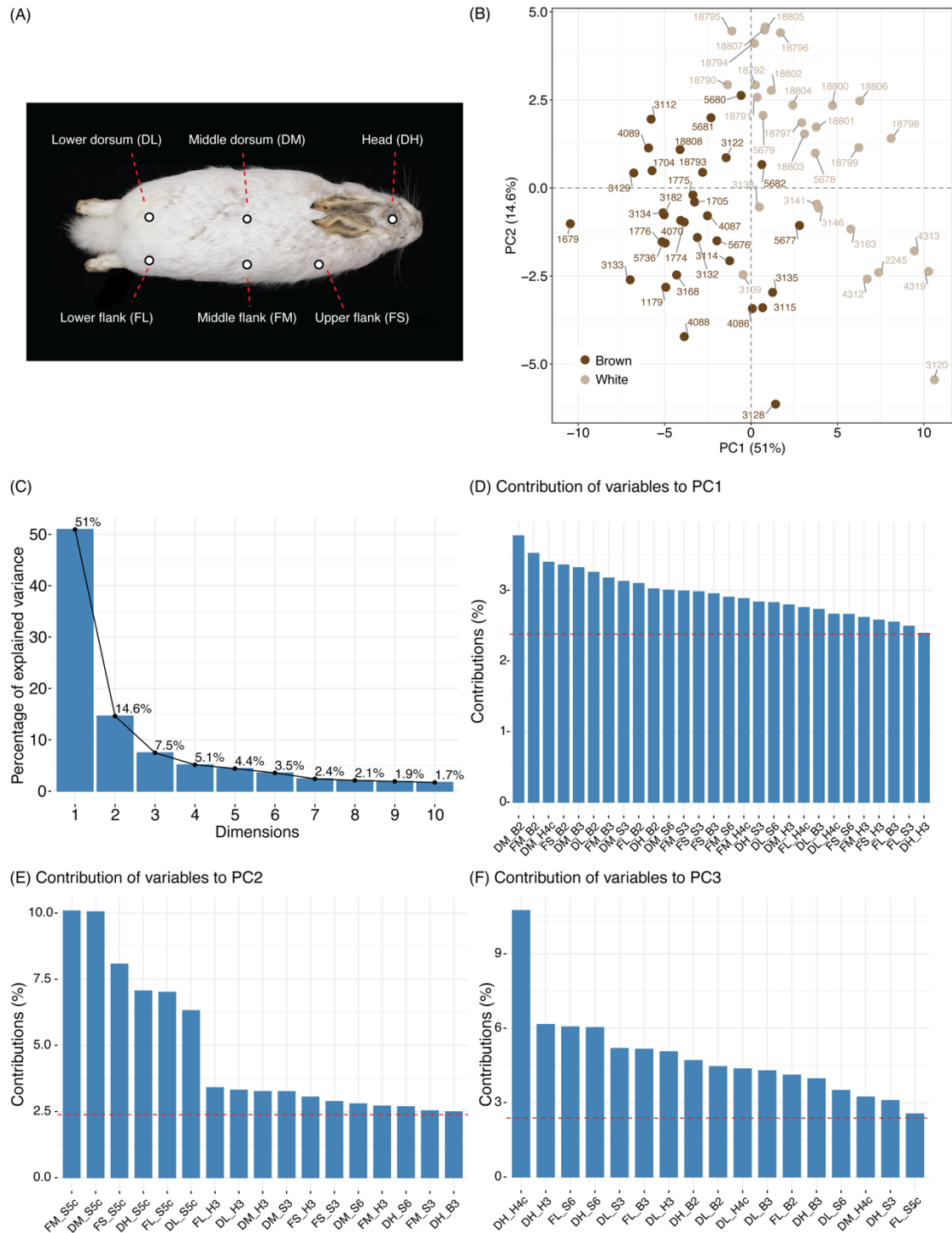


Figure S3.7 – Principal component analysis of reflectance measurements from (A) 6 dorsal regions of 61 white-tailed jackrabbit specimens from the Denver Museum of Nature and Science collection. (B) The two main principal component axes explaining variation in dorsal reflectance measurements reveal no particular clustering of the individuals, concordant with the continuous variation of all components of color. Individuals are labeled as in Table S3.3 and colored according to a discrete winter coat color classification (“white” as light-colored circles or “brown” as dark colored circles). (C) Variance explained by the first 10 principal components of the PCA. The first three components explain 77.1% of the data. (D-F) Contribution of the several reflectance measurements to the first three principal components. Variables are labeled by body region and reflectance measurement [e.g., DM_B2 corresponds to brightness (B2) of the middle dorsum]. Brightness measurements of all body regions and color components of mid-point of the dorsum load on the first dimension (D), suggesting these are the most important aspects distinguishing individual coat color. Contrast measurements load of PC2 (E) and head and rump reflectance measurements load on PC3 (F) suggesting that patterning and variation of color between body regions are also important components of winter coat color variation in white-tailed jackrabbits.

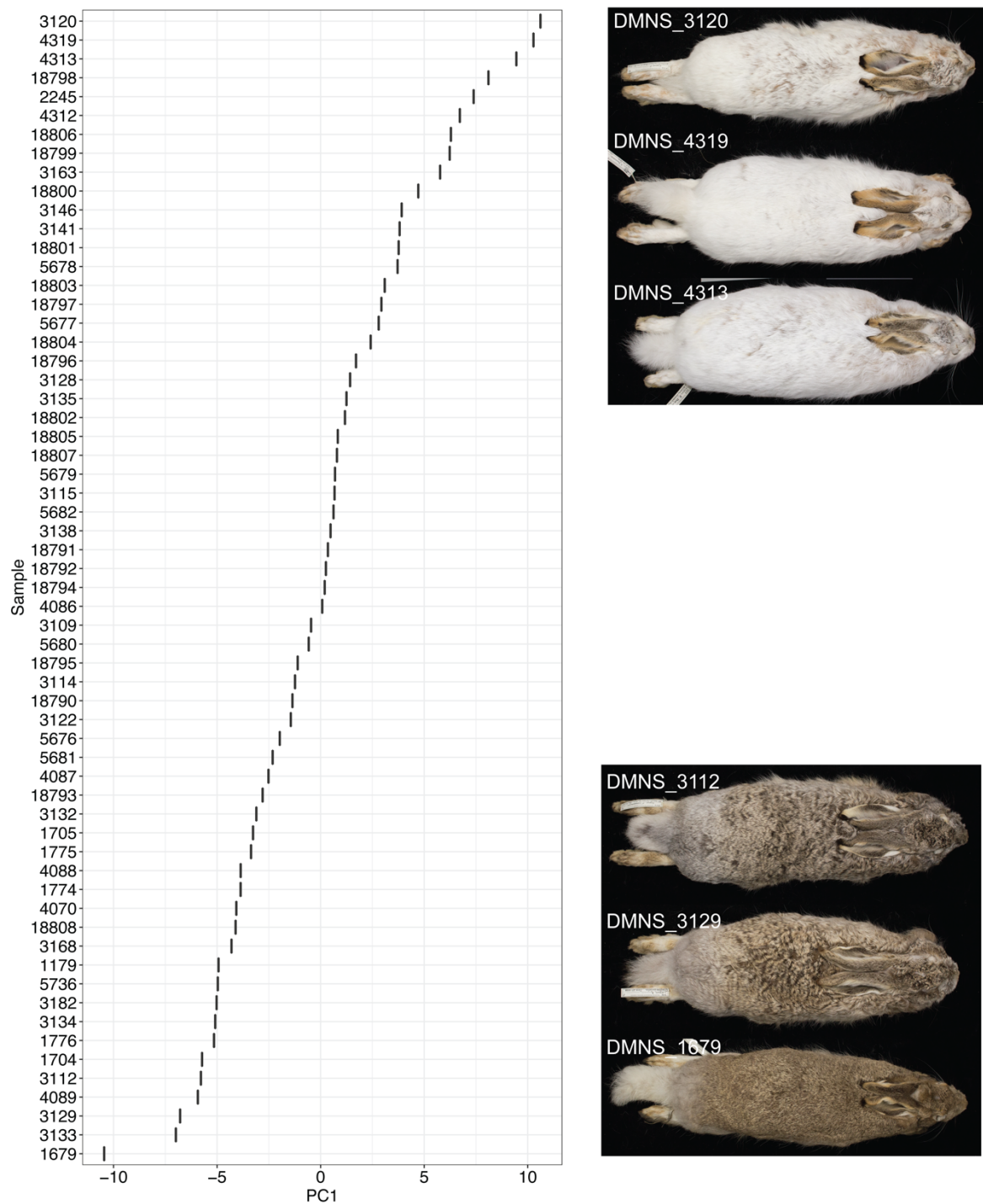


Figure S3.8 – First principal component of dorsal reflectance measurements separates white-tailed jackrabbits with contrasting coat brightness. Next to the plot, we show dorsal photographs of the three individuals with highest and lowest PC1 loadings. Individuals with high loadings are whiter/brighter than individuals with low loadings, which are browner/darker. Photographs were taken from specimens of the Denver Museum of Nature and Science, and the individual codes correspond to voucher codes in Table S3.3.

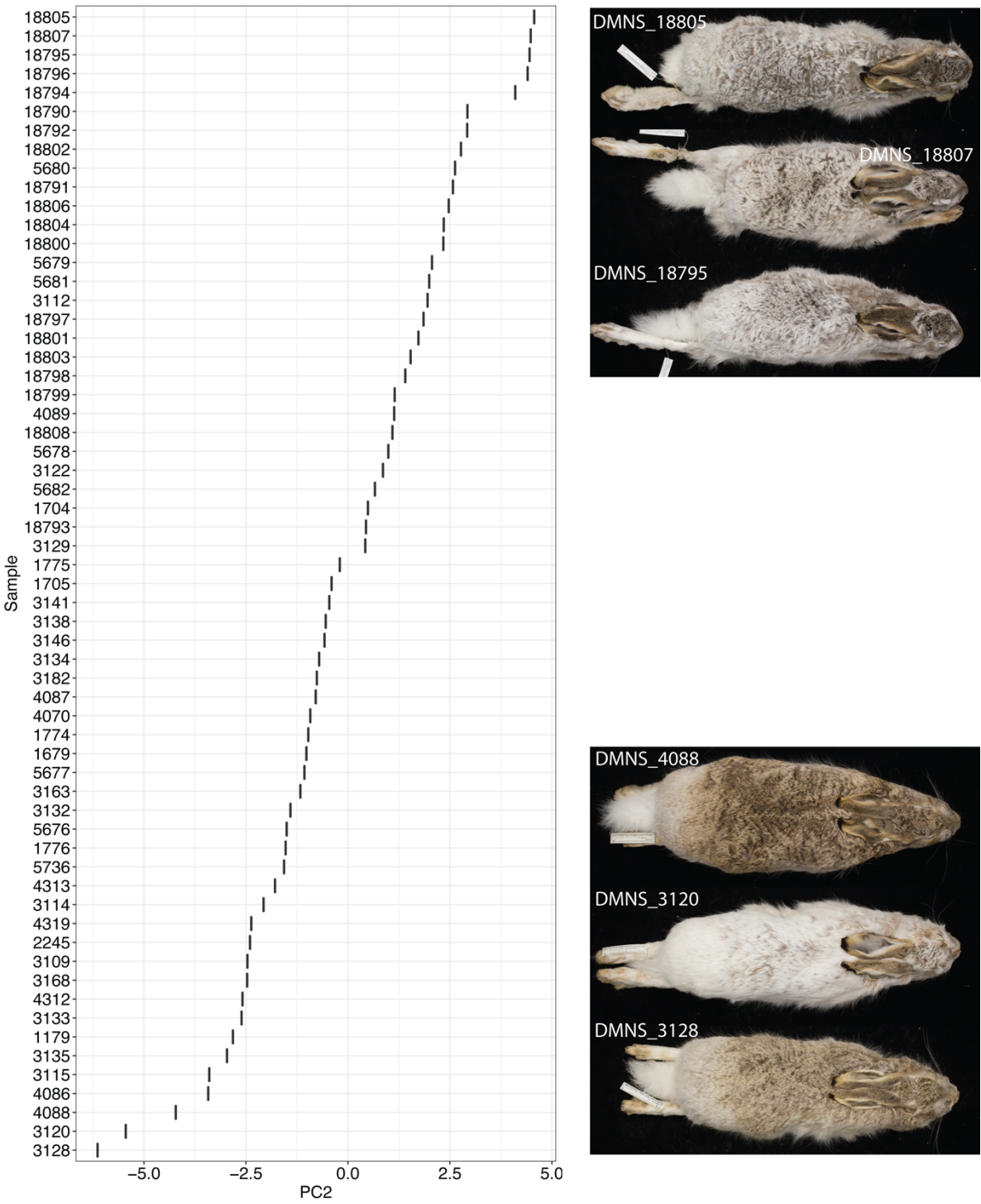


Figure S3.9 – Second principal component of dorsal reflectance measurements separates white-tailed jackrabbits with patterned coats from individuals with homogeneous coloration. Next to the plot, we show dorsal photographs of the three individuals with highest and lowest PC2 loadings. Individuals with high loadings show a patterned coat color with lighter and darker patches, while individuals with low loadings tend to be completely white or completely brown. Photographs were taken from specimens of the Denver Museum of Nature and Science, and the individual codes correspond to voucher codes in Table S3.3.

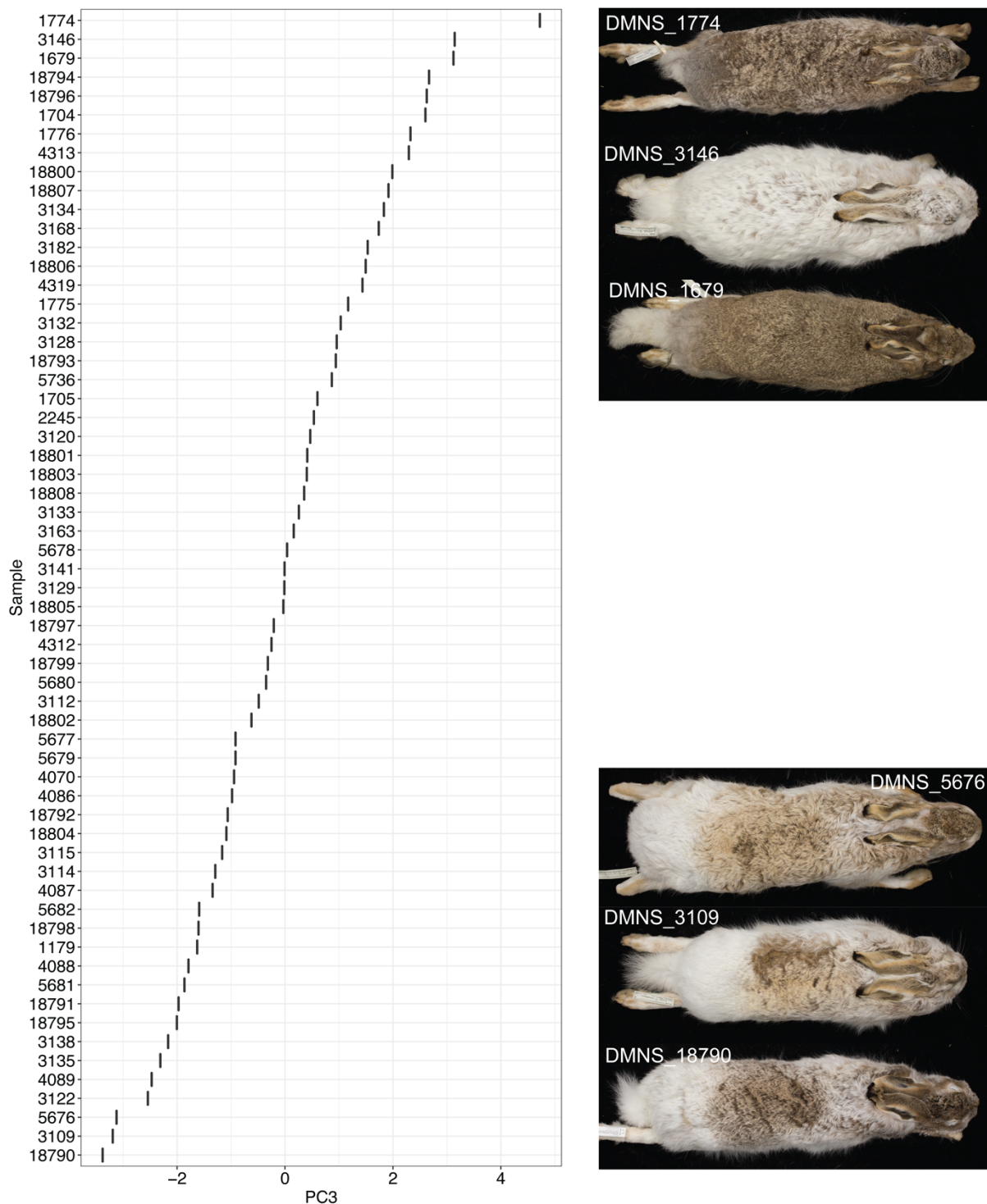
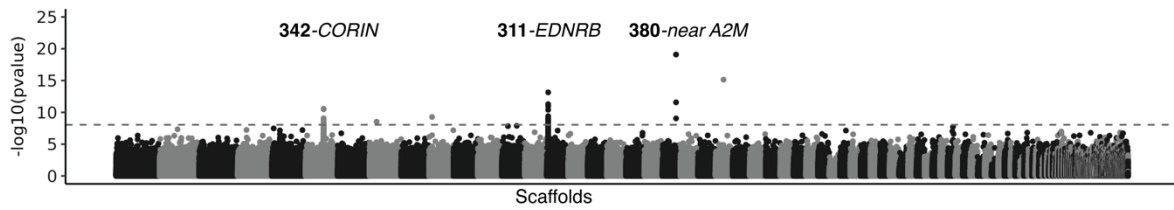
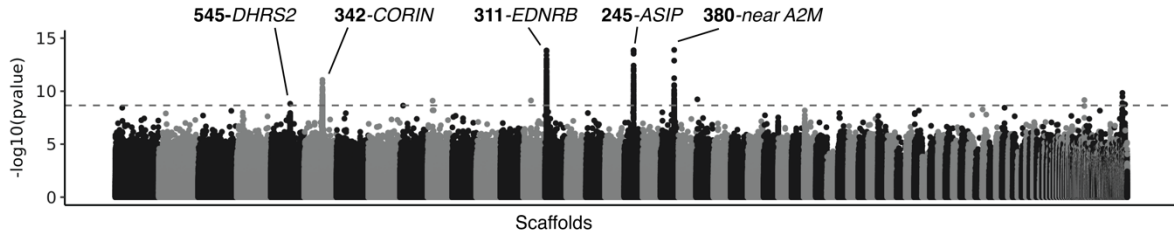


Figure S3.10 – Third principal component of dorsal reflectance measurements separates white-tailed jackrabbits with dark head and dorsum and light rump and flank from individuals with homogeneous coloration. Next to the plot, we show dorsal photographs of the three individuals with highest and lowest PC3 loadings. Individuals with low loadings show a patterned coat color with dark head and dorsum and light rump and flanks, while individuals with high loadings tend to be completely white or completely brown. Photographs were taken from specimens of the Denver Museum of Nature and Science, and the individual codes correspond to voucher codes in Table S3.3.

(A) Continuous winter coat color (61 individuals), controlling for population structure



(B) White vs. brown winter coat color (74 individuals)



(C) White vs. brown winter coat color (74 individuals), controlling for population structure

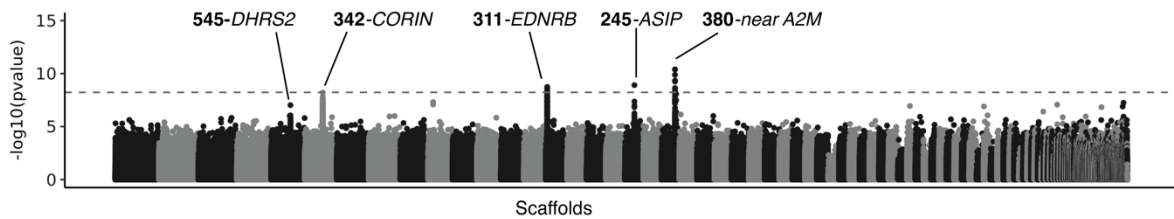


Figure S3.11 – Genome-wide association scan (GWAS) with winter coat color of white-tailed jackrabbits from Colorado. We performed **(A)** a GWAS with continuous winter coat color using the first principal component of a PCA of dorsal reflectance measurements from 61 white-tailed jackrabbits (Figure S3.7), controlling for population structure (Figure S3.4B) and **(B-C)** a GWAS with discrete winter coat color (“white” or “brown”) (Table S3.5), using genetic variation from 74 white-tailed jackrabbits **(B)** not controlling or **(C)** controlling for population structure (Figure S3.4A).

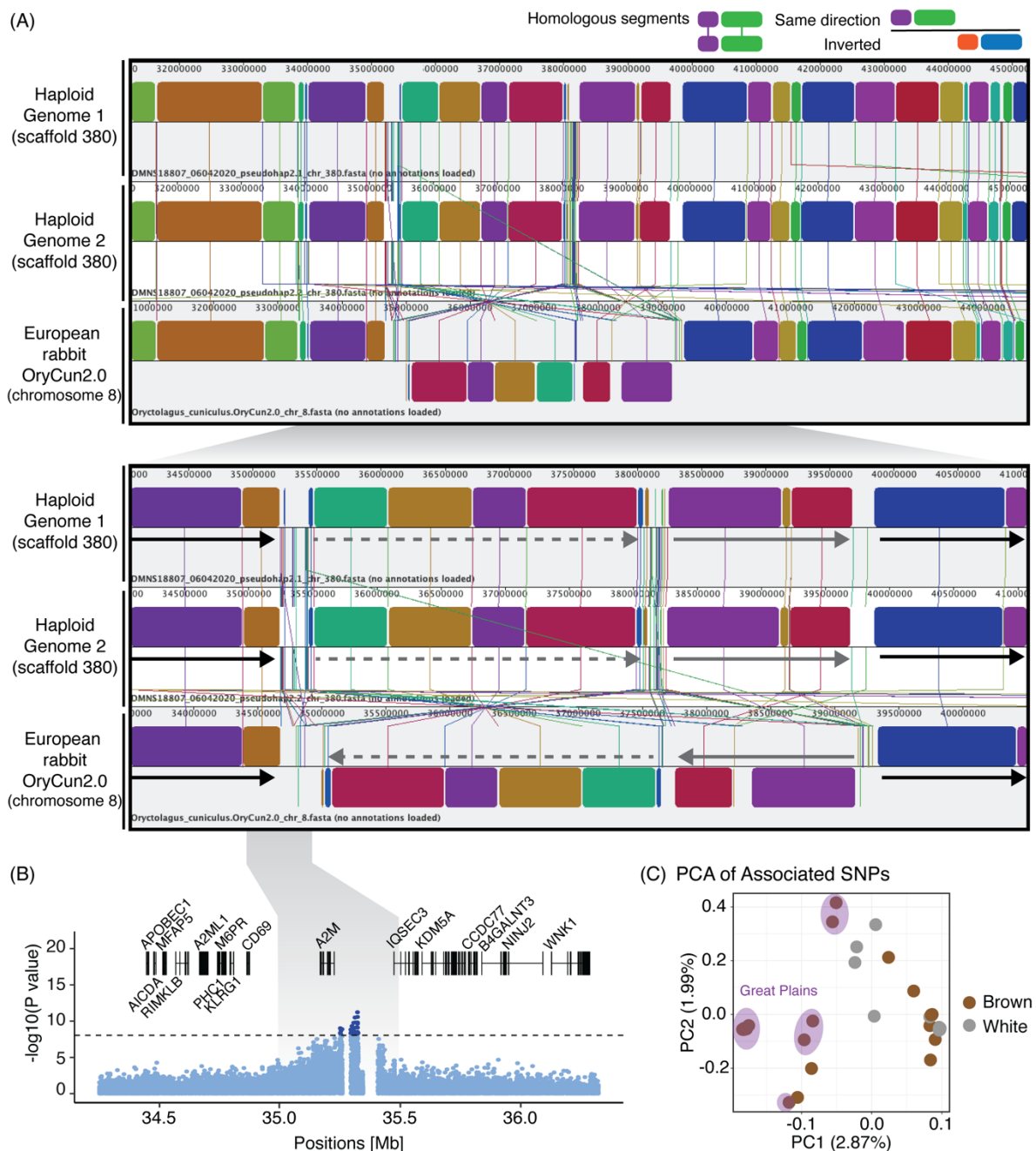


Figure S3.12 – MAUVE alignment of the two pseudo-haploid 380 scaffolds of the white-tailed jackrabbit genome and chromosome 8 of the European rabbit genome centered on the scaffold 380 association region with winter white and winter brown coat colors. **(A)** Blocks represent conserved homologous segments between haploid genome 1 (used as reference), haploid genome 2 and the European rabbit chromosome 8. Homologous segments are connected by colored lines. Blocks above and below the central line in each track are aligned in the same direction or inverted relatively to the reference, respectively. The scaffold 380 association region **(B)** coincides with the flanking region of two consecutive inversions of the white-tailed jackrabbit genome relatively to the European rabbit genome (marked with two gray arrows in panel A; black arrows indicate blocks aligned in the same direction). **(C)** A local PCA of the associated SNPs in panel B (in dark blue) reveals a separation of the brown Great Plains individuals from other individuals, suggesting a potentially spurious association with coat color.

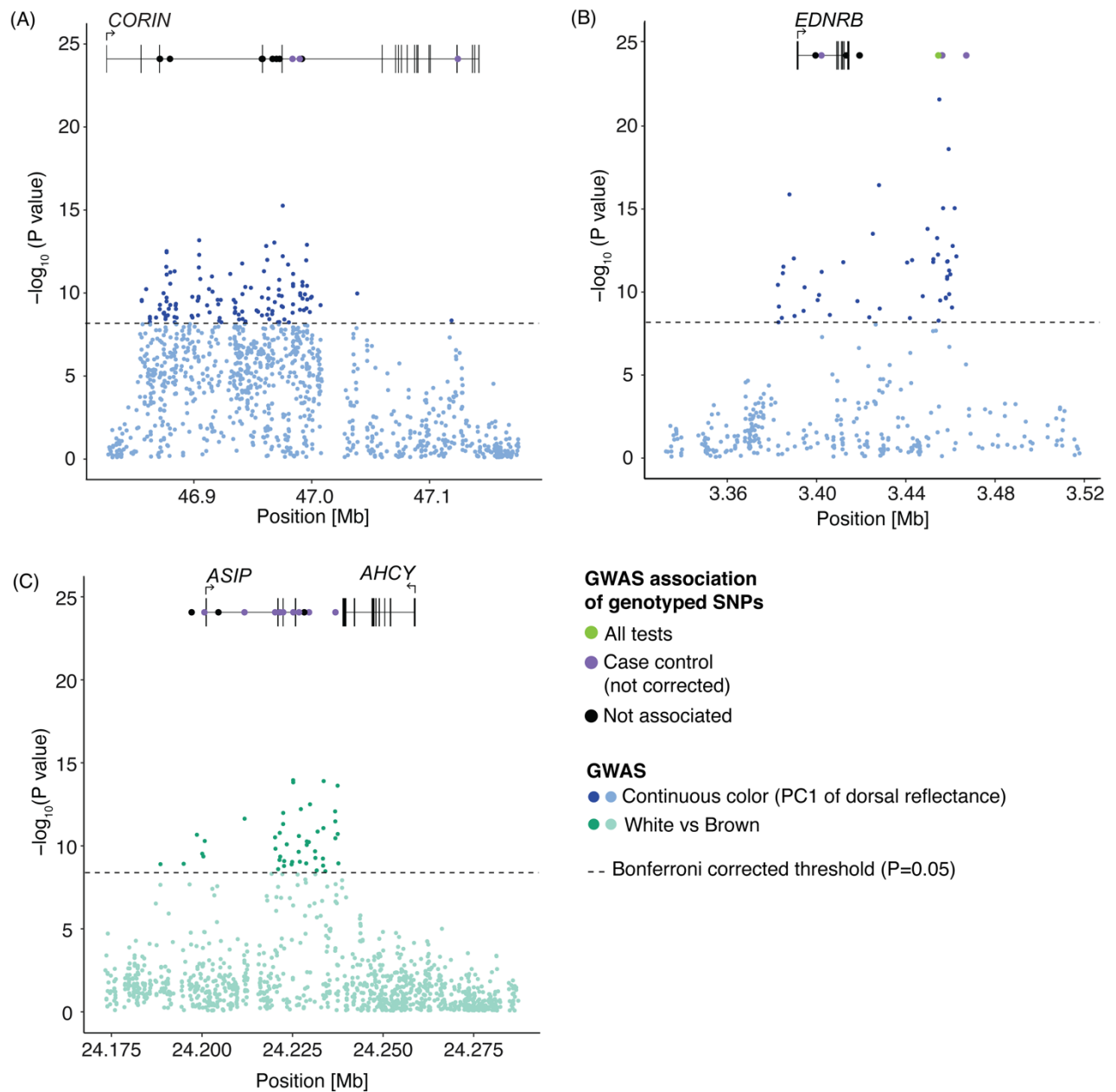
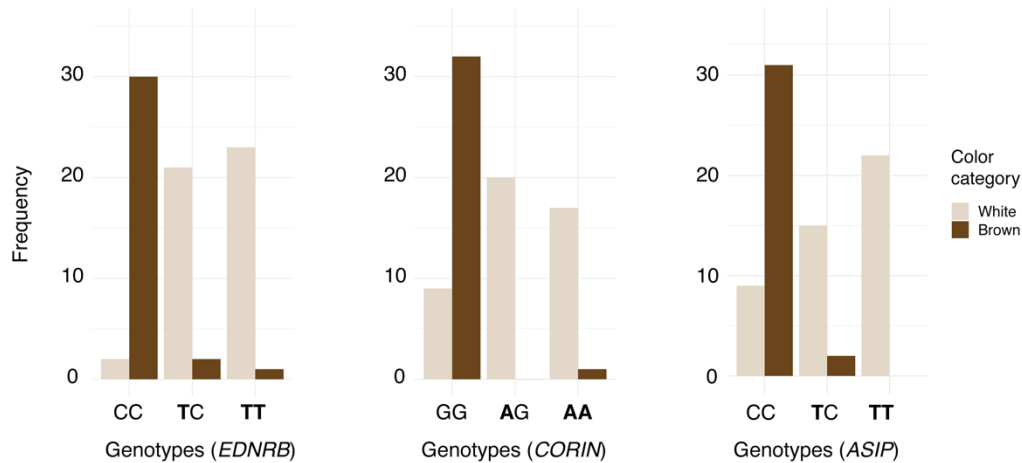


Figure S3.13 – Genome location of the 34 SNPs (green, purple or black dots) genotyped with MassARRAY® along association regions in (A) scaffold 311 containing *EDNRB*, (B) scaffold 342 containing *CORIN* and (C) scaffold 245 containing *ASIP*. Genome-wide association *P* values ($-\log_{10} P$ values) with a continuous winter coat color (PC1 of dorsal reflectance measurements for 61 individuals in blue) is shown for *EDNRB* and *CORIN* (from Figure 3.2) or with discrete color (white vs. brown color for 74 individuals in green) is shown for *ASIP* (from Figure S3.11B). Since the genotyped SNPs were selected based on the results of the association with white vs. brown coloration with data mapped to a pseudo-reference based on the European rabbit genome (Figure S3.2), we colored genotypes in green if they were also associated in all four association tests performed with sequence data mapped to the white-tailed jackrabbit genome (Figure 3.2 and Figure S3.11), in purple if they were only significantly associated with white vs. brown color not controlling for population structure (Figure S3.11B), and in black if they were not associated in any test.

(A)



(B)

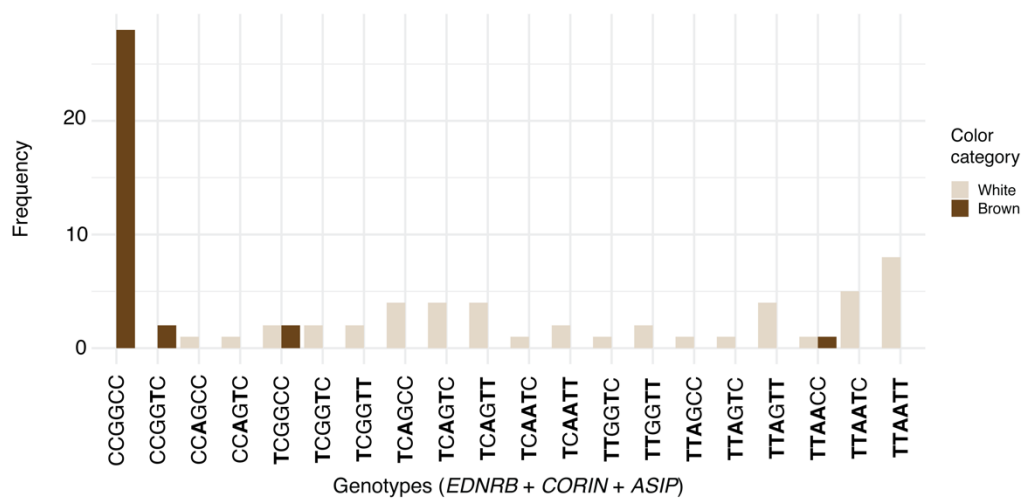


Figure S3.14 – Distribution of discrete winter coat color categories (“white” or “brown”) across *EDNRB*, *CORIN* and *ASIP* genotypes for 81 white-tailed jackrabbit individuals from Colorado. We used the SNPs with highest association with continuous winter coat color (8:79701598, 2:36361155, 4:5424640, Table S3.10) among the MassARRAY® genotyped SNPs to study **(A)** the frequency of two discrete color categories (white or brown) across *EDNRB*, *CORIN* and *ASIP* genotypes; **(B)** the frequency of two discrete color categories (white and brown) across the combined *EDNRB*, *CORIN* and *ASIP* genotypes. The white allele at each gene is highlighted in bold.

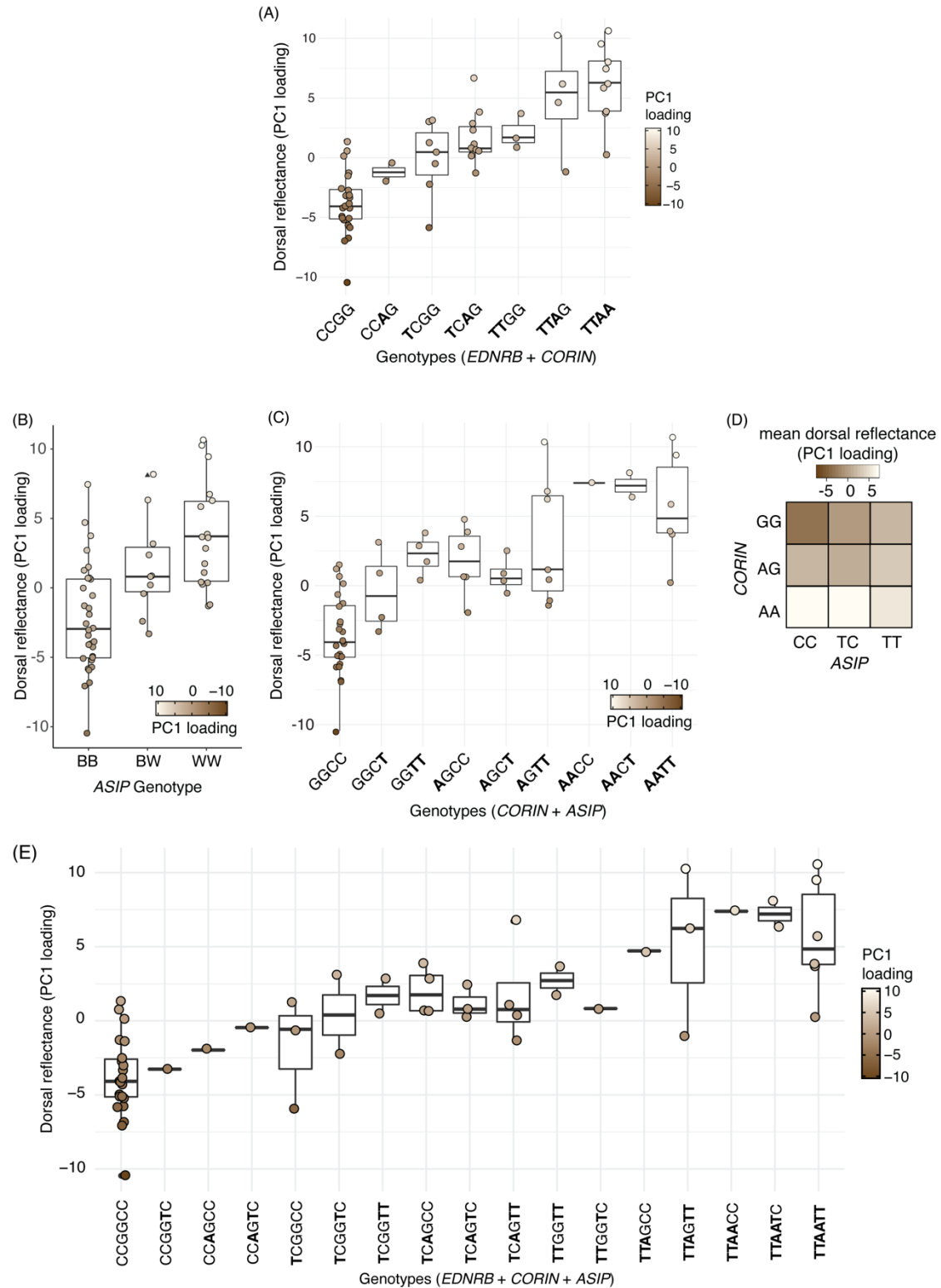


Figure S3.15 – Distribution of continuous winter coat color across *EDNRB*, *CORIN* and *ASIP* genotypes for 59 white-tailed jackrabbit individuals from Colorado. We used the SNPs with highest association with continuous winter coat color (8:79701598, 2:36361155, 4:5424640; Table S3.10) to study the (A) phenotype distribution (PC1 loading of a PCA of dorsal reflectance) across the combined *EDNRB* and *CORIN* genotypes; (B) Phenotype distribution (PC1 loading) across the three alternative genotypes (BB – homozygous brown allele; BW – heterozygous; WW – homozygous white allele) of the top associated SNP in *ASIP*; (C) Phenotype distribution (PC1 loading) across the combined *CORIN* and *ASIP* genotypes; (D) Mean phenotype (PC1 loading) across the nine possible combinations between *ASIP* and *CORIN* genotypes of the top associated SNPs; (E) Phenotype distribution (PC1 loading) across the combined genotypes of *EDNRB*, *CORIN* and *ASIP*. Genotypes are presented in the order *EDNRB*, *CORIN* and *ASIP*. The white allele at each gene is highlighted in bold.

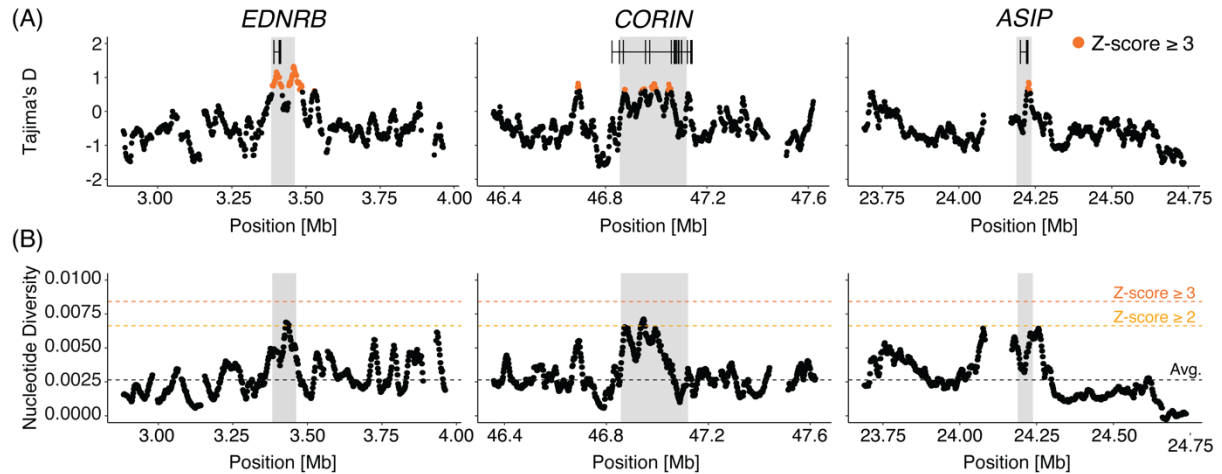


Figure S3.16 – Tajima's D (A) and nucleotide diversity (B) across the *EDNRB* (left panels), *CORIN* (middle panels) and *ASIP* (right panels) association regions (311:3382036-3468296, 342:46856651-47120426 and 245:24188393-24237751, respectively). Statistics were calculated using low-coverage sequence data of 74 white-tailed jackrabbits from Colorado. Tajima's D and nucleotide diversity were calculated in 20 kb sliding windows (2 kb step size) and windows with Z-score equal or greater than 3 are highlighted in orange. For nucleotide diversity, the dark orange, light orange and black dashed lines represent windows with Z-score above 3, Z-score above 2 and the average of the distribution, respectively.

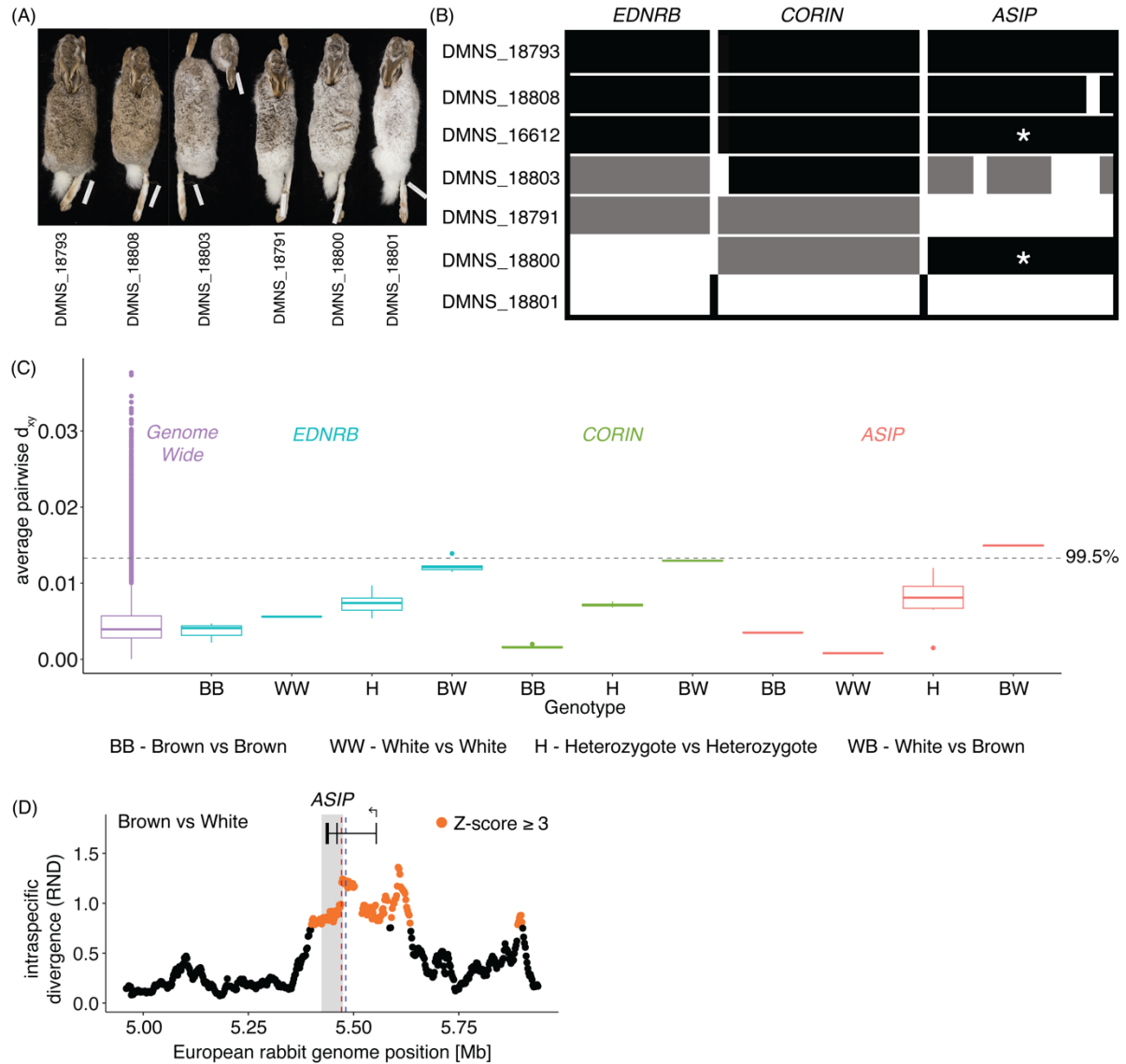


Figure S3.17 – High average pairwise genetic divergence among brown and white *EDNRB*, *CORIN* and *ASIP* alleles in white-tailed jackrabbits. Seven white-tailed jackrabbit individuals of various winter coat color shades (A) and genotypes (B) were selected to produce high-coverage whole-genome sequence data. The complete pelt of DMNS_16612 was not salvageable, but photographs of the specimen upon arrival at the DMNS suggest a brown winter coloration (<http://arctos.database.museum/guid/DMNS:Mamm:16612>), concordant with its genotype (B). Average pairwise genetic divergence (C) within the association regions between individuals of the same genotype (BB or WW comparisons), among heterozygote individuals (H) and between white or brown homozygote individuals (BW) compared against the average genome-wide divergence among the 21 possible pairwise comparisons with 7 individuals in 1,058,055 20 kb sliding windows (purple). The gray dashed line represents the 99.5% quantile of the genome-wide d_{xy} distribution for reference. (D) Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) between a winter white (DMNS_18801) and winter brown (DMNS_18808) white-tailed jackrabbits across the *ASIP* association region (gray rectangle) using high-coverage whole genome data mapped to a white-tailed jackrabbit pseudo-reference in the coordinates of the European rabbit (*OryCun* 2.0) genome. The pseudo-reference approach allows to map the entire structure of *ASIP*, including the ventral specific exon (black vertical bar outside the association region). Windows with RND values with Z-score ≥ 3 are highlighted in orange. Red and blue dashed lines highlight the position of a white-specific 1,033 bp insertion and of a brown-specific 43 kb deletion, respectively, identified in Jones et al. (2018).

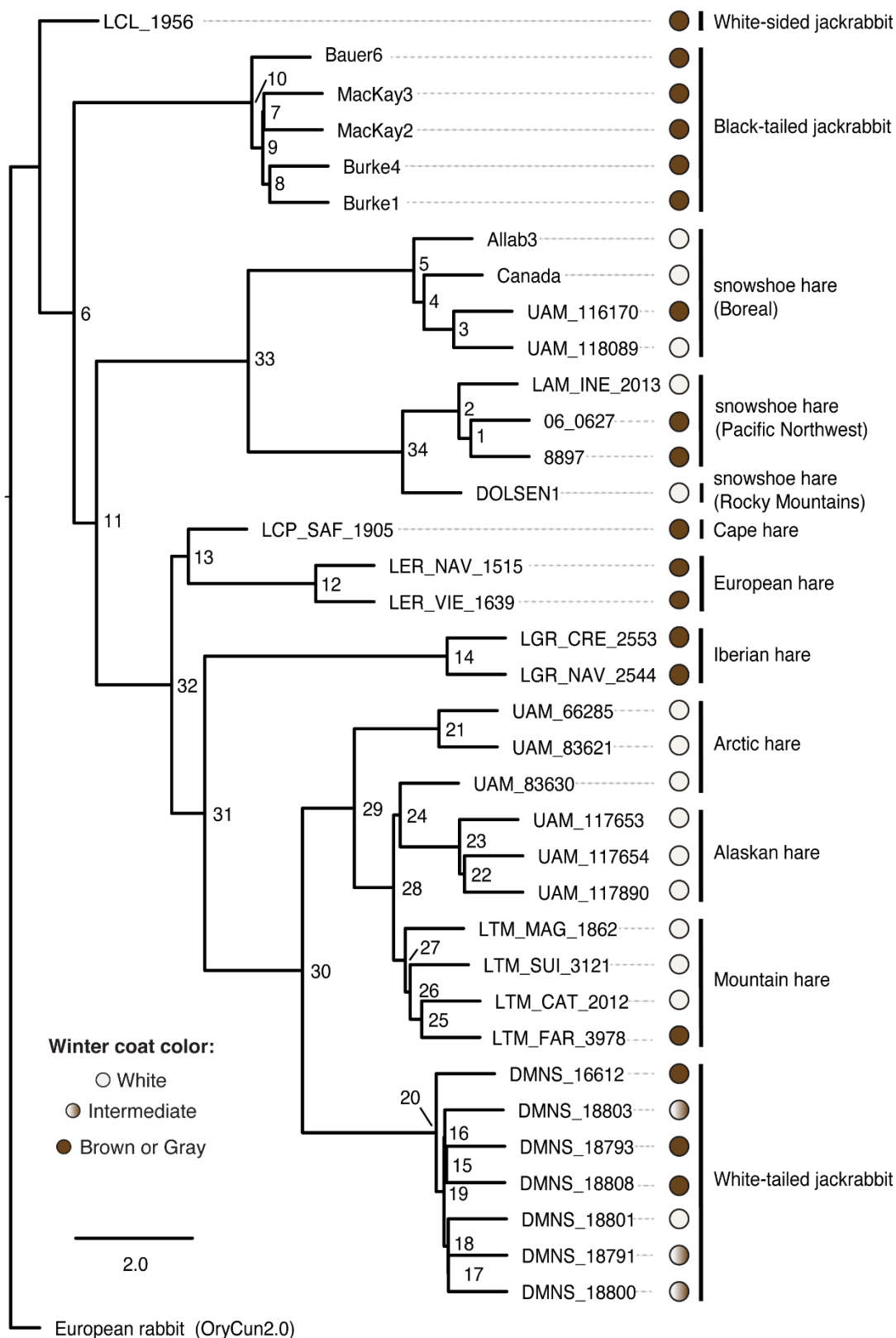


Figure S3.18 – Multispecies coalescent tree generated with *ASTRAL-III* with 43,430 gene trees. Node labels correspond to node labels in Table S3.15, containing quartet scores and posterior probabilities for each node. Data used for this analysis was mapped to species specific pseudo-references in the coordinates of the European rabbit genome. Tip labels correspond to sample labels in Table S3.3. Each individual is marked with its respective winter coat color (brown – dark/brown winter coloration; gradient – intermediate coloration; white – white winter coloration).

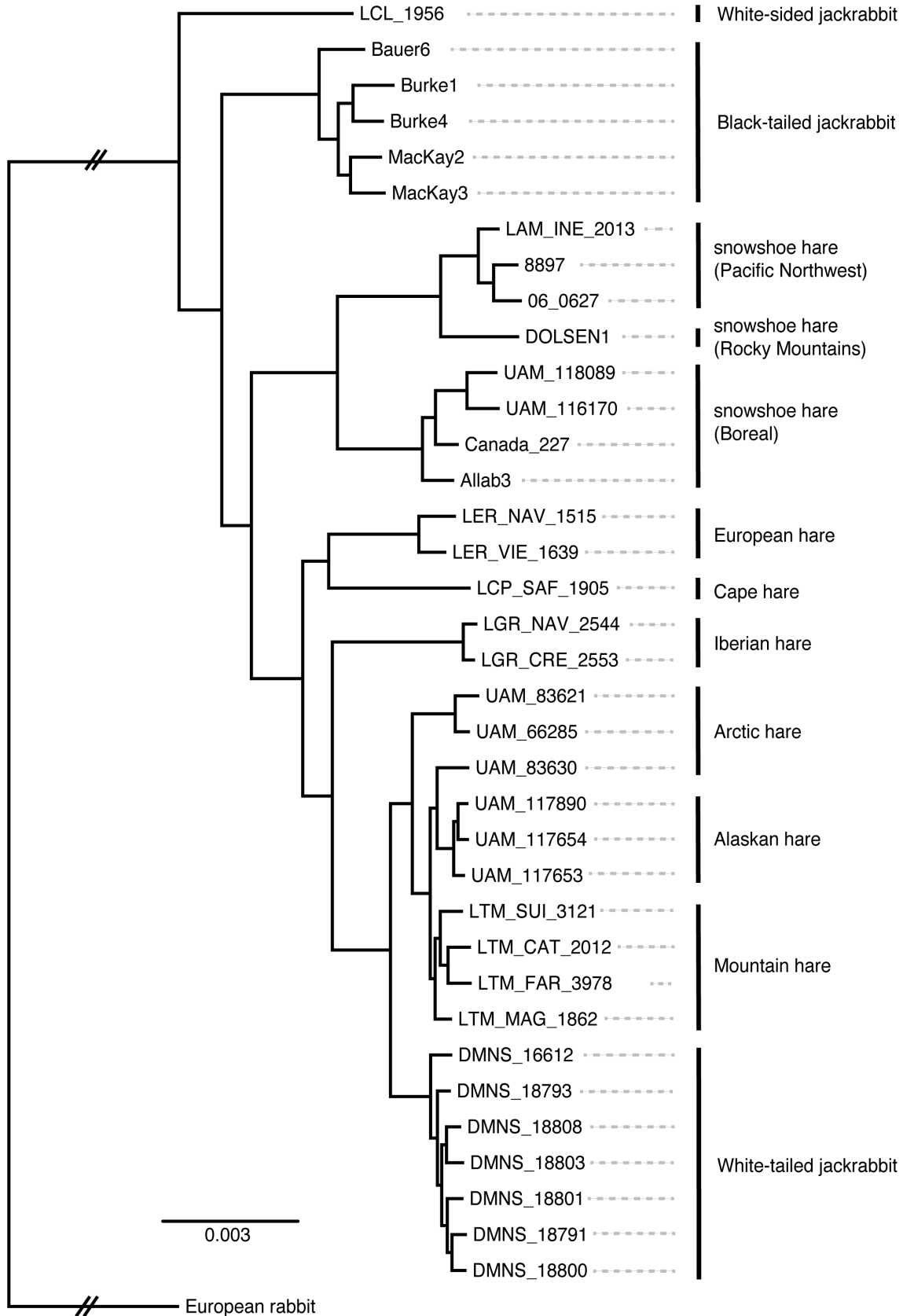


Figure S3.19 – Maximum likelihood tree for 10 hare species obtained with a genome-wide concatenated alignment with 66,166,173 positions with no missing data, from sequence data aligned to species specific pseudo-references in the coordinates of the European rabbit genome. Tip labels correspond to sample labels of Table S3.3. All branches have 100/100 bootstrap support.

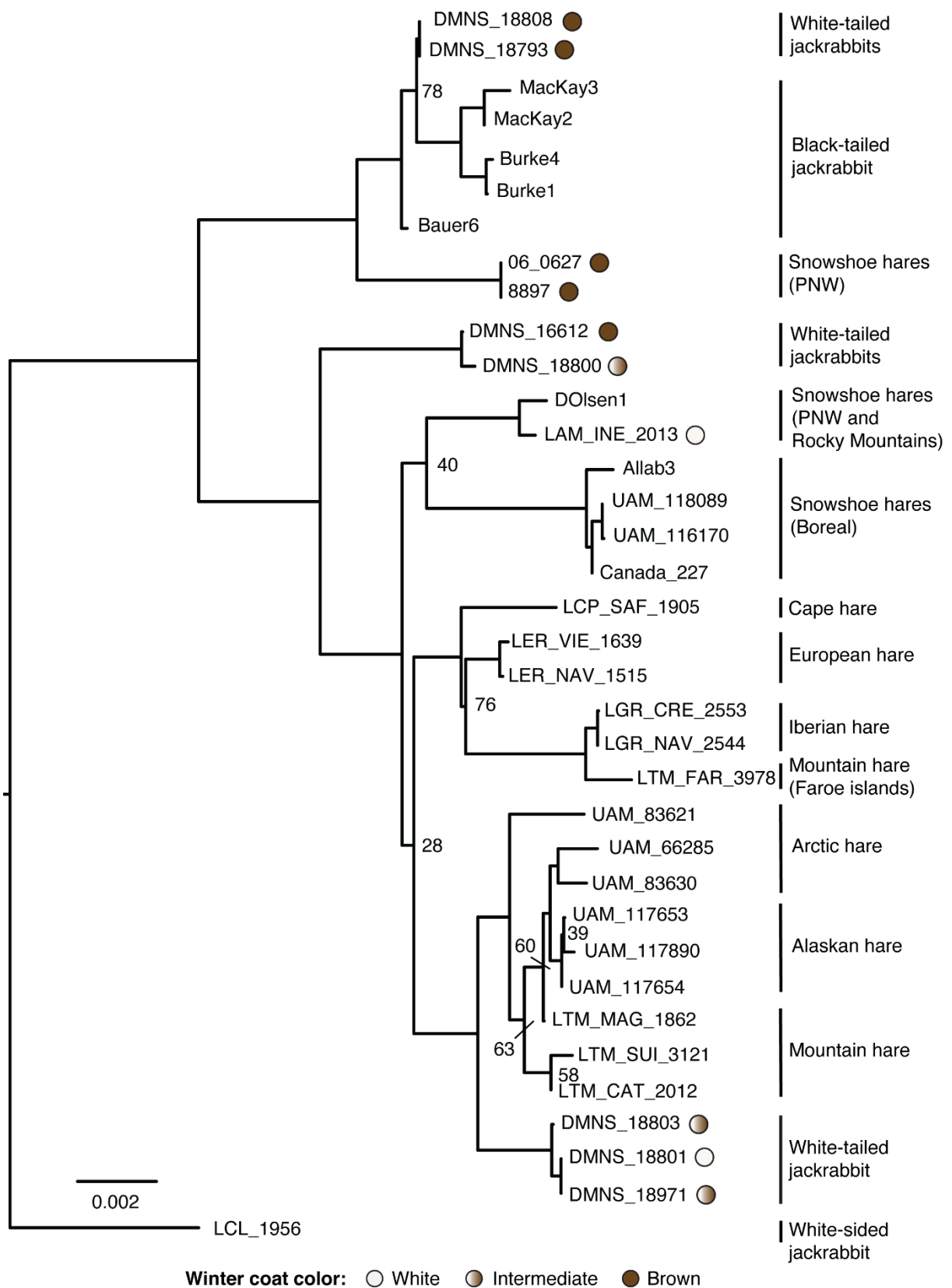


Figure S3.20 – Maximum likelihood tree of the scaffold 245 association region (245:24188393-24237751) containing *ASIP*. Alignments were generated from high-coverage data mapped to species specific pseudo-references in the coordinates of the white-tailed jackrabbit genome. Node numbers represent bootstrap support lower than 80/100.

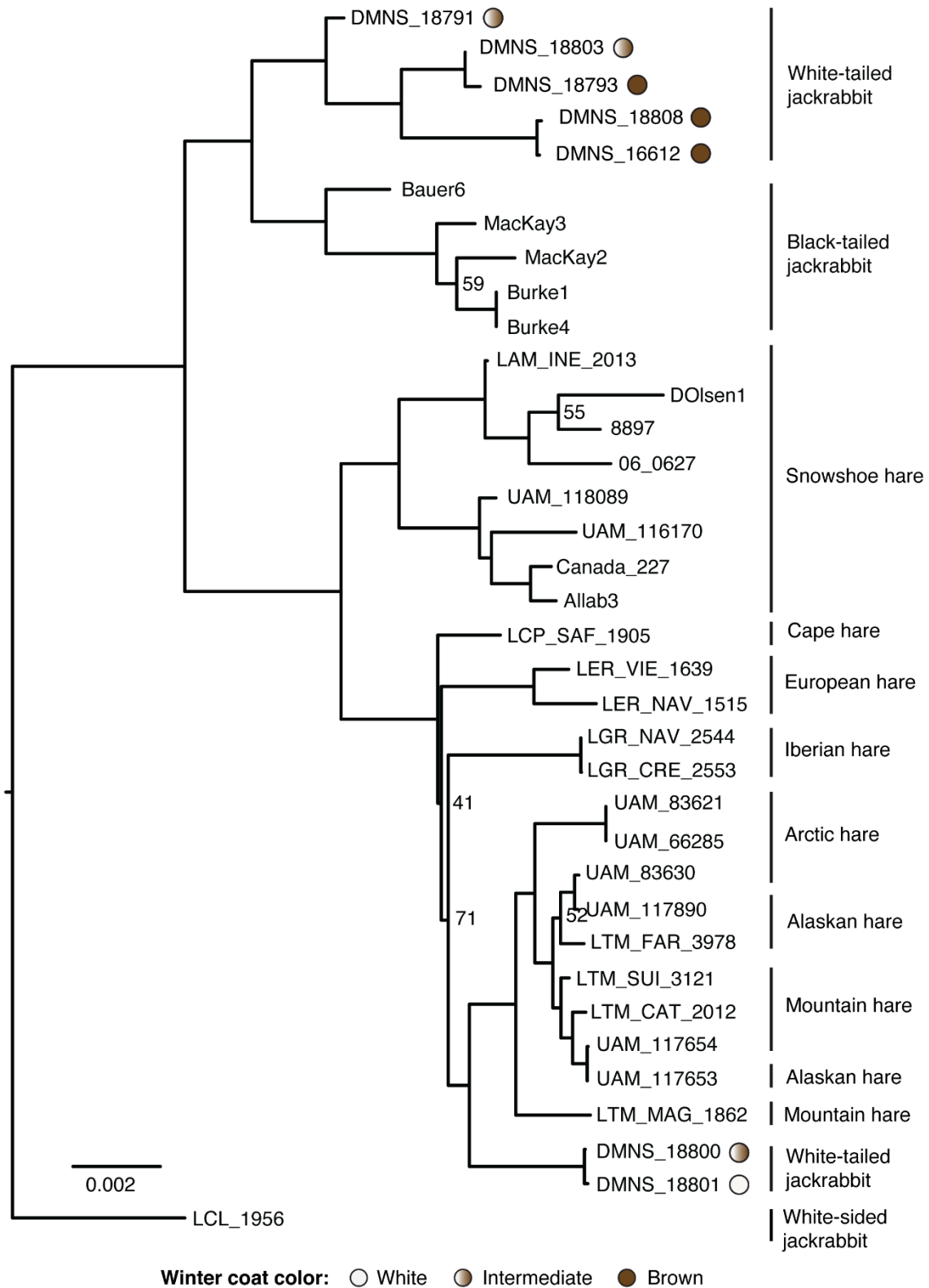


Figure S3.21 – Maximum likelihood tree of the scaffold 311 association region (311:3382036-3468296) containing *EDNRB*. Alignments were generated from high-coverage data mapped to species specific pseudo-references in the coordinates of the white-tailed jackrabbit genome. Node numbers represent bootstrap support lower than 80/100.

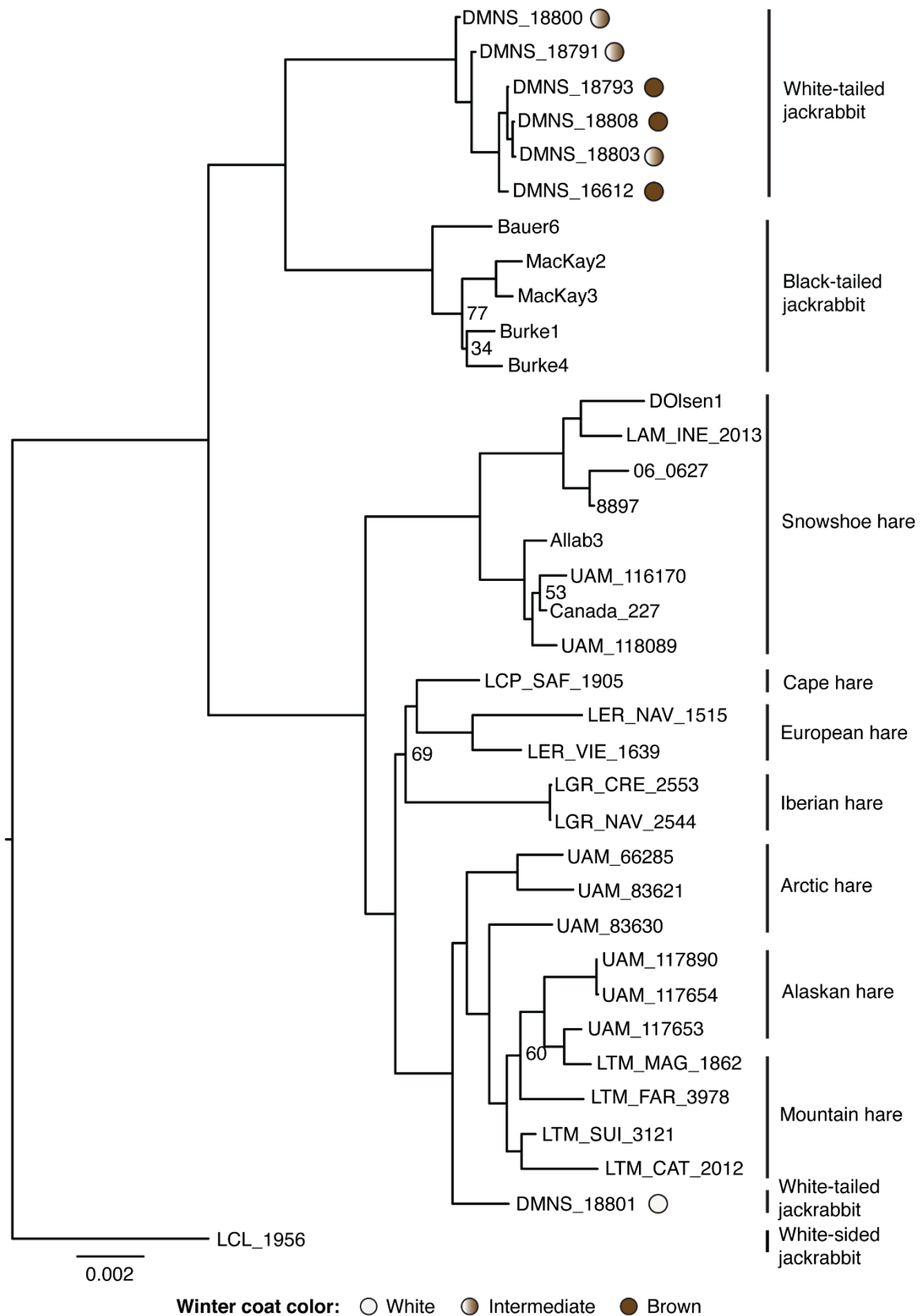


Figure S3.22 – Maximum likelihood tree of the scaffold 342 association region (342:46856651-47120426) containing *CORIN*. Alignments were generated from high-coverage data mapped to species specific pseudo-references in the coordinates of the white-tailed jackrabbit genome. Node numbers represent bootstrap support lower than 80/100.

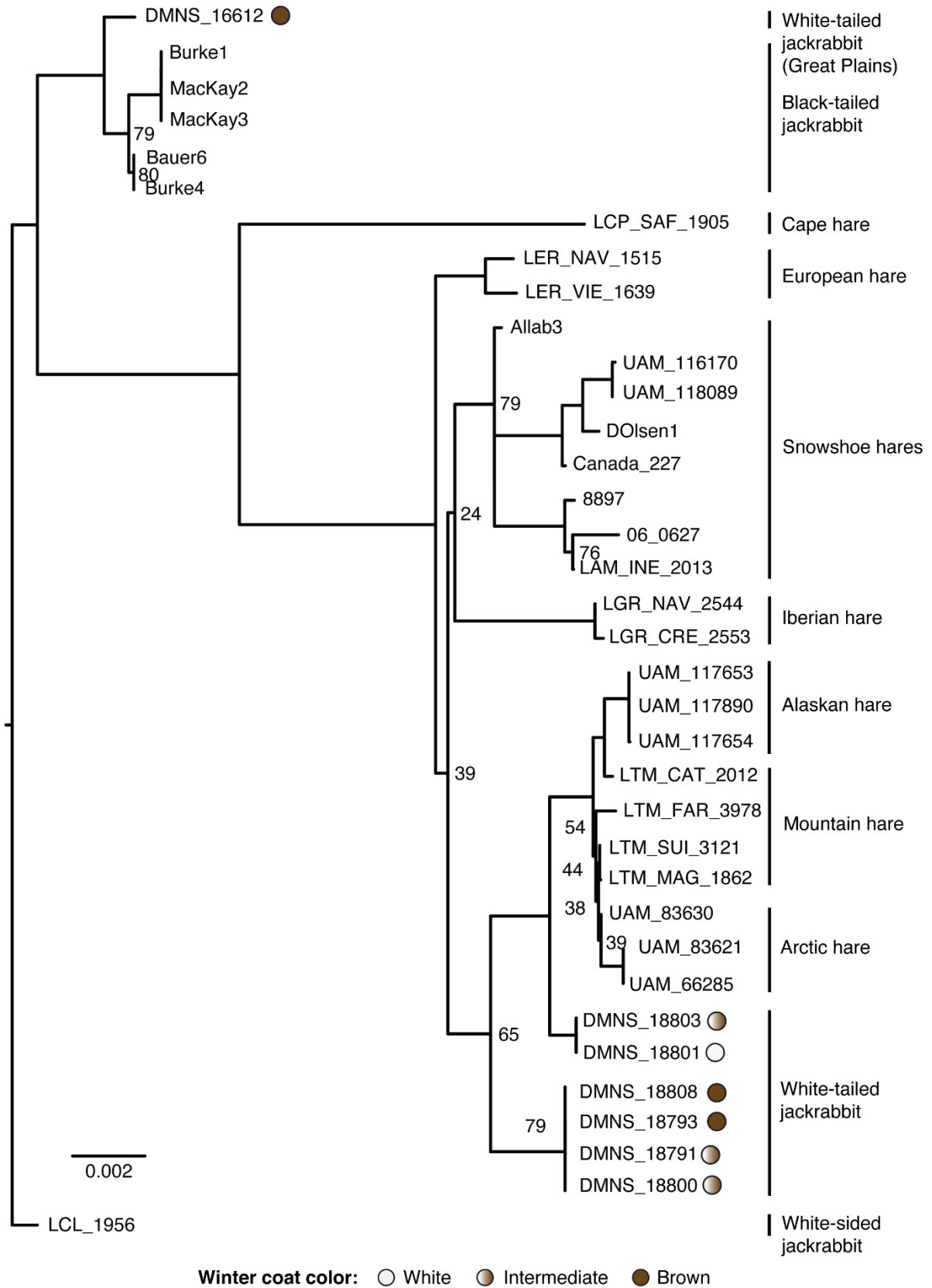
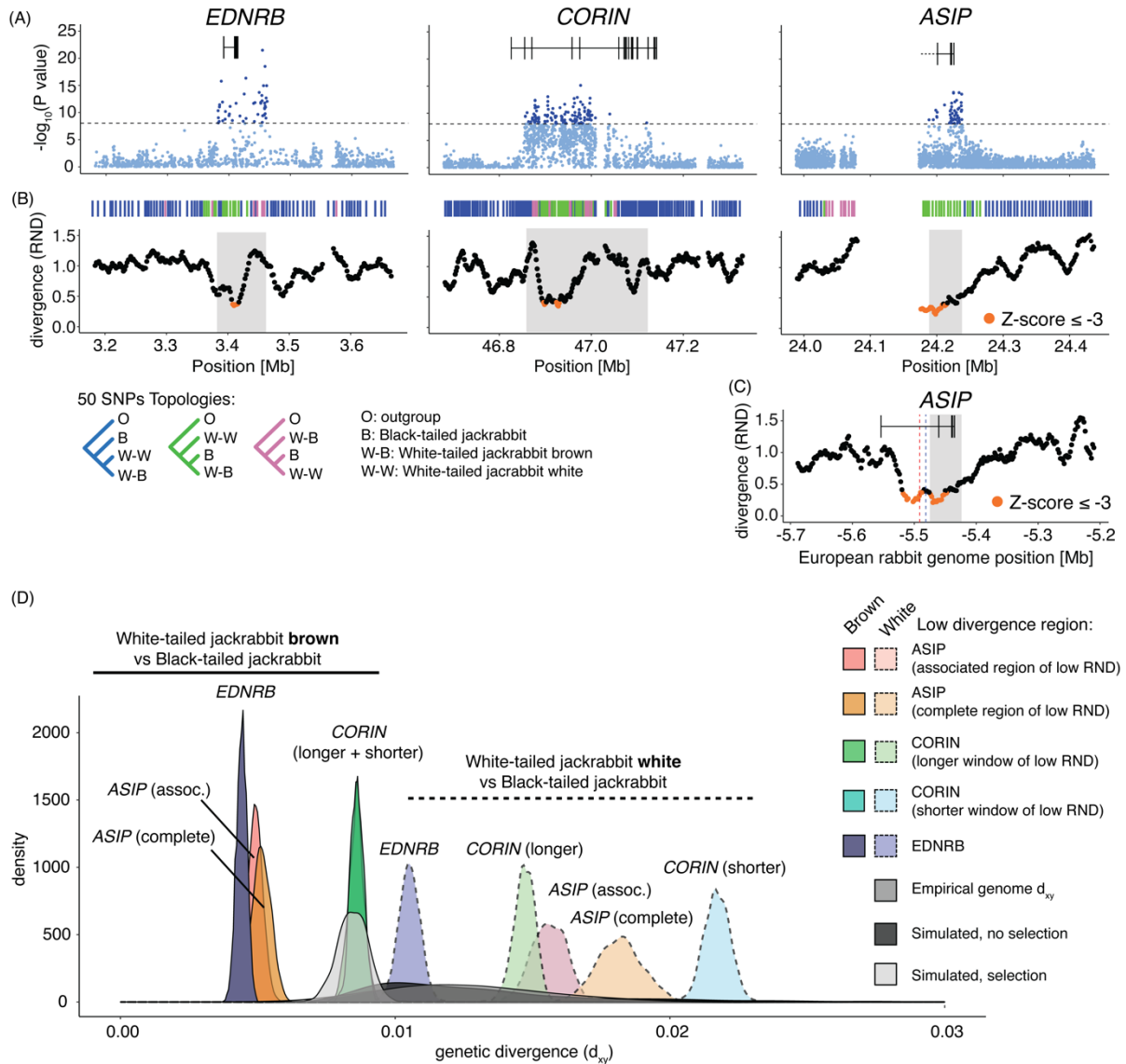


Figure S3.23 – Maximum likelihood tree of the scaffold 380 association region (380:35250713-35322089). Alignments were generated from high-coverage data mapped to species specific pseudo-references in the coordinates of the white-tailed jackrabbit genome. Node numbers represent bootstrap support lower than 80/100.



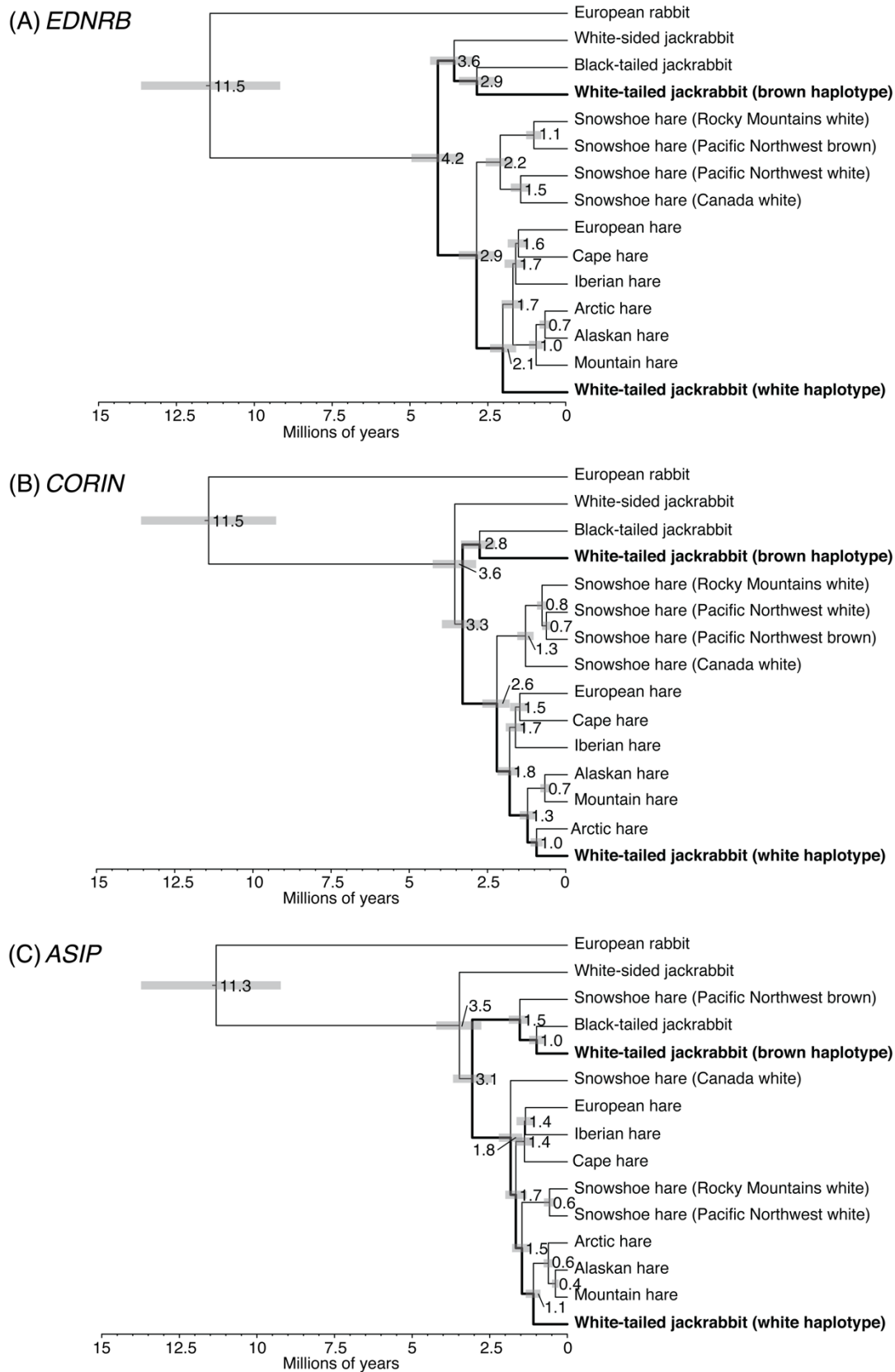


Figure S3.25 – Bayesian inference trees with divergence time estimates (millions of years) for the “white” and “brown” haplotypes of the three regions associated with winter coat color variation in white-tailed jackrabbits. One randomly phased sequence per species is included to provide context for the divergence of the “brown” and “white” haplotypes within the hare (*Lepus* spp.) radiation.

White-tailed jackrabbit (White) vs :

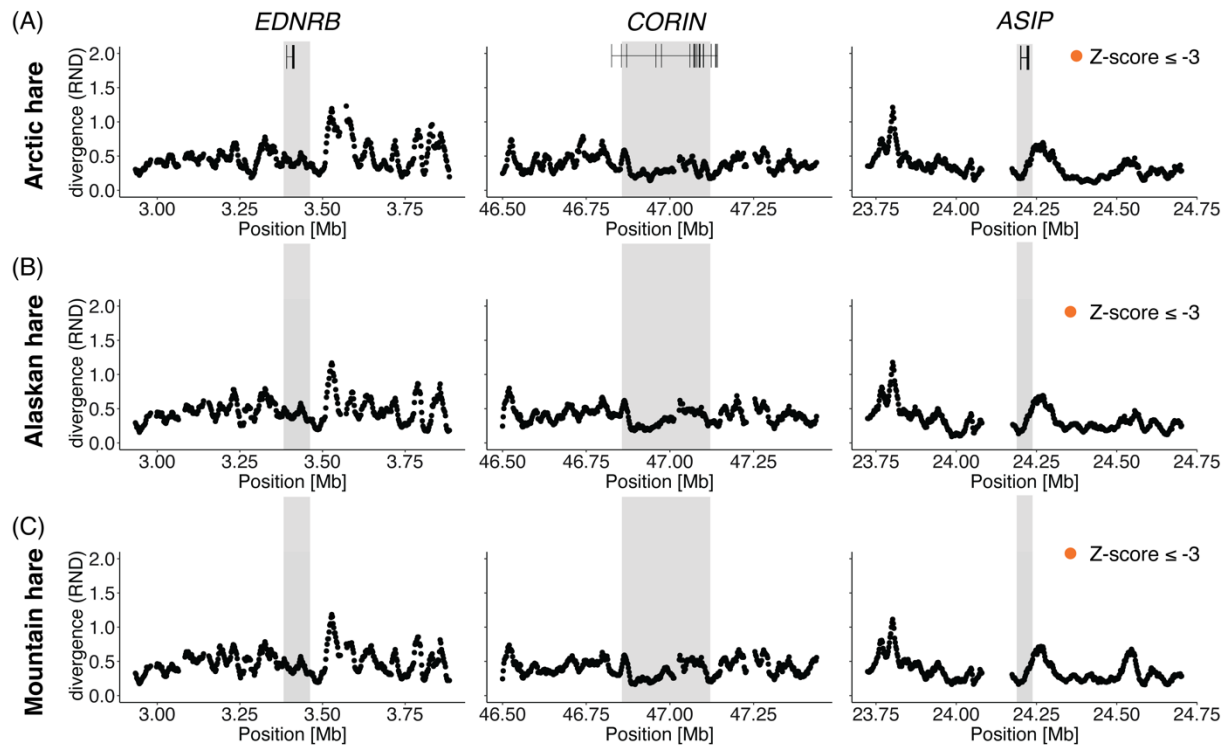


Figure S3.26 – Divergence between winter white white-tailed jackrabbits and closely related seasonal coat color changing species across the regions associated with winter coat color variation. Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) across the *EDNRB* (left panel), *CORIN* (middle panel) and *ASIP* (right panel) association regions (light gray rectangles; 311:3382036-3468296, 342:46856651-47120426 and 245:24188393-24237751, respectively). Scans were performed with whole-genome consensus sequences in the coordinates of the white-tailed jackrabbit genome of one white-tailed jackrabbit (DMNS_18801), **(A)** one Arctic hare (UAM_83621), **(B)** one Alaskan hare (UAM_117654) and **(C)** one mountain hare (LTM_CAT_2012). The white-sided jackrabbit (LCL_1956) was used as the outgroup.

White-tailed jackrabbit (White) vs Snowshoe hares

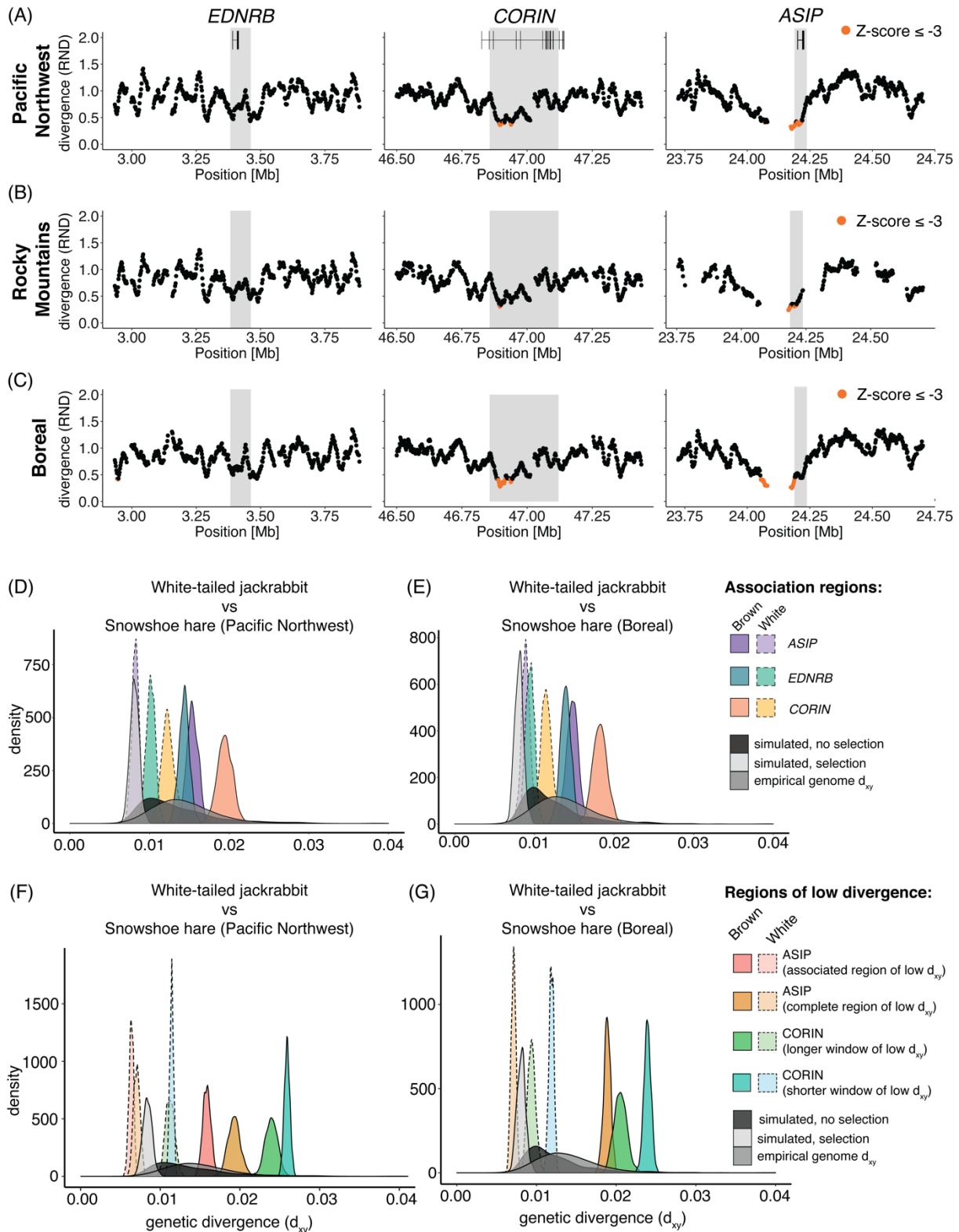


Figure S3.27 – Divergence between winter white white-tailed jackrabbits and snowshoe hare populations across the regions associated with winter coat color variation. Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) across the *EDNRB* (left panel), *CORIN* (middle panel) and *ASIP* (right panel) association regions (light gray rectangles). Scans were performed with whole-genome consensus sequences in the coordinates of the white-tailed jackrabbit genome of one white-tailed jackrabbit (DMNS_18801), **(A)** one Pacific Northwest snowshoe hare (LAM_INE_2013), **(B)** one Rocky Mountains hare (DOlsen1) and **(C)** one Boreal snowshoe hare (Canada_227). The white-sided jackrabbit (LCL_1956) was used as the outgroup. **(D-E)** Empirical and simulated distributions of d_{xy} genome-wide or for the *EDNRB*, *CORIN* and *ASIP* association regions in this

study between winter white or winter brown white-tailed jackrabbit and **(D)** Pacific Northwest snowshoe hares or **(E)** Boreal snowshoe hares. **(F-G)** Empirical and simulated distributions of d_{xy} genome-wide or for the regions of low divergence between species within or overlapping with the association regions. In **(F)** we plot empirical distributions of d_{xy} for a region of low divergence overlapping with the *ASIP* association region (245:24188393-24216000), the region of low divergence truncated by the start of the *ASIP* association region (245:24164001-24216000) and two non-overlapping regions of low divergence inside *CORIN*'s association region (342:46884001-46922000 and 342:46928001-46950000). In **(G)** we plot a region of low divergence overlapping with the *ASIP* association region [245:24044001-24198000; truncating this region by the start of the association region results in an alignment too short (< 20 kb) to generate a distribution] and two regions inside the *CORIN* association region (342:46876001-46926000 and 342:46926001-46950000). In panels **(D-G)** the distributions of d_{xy} were generated with coalescent simulations of the demographic history of the species pairs considered with and without strong selection in the ancestral population.

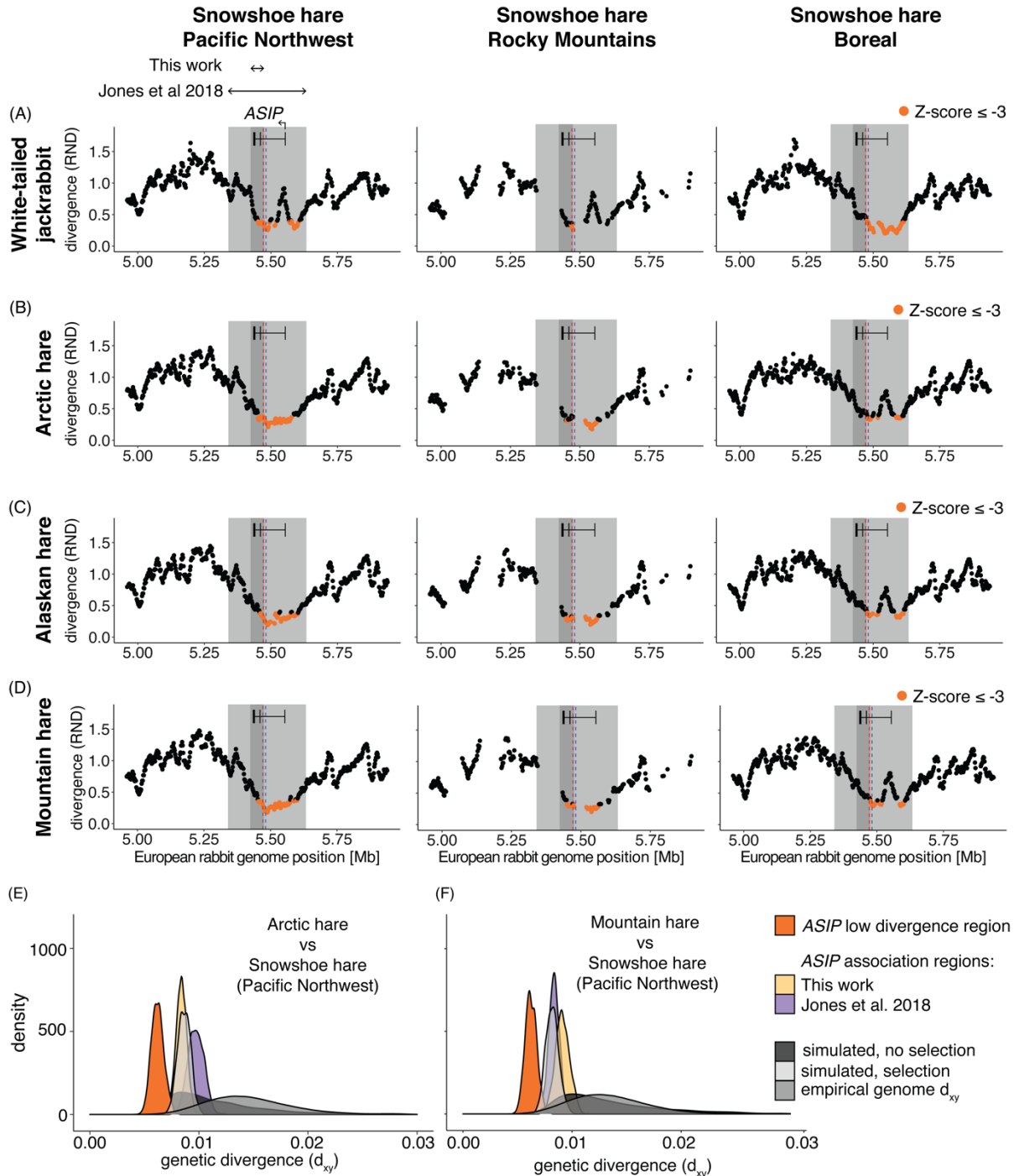
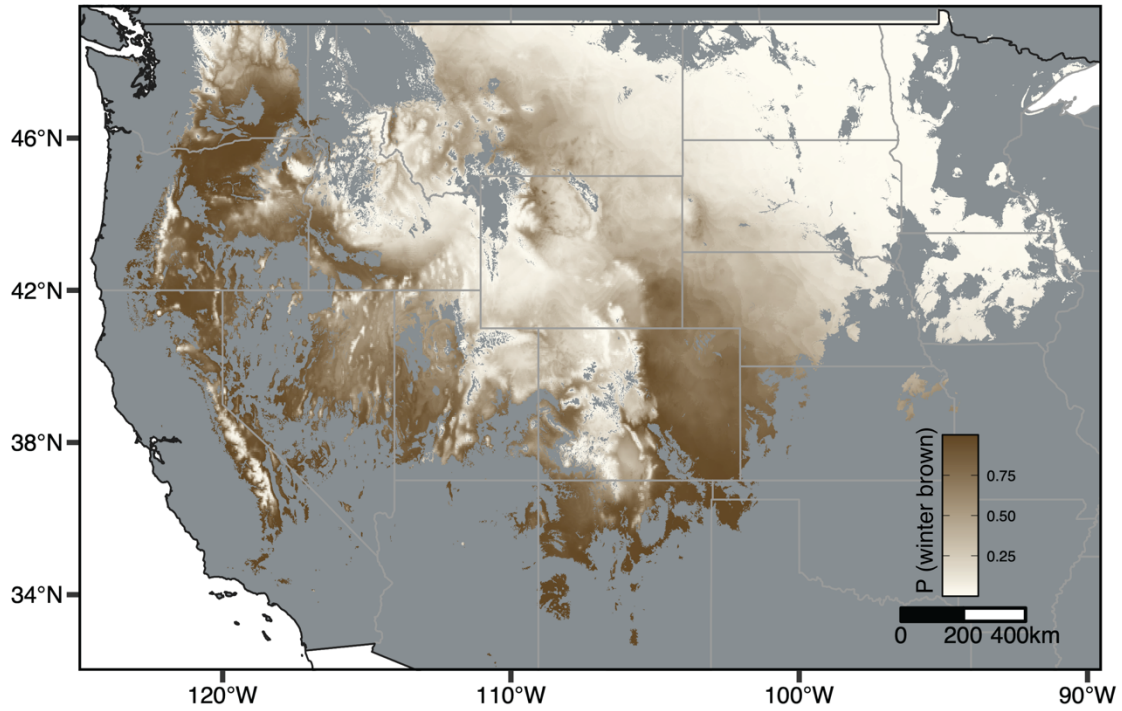


Figure S3.28 – Divergence between snowshoe hare populations and other coat color changing species in this study across the *ASIP* association region. Scaled absolute divergence (RND) in 20 kb sliding windows (2 kb step size) across the *ASIP* association region in this work (4:5423743-5474836, dark-gray narrow rectangle) or winter coat color association region from Jones et al. (2018) (4:5340275-5633020, light-gray rectangle). Scans were performed with whole-genome consensus sequences in the coordinates of the European rabbit genome of one (A) white-tailed jackrabbit (DMNS_18801), (B) one Arctic hare (UAM_83621), (C) one Alaskan hare (UAM_117654) and (D) one mountain hare (LTM_MAG_1862). The snowshoe hare individuals used were LAM_INE_2013, DOlsen1 and Canada_227, left to right. The white-sided jackrabbit (LCL_1956) was used as an outgroup. Red and blue dashed lines highlight the position of a white-specific 1,033 bp insertion and of a brown-specific 43 kb deletion, respectively, identified in Jones et al. (2018). (E-F) Simulated and empirical distributions of d_{xy} between Pacific Northwest snowshoe hares and (D) Arctic hares or (E) mountain hares, the pairs showing stronger reduction in divergence. For each pair we show empirical distributions of d_{xy} genome-wide, for the *ASIP* association region in this work (245:24188393-24237751), the *ASIP* association region in Jones et al. (2018) and the region of low divergence between species overlapping with the regions of association (4:5442001-5588000 in Arctic hares and 4:5444001-5610000 in mountain hares). The simulated distributions of d_{xy} were generated with coalescent simulations of the demographic history of the species pairs with and without strong selection in the ancestral population.

(A) Current Prob(Brown)



(B) Future Prob(Brown)

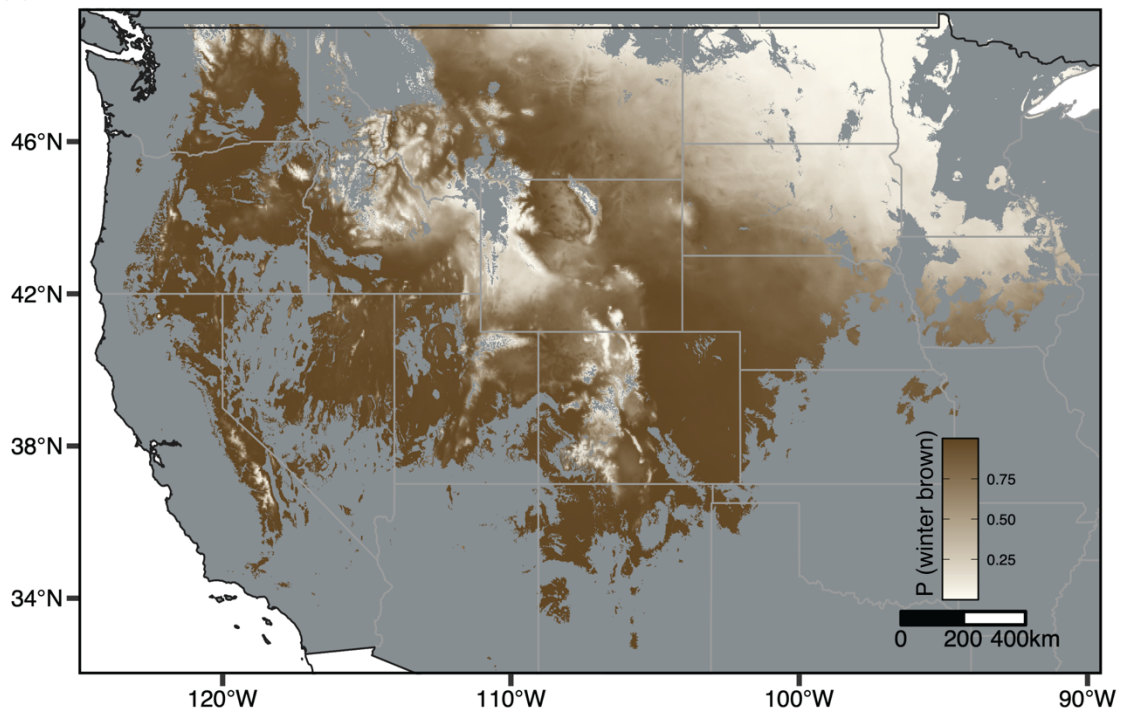


Figure S3.29 – Current (A) and future (B) predicted probability of winter brown coats using current and forecasted (2080s) distributions for mean diurnal temperature range (BIO2), isothermally (BIO3) and snow residence time (proxy of the snow cover duration variable used in the model of Figure 3.1) under a high CO₂ emissions scenario (RCP8.5).

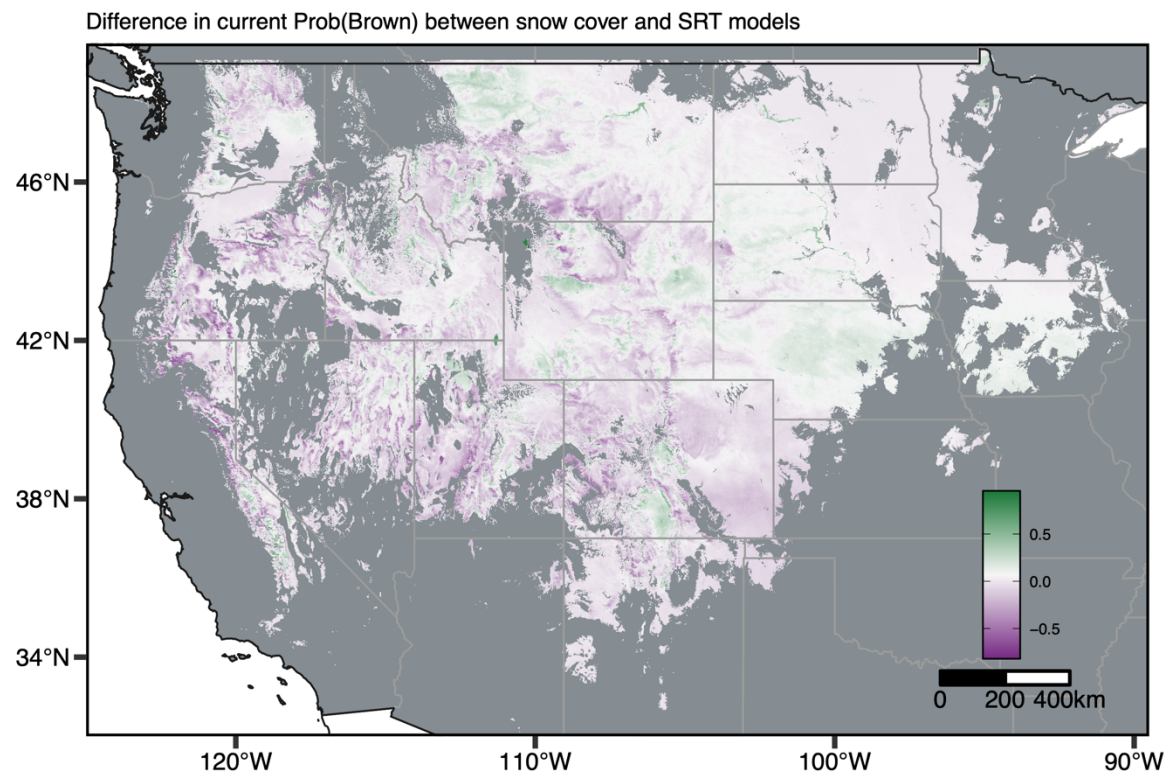


Figure S3.30 – Difference in the current distribution of probability of winter brown coats predicted with generalized linear models using current distributions of mean diurnal temperature range (BIO2), isothermally (BIO3) and either snow cover duration or snow residence time across the United States of America.

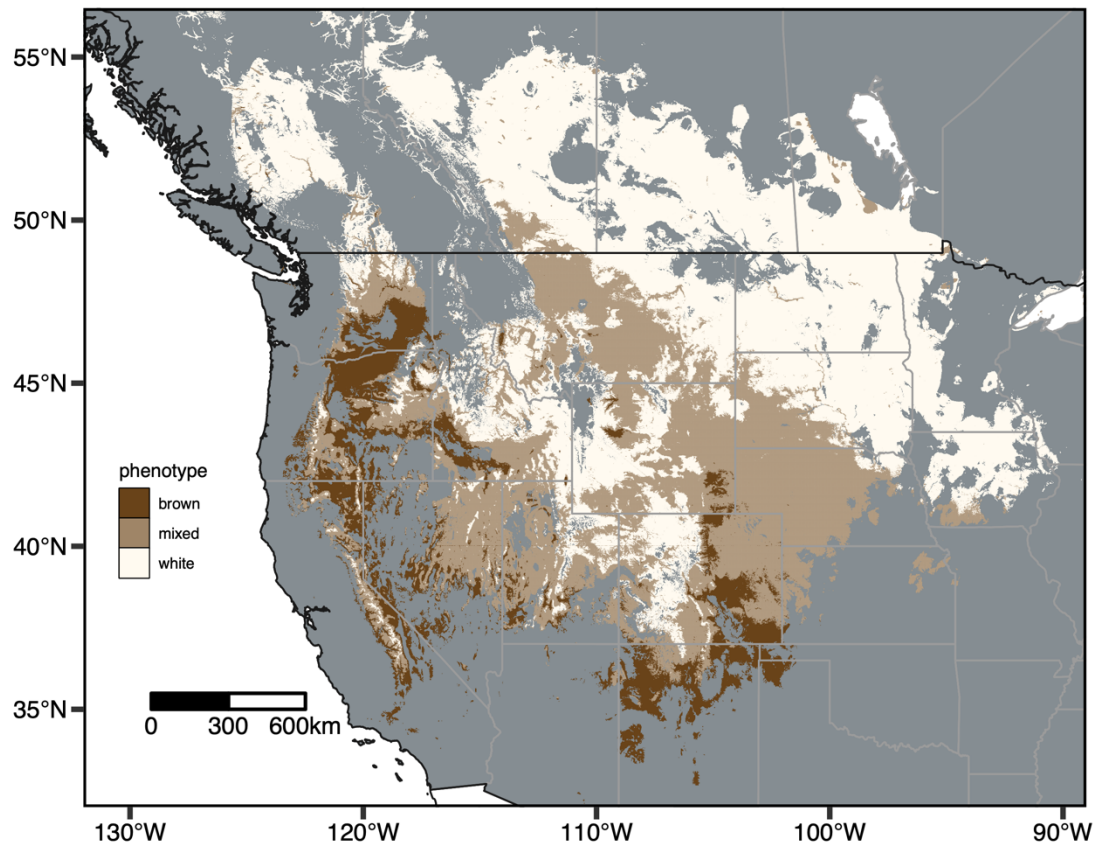


Figure S3.31 – Distribution of winter brown, winter white and polymorphic zones in coat color (mixed or intermediate) across the white-tailed jackrabbit range, defining polymorphic zones as $0.2 \leq P(\text{brown}) \leq 0.8$ following Mills et al. (2018) broad definition of polymorphic zone. Probability of winter brown was predicted using a generalized linear model with current distributions of mean diurnal temperature range (BIO2), isothermality (BIO3) and snow cover duration (Figure 3.1).

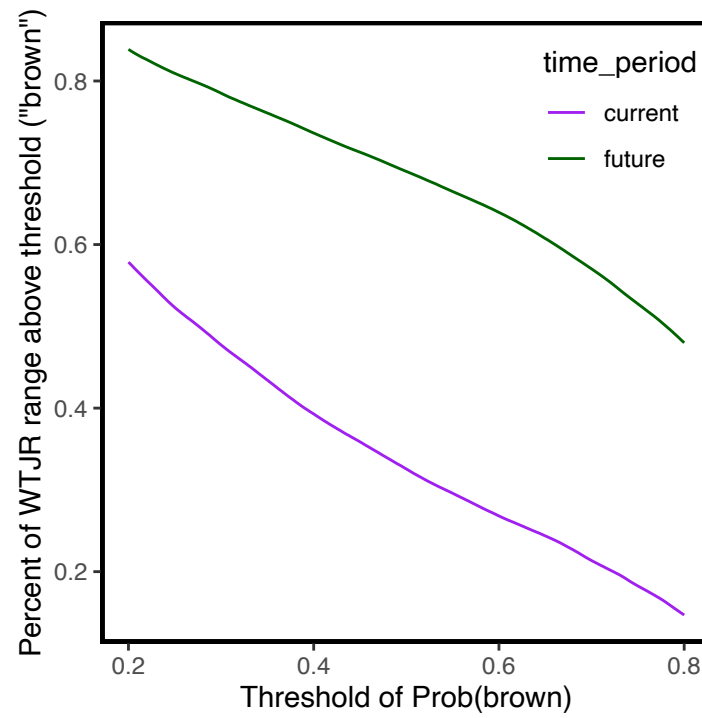


Figure S3.32 – The predicted range of winter brown white-tailed jackrabbits (WTJR) is always higher under forecasted (green line) than in current (purple line) conditions of mean diurnal temperature range (BIO2), isothermally (BIO3) and snow residence time, no matter the threshold of P(brown) used to classify an area as winter brown.

3.5.4. Supplementary Tables

Table S3.1 – Parameters of two generalized linear models predicting probability of winter brown across the distribution of white-tailed jackrabbits using mean diurnal temperature range (BIO2), isothermally (BIO3) and **(A)** snow cover duration or **(B)** snow residence time (SRT) as proxy of snow cover.

Term	Estimate (Beta)	Standard Error	Statistic	P value
A) Snow cover duration				
(Intercept)	4.11	2.30	1.79	0.07
snow cover	0.04	0.01	6.21	5.21x10 ⁻¹⁰
BIO2	0.05	0.02	2.66	0.01
BIO3	-0.36	0.09	-3.98	6.99x10 ⁻⁰⁵
B) Snow residence time				
(Intercept)	3.36	2.47	1.36	0.17
SRT	0.08	0.01	6.36	2.05x10 ⁻¹⁰
BIO2	0.03	0.02	1.89	0.06
BIO3	-0.31	0.09	-3.36	7.76x10 ⁻⁰⁴

Table S3.2 – Performance parameters for the two generalized linear models predicting the probability of winter brown across the distribution of white-tailed jackrabbits using mean diurnal temperature range (BIO2), isothermally (BIO3) and **(A)** snow cover duration or **(B)** snow residence time (SRT) as proxy of snow cover.

Null deviance	Df. Null	Log likelihood	AIC	BIC	Deviance	Df. Residual	No. of observations	Pseudo R ²
A) Snow cover duration								
267.70	195	-92.64	193.4	206.39	185.28	192	196	0.31
B) Snow residence time								
266.58	194	-93.30	194.60	207.69	186.60	191	195	0.30

Df. Null, degrees of freedom of the null model; AIC, Akaike Information Criteria; BIC, Bayesian Information Criteria; Df. Residual, degrees of freedom of the residuals.

Table S3.3 – Sampling information for specimens used to obtain genetic data.

Sample ID	Species	Museum voucher	Origin	Location (Population)	Lat.	Long.	Data
AMNH_123030	<i>Lepus townsendii</i>	M-123030	AMNH	USA/Colorado	37.33	-107.94	L/ G
DMNS_5736	<i>Lepus townsendii</i>	DMNS:Mamm:5736	DMNS	USA/Colorado	40.17	-105.10	L/ P
DMNS_1179	<i>Lepus townsendii</i>	DMNS:Mamm:1179	DMNS	USA/Colorado	40.86	-102.80	L/ G/ P
DMNS_1679	<i>Lepus townsendii</i>	DMNS:Mamm:1679	DMNS	USA/Colorado	39.37	-104.86	L/ G/ P
DMNS_1704	<i>Lepus townsendii</i>	DMNS:Mamm:1704	DMNS	USA/Colorado	37.83	-105.97	L/ G/ P
DMNS_1705	<i>Lepus townsendii</i>	DMNS:Mamm:1705	DMNS	USA/Colorado	37.83	-105.97	L/ G/ P
DMNS_1774	<i>Lepus townsendii</i>	DMNS:Mamm:1774	DMNS	USA/Colorado	37.83	-105.97	L/ P
DMNS_1775	<i>Lepus townsendii</i>	DMNS:Mamm:1775	DMNS	USA/Colorado	37.83	-105.97	L/ G/ P
DMNS_1776	<i>Lepus townsendii</i>	DMNS:Mamm:1776	DMNS	USA/Colorado	37.83	-105.97	L/ G/ P
DMNS_2245	<i>Lepus townsendii</i>	DMNS:Mamm:2245	DMNS	USA/Colorado	40.49	-107.26	L/ G/ P
DMNS_4319	<i>Lepus townsendii</i>	DMNS:Mamm:4319	DMNS	USA/Colorado	39.22	-105.97	L/ G/ P
DMNS_3109	<i>Lepus townsendii</i>	DMNS:Mamm:3109	DMNS	USA/Colorado	39.15	-105.72	L/ G/ P
DMNS_3112	<i>Lepus townsendii</i>	DMNS:Mamm:3112	DMNS	USA/Colorado	37.21	-106.21	L/ G/ P
DMNS_3114	<i>Lepus townsendii</i>	DMNS:Mamm:3114	DMNS	USA/Colorado	40.90	-104.80	L/ G/ P
DMNS_3115	<i>Lepus townsendii</i>	DMNS:Mamm:3115	DMNS	USA/Colorado	40.90	-104.80	L/ G/ P
DMNS_3120	<i>Lepus townsendii</i>	DMNS:Mamm:3120	DMNS	USA/Colorado	40.56	-106.44	L/ G/ P
DMNS_3122	<i>Lepus townsendii</i>	DMNS:Mamm:3122	DMNS	USA/Colorado	40.90	-104.80	L/ G/ P
DMNS_3128	<i>Lepus townsendii</i>	DMNS:Mamm:3128	DMNS	USA/Colorado	40.46	-104.46	L/ G/ P
DMNS_3129	<i>Lepus townsendii</i>	DMNS:Mamm:3129	DMNS	USA/Colorado	38.81	-106.21	L/ G/ P
DMNS_3132	<i>Lepus townsendii</i>	DMNS:Mamm:3132	DMNS	USA/Colorado	39.75	-104.61	L/ G/ P
DMNS_3133	<i>Lepus townsendii</i>	DMNS:Mamm:3133	DMNS	USA/Colorado	39.75	-104.61	L/ G/ P
DMNS_3134	<i>Lepus townsendii</i>	DMNS:Mamm:3134	DMNS	USA/Colorado	39.75	-104.61	L/ G/ P
DMNS_3135	<i>Lepus townsendii</i>	DMNS:Mamm:3135	DMNS	USA/Colorado	40.00	-102.42	L/ G/ P
DMNS_3138	<i>Lepus townsendii</i>	DMNS:Mamm:3138	DMNS	USA/Colorado	38.42	-105.61	L/ G/ P
DMNS_3141	<i>Lepus townsendii</i>	DMNS:Mamm:3141	DMNS	USA/Colorado	38.06	-105.36	L/ G/ P
DMNS_3146	<i>Lepus townsendii</i>	DMNS:Mamm:3146	DMNS	USA/Colorado	40.12	-106.18	L/ G/ P
DMNS_3163	<i>Lepus townsendii</i>	DMNS:Mamm:3163	DMNS	USA/Colorado	40.62	-108.20	L/ G/ P
DMNS_3168	<i>Lepus townsendii</i>	DMNS:Mamm:3168	DMNS	USA/Colorado	38.94	-103.28	L/ G/ P
DMNS_3182	<i>Lepus townsendii</i>	DMNS:Mamm:3182	DMNS	USA/Colorado	39.74	-104.32	L/ G/ P
DMNS_4070	<i>Lepus townsendii</i>	DMNS:Mamm:4070	DMNS	USA/Colorado	39.74	-104.32	L/ G/ P
DMNS_4086	<i>Lepus townsendii</i>	DMNS:Mamm:4086	DMNS	USA/Colorado	39.76	-104.43	L/ G/ P
DMNS_4087	<i>Lepus townsendii</i>	DMNS:Mamm:4087	DMNS	USA/Colorado	39.76	-104.43	L/ G/ P
DMNS_4088	<i>Lepus townsendii</i>	DMNS:Mamm:4088	DMNS	USA/Colorado	39.76	-104.43	L/ G/ P
DMNS_4089	<i>Lepus townsendii</i>	DMNS:Mamm:4089	DMNS	USA/Colorado	39.76	-104.43	L/ G/ P
DMNS_4312	<i>Lepus townsendii</i>	DMNS:Mamm:4312	DMNS	USA/Colorado	39.63	-106.04	L/ G/ P
DMNS_4313	<i>Lepus townsendii</i>	DMNS:Mamm:4313	DMNS	USA/Colorado	39.63	-106.04	L/ G/ P
DMNS_5676	<i>Lepus townsendii</i>	DMNS:Mamm:5676	DMNS	USA/Colorado	39.38	-105.80	L/ G/ P
DMNS_5677	<i>Lepus townsendii</i>	DMNS:Mamm:5677	DMNS	USA/Colorado	39.38	-105.80	L/ G/ P
DMNS_5678	<i>Lepus townsendii</i>	DMNS:Mamm:5678	DMNS	USA/Colorado	39.38	-105.80	L/ G/ P
DMNS_5679	<i>Lepus townsendii</i>	DMNS:Mamm:5679	DMNS	USA/Colorado	39.38	-105.80	L/ G/ P
DMNS_5680	<i>Lepus townsendii</i>	DMNS:Mamm:5680	DMNS	USA/Colorado	39.38	-105.80	L/ G/ P
DMNS_5681	<i>Lepus townsendii</i>	DMNS:Mamm:5681	DMNS	USA/Colorado	39.38	-105.80	L/ G/ P
DMNS_5682	<i>Lepus townsendii</i>	DMNS:Mamm:5682	DMNS	USA/Colorado	39.38	-105.80	L/ G/ P
DMNS_16612	<i>Lepus townsendii</i>	DMNS:Mamm:16612	DMNS	USA/Colorado	39.86	-104.67	L/ H/ G
DMNS_18790	<i>Lepus townsendii</i>	DMNS:Mamm:18790	Field	USA/Colorado	38.23	-106.76	L/ G/ P

The evolution and molecular bases of seasonal coat color variation in hares:
gene expression and the shared contribution of ancestral polymorphism and introgression

DMNS_18791	<i>Lepus townsendii</i>	DMNS:Mamm:18791	Field	USA/Colorado	38.22	-106.78	L/ H/ G/ P
DMNS_18792	<i>Lepus townsendii</i>	DMNS:Mamm:18792	Field	USA/Colorado	38.19	-106.70	L/ G/ P
DMNS_18793	<i>Lepus townsendii</i>	DMNS:Mamm:18793	Field	USA/Colorado	38.03	-106.21	L/ H/ G/ P
DMNS_18794	<i>Lepus townsendii</i>	DMNS:Mamm:18794	Field	USA/Colorado	38.50	-106.85	L/ G/ P
DMNS_18795	<i>Lepus townsendii</i>	DMNS:Mamm:18795	Field	USA/Colorado	38.50	-106.85	L/ G/ P
DMNS_18796	<i>Lepus townsendii</i>	DMNS:Mamm:18796	Field	USA/Colorado	38.51	-106.84	L/ G/ P
DMNS_18797	<i>Lepus townsendii</i>	DMNS:Mamm:18797	Field	USA/Colorado	38.22	-106.78	L/ G/ P
DMNS_18798	<i>Lepus townsendii</i>	DMNS:Mamm:18798	Field	USA/Colorado	38.23	-106.81	L/ G/ P
DMNS_18799	<i>Lepus townsendii</i>	DMNS:Mamm:18799	Field	USA/Colorado	38.21	-106.80	L/ G/ P
DMNS_18800	<i>Lepus townsendii</i>	DMNS:Mamm:18800	Field	USA/Colorado	38.22	-106.80	L/ H/ G/ P
DMNS_18801	<i>Lepus townsendii</i>	DMNS:Mamm:18801	Field	USA/Colorado	38.51	-106.83	L/ H/ G/ P/PsRef
DMNS_18802	<i>Lepus townsendii</i>	DMNS:Mamm:18802	Field	USA/Colorado	38.22	-106.80	L/ G/ P
DMNS_18803	<i>Lepus townsendii</i>	DMNS:Mamm:18803	Field	USA/Colorado	38.02	-106.20	L/ H/ G/ P
DMNS_18804	<i>Lepus townsendii</i>	DMNS:Mamm:18804	Field	USA/Colorado	38.28	-106.73	L/ G/ P
DMNS_18805	<i>Lepus townsendii</i>	DMNS:Mamm:18805	Field	USA/Colorado	38.19	-106.83	L/ G/ P
DMNS_18806	<i>Lepus townsendii</i>	DMNS:Mamm:18806	Field	USA/Colorado	38.19	-106.74	L/ G/ P
DMNS_18807	<i>Lepus townsendii</i>	DMNS:Mamm:18807	Field	USA/Colorado	38.20	-106.74	L/ 10X/ G/ P
DMNS_18808	<i>Lepus townsendii</i>	DMNS:Mamm:18808	Field	USA/Colorado	37.98	-106.18	L/ H/ G/ P
UCM_1194	<i>Lepus townsendii</i>	UCM:Mamm:1194	UCM	USA/Colorado	39.35	-104.46	L/ G
UCM_1195	<i>Lepus townsendii</i>	UCM:Mamm:1195	UCM	USA/Colorado	39.35	-104.46	G
UCM_13092	<i>Lepus townsendii</i>	UCM:Mamm:13092	UCM	USA/Colorado	40.52	-104.70	G
UCM_13094	<i>Lepus townsendii</i>	UCM:Mamm:13094	UCM	USA/Colorado	40.59	-105.07	G
UCM_7879	<i>Lepus townsendii</i>	UCM:Mamm:7879	UCM	USA/Colorado	38.83	-104.82	L/ G
UCM_7888	<i>Lepus townsendii</i>	UCM:Mamm:7888	UCM	USA/Colorado	38.16	-108.37	L/ G
UCM_7892	<i>Lepus townsendii</i>	UCM:Mamm:7892	UCM	USA/Colorado	38.25	-105.95	L
UCM_7894	<i>Lepus townsendii</i>	UCM:Mamm:7894	UCM	USA/Colorado	40.07	-106.10	G
UCM_7908	<i>Lepus townsendii</i>	UCM:Mamm:7908	UCM	USA/Colorado	37.65	-105.87	G
UCM_7918	<i>Lepus townsendii</i>	UCM:Mamm:7918	UCM	USA/Colorado	38.64	-107.58	G
UCM_7920	<i>Lepus townsendii</i>	UCM:Mamm:7920	UCM	USA/Colorado	38.64	-107.58	G
UCM_7921	<i>Lepus townsendii</i>	UCM:Mamm:7921	UCM	USA/Colorado	38.64	-107.58	G
UMNH_23776	<i>Lepus townsendii</i>	UMNH:Mamm:23776	UMNH	USA/Colorado	38.21	-106.81	L/ G
UMNH_23777	<i>Lepus townsendii</i>	UMNH:Mamm:23777	UMNH	USA/Colorado	38.19	-106.68	L/ G
UMNH_23778	<i>Lepus townsendii</i>	UMNH:Mamm:23778	UMNH	USA/Colorado	38.21	-106.81	L/ G
UMNH_23779	<i>Lepus townsendii</i>	UMNH:Mamm:23779	UMNH	USA/Colorado	38.21	-106.81	L/ G
UMNH_23784	<i>Lepus townsendii</i>	UMNH:Mamm:23784	UMNH	USA/Colorado	38.20	-106.83	L/ G
UMNH_23789	<i>Lepus townsendii</i>	UMNH:Mamm:23789	UMNH	USA/Colorado	38.21	-106.81	G
UMNH_23790	<i>Lepus townsendii</i>	UMNH:Mamm:23790	UMNH	USA/Colorado	38.21	-106.81	L/ G
UMNH_23804	<i>Lepus townsendii</i>	UMNH:Mamm:23804	UMNH	USA/Colorado	38.20	-106.83	G
UMNH_23805	<i>Lepus townsendii</i>	UMNH:Mamm:23805	UMNH	USA/Colorado	38.19	-106.68	L/ G
UMZM_5186	<i>Lepus townsendii</i>	UMZM:Mamm:5186	UMZM	USA/Minnesota			G
UMZM_12459	<i>Lepus townsendii</i>	UMZM:Mamm:12459	UMNH	USA/Montana			G
UMZM_15352	<i>Lepus townsendii</i>	UMZM:Mamm:15352	UMZM	USA/Montana			G
UMZM_15683	<i>Lepus townsendii</i>	UMZM:Mamm:15683	UMZM	USA/Montana			G
UMZM_15911	<i>Lepus townsendii</i>	UMZM:Mamm:15911	UMZM	USA/Montana			G
UMZM_16871	<i>Lepus townsendii</i>	UMZM:Mamm:16871	UMZM	USA/Montana			G

UMZM_19000	<i>Lepus townsendii</i>	UMZM:Mamm:19000	UMZM	USA/Montana			G
UMZM_3610	<i>Lepus townsendii</i>	UMZM:Mamm:3610	UMZM	USA/Montana			G
UMZM_426	<i>Lepus townsendii</i>	UMZM:Mamm:426	UMZM	USA/Montana			G
AMNH_129296	<i>Lepus townsendii</i>	129296	AMNH	USA/North Dakota	48.41	-97.41	G
AMNH_90911	<i>Lepus townsendii</i>	M-90911	AMNH	USA/North Dakota	48.41	-97.41	G
UMZM_14068	<i>Lepus townsendii</i>	UMZM:Mamm:14068	UMZM	USA/North Dakota			G
AMNH_40311	<i>Lepus townsendii</i>	M-40311	AMNH	USA/Oregon	44.32	-117.94	G
AMNH_40312	<i>Lepus townsendii</i>	M-40312	AMNH	USA/Oregon	44.32	-117.94	G
UAM_116170	<i>Lepus americanus</i>	UAM:Mamm:116170	Ref 1	USA/Alaska (Boreal)	58.69	-156.69	H
06_0627	<i>Lepus americanus</i>		Ref 2	Canada/ British Colombia (Pacific Northwest)			H
1_8897	<i>Lepus americanus</i>		Ref 2	USA/Oregon (Pacific Northwest)			H
Canada_227	<i>Lepus americanus</i>		Ref 2	Canada (Boreal)			H
UAM_118089	<i>Lepus americanus</i>	UAM:Mamm:118089	Ref 2	USA/ Alaska (Boreal)	59.40	-161.73	H
Allab3	<i>Lepus americanus</i>		Ref 2	USA/Pennsylvania (Boreal)			H
LAM_INE_2013	<i>Lepus americanus</i>		Ref 2	USA/Montana (Pacific Northwest)			H/ PsRef
DOlsen1	<i>Lepus americanus</i>		Ref 3	USA/Utah (Rocky Mountains)			H
UAM_83630	<i>Lepus arcticus</i>	UAM:Mamm:83630	UAM	Canada/ Nunavut	76.42	-82.90	H
UAM_66285	<i>Lepus arcticus</i>	UAM:Mamm:66285	UAM	Canada/ Nunavut	62.85	-69.87	H
UAM_83621	<i>Lepus arcticus</i>	UMA:Mamm:83621	UAM	Canada/ Quebec	58.1	-68.4	H/ PsRef
Burke1	<i>Lepus californicus</i>		Ref 2	USA/ Oregon			H
Burke4	<i>Lepus californicus</i>		Ref 2	USA/ Oregon			H
MacKay2	<i>Lepus californicus</i>		Ref 2	USA/ Nevada			H
MacKay3	<i>Lepus californicus</i>		Ref 3	USA/ Nevada			H/ PsRef
Bauer6	<i>Lepus californicus</i>		Ref 2	USA/ California			H
LCL_1956	<i>Lepus callotis</i>		CIBIO- InBIO	Mexico			H/ PsRef
LCP_SAF_1905	<i>Lepus capensis</i>		CIBIO- InBIO	South Africa			H/ PsRef
LER_NAV_1515	<i>Lepus europaeus</i>		Ref 4	Spain/Navarra			H/ PsRef
LER_VIE_1639	<i>Lepus europaeus</i>		Ref 4	Austria/Vienna			H
LGR_CRE_2553	<i>Lepus granatensis</i>		Ref 5	Spain/Ciudad Real			H
LGR_NAV_2544	<i>Lepus granatensis</i>		Ref 5	Spain/Navarra			H/ PsRef
UAM_117890	<i>Lepus othus</i>	UAM:Mamm:117890	UAM	USA/Alaska	59.45	-161.70	H
UAM_117653	<i>Lepus othus</i>	UAM:Mamm:117653	UAM	USA/Alaska	59.45	-161.70	H
UAM_117654	<i>Lepus othus</i>	UAM:Mamm:117654	UAM	USA/Alaska	59.45	-161.71	H/ PsRef
LTM_MAG_1862	<i>Lepus timidus</i>		CIBIO- InBIO	Russia/Magadan			H
LTM_FAR_3978	<i>Lepus timidus</i>		Ref 6	Faroe Islands/Signabør			H
LTM_CAT_2012	<i>Lepus timidus</i>		Ref 5	Finland/ Captivity			H/ PsRef
LTM_SUI_3121	<i>Lepus timidus</i>		CIBIO- InBIO	Switzerland/ Alps			H

Museums abbreviations: DMNS, Denver Museum of Nature and Science; UCM, University of Colorado Museum of Natural History; AMNH, American Museum of Natural History, UMNH, Utah Museum of Natural History, UMZM, Philip L. Wright Zoological Museum; UAM, University of Alaska Museum. References: Ref 1, Jones et al. (2020), Evolution; Ref 2, Jones et al. (2020), Am Nat; Ref 3, Jones et al. (2018), Science; Ref 4, Seixas (2017), PhD Thesis; Ref 5, Seixas et al. (2018), Genome Biol; Ref 6, Giska et al. (2019), PNAS; Data abbreviations: L, Low-coverage whole-genome data; H, High-coverage whole-genome data; 10 X, Chromium linked-read data; G, MassARRAY® genotype data; P, Dorsal reflectance measurements; PsRef, used to generate a species-specific pseudo-reference. Species: *L. townsendii*, white-tailed jackrabbit; *L. americanus*, snowshoe hare; *L. arcticus*,

The evolution and molecular bases of seasonal coat color variation in hares:
gene expression and the shared contribution of ancestral polymorphism and introgression

Arctic hare; *L. californicus*, black-tailed jackrabbits; *L. callotis*, white-sided jackrabbit; *L. capensis*, Cape hare; *L. europaeus*, European hare; *L. granatensis*, Iberian hare; *L. othus*, Alaskan hare; *L. timidus*, mountain hare.

Table S3.4 – Chromium linked-read data and assembly statistics. Statistics marked with an asterisk (*) were obtained from *Supernova* and statistics marked with a plus (+) sign were obtained from *QUAST*.

Input read data	
Number of Reads *	1265,98 million
Mean read length *	139.50 bps
Raw coverage *	62.50 X
Effective read coverage *	44.63 X
Fraction of Q30 bases in read 2 *	88.13%
Median insert size *	337.00 bps
Fraction of proper read pairs *	90.77%
Fraction of barcodes used *	1
Estimated genome size *	3.06 Gbs
Genome repetitivity index *	9.08%
High AT index *	0.09%
GC content of assembly *	43.76%
Dinucleotide content *	0.33%
Weighted mean molecule size *	141.44 Kbs
Molecule count extending 10 kbs on both sides *	235.65
Mean distanced between heterozygous SNPs *	247.00 bps
Fraction of reads that are not barcoded *	8.62%
N50 reads per barcode *	1,090
Fraction of reads that are duplicates *	18.05%
Nonduplicate and phased reads *	53.81%
Output assembly statistics	
Long scaffolds *	4,660
N50 edge size *	18.53 Kbs
N50 contig size *	104.25 Kbs
N50 phase block size *	19.52 Mbs
N50 scaffold size *	48.03 Mbs
Percentage of base assembly missing from scaffolds *	4.44%
Assembly size (only scaffolds >=10 kbs) *	2.53 Gbs
Number of contigs (>= 0 bps) +	45,933
Number of contigs (>= 1,000 bps) +	45,933
Number of contigs (>= 5,000 bps) +	1,2329
Number of contigs (>= 10,000 bps) +	4,712
Number of contigs (>= 25,000 bps) +	1,084
Number of contigs (>= 50,000 bps) +	523
Largest contig +	113,291,978
Total length +	28.01 Gbs
GC (%) +	43.70
Number of missing (N's) per 100 kbs +	5270.77

bps, base pairs; Gbs, Gigabase pairs; Kbs, Kilobase pairs; Mbs, Megabase pairs

Table 3.5 – Sequencing statistics for low coverage whole-genome data of 74 white-tailed jackrabbit individuals from Colorado. Each sample was mapped to a white-tailed jackrabbit pseudo-reference in the coordinates of the European rabbit genome (66 samples) or to the *de novo* assembled white-tailed jackrabbit genome (74 samples). Individuals marked with an "I" were alternatively classified as having an intermediate coat color. NA, individual not mapped to the white-tailed jackrabbit pseudo-reference.

Sample ID	Color classification	Raw Reads	White-tailed jackrabbit pseudo-reference	White-tailed jackrabbit genome
AMNH_123030	White	169429981	1.7	2.0
DMNS_1179	Brown	256427729	2.3	2.6
DMNS_16612	Brown	13701435	1.1	1.2
DMNS_1679	Brown	99289394	NA	0.8
DMNS_1704	Brown	585443400	3.1	4.9
DMNS_1705	Brown	168026511	2.1	2.3
DMNS_1774	Brown	84180426	NA	0.6
DMNS_1775	Brown	95501928	NA	0.7
DMNS_1776	Brown	200311266	1.6	1.9
DMNS_18790	White(I)	25582777	2.1	2.3
DMNS_18791	White(I)	24601295	2.0	2.2
DMNS_18792	White(I)	18767659	1.5	1.7
DMNS_18793	Brown	12877146	1.0	1.2
DMNS_18794	White	22343109	1.8	2.0
DMNS_18795	White	13334481	1.1	1.2
DMNS_18796	White	19158462	1.5	1.7
DMNS_18797	White	12704083	1.0	1.2
DMNS_18798	White	22108893	1.9	2.0
DMNS_18799	White	20686569	1.7	1.9
DMNS_18800	White	19844224	1.6	1.8
DMNS_18801	White	11420186	0.9	1.0
DMNS_18802	White	18720862	1.5	1.7
DMNS_18803	White(I)	16503228	1.4	1.5
DMNS_18804	White(I)	16450649	1.3	1.5
DMNS_18805	White	12620722	1.0	1.1
DMNS_18806	White	26862511	2.2	2.4
DMNS_18807	White(I)	34305248	2.7	3.0
DMNS_18808	Brown	15084876	1.2	1.4
DMNS_2245	White	128480397	1.5	1.8
DMNS_3109	White(I)	68822719	1.3	1.5
DMNS_3112	Brown	48317387	1.0	1.1
DMNS_3114	Brown(I)	133743773	2.4	2.7
DMNS_3115	Brown(I)	106680935	2.0	2.2
DMNS_3120	White	52590295	1.3	1.4
DMNS_3122	Brown(I)	62059467	1.5	1.7
DMNS_3128	Brown	79218006	NA	1.2
DMNS_3129	Brown	115348088	NA	2.9
DMNS_3132	Brown(I)	73918231	1.5	1.6
DMNS_3133	Brown	74201845	1.4	1.6
DMNS_3134	Brown	43791892	1.1	1.3
DMNS_3135	Brown(I)	47630371	1.1	1.2
DMNS_3138	White(I)	64581014	NA	1.2
DMNS_3141	White	45339342	1.1	1.2
DMNS_3146	White	63742183	1.2	1.4
DMNS_3163	White	60042967	1.2	1.4
DMNS_3168	Brown	61550264	1.3	1.5
DMNS_3182	Brown	68341038	1.6	1.7
DMNS_4070	Brown	36101494	1.0	1.1
DMNS_4086	Brown	66102247	1.7	1.9
DMNS_4087	Brown	92935497	2.3	2.6

DMNS_4088	Brown	108414878	NA	2.7
DMNS_4089	Brown(I)	66169300	1.8	2.0
DMNS_4312	White(I)	72520025	1.6	1.7
DMNS_4313	White	60962683	1.4	1.5
DMNS_4319	White	88390888	1.4	1.6
DMNS_5676	Brown(I)	92629126	1.7	2.0
DMNS_5677	Brown(I)	64246210	1.7	1.9
DMNS_5678	White(I)	42637950	1.1	1.2
DMNS_5679	White(I)	54828989	1.2	1.3
DMNS_5680	Brown(I)	60096832	1.5	1.7
DMNS_5681	Brown(I)	58519883	1.4	1.6
DMNS_5682	Brown(I)	64533073	1.9	2.1
DMNS_5736	Brown	105018278	NA	0.7
UCM_1194	Brown	321150465	2.1	2.4
UCM_7879	Brown	225446791	2.1	2.5
UCM_7888	Brown(I)	88955501	1.6	1.8
UCM_7892	Brown	161140510	2.1	2.4
UMNH_23776	White	124707852	1.9	2.1
UMNH_23777	White	85442156	1.5	1.7
UMNH_23778	White(I)	125806703	2.2	2.4
UMNH_23779	White	96428064	1.5	1.7
UMNH_23784	White	125224542	1.6	1.8
UMNH_23790	White(I)	115829936	1.8	2.0
UMNH_23805	White	70185802	1.1	1.2

Table S3.6 – Sequencing statistics for high coverage whole-genome data from 36 individuals of 10 hare species. Each sample was mapped to a pseudo-reference with the coordinates of the white-tailed jackrabbit (WTJR) genome or European rabbit genome.

Species (Population)	Sample ID	Pseudo-reference WTJR coordinates (X coverage)	Pseudo-reference Rabbit coordinates (X coverage)
Alaskan hare	UAM_117653	10.8	9.7
Alaskan hare	UAM_117654	13.2	12.0
Alaskan hare	UAM_117890	11.7	10.7
Arctic hare	UAM_66285	10.4	9.2
Arctic hare	UAM_83621	12.4	11.0
Arctic hare	UAM_83630	7.6	6.9
Black-tailed jackrabbit	Bauer6	12.8	12.2
Black-tailed jackrabbit	Burke1	12.2	11.0
Black-tailed jackrabbit	Burke4	11.7	10.6
Black-tailed jackrabbit	MacKay2	11.5	10.4
Black-tailed jackrabbit	MacKay3	7.9	8.1
Cape hare	LCP_SAF_1905	14.9	13.1
European hare	LER_NAV_1515	13.7	13.6
European hare	LER_VIE_1639	12.6	11.6
Iberian hare	LGR_CRE_2553	23.7	21.9
Iberian hare	LGR_NAV_2544	25.2	23.3
Mountain hare	LTM_CAT_2012	21.0	19.4
Mountain hare	LTM_FAR_3978	7.9	7.4
Mountain hare	LTM_MAG_1862	12.0	10.8
Mountain hare	LTM_SUI_3121	22.7	21.0
Snowshoe hare (Boreal)	Allab3	11.2	10.0
Snowshoe hare (Boreal)	Canada_227	11.7	10.6
Snowshoe hare (Boreal)	UAM_116170	15.5	14.2
Snowshoe hare (Boreal)	UAM_118089	11.1	10.2
Snowshoe hare (PNW)	8897	14.6	13.4
Snowshoe hare (PNW)	06_0627	9.7	8.8
Snowshoe hare (PNW)	LAM_INE_2013	33.5	31.0
Snowshoe hare (Rocky Mountains)	DOlsen1	7.6	7.0
White-sided jackrabbit	LCL_1956	9.4	8.5
White-tailed jackrabbit	DMNS_16612	15.5	13.9
White-tailed jackrabbit	DMNS_18791	12.2	11.3
White-tailed jackrabbit	DMNS_18793	16.4	14.8
White-tailed jackrabbit	DMNS_18800	9.6	8.6
White-tailed jackrabbit	DMNS_18801	11.2	10.1
White-tailed jackrabbit	DMNS_18803	9.3	8.7
White-tailed jackrabbit	DMNS_18808	11.7	10.9

PNW, Pacific Northwest.

Table S3.7 – Weighted (bellow diagonal) and unweighted (above diagonal) fixation indexes (F_{st}) among the white-tailed jackrabbit population clusters identified with *NGSadmix* under a model with three ancestral populations (Figure S3.5).

	Northwestern Colorado & Great Plains	Central Colorado & San Luis Valley	Cochetopa Dome
Northwestern Colorado & Great Plains		0.035	0.047
Central Colorado & San Luis Valley	0.037		0.031
Cochetopa Dome	0.050	0.031	

The evolution and molecular bases of seasonal coat color variation in hares:
gene expression and the shared contribution of ancestral polymorphism and introgression

Table S3.8 – Genotypes at selected SNPs located in three association regions with winter coat color detected with genome-wide associations with a quantitative (dorsal reflectance (PC1 loading); -999 is missing data) or discrete (white “W” or brown “B”) color classification, using samples in bold. Positions are in the European rabbit genome (OryCun2.0) coordinates and 5'-3' direction. Genotypes are colored as black: homozygous brown; dark gray: heterozygous; light gray: homozygous white; and white: missing data (0).

State	Sample	Dorsal reflectance (PC1)	Color	Chromosome 2 (CORIN)												Chromosome 4 (ASIP)												Chromosome 8 (EDNRB)											
				36198394	36337394	36337692	36339237	36345330	36355364	36357641	36361155	36370674	36371255	36447917	36457059	36457316	5424640	5431934	5433246	5434348	5436267	5438311	5439658	5440519	5445968	5457518	5461010	5464507	79633762	79646450	79646532	79648139	79695542	79695631	79701598	79713008	79721287		
CO	UCM_7918	-999	W	0	0	0	0	GG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	TT	AA	0	0	0	0	0	0	CC	0		
CO	UCM_7921	-999	W	0	0	0	0	GG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	TT	0	0	0	0	0	0	0	CC	0		
CO	UCM_1195	-999	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC	CC		
CO	UCM_13094	-999	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC	CC		
CO	UCM_7879	-999	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_16612	-999	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC	CC		
CO	UCM_7908	-999	B	TT	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	AT	GG	CC	CT	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_1679	-10.5	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3129	-6.79	B	GT	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3133	-6.99	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3182	-5.02	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_1704	-5.73	B	GT	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3168	-4.3	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_1776	-5.15	B	GG	CC	CC	TT	TT	CC	0	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	0	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_18808	-4.11	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	0	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_1179	-4.93	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_4088	-3.86	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_4087	-2.52	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_4070	-4.07	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_18793	-2.8	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3122	-1.44	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_4086	0.078	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3114	-1.24	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3128	1.422	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3115	0.672	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	GT	CT	AG	GA	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3134	-5.09	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	GC	CC		
CO	UCM_13092	-999	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	TC	CT	CC	CC	CC		
CO	DMNS_3112	-5.78	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	0	GG	AA	GG	CC	CC	TC	CT	CC	CC	CC		
CO	DMNS_1775	-3.36	B	0	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	0	CA	CA	CC	CC	CC	CC	CC		
CO	DMNS_3132	-3.11	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	TC	CT	CC	CC	CC		
CO	UCM_1194	-999	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	TT	GA	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_1705	-3.26	B	GG	CC	CC	TT	TT	CC	0	GG	GG	CC	AA	CC	GG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AA	GA	AA	GG	CC	CC	CC	CC	CC	CC	GC	CC	
CO	DMNS_5676	-1.97	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3109	-0.46	W	GT	TC	CT	GT	GT	CC	GT	AG	GA	TC	CA	TC	CG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	TT	GA	AA	GG	CC	CC	CC	CC	CC	CC	CC		
CO	DMNS_3135	1.249	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	GG	CC	CA	TC	CT	TC	GC	CC		
CO	UMNH_23778	-999	W	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TC	GC	TC		
CO	DMNS_4089	-5.93	B	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TC	GC	TC		

The evolution and molecular bases of seasonal coat color variation in hare:
gene expression and the shared contribution of ancestral polymorphism and introgression

CO	DMNS_5680	-0.57	W	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_5681	-2.32	W	GT	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AT	GA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_18803	3.094	W	TT	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	TC	AT	GT	TT	CT	CT	AG	GA	AA	0	AA	GA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_3138	0.479	W	GT	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	GA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_18797	2.936	W	GT	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	TA	AG	CA	CA	TC	CT	TC	GC	TC	
CO	DMNS_5679	0.694	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_5682	0.623	W	GT	TC	CT	GT	TT	CT	GT	AG	GA	TC	CA	TC	CG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_5677	2.806	W	GT	TC	CT	GT	TT	CT	GT	AG	GA	TC	CA	TC	CG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_3141	3.815	W	GT	TC	CT	GT	TT	CT	GT	AG	GA	TC	CA	TC	CG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	UMNH_23790	-999	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AA	GA	TA	AG	CA	CA	TT	TT	TC	GC	TC
CO	DMNS_18804	2.415	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AT	GA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_18792	0.255	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AT	GA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_18807	0.789	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TC	AT	GT	TC	CT	CT	AG	GA	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_4312	6.723	W	TT	TC	CT	GT	GT	CC	GT	AG	GA	TC	CA	TC	CG	TT	AT	GT	TC	CT	CT	AG	GA	GA	AG	AT	GA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_18802	1.174	W	TT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_18790	-1.36	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	DMNS_18791	0.352	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	UMNH_23789	-999	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AT	GA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	UMNH_23779	-999	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TA	AG	CA	CA	TC	CT	TC	GC	TC
CO	UCM_7894	-999	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TA	AG	CA	CC	TC	CT	TC	GC	TC
CO	DMNS_18805	0.828	W	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AA	GA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_18796	1.707	W	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_5678	3.716	W	GG	CC	CC	TT	TT	CC	GG	GG	GG	CC	AA	CC	GG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	TT	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_18800	4.715	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	UMNH_23805	-999	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AA	GA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_4319	10.28	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TC	AA	TT	TT	TT	TT	AA	AA	AA	AA	TT	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_18799	6.233	W	TT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_18795	-1.11	W	TT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	UMNH_23784	-999	W	GT	TC	CT	GT	GT	CT	GT	AG	GA	TC	CA	TC	CG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	UCM_7888	-999	B	TT	TC	TT	GT	GT	CT	TT	AA	GA	TC	CA	TC	CG	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TA	AG	CA	CA	TC	CT	TT	GC	TC
CO	DMNS_2245	7.39	W	GT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	CC	TT	GG	CC	CC	CC	GG	GG	GG	GG	TT	GG	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_18806	6.291	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AT	GA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_18798	8.104	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AA	GA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	AMNH_123030	-999	W	TT	TT	TT	GG	GG	TT	0	AA	AA	TT	CC	TT	CC	TC	AT	GT	TC	CT	CT	AG	GA	GG	AG	AA	GA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	UMNH_23804	-999	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TC	AT	GT	TT	CT	CT	AG	GA	AA	0	AA	GA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	UCM_7920	-999	W	GT	TT	TT	GG	0	TT	TT	AA	AA	TT	CC	TT	CC	TC	AT	GT	TC	CT	CT	AG	GA	GA	AG	AT	GA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_3163	5.771	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CA	TC	CG	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_18794	0.196	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_18801	3.769	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_3146	3.915	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_4313	9.451	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	UMNH_23776	-999	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	UMNH_23777	-999	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT
CO	DMNS_3120	10.61	W	TT	TT	TT	GG	GG	TT	TT	AA	AA	TT	CC	TT	CC	TT	AA	TT	TT	TT	TT	AA	AA	AA	AA	AA	AA	TT	AA	AA	AA	TT	TT	TT	GG	TT

The evolution and molecular bases of seasonal coat color variation in hares: gene expression and the shared contribution of ancestral polymorphism and introgression

OR	AMNH_40311	-999	W	0	0	0	0	GG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
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CO, Colorado; OR, Oregon; MT, Montana; ND, North Dakota. B, Brown; W, White.

Table S3.9 – Genotypes of the European rabbit genome reference (haploid), and black-tailed and white-sided jackrabbits, and Arctic, Alaskan and mountain hares. Genotypes are in the European rabbit coordinates and 5'-3' direction. Genotypes are colored as black: homozygous brown; dark gray: heterozygous; light gray: homozygous white. Blue genotypes represent a third allele and N represents missing data.

Rabbit genome coordinates	White-tailed jackrabbit coordinates	Brown haplotype	White haplotype	Rabbit	White-sided jackrabbit (LCL_1956)	Black-tailed jackrabbit			Arctic hare			Alaskan hare			Mountain hares		
						Bauer6	Mackay2	Burckel	UAM_66285	UAM_83630	UAM_83621	UAM_117653	UAM_117654	UAM_117890	LTM_MAG_1862	LTM_SUI_3121	LTM_CAT_2012
2:36198394	342:47124004	GG	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT
2:36337394	342:46991691	CC	TT	CC	CC	CC	CC	CC	TT	TT	TT	TT	TT	TT	CT	TT	TT
2:36337692	342:46991393	CC	TT	AA	TT	TT	TT	CT	TT	TT	TT	TT	TT	TT	TT	TT	TT
2:36339237	342:46989842	TT	GG	TT	TT	TT	TT	TT	GG	GG	GG	GG	GG	GG	GG	GG	GG
2:36345330	342:46983691	TT	GG	GG	GG	GG	GT	GG	GG	AA	GG	AA	AA	AA	AA	AA	AA
2:36355364	342:46972767	CC	TT	C	C	C	C	C	TT	TT	TT	TT	TT	TT	TT	TT	TT
2:36357641	342:46970080	GG	TT	TT	TT	TT	TT	TT	N	N	TT	TT	N	N	TT	TT	TT
2:36361155	342:46966737	GG	AA	AA	AA	AA	AG	AG	AA	AA	AA	AA	AA	AA	AA	AA	AA
2:36370674	342:46958289	GG	AA	AA	N	AA	AA	AA	N	AA	AA	N	AA	AA	AA	AA	AA
2:36371255	342:46957711	CC	TT	CC	CC	CC	CC	CC	TT	TT	TT	TT	TT	TT	TT	TT	TT
2:36447917	342:46879687	AA	CC	AA	AA	AA	AA	AA	CC	N	CC	AA	CC	AC	AA	AC	AA
2:36457059	342:46871251	CC	TT	TT	CC	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT
2:36457316	342:46870994	GG	CC	AA	GG	GG	GG	GG	CC	CC	N	CC	N	CC	CC	CC	CC
4:5424640	245:24236852	CC	TT	CC	N	N	CC	CC	TT	N	TT	N	TT	N	TT	TT	TT
4:5431934	245:24229551	TT	AA	AA	AA	AT	AT	TT	AA	AA	AA	AA	AA	AA	AA	AA	AA
4:5433246	245:24228244	GG	TT	GG	N	GG	GG	GG	TT	TT	TT	TT	TT	TT	TT	TT	TT
4:5434348	245:24226798	CC	TT	CC	TT	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC
4:5436267	245:24225226	CC	TT	CC	N	CC	CC	CC	TT	TT	TT	N	TT	TT	TT	TT	TT
4:5438311	245:24222426	CC	TT	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC
4:5439658	245:24221492	GG	AA	AA	N	GG	GG	GG	AA	AA	AA	N	AA	AA	AA	AA	AA
4:5440519	245:24220190	GG	AA	GG	GG	GG	GG	GG	AA	AA	AA	AA	AA	AA	AA	AA	AA
4:5445968	245:24211740	GG	AA	GG	AA	GG	GG	GG	AA	AA	AA	AA	AA	AA	AA	AA	AA
4:5457518	245:24204540	GG	AA	GG	GG	GG	GG	GG	AA	AA	N	N	AA	AA	AA	AA	AA
4:5461010	245:24200684	TT	AA	TT	N	TT	TT	TT	TT	N	TT	TT	TT	TT	TT	TT	TT
4:5464507	245:24197147	GG	AA	GG	GG	GG	GG	GG	AA	N	AA	N	AA	AA	AA	AA	AA
8:79633762	311:3467105	AA	TT	AA	AA	AT	TT	TT	N	N	TT	N	TT	TT	TT	TT	TT
8:79646450	311:3456263	GG	AA	GG	GG	GG	GG	GG	AA	GG	AA	GG	GG	GG	GG	GG	GG
8:79646532	311:3456181	CC	AA	CC	CC	CC	CC	CC	N	CC	N	CC	CC	CC	CC	CC	CC
8:79648139	311:3454568	CC	AA	CC	CC	CC	CC	CC	N	N	AA	AA	AA	AA	AA	AA	AA
8:79695542	311:3419309	CC	TT	CC	CC	CC	CC	CC	N	N	TT	TT	TT	N	TT	TT	TT
8:79695631	311:3419220	CC	TT	CC	CC	CC	CC	CC	N	N	TT	TT	TT	TT	TT	TT	TT
8:79701598	311:3413288	CC	TT	TT	TT	TT	CC	N	N	TT	TT	TT	TT	TT	TT	TT	TT
8:79713008	311:3402184	CC	GG	CC	CC	N	CC	CC	GG	GG	GG	GG	GG	GG	GG	GG	GG
8:79721287	311:3399522	CC	TT	CC	CC	CC	CC	CC	N	N	TT	TT	TT	TT	TT	TT	TT

Table S3.10 – Association test between genotyped SNPs and the first principal component of a PCA of dorsal reflectance (Figure S3.7). We performed linear regressions in R (function *lm()*) or *PLINK* 1.9 (--linear) of the effect of each SNP on the phenotype assuming an additive model between the alleles at each SNP (0, 1 and 2). All tests controlled for population structure using the main principal component axes partitioning genetic variation of 240,109 independent positions segregating in 61 individuals as covariates (Figure S3.4B). Beta reflects the effect of adding an extra white allele on phenotype.

Rabbit genome coordinates	White-tailed jackrabbit coordinates	Linear Regression in R (<i>lm()</i>)					Linear Regression in PLINK		
		Beta	Std. Error	T value	P value	PVE (R ²)	Beta	T value	P value
2:36198394	342:47124004	3.96	0.70	5.64	6.36E-07	0.40	3.96	5.64	6.36E-07
2:36337394	342:46991691	4.27	0.62	6.84	6.82E-09	0.48	4.27	6.84	6.90E-09
2:36337692	342:46991393	4.27	0.62	6.84	6.82E-09	0.48	4.27	6.84	6.90E-09
2:36339237	342:46989842	4.27	0.62	6.84	6.82E-09	0.48	4.27	6.84	6.90E-09
2:36345330	342:46983691	4.10	0.64	6.39	3.68E-08	0.45	4.10	6.40	3.66E-08
2:36355364	342:46972767	4.08	0.64	6.37	4.10E-08	0.45	4.08	6.37	4.10E-08
2:36357641	342:46970080	4.17	0.64	6.50	2.89E-08	0.47	4.17	6.49	2.93E-08
2:36361155*	342:46966737*	4.27	0.62	6.84	6.82E-09	0.48	4.27	6.84	6.90E-09
2:36370674	342:46958289	4.27	0.62	6.84	6.82E-09	0.48	4.27	6.84	6.90E-09
2:36371255	342:46957711	4.27	0.62	6.84	6.82E-09	0.48	4.27	6.84	6.90E-09
2:36447917	342:46879687	4.38	0.66	6.64	1.48E-08	0.47	4.38	6.64	1.49E-08
2:36457059	342:46871251	4.38	0.66	6.64	1.48E-08	0.47	4.38	6.64	1.49E-08
2:36457316	342:46870994	4.38	0.66	6.64	1.48E-08	0.47	4.38	6.64	1.49E-08
4:5424640*	245:24236852*	3.11	0.65	4.76	1.44E-05	0.32	3.11	4.76	1.45E-05
4:5431934	245:24229551	3.09	0.68	4.56	2.87E-05	0.30	3.09	4.56	2.88E-05
4:5433246	245:24228244	3.09	0.68	4.56	2.87E-05	0.30	3.09	4.56	2.88E-05
4:5434348	245:24226798	3.06	0.67	4.60	2.52E-05	0.31	3.06	4.60	2.54E-05
4:5436267	245:24225226	3.12	0.67	4.67	1.98E-05	0.31	3.12	4.67	1.98E-05
4:5438311	245:24222426	3.12	0.67	4.67	1.98E-05	0.31	3.12	4.67	1.98E-05
4:5439658	245:24221492	3.12	0.67	4.67	1.98E-05	0.31	3.12	4.67	1.98E-05
4:5440519	245:24220190	3.12	0.67	4.67	1.98E-05	0.31	3.12	4.67	1.98E-05
4:5445968	245:24211740	2.93	0.65	4.49	3.68E-05	0.30	2.93	4.49	3.70E-05
4:5457518	245:24204540	2.96	0.67	4.42	4.72E-05	0.29	2.95	4.42	4.75E-05
4:5461010	245:24200684	2.12	0.75	2.85	6.28E-03	0.19	2.12	2.85	6.25E-03
4:5464507	245:24197147	2.96	0.66	4.46	4.13E-05	0.30	2.95	4.46	4.15E-05
8:79633762	311:3467105	4.66	0.52	9.01	2.04E-12	0.61	4.66	9.00	2.08E-12
8:79646450	311:3456263	4.61	0.55	8.46	1.82E-11	0.59	4.61	8.46	1.82E-11
8:79646532	311:3456181	4.61	0.55	8.36	2.29E-11	0.58	4.61	8.36	2.23E-11
8:79648139	311:3454568	4.66	0.54	8.70	6.29E-12	0.60	4.66	8.71	6.08E-12
8:79695542	311:3419309	4.52	0.55	8.15	4.88E-11	0.57	4.52	8.15	4.99E-11
8:79695631	311:3419220	4.52	0.55	8.15	4.88E-11	0.57	4.52	8.15	4.99E-11
8:79701598*	311:3413288	4.66	0.52	9.01	2.04E-12	0.61	4.66	9.00	2.08E-12
8:79713008	311:3402184	4.54	0.55	8.23	3.73E-11	0.57	4.54	8.22	3.82E-11
8:79721287	311:3399522	4.61	0.53	8.65	7.73E-12	0.59	4.60	8.64	7.89E-12

Table S3.11 – Linear regression testing association with continuous winter coat color considering the strongest associated SNPs at *EDNRB* and *CORIN* (Table S3.10) as factors. For each *ASIP* SNP, the ADD row gives the association coefficient and *P* value for the *ASIP* SNP while the two other rows give the association parameters and *P* value for the *EDNRB* (8:79701598) and *CORIN* (2:36361155) SNPs. Numbers in bold highlight association where the *P* value is smaller than 0.05. All tests controlled for population structure using the main principal component axes partitioning genetic variation of 240,109 independent positions segregating in 61 individuals as covariates (Figure S3.4B).

Rabbit genome coordinates	Test/ Covariate	Beta	T value	P value
4:5424640	ADD	0.54	0.92	0.36
4:5424640	chr8_79701598	3.14	4.20	1.02E-04
4:5424640	chr2_36361155	1.84	2.69	9.53E-03
4:5431934	ADD	0.36	0.58	0.56
4:5431934	chr8_79701598	3.25	4.29	7.64E-05
4:5431934	chr2_36361155	1.86	2.70	9.27E-03
4:5433246	ADD	0.36	0.58	0.56
4:5433246	chr8_79701598	3.25	4.29	7.64E-05
4:5433246	chr2_36361155	1.86	2.70	9.27E-03
4:5434348	ADD	0.52	0.87	0.39
4:5434348	chr8_79701598	3.14	4.14	1.24E-04
4:5434348	chr2_36361155	1.87	2.74	8.42E-03
4:5436267	ADD	0.55	0.92	0.36
4:5436267	chr8_79701598	3.14	4.21	1.01E-04
4:5436267	chr2_36361155	1.85	2.70	9.19E-03
4:5438311	ADD	0.55	0.92	0.36
4:5438311	chr8_79701598	3.14	4.21	1.01E-04
4:5438311	chr2_36361155	1.85	2.70	9.19E-03
4:5439658	ADD	0.55	0.92	0.36
4:5439658	chr8_79701598	3.14	4.21	1.01E-04
4:5439658	chr2_36361155	1.85	2.70	9.19E-03
4:5440519	ADD	0.55	0.92	0.36
4:5440519	chr8_79701598	3.14	4.21	1.01E-04
4:5440519	chr2_36361155	1.85	2.70	9.19E-03
4:5445968	ADD	0.47	0.81	0.42
4:5445968	chr8_79701598	3.16	4.15	1.20E-04
4:5445968	chr2_36361155	1.88	2.75	8.19E-03
4:5457518	ADD	0.29	0.50	0.62
4:5457518	chr8_79701598	3.18	4.21	1.02E-04
4:5457518	chr2_36361155	2.04	2.95	4.76E-03
4:5461010	ADD	0.05	0.09	0.93
4:5461010	chr8_79701598	3.34	4.55	3.42E-05
4:5461010	chr2_36361155	1.85	2.62	1.18E-02
4:5464507	ADD	0.31	0.53	0.60
4:5464507	chr8_79701598	3.27	4.30	7.46E-05
4:5464507	chr2_36361155	1.86	2.71	8.99E-03

Table S3.12 – Linear regression testing association of additive effects of allele dosage (ADD), dominance deviation from additivity (DOMDEV) and a 2 degree of freedom joint test of both additivity and dominance (GENO_2DF) with continuous winter coat color. All tests controlled for population structure using the main principal component axes partitioning genetic variation of 240,109 independent positions segregating in 61 individuals as covariates (Figure S3.4B).

Rabbit genome coordinates	White-tailed jackrabbit coordinates	Additivity (ADD)			Dominance deviation (DOMDEV)			GENO_2DF (ADD + DOMDEV)	
		Beta	T value	P value	Beta	T value	P value	T value	P value
2:36198394	342:47124004	3.96	5.58	8.43E-07	-0.12	-0.10	0.92	31.25	1.63E-07
2:36337394	342:46991691	4.30	6.64	1.58E-08	-0.24	-0.20	0.84	46.01	1.02E-10
2:36337692	342:46991393	4.30	6.64	1.58E-08	-0.24	-0.20	0.84	46.01	1.02E-10
2:36339237	342:46989842	4.30	6.64	1.58E-08	-0.24	-0.20	0.84	46.01	1.02E-10
2:36345330	342:46983691	4.18	6.30	5.52E-08	-0.67	-0.54	0.59	40.67	1.47E-09
2:36355364	342:46972767	4.18	6.29	5.84E-08	-0.76	-0.61	0.54	40.44	1.66E-09
2:36357641	342:46970080	4.21	6.38	4.80E-08	-0.45	-0.37	0.72	41.62	9.17E-10
2:36361155	342:46966737	4.30	6.64	1.58E-08	-0.24	-0.20	0.84	46.01	1.02E-10
2:36370674	342:46958289	4.30	6.64	1.58E-08	-0.24	-0.20	0.84	46.01	1.02E-10
2:36371255	342:46957711	4.30	6.64	1.58E-08	-0.24	-0.20	0.84	46.01	1.02E-10
2:36447917	342:46879687	4.35	6.27	6.19E-08	0.18	0.15	0.88	43.26	4.04E-10
2:36457059	342:46871251	4.35	6.27	6.19E-08	0.18	0.15	0.88	43.26	4.04E-10
2:36457316	342:46870994	4.35	6.27	6.19E-08	0.18	0.15	0.88	43.26	4.04E-10
4:5424640	245:24236852	3.15	4.78	1.38E-05	1.02	0.69	0.49	22.91	1.06E-05
4:5431934	245:24229551	3.13	4.63	2.38E-05	1.48	1.04	0.30	21.92	1.74E-05
4:5433246	245:24228244	3.13	4.63	2.38E-05	1.48	1.04	0.30	21.92	1.74E-05
4:5434348	245:24226798	3.11	4.66	2.12E-05	1.39	0.95	0.35	22	1.67E-05
4:5436267	245:24225226	3.13	4.69	1.91E-05	1.32	1.00	0.32	22.8	1.12E-05
4:5438311	245:24222426	3.13	4.69	1.91E-05	1.32	1.00	0.32	22.8	1.12E-05
4:5439658	245:24221492	3.13	4.69	1.91E-05	1.32	1.00	0.32	22.8	1.12E-05
4:5440519	245:24220190	3.13	4.69	1.91E-05	1.32	1.00	0.32	22.8	1.12E-05
4:5445968	245:24211740	2.95	4.50	3.65E-05	1.23	0.81	0.42	20.68	3.23E-05
4:5457518	245:24204540	2.96	4.42	4.88E-05	1.21	0.79	0.43	20.05	4.43E-05
4:5461010	245:24200684	2.14	2.86	0.006152	1.24	0.61	0.54	8.402	0.01498
4:5464507	245:24197147	2.97	4.46	4.14E-05	1.32	0.91	0.37	20.61	3.34E-05
8:79633762	311:3467105	4.66	8.92	3.30E-12	0.05	0.05	0.96	79.6	5.18E-18
8:79646450	311:3456263	4.62	8.36	2.95E-11	0.06	0.06	0.95	70.21	5.68E-16
8:79646532	311:3456181	4.60	8.26	3.76E-11	-0.22	-0.22	0.83	68.8	1.15E-15
8:79648139	311:3454568	4.66	8.63	9.59E-12	-0.18	-0.20	0.84	74.65	6.17E-17
8:79695542	311:3419309	4.52	8.12	6.26E-11	-0.71	-0.78	0.44	66.53	3.58E-15
8:79695631	311:3419220	4.52	8.12	6.26E-11	-0.71	-0.78	0.44	66.53	3.58E-15
8:79701598	311:3413288	4.66	8.92	3.30E-12	0.05	0.05	0.96	79.6	5.18E-18
8:79713008	311:3402184	4.54	8.19	4.91E-11	-0.66	-0.73	0.47	67.52	2.18E-15
8:79721287	311:3399522	4.61	8.56	1.25E-11	0.04	0.05	0.96	73.36	1.18E-16

Table S3.13 – Linear regression testing for dominance deviation (DOMDEV) from an Additive model (ADD) using the top associated SNPs at the two other candidate regions as covariates (i.e., Response ~ SNP + top SNP1 + top SNP2 + PC1 + PC2; Table S3.10). The SNPs used as covariates are marked with an asterisk (*). All tests controlled for population structure using the main principal component axes partitioning genetic variation of 240,109 independent positions segregating in 61 individuals as covariates (Figure S3.4B).

Rabbit genome coordinates	White-tailed jackrabbit coordinates	Additivity (ADD)			Dominance deviation (DOMDEV)			GENO_2DF (ADD + DOMDEV)	
		Beta	T value	P value	Beta	T value	P value	T value	P value
2:36198394	342:47124004	1.52	2.11	0.04	0.61	0.62	0.54	5.11	0.08
2:36337394	342:46991691	1.65	2.29	0.03	0.86	0.87	0.39	7.95	0.02
2:36337692	342:46991393	1.65	2.29	0.03	0.86	0.87	0.39	7.95	0.02
2:36339237	342:46989842	1.65	2.29	0.03	0.86	0.87	0.39	7.95	0.02
2:36345330	342:46983691	1.47	2.03	0.05	0.64	0.60	0.55	5.78	0.06
2:36355364	342:46972767	1.46	1.94	0.06	0.30	0.29	0.77	4.53	0.10
2:36357641	342:46970080	1.65	2.25	0.03	0.84	0.81	0.42	7.55	0.02
2:36361155*	342:46966737*	1.65	2.29	0.03	0.86	0.87	0.39	7.95	0.02
2:36370674	342:46958289	1.65	2.29	0.03	0.86	0.87	0.39	7.95	0.02
2:36371255	342:46957711	1.65	2.29	0.03	0.86	0.87	0.39	7.95	0.02
2:36447917	342:46879687	1.75	2.42	0.02	0.74	0.78	0.44	8.24	0.02
2:36457059	342:46871251	1.75	2.42	0.02	0.74	0.78	0.44	8.24	0.02
2:36457316	342:46870994	1.75	2.42	0.02	0.74	0.78	0.44	8.24	0.02
4:5424640*	245:24236852*	0.58	0.98	0.33	1.03	0.97	0.34	1.78	0.41
4:5431934	245:24229551	0.39	0.65	0.52	1.51	1.50	0.14	2.60	0.27
4:5433246	245:24228244	0.39	0.65	0.52	1.51	1.50	0.14	2.60	0.27
4:5434348	245:24226798	0.56	0.94	0.35	1.10	1.04	0.30	1.85	0.40
4:5436267	245:24225226	0.51	0.87	0.39	1.80	1.95	0.06	4.67	0.10
4:5438311	245:24222426	0.51	0.87	0.39	1.80	1.95	0.06	4.67	0.10
4:5439658	245:24221492	0.51	0.87	0.39	1.80	1.95	0.06	4.67	0.10
4:5440519	245:24220190	0.51	0.87	0.39	1.80	1.95	0.06	4.67	0.10
4:5445968	245:24211740	0.48	0.83	0.41	1.02	0.95	0.35	1.56	0.46
4:5457518	245:24204540	0.30	0.50	0.62	1.13	1.06	0.29	1.37	0.50
4:5461010	245:24200684	0.07	0.12	0.91	0.84	0.61	0.55	0.38	0.83
4:5464507	245:24197147	0.31	0.53	0.60	1.46	1.43	0.16	2.32	0.31
8:79633762	311:3467105	3.12	4.13	1.30E-04	0.36	0.42	0.68	17.55	1.54E-04
8:79646450	311:3456263	2.99	3.72	5.00E-04	0.25	0.26	0.80	13.85	9.82E-04
8:79646532	311:3456181	2.86	3.63	6.45E-04	0.09	0.10	0.92	13.21	1.35E-03
8:79648139	311:3454568	3.01	4.01	1.93E-04	0.26	0.30	0.77	16.52	2.59E-04
8:79695542	311:3419309	2.76	3.64	6.22E-04	-0.13	-0.15	0.88	13.39	1.24E-03
8:79695631	311:3419220	2.76	3.64	6.22E-04	-0.13	-0.15	0.88	13.39	1.24E-03
8:79701598*	311:3413288*	3.12	4.13	1.30E-04	0.36	0.42	0.68	17.55	1.54E-04
8:79713008	311:3402184	2.82	3.64	6.38E-04	-0.14	-0.16	0.87	13.26	1.32E-03
8:79721287	311:3399522	2.98	3.75	4.52E-04	0.22	0.23	0.82	14.06	8.83E-04

Table S3.14 – Linear regression testing the interaction among all the SNPs genotyped across the *EDNBR*, *CORIN* and *ASIP* association regions. Only interactions with *P* value ≤ 0.05 (not corrected for multiple tests) are shown. For each pair of SNPs, we present the regression coefficient (Beta) and *P* value of the interaction term in the regression analysis in the format Beta/ *P* value.

SNP1/ SNP2	4:5424640	4:5431934	4:5433246	4:5434348	4:5436267	4:5438311	4:5439658	4:5440519	4:5445968	4:5457518	4:5461010	4:5464507
2:36337394	-1.75/ 9.62E-03	-1.82/ 7.00E-03	-1.82/ 7.00E-03	-1.83/ 5.09E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.83/ 4.67E-03	-1.75/ 8.37E-03	-1.42/ 3.46E-02	-1.82/ 6.46E-03
2:36337692	-1.75/ 9.62E-03	-1.82/ 7.00E-03	-1.82/ 7.00E-03	-1.83/ 5.09E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.83/ 4.67E-03	-1.75/ 8.37E-03	-1.42/ 3.46E-02	-1.82/ 6.46E-03
2:36339237	-1.75/ 9.62E-03	-1.82/ 7.00E-03	-1.82/ 7.00E-03	-1.83/ 5.09E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.83/ 4.67E-03	-1.75/ 8.37E-03	-1.42/ 3.46E-02	-1.82/ 6.46E-03
2:36345330	NA	NA	NA	-1.5/ 4.69E-02	-1.57/ 4.12E-02	-1.57/ 4.12E-02	-1.57/ 4.12E-02	-1.57/ 4.12E-02	-1.49/ 4.41E-02	NA	NA	NA
2:36355364	-2.03/ 2.25E-03	-1.93/ 5.75E-03	-1.93/ 5.75E-03	-1.92/ 4.60E-03	-2.02/ 3.30E-03	-2.02/ 3.30E-03	-2.02/ 3.30E-03	-2.02/ 3.30E-03	-1.92/ 4.15E-03	-1.87/ 6.63E-03	-1.49/ 3.55E-02	-1.93/ 5.19E-03
2:36357641	-1.76/ 1.02E-02	-1.84/ 7.55E-03	-1.84/ 7.55E-03	-1.84/ 5.64E-03	-1.93/ 4.22E-03	-1.93/ 4.22E-03	-1.93/ 4.22E-03	-1.93/ 4.22E-03	-1.83/ 5.18E-03	-1.76/ 9.05E-03	-1.58/ 2.09E-02	-1.83/ 6.95E-03
2:36361155	-1.75/ 9.62E-03	-1.82/ 7.00E-03	-1.82/ 7.00E-03	-1.83/ 5.09E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.83/ 4.67E-03	-1.75/ 8.37E-03	-1.42/ 3.46E-02	-1.82/ 6.46E-03
2:36370674	-1.75/ 9.62E-03	-1.82/ 7.00E-03	-1.82/ 7.00E-03	-1.83/ 5.09E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.83/ 4.67E-03	-1.75/ 8.37E-03	-1.42/ 3.46E-02	-1.82/ 6.46E-03
2:36371255	-1.75/ 9.62E-03	-1.82/ 7.00E-03	-1.82/ 7.00E-03	-1.83/ 5.09E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.92/ 3.84E-03	-1.83/ 4.67E-03	-1.75/ 8.37E-03	-1.42/ 3.46E-02	-1.82/ 6.46E-03
2:36447917	-1.77/ 1.10E-02	-1.85/ 8.05E-03	-1.85/ 8.05E-03	-1.85/ 6.09E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.84/ 5.62E-03	-1.77/ 9.73E-03	-1.43/ 3.82E-02	-1.84/ 7.46E-03
2:36457059	-1.77/ 1.10E-02	-1.85/ 8.05E-03	-1.85/ 8.05E-03	-1.85/ 6.09E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.84/ 5.62E-03	-1.77/ 9.73E-03	-1.43/ 3.82E-02	-1.84/ 7.46E-03
2:36457316	-1.77/ 1.10E-02	-1.85/ 8.05E-03	-1.85/ 8.05E-03	-1.85/ 6.09E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.95/ 4.49E-03	-1.84/ 5.62E-03	-1.77/ 9.73E-03	-1.43/ 3.82E-02	-1.84/ 7.46E-03

Table S3.15 – Information for the nodes in the multispecies coalescent tree estimated with *ASTRAL-III* in Figure 3.3B and S3.18. For each node, we indicate the quartet score for the first (q1), second (q2) and third (q3) most prevalent topologies, number of quartet trees that support the first (f1), second (f2) and third (f3) most prevalent topologies, the posterior probability for the first (pp1), second (pp2) and third (pp3) most prevalent topologies, the total number of quartets defined around each branch (QC) and the effective number of genes for the branch (EN).

Node	q1	q2	q3	f1	f2	f3	pp1	pp2	pp3	QC	EN
1	0.457	0.222	0.321	19850	9620	13960	1.000	0.000	0.000	34	43430
2	0.747	0.129	0.125	32422	5585	5424	1.000	0.000	0.000	66	43430
3	0.601	0.195	0.205	26081	8461	8888	1.000	0.000	0.000	34	43430
4	0.440	0.261	0.299	19119	11330	12981	1.000	0.000	0.000	66	43430
5	0.961	0.017	0.022	41742	745	943	1.000	0.000	0.000	348	43430
6	0.630	0.128	0.242	27353	5572	10506	1.000	0.000	0.000	150	43430
7	0.347	0.336	0.317	15072	14589	13769	1.000	0.000	0.000	66	43430
8	0.409	0.294	0.297	17784	12750	12896	1.000	0.000	0.000	66	43430
9	0.447	0.284	0.269	19402	12337	11691	1.000	0.000	0.000	128	43430
10	0.969	0.015	0.017	42065	638	727	1.000	0.000	0.000	240	43430
11	0.547	0.206	0.246	23761	8968	10701	1.000	0.000	0.000	1760	43430
12	0.925	0.046	0.028	40176	2018	1237	1.000	0.000	0.000	34	43430
13	0.500	0.368	0.133	21712	15962	5756	1.000	0.000	0.000	570	43430
14	0.990	0.006	0.004	42979	266	186	1.000	0.000	0.000	306	43430
15	0.352	0.327	0.321	15294	14201	13935	1.000	0.000	0.000	34	43430
16	0.342	0.332	0.326	14850	14412	14168	1.000	0.000	0.000	186	43430
17	0.337	0.336	0.326	14640	14614	14176	0.597	0.384	0.019	34	43430
18	0.379	0.307	0.315	16448	13323	13659	1.000	0.000	0.000	186	43430
19	0.417	0.288	0.294	18123	12522	12785	1.000	0.000	0.000	270	43430
20	0.933	0.032	0.035	40515	1384	1531	1.000	0.000	0.000	1200	43430
21	0.844	0.045	0.111	36653	1952	4825	1.000	0.000	0.000	216	43430
22	0.386	0.286	0.328	16784	12418	14228	1.000	0.000	0.000	34	43430
23	0.760	0.120	0.120	33010	5202	5218	1.000	0.000	0.000	66	43430
24	0.403	0.352	0.244	17521	15299	10609	1.000	0.000	0.000	348	43430
25	0.453	0.239	0.309	19663	10368	13399	1.000	0.000	0.000	34	43430
26	0.387	0.328	0.285	16817	14253	12360	1.000	0.000	0.000	66	43430
27	0.454	0.247	0.299	19717	10715	12998	1.000	0.000	0.000	348	43430
28	0.660	0.118	0.222	28681	5108	9641	1.000	0.000	0.000	864	43430
29	0.726	0.075	0.199	31545	3263	8623	1.000	0.000	0.000	2240	43430
30	0.875	0.105	0.020	38020	4561	849	1.000	0.000	0.000	2520	43430
31	0.622	0.135	0.243	27029	5865	10536	1.000	0.000	0.000	1530	43430
32	0.817	0.067	0.117	35465	2891	5074	1.000	0.000	0.000	3192	43430
33	0.951	0.025	0.025	41291	1067	1072	1.000	0.000	0.000	2464	43430
34	0.953	0.019	0.028	41385	838	1207	1.000	0.000	0.000	348	43430

Table S3.16 – Demographic parameters inferred with *G-PhoCs* for the history of divergence between white-tailed jackrabbits and (A) black-tailed jackrabbits; (B) snowshoe hares from the Boreal population; (C) snowshoe hares from Pacific Northwest population and (D) Arctic hares, and between Pacific Northwest snowshoe hares and (E) Arctic hares and (F) mountain hares. Raw estimates of effective population size (θ), population divergence time (τ) and migration rate (M) were converted assuming a mutation rate (μ) of 2.8×10^{-9} and generation time of 2 years. Mean values for each parameter are presented along with 95% highest posterior density (HPD) interval.

G-PhoCs parameters	Demographic parameters (95% HPD interval)
<i>A) White-tailed jackrabbits and Black-tailed jackrabbits</i>	
theta white-tailed jackrabbit	363,767 (356,372; 371,396) diploid individuals
theta black-tailed jackrabbit	811,232 (794,037; 828,603) diploid individuals
theta ancestral	424,912 (409,849; 440,240) diploid individuals
Tau ancestral	1,495,296 (1,466,118; 1,524,325) generations
migration WTJ -> BTJ	8.56×10^{-08} (7.72×10^{-08} ; 9.40×10^{-08}) migrants/ generation
migration BTJ -> WTJ	4.83×10^{-08} (4.06×10^{-08} ; 5.61×10^{-08}) migrants/ generation
<i>B) White-tailed jackrabbits and Snowshoe hares (Boreal population)</i>	
theta white-tailed jackrabbit	342,448 (335,041; 349,940) diploid individuals
theta snowshoe hare (Boreal)	610,924 (597,319; 624,364) diploid individuals
theta ancestral	382,601 (368,719; 396,402) diploid individuals
Tau ancestral	1,415,636 (1,391,071; 1,439,793) generations
migration WTJ -> SSH_B	6.43×10^{-09} (3.30×10^{-09} ; 9.59×10^{-09}) migrants/ generation
migration SSH_B -> WTJ	8.23×10^{-09} (4.16×10^{-09} ; 1.26×10^{-08}) migrants/ generation
<i>C) White-tailed jackrabbits and Snowshoe hares (Pacific Northwest population)</i>	
theta white-tailed jackrabbit	388,115 (381,063; 395,241) diploid individuals
theta snowshoe hare (PNW)	332,223 (326,179; 338,413) diploid individuals
theta ancestral	491,289 (475,523; 507,057) diploid individuals
Tau ancestral	1,438,150 (1,412,479; 1,463,604) generations
migration WTJ -> SSH_PNW	1.32×10^{-08} (9.10×10^{-09} ; 1.75×10^{-08}) migrants/ generation
migration SSH_PNW -> WTJ	4.00×10^{-09} (1.72×10^{-09} ; 6.35×10^{-09}) migrants/ generation
<i>D) White-tailed jackrabbits and Arctic hares</i>	
theta white-tailed jackrabbit	570,009 (549,286; 591,009) diploid individuals
theta Arctic hare	68,991 (64,545; 73,482) diploid individuals
theta ancestral	426,714 (410,420; 443,321) diploid individuals
Tau ancestral	577,000 (534,893; 617,714) generations
migration WTJ -> ARH	1.38×10^{-06} (1.23×10^{-06} ; 1.54×10^{-06}) migrants/ generation
migration ARH -> WTJ	2.30×10^{-09} (2.80×10^{-14} ; 1.45×10^{-08}) migrants/ generation
<i>E) Arctic hares and Snowshoe hares (Pacific Northwest population)</i>	
theta Arctic hare	157,981 (153,578; 162,404) diploid individuals
theta snowshoe hare (PNW)	403,132 (393,567; 412,616) diploid individuals
theta ancestral	535,088 (510,795; 559,336) diploid individuals
Tau ancestral	1,509,779 (1,465,832; 1,554,946) generations
migration ARH -> SSH_PNW	1.95×10^{-10} (0.00; 1.04×10^{-09}) migrants/ generation
migration SSH_PNW -> ARH	3.72×10^{-08} (2.78×10^{-08} ; 4.64×10^{-08}) migrants/ generation
<i>F) Mountain hares and Snowshoe hares (Pacific Northwest population)</i>	
theta mountain hares	299,404 (294,440; 304,446) diploid individuals
theta snowshoe hare (PNW)	329,079 (323,541; 334,617) diploid individuals
theta ancestral	462,179 (448,398; 476,335) diploid individuals
Tau ancestral	1,437,304 (1,414,307; 1,459,579) generations
migration MTH -> SSH_PNW	8.50×10^{-09} (5.64×10^{-09} ; 1.15×10^{-08}) migrants/ generation
migration SSH_PNW -> MTH	4.62×10^{-10} (0.00; 1.44×10^{-09}) migrants/ generation

Table S3.17 – Genome-wide tests of admixture with D-statistic among mountain hares, Arctic hares, Alaskan hares, white-tailed jackrabbits, black-tailed jackrabbits and three snowshoe hare populations (Pacific Northwest, Boreal and Rocky Mountains). Pairs of species highlighted in bold show significant (P value $\leq 2.00 \times 10^{-3}$, after Bonferroni multiple test correction) admixture.

P1	P2	P3	Positions	D statistic	P value
Snowshoe hare (Boreal)	Snowshoe hare (Pacific Northwest)	Arctic hare	13147376	0.04	3.14×10^{-35}
Snowshoe hare (Boreal)	Snowshoe hare (Pacific Northwest)	Alaskan hare	17164076	0.04	2.71×10^{-29}
Snowshoe hare (Boreal)	Snowshoe hare (Pacific Northwest)	mountain hare	18001852	0.04	6.41×10^{-28}
Snowshoe hare (Boreal)	Snowshoe hare (Pacific Northwest)	White-tailed jackrabbit	18697358	0.05	3.40×10^{-47}
Snowshoe hare (Boreal)	Snowshoe hare (Rocky Mountains)	Arctic hare	7568040	0.06	6.8×10^{-42}
Snowshoe hare (Boreal)	Snowshoe hare (Rocky Mountains)	Alaskan hare	11326510	0.05	6.14×10^{-34}
Snowshoe hare (Boreal)	Snowshoe hare (Rocky Mountains)	Mountain hare	11920483	0.05	7.58×10^{-33}
Snowshoe hare (Boreal)	Snowshoe hare (Rocky Mountains)	White-tailed jackrabbit	12479108	0.07	4.47×10^{-50}
Snowshoe hare (Rocky Mountains)	Snowshoe hare (Pacific Northwest)	Arctic hare	7695399	-0.02	6.34×10^{-06}
Snowshoe hare (Rocky Mountains)	Snowshoe hare (Pacific Northwest)	Alaskan hare	11506290	-0.02	5.18×10^{-04}
Snowshoe hare (Rocky Mountains)	Snowshoe hare (Pacific Northwest)	Mountain hare	12114599	-0.02	3.22×10^{-04}
Snowshoe hare (Rocky Mountains)	Snowshoe hare (Pacific Northwest)	White-tailed jackrabbit	12686784	-0.02	2.42×10^{-05}
White-tailed jackrabbit	Arctic hare	Snowshoe hare (Boreal)	13093430	0.06	2.02×10^{-30}
White-tailed jackrabbit	Arctic hare	Snowshoe hare (Pacific Northwest)	13325780	0.04	1.52×10^{-13}
White-tailed jackrabbit	Arctic hare	Snowshoe hare (Rocky Mountains)	7638069	0.04	4.67×10^{-07}
White-tailed jackrabbit	Alaskan hare	Snowshoe hare (Boreal)	17112906	0.07	1.68×10^{-30}
White-tailed jackrabbit	Alaskan hare	Snowshoe hare (Pacific Northwest)	17385477	0.04	2.63×10^{-11}
White-tailed jackrabbit	Alaskan hare	Snowshoe hare (Rocky Mountains)	11431490	0.04	5.46×10^{-06}
Mountain hare	White-tailed jackrabbit	Snowshoe hare (Boreal)	17940941	-0.07	2.94×10^{-29}
Mountain hare	White-tailed jackrabbit	Snowshoe hare (Pacific Northwest)	18237884	-0.04	2.10×10^{-11}
Mountain hare	White-tailed jackrabbit	Snowshoe hare (Rocky Mountains)	12030867	-0.04	2.05×10^{-06}
Mountain hare	Arctic hare	White-tailed jackrabbit	13007776	0.16	6.60×10^{-206}
Mountain hare	Alaskan hare	White-tailed jackrabbit	16838824	0.04	2.11×10^{-14}
Mountain hare	White-tailed jackrabbit	Black-tailed jackrabbit	12613388	0.19	4.4×10^{-143}

Table S3.18 – Admixture proportion (f_G ; Martin et al. 2015) between white-tailed jackrabbits and black-tailed jackrabbits.

P1	P2	P3	Admixture proportion	No. Positions	P value
Mountain hare	White-tailed jackrabbit	Black-tailed jackrabbit	0.04	9842714	1.20×10^{-80}

3.5.5. References

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Chapter IV

Transcriptomic Regulation of Seasonal Coat Color Change in Hares

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4.1. Abstract

Color molts from summer brown to winter white coats have evolved in several species to maintain camouflage year-round in environments with seasonal snow. Despite the evolutionary relevance of this key phenological adaptation, its molecular regulation has only recently begun to be addressed. Here, we analyze skin transcription changes during the autumn molt of the mountain hare (*Lepus timidus*) and integrate the results with an established model of gene regulation across the spring molt of the closely related snowshoe hare (*L. americanus*). We quantified differences in gene expression among three stages of molt progression – “brown” (early molt), “intermediate” and “white” (late molt). We found 632 differentially expressed genes, with a major pulse of expression early in the molt, followed by a milder one in late molt. The functional makeup of differentially expressed genes anchored the sampled molt stages to the developmental timeline of the hair growth cycle, associating anagen to early molt and the transition to catagen to late molt. The progression of color change was characterized by differential expression of genes involved in pigmentation, circadian and behavioral regulation. We found significant overlap between differentially expressed genes across the seasonal molts of mountain and snowshoe hares, particularly at molt onset, suggesting conservatism of gene regulation across species and seasons. However, some discrepancies suggest seasonal differences in melanocyte differentiation and the integration of nutritional cues. Our established regulatory model of seasonal coat color molt provides an important mechanistic context to study the functional architecture and evolution of this crucial seasonal adaptation.

Keywords

Seasonal coat color change, Molt cycle, Developmental timeline, Transcriptomics, Gene expression

4.2. Introduction

Seasonal environments impose numerous challenges to organismal survival. To track seasonality, individuals cycle important biological processes, known as phenologies (e.g., reproduction, migration, hibernation or molt), allowing a better phenotypic match with seasonal selective pressures (Visser et al. 2010; Helm et al. 2013). Accurate timing of physiological and behavioral changes is important for fitness. Synchronizing phenotypes with the environment requires a precise perception of predictable cues (e.g., variation in day length) that anticipate seasonal change and trigger regulatory cascades and shifts in gene expression, with consequent phenotypic change (Bradshaw and Holzapfel 2007). Quantifying gene expression changes underlying phenological traits is therefore crucial to understand how organisms synchronize their phenotypes with the surrounding environment (Schwartz and Andrews 2014; Ferreira et al. 2017; Jones et al. 2018; Giska et al. 2019).

Discrete seasonal molts have evolved as energy-efficient phenological adaptations to regenerate hair layers and produce coats with insulating properties adequate for each season (Hart 1956; Ling 1972; Geyfman et al. 2015). In at least 20 vertebrate species, seasonal molts are accompanied by the change from summer brown to winter white coats, allowing camouflage in snow-covered environments (Mills et al. 2013, 2018). As in other phenological traits, seasonal molting is mostly triggered by annual changes in day length (photoperiod) (Bissonnette and Bailey 1944; Hoffmann 1978; Lincoln et al. 2006; Zimova et al. 2018). Other environmental factors, such as temperature or snow presence, have been suggested to influence the rate and completeness of the molt in different mammals (Zimova et al., 2018 and references therein). However, molt onset seems to have minimal plasticity (Zimova et al. 2014), suggesting that photoperiod is the most important cue entraining annual molt timing. Consequently, since animals coordinate molt with the fixed variation in photoperiod, the reductions of annual snow cover associated with climate change are predicted to lead to increased mismatches between coat and background colors, disrupting camouflage and negatively affecting the fitness of seasonally color changing species (Zimova et al. 2016; Atmeh et al. 2018). Predicting how color changing molts will respond to environmental change requires a better understanding of the internal mechanisms that regulate the change of hair and color (Helm and Stevenson 2014).

In mammals, pathways and genes involved in the perception of the photoperiodic signal, such as the circadian clock and melatonin production and reception, hair growth cycle, and melanogenesis are expected to be involved in the regulation of seasonal coat color changing molts (Allain and Rougeot 1980; Duncan and Goldman 1984a; Balsalobre 2002; Slominski et al. 2004; Lincoln et al. 2006; Lin et al. 2009; Ferreira et al. 2017). The molecular

mechanisms underlying the hair growth cycle (Schlake et al. 2004; Slominski et al. 2004; Lin et al. 2009), and the basis of static melanin-based coloration have been thoroughly studied (Allain and Rougeot 1980; Logan and Weatherhead 1980; Duncan and Goldman 1984a). However, the specific regulatory mechanisms of coat color changing molts have only been addressed recently. We previously studied the regulatory patterns of seasonal changing molts in snowshoe hares, inferring gene expression changes in the skin during the spring white-to-brown molt in the species using RNA-sequencing (Ferreira et al. 2017). This work established a genic regulatory model for the seasonal molt of snowshoe hares characterized primarily by genes involved in the hair growth cycle, circadian rhythms, and pigmentation. For example, among the differentially expressed genes along the molt, a pulse of expression of the agouti signaling protein (*ASIP*) gene was detected at the onset of the molt. The ASIP peptide signals the switch between darker and lighter melanin pigment, suggesting a role of the melanin production pathway in the regulation of seasonal color change (Ferreira et al. 2017). Furthermore, *cis*-regulatory variation at *ASIP* has been linked to polymorphism in winter coat color in snowshoe hares (*Lepus americanus*) (Jones et al. 2018) and mountain hares (*L. timidus*) (Giska et al. 2019). It remains unclear how generalizable these results are across species.

Here, we study the transcriptional landscape of the autumn molt in the mountain hare (*L. timidus*), and integrate the results with the previously quantified gene expression landscape of the spring molt of snowshoe hares (Ferreira et al. 2017). Mountain hares are widely distributed across Eurasia, from Western Europe to Eastern Russia, with some isolated populations in the Alps, Ireland, British and Faroe Islands (Angerbjörn 2018). Throughout most of their range, mountain hares alternate between summer brown and winter white coats, with some exception, such as in Ireland and the Faroe Islands (Bergengren, 1969; Schai-Braun & Hackländer, 2016; Angerbjörn, 2018; Mills et al., 2018). Here we test the generality of the snowshoe hare seasonal molt regulatory model (Ferreira et al. 2017), placing hair color change in the stages of the hair growth cycle, and pin-pointing seasonal differences in regulatory mechanisms between the two species. While snowshoe and mountain hares are not sister species (Matthee et al. 2004; Melo-Ferreira et al. 2012), it is yet unclear to what degree ancestral variation or ancient introgression may explain sharing of the trait (Jones et al. 2018). We predict that the major expression waves related with pelage molt are conserved in these two species given the conservation of hair growth cycle mechanism in mammals (Stenn and Paus 2001; Lin et al. 2004; Schlake et al. 2004). Possible differences may then be related to species-specific differences or seasonal differences in hair color and composition, or to the integration of seasonal specific cues onto the regulation of hair growth. By integrating results across both species, we expand the model of genic regulation during both autumn and

spring seasonal coat color change in hares and provide a context to understand the functional mechanisms controlling this crucial seasonal adaptation.

4.3. Materials and methods

4.3.1. Sampling, laboratory procedures and sequencing

Sampling was performed following the reasoning of Ferreira et al., (2017). Briefly, the spatial progression of the molt across the body of a single molting individual results in the simultaneous occurrence of different skin patches that are expected to be in different molting stages – proliferative stage of active hair growth “anagen”, the follicle regression stage “catagen” and a final resting stage “telogen” (Lin et al. 2004; Schlake et al. 2004; Geyfman et al. 2012) (Figure 4.1). Given an expected lag between expression changes and the visible changes in hair color used as proxy of molting stage (Ferreira et al. 2017), we expect that early molt (“brown”) captures mostly anagen related processes and possibly late telogen, and late molt (“white”) captures late anagen and the transition to catagen (Figure 4.1B). Four mountain hare (*L. timidus*) specimens were collected from hunting bags during regular permit hunting in Grisons, Switzerland (Table S4.1). Hares were undergoing pelage molt, from brown to white, which starts on the extremities and ends in the head and dorsum, a pattern that is reversed during spring molt (Hewson 1958). Sampling different molting stages in the same individual allows controlling for individual variation in gene expression (Ferreira et al. 2017), and only individuals with three identifiable patches of skin corresponding to the stages of molt progression were used in this study: early molt (brown hair), intermediate stage (both brown and white hair) and late molt (white hair) (Figure 4.1), resulting in a total of 12 tissue samples. Skin patches of 2 cm² were collected from the animals’ dorsum for each skin type immediately after death and stored first in RNeasy® (ThermoFisher) and then at -80°C until RNA extraction.

From each skin sample, total RNA was extracted using the RNeasy® Mini-Kit (QIAGEN) and strand-specific cDNA libraries were synthesized using the SureSelect Strand-Specific RNA Library Prep for Illumina Multiplexed Sequencing (Agilent Technologies). Library sizes were inferred using Bioanalyzer 2100 and pooled in equal molar concentrations using the KAPA Library quantification kit (KAPA BIOSYSTEMS). Pooled libraries were sequenced in two separate Illumina HiSeq runs: ~3% of two lanes of 100 base pairs (bp) paired-end reads of an HiSeq 2000, at University of California Berkeley, and then three lanes of 101 bp paired-end reads in a HiSeq 2500 at Utah Microarray Core.

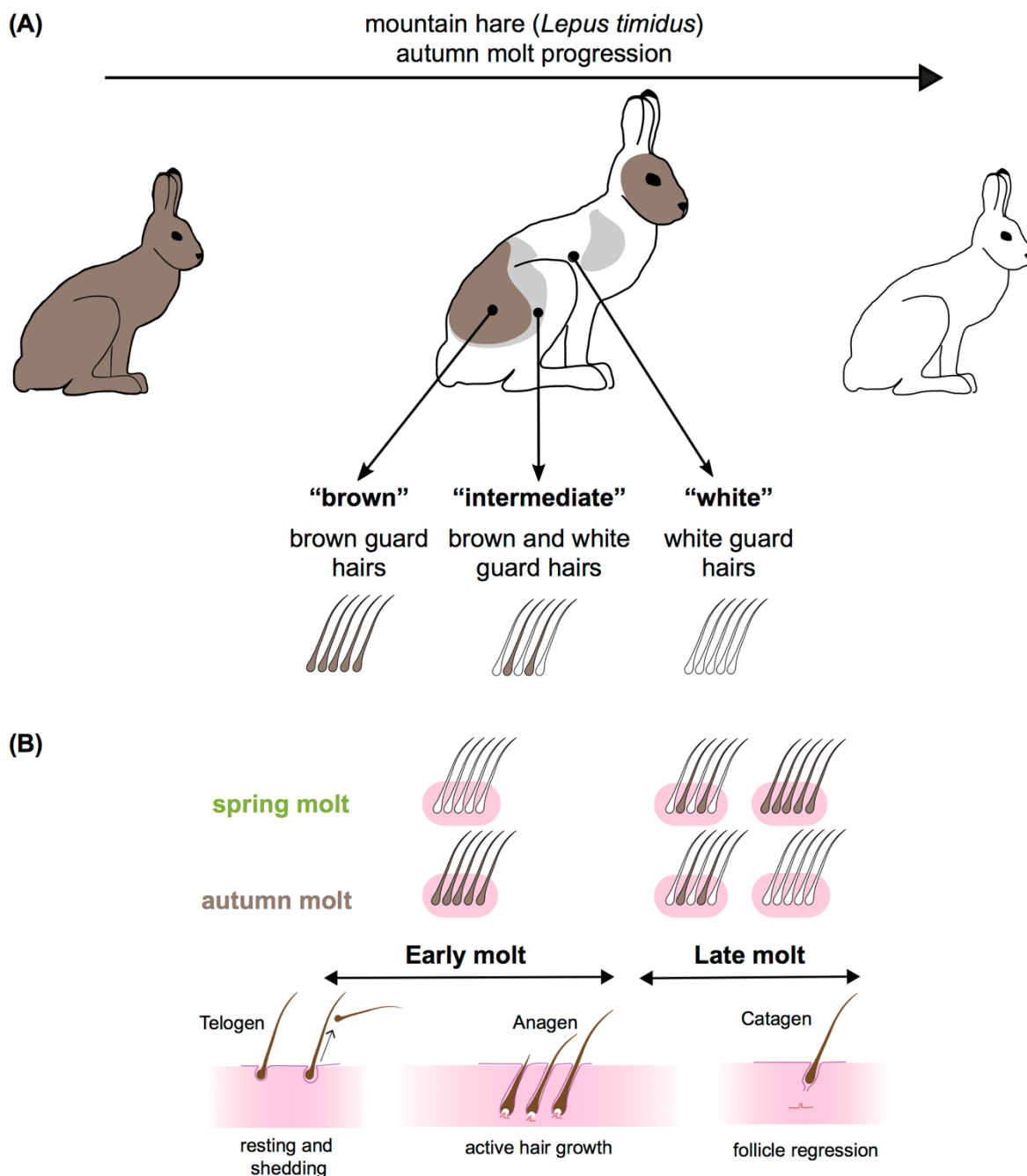


Figure 4.1 – Mountain hare autumn molt and sampling scheme. (A) Three patches of skin were sampled from individuals undergoing autumn molt, classified according to the color of guard hairs: “brown”, “intermediate” (both brown and white hairs) and “white”. (B) Parallel between the molt stages of our sampling scheme and the stages of the hair growth cycle, as established for the spring molt in Ferreira et al., (2017), and hypothesized for the autumn molt (this work).

4.3.2. Quality control and transcriptome assembly

Reads failing quality control, flagged by the Illumina Casava-1.8 Fastq Filter (Gordon 2011), were discarded and adapters removed using *Cutadapt* 1.3. *Trimmomatic* 0.32 was used to retain a minimum phred quality score of 15 across 4 bp sliding windows, to trim the

first 13 base pairs from every read, which showed a nucleotide composition bias (Hansen et al. 2010), and, finally to remove trimmed reads smaller than 23 bp.

A *de novo* skin transcriptome assembly for *L. timidus* was performed to allow an independent unbiased comparison with the results of Ferreira et al., (2017). The transcriptome assembly was performed with *Trinity* v2.6.6 (Grabherr et al. 2011), using filtered and properly paired reads and specifying “RF” for strand-specific assembly. *Transrate* 1.0.2 (Smith-Unna et al. 2016) was used to assess the quality of the transcriptome, filtering erroneously assembled contigs. We then annotated this filtered transcriptome using the rabbit (OryCun2.0) and mouse (GRCm38.p6) ENSEMBL 92 peptide references applying the reciprocal blast implemented in *Transrate*. The mouse annotation was only used when no rabbit annotation was produced for all analyses, except for the Reactome pathway enrichment analysis (see below).

4.3.3. Differential expression analysis

Reads were mapped to the *de novo* assembled transcriptome using *bowtie2* (v2.3.4.1) with the following flags: -q, --phred33, -D 20, -R 3, -N 1, -L 20, -i S,1,0.50, --dpad 0, --gbar 9999999, --mp 1,1, --np 1, --score-min L,0,-0.1, -I 1, -X 1000, --no-mixed, --no-discordant, --nofw, -p 1, -k 200, --fr, -x. To generate *bowtie2* indices, we used the *extract-transcript-to-gene-map-from-trinity* script and then *rsem-prepare-reference* script from *RSEM* 1.3.0. Finally, the outputs from *bowtie2* were used as inputs for *rsem-calculate-expression* to generate relative abundances for all genes across the transcriptome. The transcriptome annotation was used to filter the *RSEM* output, removing *Trinity* “genes” (putative genes) with annotation to multiple genes (i.e., assembled putative isoforms annotated to different ENSEMBL genes), or with no annotation.

Using the filtered *RSEM* output, differential expression was inferred between molt stages (“brown”, “intermediate” and “white”) using *edgeR* (3.20.9) (Robinson et al. 2010). Only *Trinity* genes expressed at a minimum of one count per million (CPM) mapped reads in at least 4 samples were retained. The trimmed mean of M-values was used to normalize data across libraries, and a Cox-Reid profile-adjusted likelihood was used to calculate common, trended and tagwise dispersions, as described in Robinson et al., (2010). The biological coefficient of variation (BCV) was estimated as the square root of the common dispersion (McCarthy et al. 2012). To inspect the relationship between expression counts in each sample, we plotted a multidimensional scaling plot (MDS plot) in *R* using the 500 genes with highest dispersion between each sample pair. Given an aberrant expression pattern estimated for one individual, which did not result in the individualization of molting stages (Figure S4.1) expected

from our sampling strategy (Ferreira et al. 2017), we evaluated the BCV and estimated the expected power to detect gene expression changes in the 4 versus 3-individual dataset using *RNASeqPower* (Hart et al. 2013). For comparison, we also estimated the expected power for the snowshoe hare dataset from Ferreira et al., (2017).

The inspection of the MDS plot showed that replicates tend to cluster primarily by molting stage and secondarily by individual (Figure S4.2), suggesting that individual is a significant contributor of variation to gene expression differences. To incorporate this factor in the analysis, we used “individual” as a blocking factor in the statistical test for differential expression, to control for individual variation in gene expression. Differential expression between molting stages was inferred using a likelihood ratio test for 3 pairwise comparisons. After a Benjamin-Hochberg multiple test correction, all genes with a false discovery rate (FDR) smaller than 0.05 were considered differentially expressed. We tested for significant overlap between mountain hare and snowshoe hare set of upregulated genes in each molt stage, by collapsing the upregulated genes for the same stage from each pairwise comparison, and using a Fisher exact test in R, considering as background all common unique ENSEMBL gene annotations between the transcriptomes of the two species.

A clustering analysis was then performed to identify major expression patterns across genes. We converted CPM levels for each gene to fragments per kilobase per million reads mapped (FPKM) and further $\log_2$ transformed and mean centered these values. The $\log_2$ mean centered FPKM value for each differentially expressed gene was used as input for two clustering methods. First, a complete linkage hierarchical clustering was performed using the Pearson’s correlation coefficient between samples and Euclidian distances between genes with the R package *gplots* 3.0. Second, a partition clustering analysis was done using a gap statistic to calculate the optimal number of clusters (k) using *clusGap()* from the R package *cluster* (v2.0.7). The optimal k of 9 was used in a partitioning around medoids (pam) clustering analysis using the *pam()* from R package *cluster* (Figure S4.3). The k clusters were then summarized in 3 major general patterns of expression, and the pam clustering was repeated with k=3. Finally, the expression levels and mean expression level per cluster were plotted using a modified version of the *plot_expression_patterns.pl* script from the *Trinity* pipeline.

4.3.4. Functional enrichment analysis

The functions of genes within each cluster of distinct expression signature were investigated using a Gene Ontology enrichment analysis using *Ontologizer* 2.1 (Bauer et al. 2008). A custom gene association file was built using custom scripts *make_input_4_gaf.py* and *do_Gaf_file_new_version.py* (available at

https://github.com/evochange/coat_color_change_transcriptomics) and using the GO term ID for each ENSEMBL ID present in the transcriptome assembly. Enrichment was tested (FDR < 0.05) using a parent-child-union test with the entire skin transcriptome as background and the Benjamin-Hochberg correction for multiple tests. Furthermore, a Reactome pathway enrichment analysis was done using the Reactome database (version 65; <https://reactome.org/PathwayBrowser/#TOOL=AT>) and the mouse annotation for each gene (the rabbit is not supported in the database). Enrichment was tested using the binomial test.

Finally, we investigated the expression profiles of genes annotated with Gene Ontology terms potentially related with seasonal coat color change: “molting cycle” (GO:0042303), “circadian rhythm” (GO:0007623), “pigmentation” (GO:0043473) or one of the child terms. The child terms were identified using the GOOSE tool at the Gene Ontology Consortium website (<http://amigo.geneontology.org/goose>)

4.3.5. Quantitative PCR for Agouti gene expression

Given its importance as a marker of anagen and for coat color regulation (Ferreira et al. 2017; Jones et al. 2018; Giska et al. 2019), we further investigated *ASIP*'s expression pattern. We quantified the expression of *ASIP* hair cycle isoform and reference housekeeping genes *SDHA* and *ACTB* for the three individuals and nine skin samples included in the differential gene expression analyses. Total RNA was extracted from 30 mg of each skin sample with the RNeasy® Mini Kit (QIAGEN). Tissue was homogenized in a rotor-stator homogenizer (Mixer Mill MM400, Retsch) at 30 Hz for 10 min. The RNA integrity was checked with the Agilent Technologies TapeStation for a minimum RIN > 7. First-strand cDNA synthesis was performed using oligo (dT) primers and the GRS cDNA synthesis kit (GRISP) using 400 ng of RNA from each skin sample. Relative expression was quantified based on quantitative PCR (qPCR) of the gene of interest normalized across individuals to the reference genes (primers are indicated in Table S4.2). Their amplification efficiencies were calculated based on the slope of a regression line fitted to C_t values of five samples from 2-fold serial dilutions ($E = 10^{(-1/\text{slope})} - 1$), using two replicates per sample. Then, to quantify *ASIP* expression across the molt, three replicates of qPCR reactions per skin sample were performed using 1X iTaq™ universal SYBR® green supermix (Bio-Rad), 0.4 μM each primer and 1 μl cDNA (2-fold dilution of stock cDNA), total volume 10 μl, and the thermal conditions shown in Table S4.3. The raw qPCR results, presented as the threshold cycle (C_t), were used to calculate expression of the *ASIP* hair cycle isoform, normalized by the expression of the *ACTB* or *SDHA* gene according to the formula $2^{-\Delta C_t}$, where $\Delta C_t = C_t^{\text{Agouti}} - C_t^{\text{ref gene}}$. We tested for differences in expression between molt stages using the linear regression (*pcr_lm()*) test from *R* package

pcr (Ahmed and Kim 2018), using “individual” and “molt stage” as factors, and *ACTB* as the reference gene.

4.4. Results

4.4.1. Assembly, annotation and differential expression

A total of 139,005,571 paired-end reads were obtained, averaging 11,583,798 read pairs per individual (range = 10,974,557 – 12,255,152). After adapter and quality-based trimming, the final dataset comprised 124,393,164 read pairs (averaging = 10,366,097 read pairs per individual, range = 9,916,123 – 10,946,710), varying in length from 23 to 87 base pairs (Table S4.4). These reads were then used to produce a strand-specific assembly using *Trinity*, and the resulting raw transcriptome consisted of 173,848 *Trinity* “genes” and 233,182 *Trinity* “transcripts” (Table S4.5). After quality control using *Transrate*, the transcriptome contained 159,769 “genes” and 202,135 “transcripts” (Tables S4.5 and S4.6). Finally, the annotation-based filter resulted in 36,101 *Trinity* “genes” (Table S4.6).

The inspection of the MDS plot revealed that the skin samples of one individual consistently deviated from the remaining samples and did not capture the individualization of the sampled molt stages aimed with our sampling strategy (Figure S4.1) (Ferreira et al. 2017). This suggests an artificial bias during sampling that increased the variance in the dataset. The 3-individuals’ dataset provided higher power (0.82 vs 0.72 to detect a 1.5-fold change for a probability of false positives of 0.05) and lower BCV (~0.16 vs. ~0.21) when compared to the 4-individuals’ dataset. This estimated power is comparable to that of the snowshoe hare dataset from Ferreira et al., (2017) (0.85 to detect 1.5-fold changes in expression), and adequate to detect differential expression for lowly expressed genes (Todd et al. 2016). Therefore, hereafter we report the results from the analyses of the 3-individuals’ dataset. After filtering for multiple annotated and lowly expressed genes, our transcriptome consisted of 17,270 genes, corresponding to 12,070 annotated ENSEMBL genes (11,223 rabbit genes and 847 mouse genes) (Tables S4.6 and S4.7). Of the annotated genes, 75% (9,091) were also present among the 10,345 genes annotated in the snowshoe hare transcriptome (Ferreira et al. 2017).

Differential expression was tested in three pairwise comparisons between the “brown”, “intermediate” and “white” molt stages, and 632 genes, corresponding to 514 unique ENSEMBL annotations, were found differentially expressed in at least one pairwise test (Figure 4.2; Table S4.8). Of these, 131 (25%) were also found differentially expressed in the

snowshoe hare dataset (total of 754 unique ENSEMBL annotations) in any stage, representing a significant overlap (Fisher exact test, odds ratio = 4.37, $p = 2.2\text{E-}16$; Table S4.9). A significant overlap was also found among genes upregulated early in molt in both mountain and snowshoe hares (102 unique ENSEMBL annotations of 379 and 547 upregulated early in mountain and snowshoe hare respectively; Fisher exact test, odds ratio = 6.83, $p = 2.20\text{E-}16$), but no overlap or significant overlap among genes upregulated in intermediate or late in molt, respectively ($p > 0.05$; Table S4.9). As in Ferreira et al., (2017), most upregulated genes were found in the early molt (“brown” in the autumn molt), whereas “intermediate” was the stage with the lowest number of upregulated genes (Figures 4.2, 4.3A and S4.4).

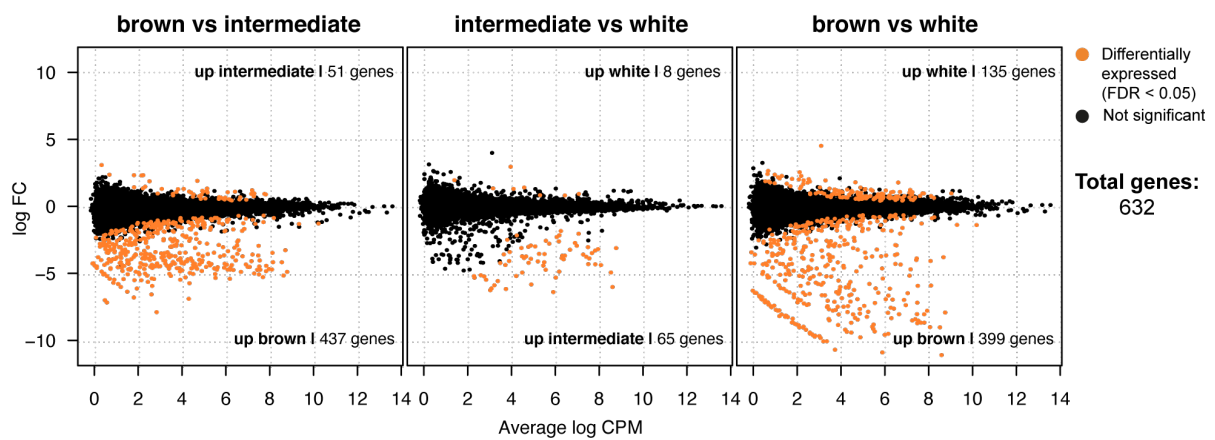


Figure 4.2 – Smear plots showing the number of differentially expressed genes (FDR < 0.05) in three pairwise comparisons between molt stages. Average log counts per million for each gene (dots) are plotted against log fold change. For each comparison, the number of upregulated genes in each stage is shown.

To inspect the pattern of expression of these genes across the molt, we plotted mean centered $\log_2$ FPKM expression levels in a heatmap (left panel of Figure 4.3A), showing that most genes tend to be upregulated early in the molt and that levels of expression tend to decrease towards the end of molt. However, a smaller proportion of genes showed higher levels of expression at the end of molt. The hierarchical clustering analyses showed that sampled “brown” and “white” skin tissues clustered according to molt stage, while “intermediate” samples were split between the two clusters. Furthermore, the partitioning clustering analysis resulted in three clusters of genes with similar expression patterns: 1) 239 genes that decrease their expression gradually towards the end of molt, from “brown” to “white”; 2) 239 genes decrease their expression from “brown” to “intermediate” and retain a constant level of expression towards “white” and; 3) 154 genes increase their expression gradually from “brown” to “white” (left panel of Figures 4.3A and S4.4). Both clusters 1 and 2 group genes with higher expression early in the molt, which was also found in snowshoe hares (right panel of Figure 4.3A).

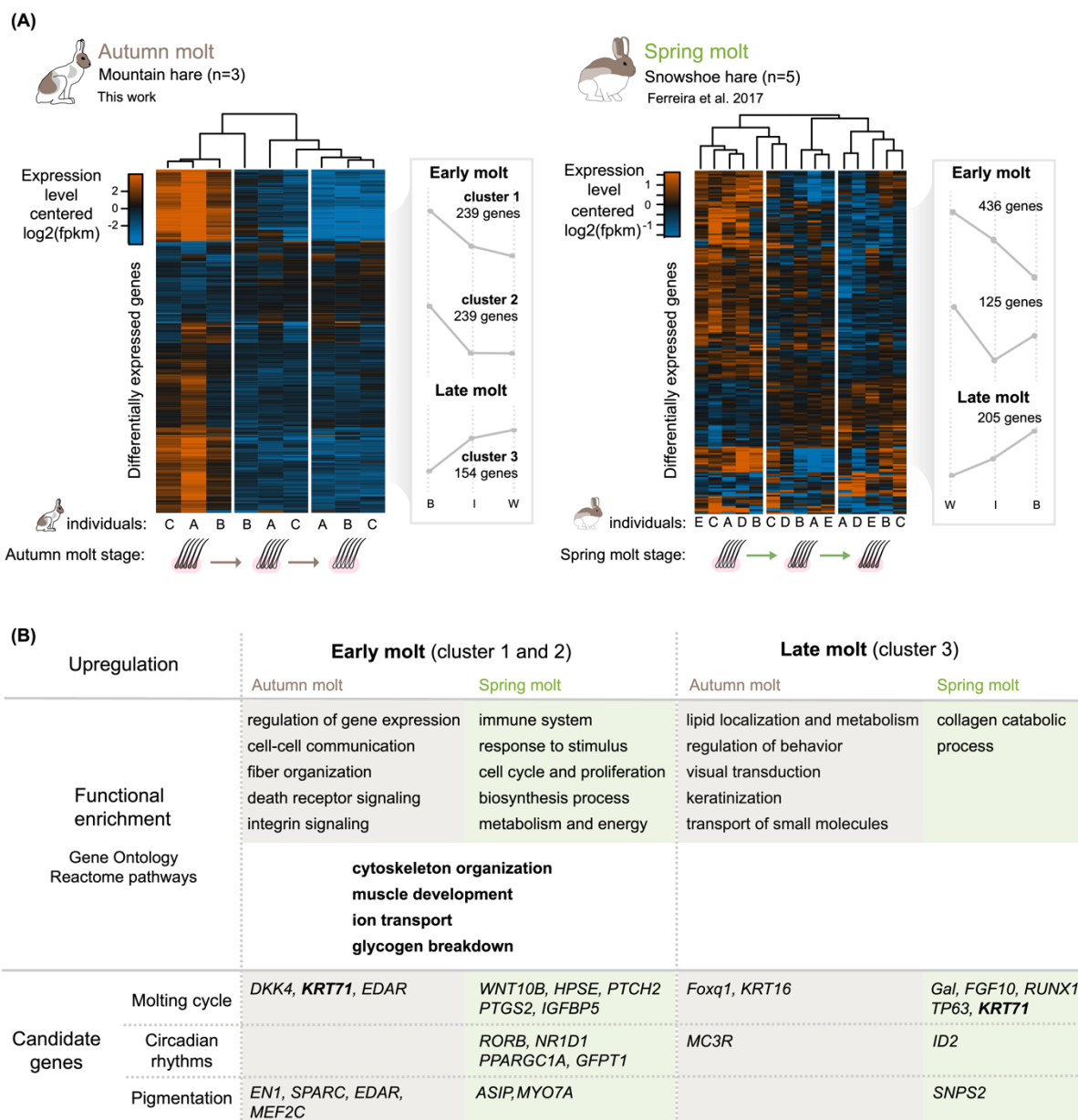


Figure 4.3 - A model of the transcriptional landscape of seasonal coat color change. Left panels show the results of this work on the autumn molt of the mountain hare and right panels show the corresponding results on the spring molt of the snowshoe hare, adapted from Ferreira et al., (2017). (A) Patterns of expression of differentially expressed genes in the mountain hare and snowshoe hare datasets are shown as heatmaps of $\log_2$ transformed, mean centered FPKM levels of expression per differentially expressed gene (row) and sample (column; A-C and A-E). Dendrograms represent the complete linkage hierarchical clustering of Pearson's correlation coefficient between samples. Clusters result from PAM clustering of individual gene expression levels for $k=3$ (B – "brown"; I – "intermediate"; W – "white"). The gray line connects the mean expression value of genes' expression in "brown" (B), "intermediate" (I) and "white" (W). (B) Functional enrichment and candidate gene expression at early and late molt. For clusters in (A), a Gene Ontology and Reactome enrichment analysis was performed and the presence of candidate genes, known to have functions related with molting cycle, circadian rhythms and pigmentation regulation was inspected. Terms and genes specific to mountain hare (this work) and snowshoe hare (Ferreira et al. 2017) are highlighted in brown and green, respectively. Bold black indicates common terms to both species.

4.4.2. Gene-ontology term and Reactome pathway analyses

To understand if genes with different expression patterns could be distinguished by their functions, we tested the enrichment of Gene Ontology (GO) terms and Reactome pathways in each gene cluster obtained from partitioning clustering analysis (Tables S4.10 to S4.15). We found 65, 4, and 2 GO biological process terms enriched in cluster 1, 2, and 3, respectively (Figure 4.3B, Tables S4.10 to S4.12). Furthermore, 10 Reactome pathways were found enriched in cluster 1, 18 in cluster 2 and 9 in cluster 3 (Figure 4.3B, Tables S4.13 to S4.15). In general, the enrichment analysis revealed functions and pathways that are expected in the molting skin (Ferreira et al. 2017). Both clusters with higher levels of expression in “brown” (1 and 2; Figure 4.3B) showed a similar enrichment of GO terms that were related with muscle development, cytoskeleton organization, transmembrane transport, circulatory system development, cell-cell communication and adhesion and regulation of gene expression and protein synthesis (Figure 4.3B, Tables S4.10 and S4.11). Pathway enrichment in both clusters was consistent with the GO enrichment; muscle related pathways enriched in cluster 1, and metabolism of proteins and RNA, and signaling transduction were enriched in cluster 2 (Figure 4.3B, Tables S4.13 and S4.14). In cluster 3 (genes increasing their expression towards the end of molt), only two GO biological process terms were found enriched: “lipid localization” and “regulation of behavior” (Figure 4.3B, Table S4.12). Pathways found enriched in cluster 3 are involved in keratinization, lipid metabolism, the retinoid cycle and transport of small molecules (Figure 4.3B, Table S4.15). Next, we compared patterns of functional enrichment detected in mountain hares with patterns found during the snowshoe hare molt (Ferreira et al. 2017). Overlapping GO terms and Reactome pathways were only detected for gene clusters showing higher expression early in the molt cycle including muscle tissue development, ion transport, cytoskeleton organization and glycogen breakdown (Figures 4.3B and S4.5).

In addition, we inspected the expression of genes with functions expected to be involved in seasonal coat color change. We found nine candidate genes that were differentially expressed across the molt with the GO terms “pigmentation”, “molting cycle” and “circadian rhythm” (12 Trinity genes; Figure 4.3B, Table S4.16). Six of these genes (*EN1*, *SPARC*, *MEF2C*, *DKK4*, *KRT71*, *EDAR*) showed higher expression at early molt (cluster 2) and were found to be upregulated in “brown”, while three (*Foxq1*, *KRT16*, *MC3R*) increased their expression towards the end of molt (cluster 3) and were up regulated either in “white” or “intermediate” (Figure 4.3B, Table S4.16). Genes *Foxq1*, *DKK4*, *KRT71*, *KRT16*, *EDAR* are putatively involved in molting cycle-related functions. Genes *EN1*, *SPARC*, *EDAR* and *MEF2C* are annotated to the Gene Ontology term “pigmentation” and the child term “melanosome

differentiation”, being all upregulated in “brown”. Finally, one gene, *MC3R*, annotated to child terms of “circadian rhythm” (“circadian regulation of gene expression” and “locomotor rhythm”), showed higher expression in “white” and “intermediate”. *KTR71* was the only candidate gene found differentially expressed both here and in Ferreira et al., (2017) (an overlap not expected by chance; odds ratio=66.3, $p=0.02$; Table S4.9), though its late molt upregulation contrasts with the higher expression early in the molt in the snowshoe hare (Figure 4.3B).

Using qPCR, we note a tendency for higher expression of *ASIP* hair cycle isoform early in the molt of individual A, a marginal difference between early and the other stages for individual B, and no difference for individual C, though none of these patterns was significant (Figure S4.6; Table S4.17; Table S4.18). No significant differences in *ASIP*’s expression along the molt were detected based on RNA-sequencing data (Figure S4.6).

4.5. Discussion

Understanding how changes at different levels of molecular organization (e.g., gene, protein or pathway) affect phenotype variation is critical to deciphering the evolution of adaptive phenotypes (e.g., Dalziel et al., 2009). When possible, mechanistic studies of phenotypes should be based on natural populations so that molecular variation can be tied to the natural conditions that individuals experience (Alvarez et al. 2014) and, ultimately, to fitness (Barrett and Hoekstra 2011). However, field-based quantitative studies are challenging given the multiplicity of uncontrolled factors, including individual variation, that may hinder biologically meaningful patterns (Todd et al. 2016). We leveraged the ability to sample different stages of the developmental timeline of seasonal coat color change in single molting hares to control for individual variation, and then combine transcriptional data from mountain and snowshoe hares to build a working model of the genic regulation of this crucial seasonal adaptation.

4.5.1. The transcriptional landscape of the molt in the context of the hair growth cycle

In keeping with the results in snowshoe hares (Ferreira et al. 2017), we found that most genes show a pattern of expression that is correlated with early (“brown”) or late (“white”) molt (Figure 4.3A), underlining the difficulty in discretizing the “intermediate” stage (Figures 4.2, 4.3 and S4.4). As in the snowshoe hare spring molt, most of the upregulated genes were detected early in the molt. This pattern of induction is expected during the proliferative stage of the hair

growth cycle, anagen (Lin et al. 2004; Schlake et al. 2004), when melanogenesis occurs (Slominski and Paus 1993). Genes upregulated in brown/early molt (cluster 1 and 2 of Figure 4.3A), showed enrichment of functional categories that are characteristic of anagen (Figure 4.3B and Tables S4.10, S4.11, S4.13 and S4.14). For example, genes in cluster 1 and 2 were enriched in functions related with tissue organization and morphogenesis (e.g., “cytoskeleton organization”, “supramolecular fiber organization”, “tissue development”; Lin et al., 2004), gene expression and protein synthesis (e.g., “positive regulation of gene expression”, “metabolism of RNA”; Geyfman et al., 2012) cell-cell communication and adhesion (e.g., “regulation of cell communication by electrical coupling”; pathway “integrin cell surface interactions”; Mayer et al., 1993; Botchkarev and Kishimoto, 2003) and metabolism (e.g., “glycogen metabolism”; Shipman et al., 1955; Geyfman et al., 2015). An inspection of candidate genes for molt, circadian rhythm and pigment regulation, revealed that genes with known anagen-related expression were also highly expressed early in the molt, such as hair keratins (*KRT71*; Stark et al., 1987; Porter et al., 2001), hair proliferation and shape-determining genes (*EDAR* and *DKK4*; Millar, 2002; Fliniaux et al., 2008; Adhikari et al., 2016), genes involved in the incorporation of collagen to maintain skin integrity (*SPARC*, Table S4.16; collagen genes *IIA1*, *IIIA1* and *VIA2* annotated to “integrin cell surface interactions”, Table S4.14; Parakkal, 1969; Mayer et al., 1993; Strandjord et al., 1999) and pigmentation genes (*EN1*, *SPARC*, *EDAR* and *MEF2C*; Table S4.16; Crocker and Cattanach, 1979; Slominski and Paus, 1993; Loomis et al., 1998; Agarwal et al., 2011; Ditommaso et al., 2014).

Thus, the brown early molt stage largely recovers anagenic processes. However, we also detected some clear exceptions, such as the upregulation of pathways related to cell death usually characteristic of catagen (Stenn and Paus 2001; Lin et al. 2004), and the upregulation of muscle-related genes at the beginning of molt (Tables S4.10, S4.11, S4.13 and S14). Notably, muscle-related genes were also differentially upregulated early in the spring molt of the snowshoe hare (Ferreira et al., 2017; Figure 4.3B), which could be related with the remodeling of tissue types that surround the hair follicle during hair growth cycle (Schmidt and Horsley 2012).

Our white/late molt skin samples showed expression signatures consistent with hair follicles transitioning into late anagen and possibly catagen (Figures 4.2 and 4.3, Tables S4.12 and S4.15), mirroring patterns of the snowshoe hare molt (Figure 4.3; Ferreira et al., 2017). For instance, genes important for hair shaft differentiation and development (Table S4.16; Lin et al., 2009), keratinization (Pinkus et al. 1981), and lipid related functions (Tables S4.12 and S4.15) (Lin et al. 2009) were all enriched for late upregulation. Finally, catagen induction is signaled by the enrichment of retinoid related pathways (Table S4.15) and late molt upregulation of *STRA6* (annotated to “regulation of behavior”; Table S4.12), responsible for

the uptake of vitamin A into the cell which seems to be important for catagen initiation (Foitzik et al. 2005; Everts 2012).

Despite the natural noise of expression studies performed in wild specimens, we were able to recover strong signatures that associate visible markers of molt progression (i.e., change in hair color) with the underlying hair cycle stages of anagen and catagen. Among this and the snowshoe hare study (Ferreira et al. 2017), we found significant overlaps among genes expressed in the molting skin (75%), among genes with stronger expression early in the molt (25% overlap), and among candidate genes for regulation of molt functions. These results suggest conservatism of the pathways and genes involved in skin processes, molt phenology, hair cycle activation (early molt) and regulation (candidate genes) in mountain and snowshoe hares, despite differences in the sampled species and molting season. The conservation of regulatory processes underlying pelage molts in these species is expected given the general conservatism of the hair growth cycle mechanism in mammals (Stenn and Paus 2001) and the recent divergence between mountain and snowshoe hares [4 million years (Matthee et al. 2004); ~1% uncorrected pairwise sequence divergence, (Jones et al., 2018)] (Voolstra et al. 2007; Uebbing et al. 2016). The developmental framework used here should be transferable to other seasonally molting species, including other species that have evolved seasonal camouflage, thus contributing to the general understanding of the regulatory underpinnings of this adaptive phenotype across taxa (Manceau et al. 2010; Rosenblum et al. 2014).

4.5.2. Candidate genes associated with circadian rhythm and pigmentation

To better understand the mechanistic regulation of brown to white autumn color change in mountain hares, we also inspected the expression of candidate genes associated with circadian rhythm regulation and pigmentation. While circadian gene expression is usually associated with late telogen and early anagen (Lin et al. 2009; Geyfman et al. 2015), in our work only one gene with “circadian rhythm” GO term annotation, *MC3R*, was found differentially expressed and upregulated towards the end of molt (Figure 4.3B; Table S4.16). *MC3R* is not a central clock gene and rather acts as a reinforcer of rhythm behavior [reviewed in Girardet et al., (2014)] by regulating the expression of clock genes in response to nutrient availability and light cues (Sutton et al. 2008). Likewise, genes related to rhythmic gene expression in response to metabolic cues (Slominski et al. 2003; Xu et al. 2009; Helfer et al. 2012; Oh et al. 2016) were differentially expressed and upregulated late in molt (e.g., *APOE*, *STRA6*, and *HTR2B* in Table S4.12). Collectively, these genes may integrate the response to external or internal cues, such as food intake or nutritional condition, and cyclic phenotypic

changes in the body, such as the molt cycle. In snowshoe hares, most of the differentially expressed circadian rhythm genes were upregulated early in the molt, and only one is known to be involved in the regulation of the circadian clock in response to nutrient intake (*GFPT1* in Figure 4.3B; Ferreira et al., 2017).

Given that the autumn molt of mountain hares is accompanied by growing hair changing from brown to white, we looked for expression changes of known pigmentation genes. We first focus on the pigmentation gene *ASIP* (Lu et al. 1994; Manceau et al. 2011), which tracks the onset of melanogenesis during the spring molt of the snowshoe hare (Ferreira et al. 2017) and determines the expression of alternative winter coat colors in snowshoe and mountain hares (Jones et al. 2018; Giska et al. 2019). *ASIP*'s expression trended higher early in the molt (Figure S4.6), consistent with activation of melanogenesis (Slominski and Paus 1993). However, there was no significant difference in *ASIP*'s expression among stages, both using RNA-Sequencing and targeted qPCR data (Figure S4.6) and, therefore, the relevance of the trend needs further investigation. The fact that *ASIP* is upregulated over a narrow window of 2-3 days during the hair growth cycle (Bultman et al. 1992; Miller et al. 1993) may explain failure to detect the expression peak in some individuals.

Additionally, we detected early upregulation of four genes involved in pigmentation (*EN1*, *SPARC* and *EDAR*) or melanocyte differentiation (*MEF2C*) (Figure 4.3B; Table S4.16). *SPARC* and *EDAR* play major roles in hair follicle and skin related processes (Strandjord et al. 1999; Millar 2002; Fliniaux et al. 2008; Adhikari et al. 2016). *MEF2C* is involved in the differentiation of follicular melanocytes of the new hair cycle (Tobin et al. 1999), and its loss results in decreased pigmentation (Agarwal et al. 2011). *EN1* is involved in establishing the boundary of expression of dorsal genes (Davis et al. 1991; Cygan et al. 1997; Candille et al. 2004) and limiting the migration and proliferation of melanocytes in the ventral limb (Cygan et al. 1997). *EN1* typically shows ventral expression and thus the upregulation early in the molt in our dorsal samples appears surprising. It is possible that transient expression of *EN1* contributes to winter whitening, by allowing dorsal expression of the ventral isoform of *ASIP*. However, the expression of ventral *ASIP* isoform in the dorsal skin was negligible (Figure S4.6C; Table S4.17). Thus, as with snowshoe hares (Jones et al. 2018), expression of ventral *ASIP* isoform does not appear to be involved in the evolution of dorsal winter whitening in mountain hares.

There was no overlap of pigmentation candidate genes differentially expressed in mountain hares and snowshoe hares (Figure 4.3B). This may be due to season or species-specific differences in expression. However, since we found a general conservation of the genic regulation of the early molt across species, we hypothesize that this difference may be related with the mechanisms of production of brown or white hairs across seasons. For

example, we found upregulation of genes related with melanocyte differentiation early in autumn molt, which were not detected in spring molt (Figure 4.3B; Table S4.16; Ferreira et al., 2017), suggesting that the production of winter white coats may involve an impact on melanocyte differentiation. Whether this hypothesis can indeed be generalized from mountain to snowshoe hare must await the study of the autumn molt in the latter species, and a better understanding of the genetic basis of the evolution of winter white coats in *Lepus*.

4.6. Conclusions and Significance

Coat color change is a complex adaptation to environmental seasonality that involves the regulation of circadian response, hair shedding and regrowth, and pigmentation. The joint analysis of the transcriptional landscape of the coat color molt in snowshoe and mountain hares shows that hair color in molting animals broadly delimits the underlying developmental timeline of seasonal molts. Particularly, early molt is the most transcriptionally active stage, marking the activation of circadian clock genes and inducing hair growth and pigmentation. As this inductive wave recedes, color change becomes noticeable, and in late molt a second transcription wave occurs, marking the transition from anagen to catagen. We now show that, in autumn, the upregulation of genes related with integration of nutritional cues into the circadian clock late in molt may further adjust molt rate in response to nutritional condition. This comprehensive mechanistic baseline will be a useful tool to guide functional molecular studies on the control of seasonal color change in hares and other species and to interpret quantitative genotype-to-phenotype studies on the genetic basis of winter coat color determination.

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4.8. Competing interests

The authors declare no conflict of interests.

4.9. Author Contributions

JM-F, JMG, KH, LSM and PCA conceived the study; JM-F, JMG and MSF designed the work; HJ and KH organized and performed field work; CMC and LF performed laboratory work; MSF analyzed data; MSF and IG analyzed qPCR data; MSF, JM-F and JMG wrote the manuscript with comments from the other authors; all authors read, revised and approved the final manuscript.

4.10. Data Accessibility

Raw reads were deposited at the Sequence Read Archive (SRA) under BioProject PRJNA590529. Raw and filtered transcriptome assembly, the blast output annotations of the transcriptome to the ENSEMBL 92 rabbit (OryCun2.0) and mouse (GRCm38) references and

gene and isoform level counts were deposited at Dryad (<https://doi.org/10.5061/dryad.x0k6djhfh>). The pipeline and custom scripts used to generate and annotate the assembly, generate bam files and do a Gene Ontology analysis with a custom annotation are available at https://github.com/evochange/coat_color_change_transcriptomics. All supplementary materials can also be accessed at the end of this chapter or at <https://doi.org/10.1002/ece3.5956>.

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4.12. Supplementary Materials

4.12.1. Supplementary Figures

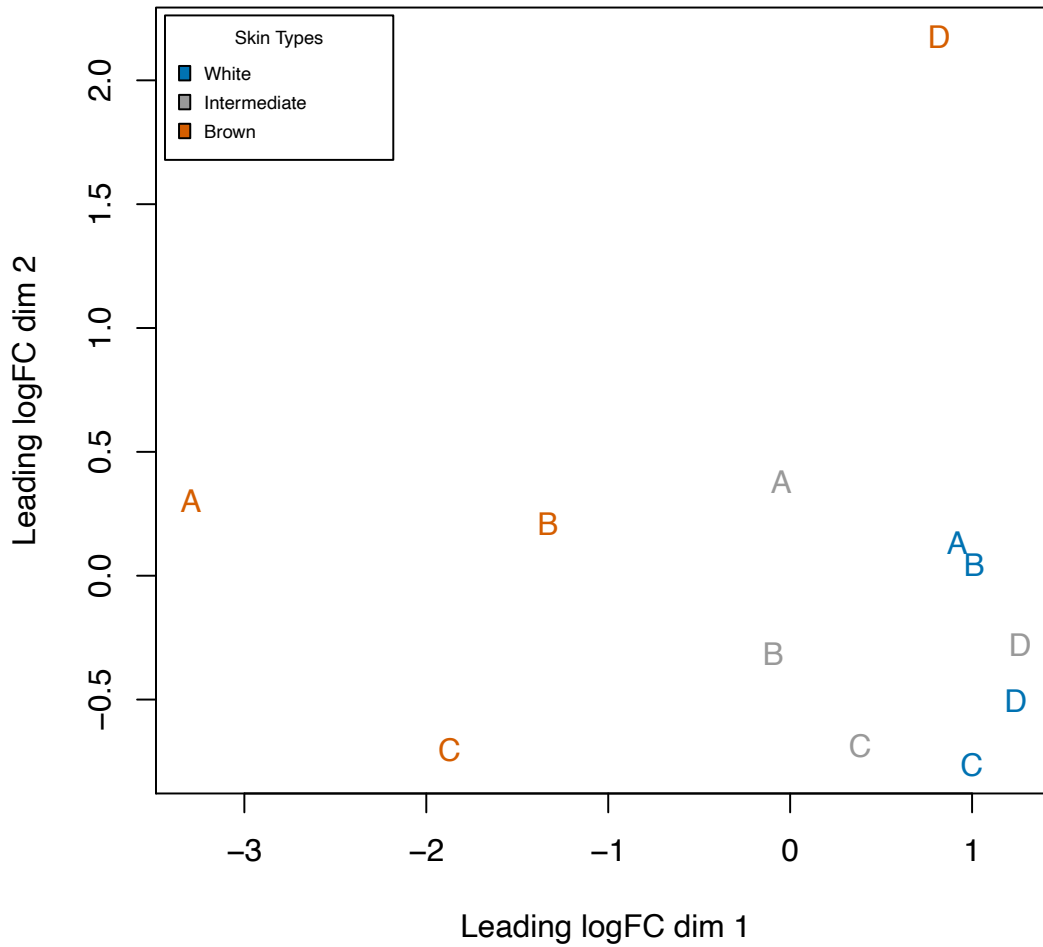


Figure S4.1 – Multidimensional scaling plot with 4 individuals where distance between samples was calculated using the 500 genes with higher dispersion between each sample pair. Distance between samples is the Euclidian distance (average root-mean-square) of the 500 genes' absolute $\log_2$ fold change between samples. Letters identify each individual sample, and colors identify each molting stage.

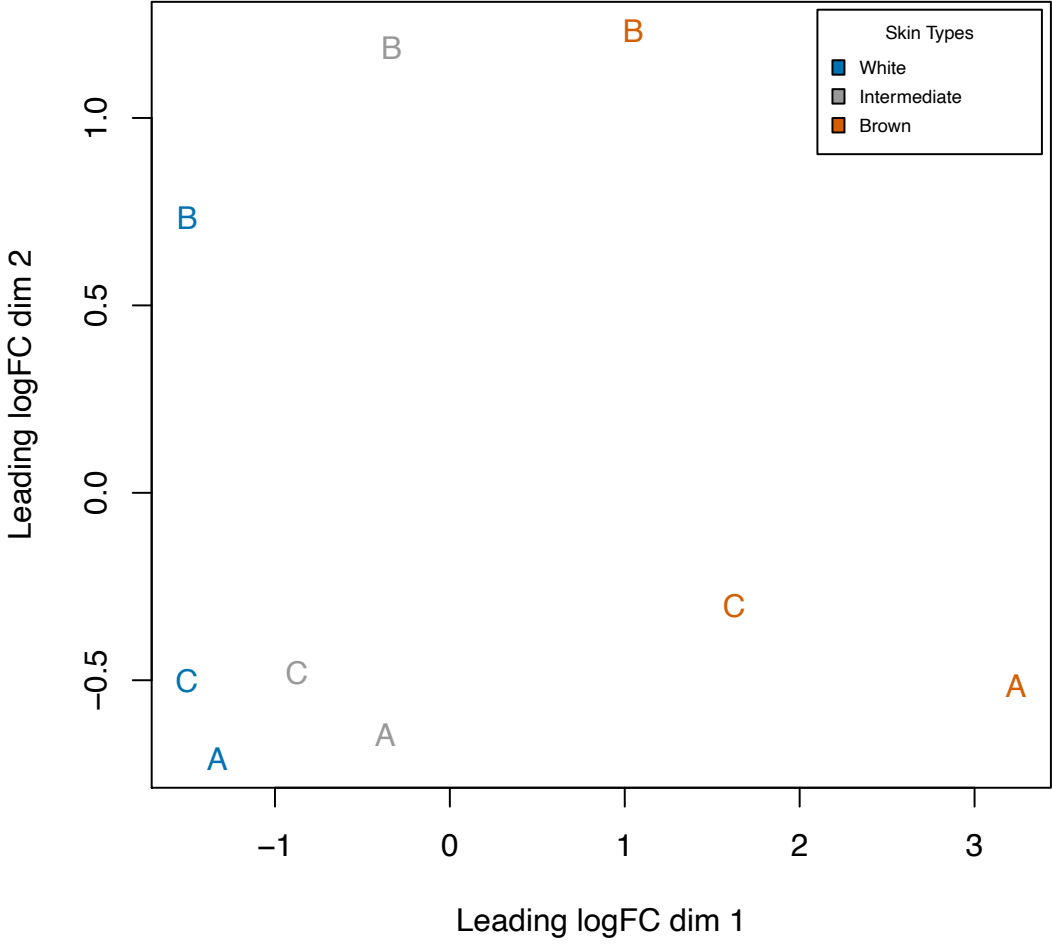


Figure S4.2 – Multidimensional scaling plot with 3 individuals where distance between samples was calculated using the 500 genes with higher dispersion between each sample pair. Distance between samples is the Euclidian distance (average root-mean-square) of the 500 genes’ absolute $\log_2$ fold change between samples. Letters identify each individual sample, and colors identify each molting stage.

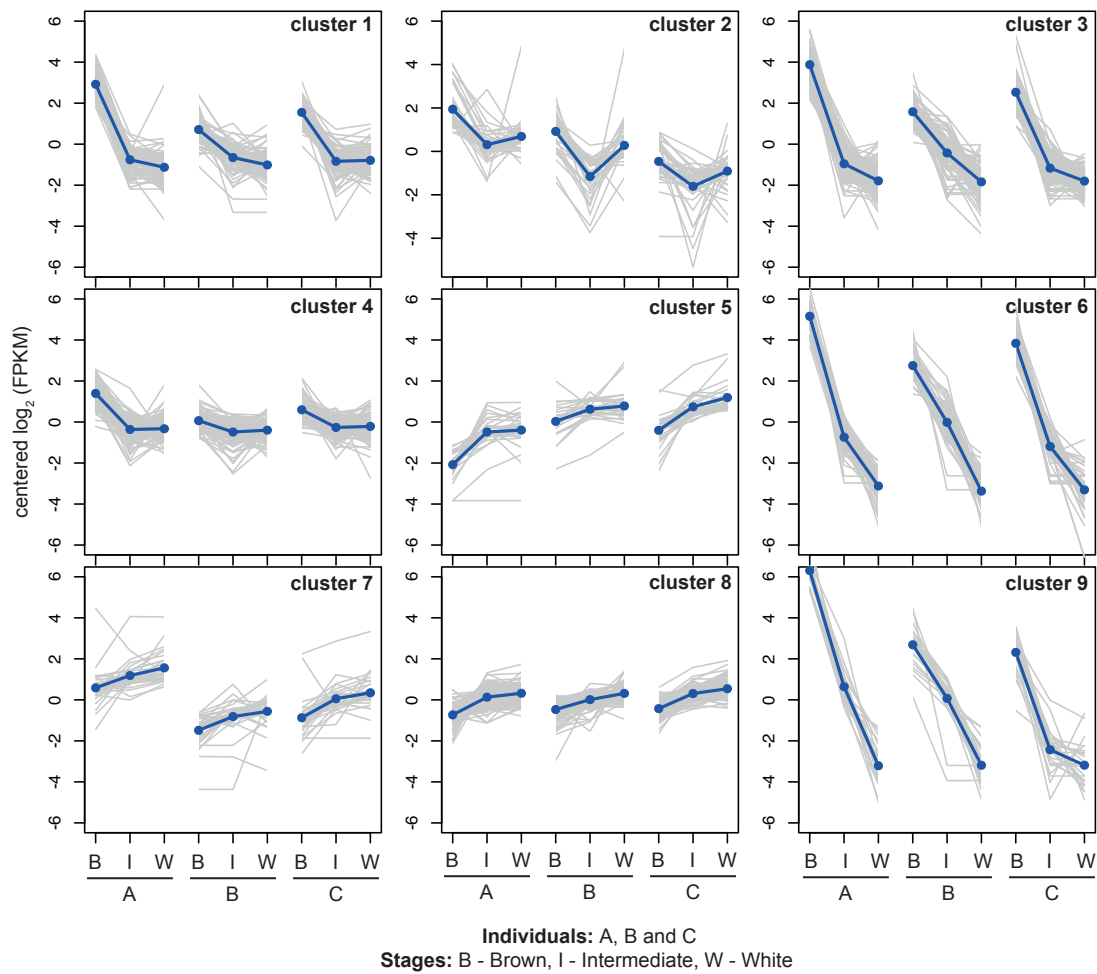


Figure S4.3 – Clusters are resultant from PAM clustering of individual gene expression levels for $k=9$. The average expression level for the group of genes in each cluster is displayed in black. Letters A, B and C represent mountain hare individuals and letters B, I and W represent the “brown”, “intermediate” and “white” molting stages, respectively.

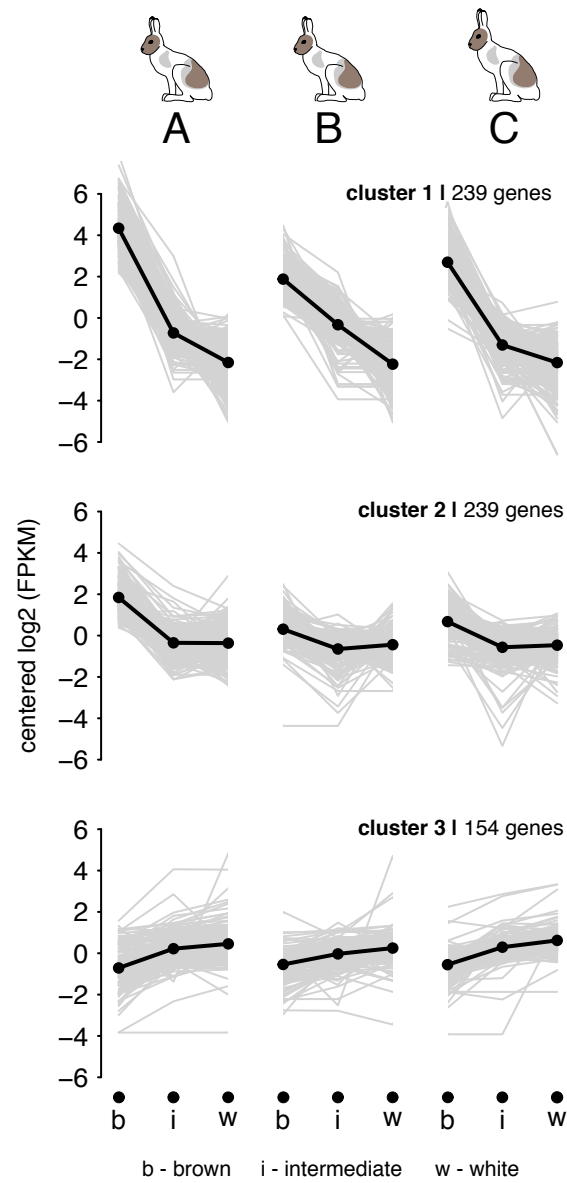


Figure S4.4 – Clusters are resultant from PAM clustering of individual gene expression levels for $k=3$. The average expression level for the group of genes in each cluster is displayed in black. Letters A, B and C represent mountain hare individuals and letters b, i and w represent the “brown”, “intermediate” and “white” molting stages, respectively.

The evolution and molecular bases of seasonal coat color variation in hares:
gene expression and the shared contribution of ancestral polymorphism and introgression

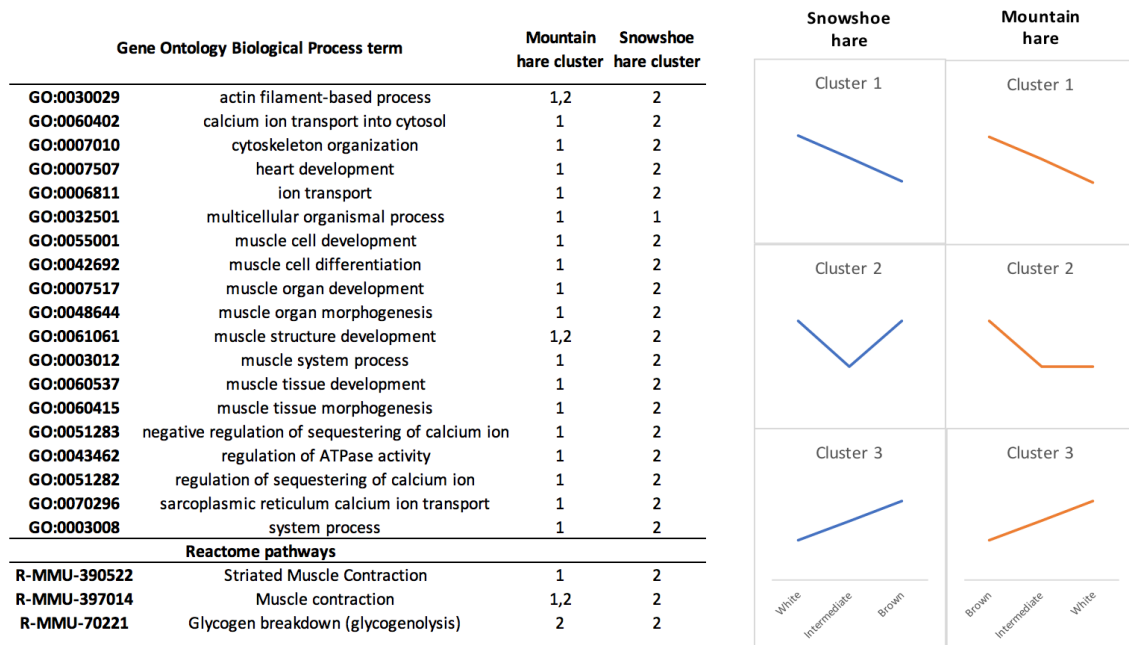


Figure S4.5 – Functional overlap between the mountain hare autumn molt and snowshoe hare spring molt. Overlapping Gene Ontology Biological Process terms and Reactome pathways and the clusters where they are enriched in both species' datasets.

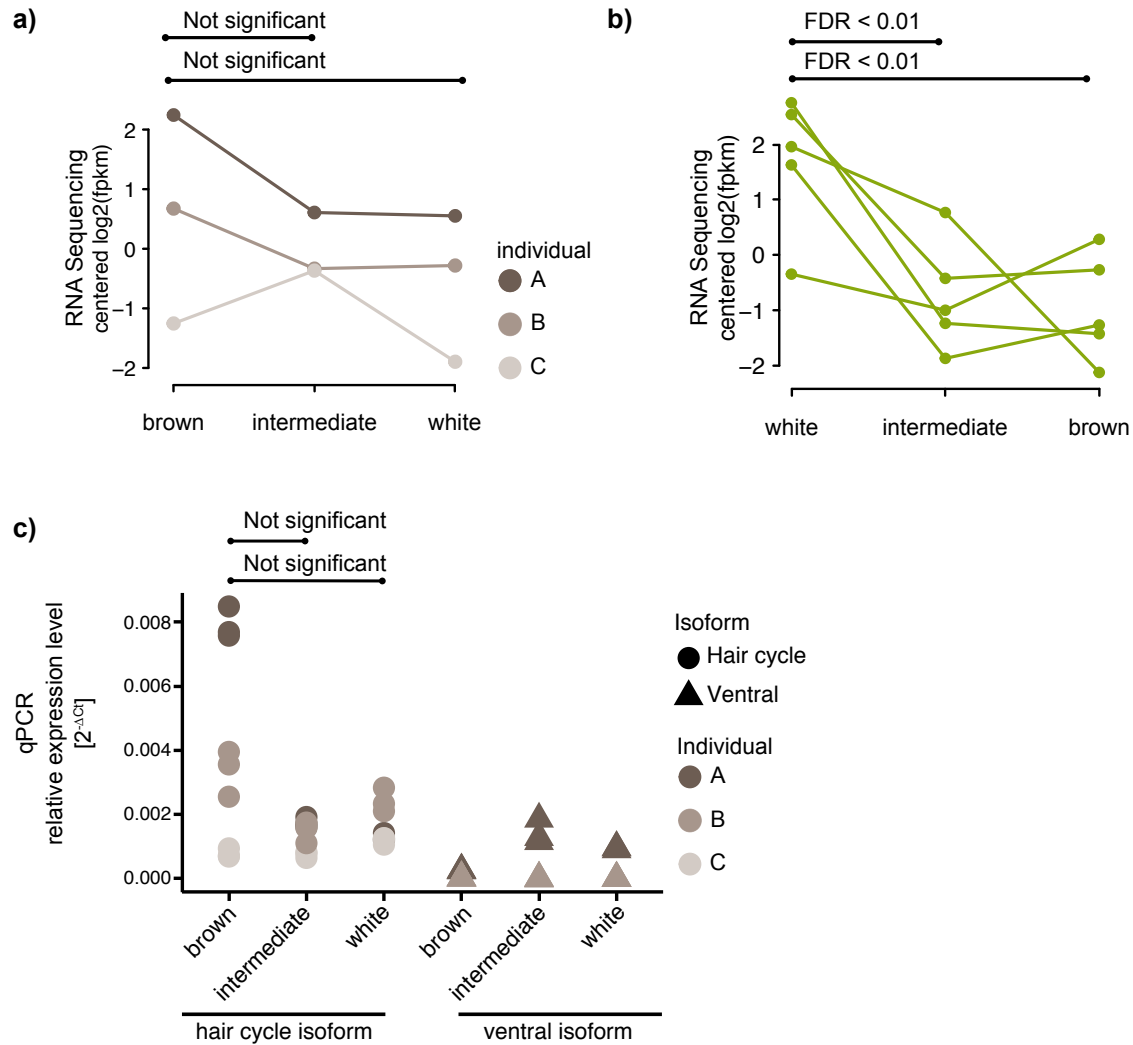


Figure S4.6 – The expression of candidate agouti signaling peptide (*ASIP*) gene across the autumn molt of (A, C) mountain hares (this work) and (B) spring molt of snowshoe hares (recalculated from Ferreira et al. 2017). For both species (A, B), expression levels are shown in mean centered log₂ FPKM for all individuals in each dataset, derived from RNA-sequencing. For mountain hares, (C) relative expression levels estimated from qPCR (2^{-ΔCt}) for the *ASIP* hair cycle and ventral isoform are shown for individuals and tissue types, using *ACTB* as a reference gene.

4.12.2. Supplementary Tables

Table S4.1 – Sampling details for the individuals used in this study.

Individual	Database ID	Sampling location	Sex	% White
A	3112	Falein, Filisur, Switzerland	F	10%
D	3113	Falein, Filisur, Switzerland	F	5%
B	3114	Trimmis, Stams, Switzerland	M	25%
C	3116	Aienfeld, Guscha, Switzerland	F	40%

Table S4.2 – Sequences, sequencing efficiencies and expected PCR product length of the primers used for qPCR expression analysis of the ventral and hair cycle *ASIP* isoforms from Jones et al. (2018), Science, and reference genes *ACTB* (from Fabiana Neves, CIBIO-InBIO, University of Porto) and *SDHA* (from Giska et al. (2019), PNAS).

Product	Primer	Sequence (5'-3')	Efficiency [%]	PCR product [bp]
<i>ASIP</i> hair cycle isoform	agouti_hair_F3	GGACAGAAAAGACGCTCTGC	96% ($R^2 = 0.9968$)	180
	agouti_trscpt_R2	TGGTCATCAGTGGGTTTCTCC		
<i>ASIP</i> ventral specific isoform	agouti_vent_F4	TGGAAGAGGGAGTCATTAGCTG	107% ($R^2 = 0.9934$)	163
	agouti_trscpt_R2	TGGTCATCAGTGGGTTTCTCC		
<i>ACTB</i>	LTM_actb_F	GAAGATCTGGCACCACACCTTC	118% ($R^2 = 0.924$)*	162
	LTM_actb_R	CCTGGATGGCCACGTACATG		
<i>SDHA</i>	LTM_sdha_F	CCCTTGTA GTTGGTAGGAATGC	114% ($R^2 = 0.971$)	181
	LTM_sdha_R	CCCGACAAGGATCACGTCTA		

* efficiency estimated simultaneously with analysis from Giska et al. (2019), PNAS.

Table S4.3 – Thermal conditions for quantitative PCR used to amplify *ASIP* isoforms and reference genes.

	Temperature (°C)	Time (s)
	95	30
40 cycles	95	5
	62	30
melt curve analysis	65 to 95, in 0.5°C increments	

Table S4.4 – Number of reads for each individual skin sample, before and after filtering for quality.

Samples	Number of raw reads	Number of filtered reads
A brown skin	11 225 550	10 064 999
A intermediate skin	11 534 533	10 395 094
A white skin	11 622 112	10 349 562
B brown skin	11 361 420	10 171 463
B intermediate skin	11 682 515	10 423 361
B white skin	12 255 152	10 946 710
C brown skin	12 056 015	10 670 922
C intermediate skin	11 747 125	10 526 823
C white skin	11 799 652	10 518 387
D brown skin	11 328 248	10 180 415
D intermediate skin	11 418 692	10 229 305
D white skin	10 974 557	9 916 123
Total	139 005 571	124 393 164

Table S4.5 - *Transrate* metrics for the raw transcriptome produced with *Trinity*.

Transrate Metrics	Raw Transcriptome Value
No. Contigs	233182
Size Smallest Contig	201
Size Largest Contig	20708
No. Assembled bases	183897734
Contig mean length	788.64464
No. contigs shorter than 200bp	0
No. contigs longer than 1k bp	47338
No. contigs longer than 10k	158
No. contigs with Open Reading Frame	50920
% of contig length covered by ORF	58.63364
N90	271
N70	698
N50	1714
N30	2994
N10	5251
% GC bases	0.51768
GC skew	0.01606
AT skew	0.01445
Count o CpG sites relative to expected	1.55914
No. of N bases	0
Proportion of N Bases	0
Linguistic Complexity	0.13407
No. read pairs provided	124393164
No. of read pairs mapped	106605225
Proportion of mapped reads	0.857
No. Of read pairs mapped indicative of good assembly	77318085
Proportion of read pairs mapped indicative of good assembly	0.62156
No. Of read pairs mapped indicative of bad assembly	29287140
No. Of potential links between contigs	43755
No. Bases not covered by any reads	21364227
Proportion of bases not covered by any reads	0.11617
No. of contigs that contain bases with no read coverage	89989
Prop. of contigs that contain bases with no read coverage	0.38592
No. of contigs that contain bases with mean-read coverage <1	18509
Prop. of contigs that contain bases with mean-read coverage <1	0.07938
No. of contigs that contain bases with mean-read coverage <10	181553
Prop. of contigs that contain bases with mean-read coverage <10	0.77859
the number of contigs that have >=50% estimated chance of being segmented	17258
the proportion of contigs that have >=50% estimated chance of being segmented	0.07401
Assembly score	0.22641
Optimal assembly score	0.30087
Cutoff	0.15904
weighted	0.83561
Number of good contigs	202135
Proportion of good contigs	0.87

Table S4.6 - Filtering steps applied to the transcriptome, and the number of *Trinity* genes retained in each step.

Filtering steps	Trinity genes
Raw transcriptome	173848
Transrate good transcriptome	159769
Annotated transcriptome	36101
Expression filter	17270

Table S4.7 - List of *Trinity* genes in the transcriptome, their annotation to either Rabbit and/or Mouse ENSEMBL gene database and Associated gene name. NA = Non-Applicable

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.8 - List of differentially expressed *Trinity* genes in at least one of the pairwise comparisons performed. For each *Trinity* gene, log fold change (logFC) and false discovery rate (FDR) is indicated, as well as the ENSEMBL annotation to rabbit and/or mouse, associated gene name and Gene Ontology ID. NA=Non-Applicable.

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.9 - Fisher exact test of overlap between the number of unique ENSEMBL annotations for each group of genes in mountain hare and snowshoe hare molt cycle, considering a background of 9,091 common genes between the skin transcriptomes of both species, calculated with *fisher.test()* in R.

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.10 - Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 1 resultant from partitioning cluster analysis.

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.11 - Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 2 resultant from partitioning cluster analysis.

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.12 - Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 3 resultant from partitioning cluster analysis.

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.13 – Reactome pathways significantly enriched in the genes of cluster 1 resultant from the partitioning cluster analysis.

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.14 – Reactome pathways significantly enriched in the genes of cluster 2 resultant from the partitioning cluster analysis.

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.15 – Reactome pathways significantly enriched in the genes of cluster 3 resultant from the partitioning cluster analysis.

* This table is available in Appendix and online (<https://doi.org/10.1002/ece3.5956>).

Table S4.18 – Linear regression test results modeling the contribution of "Individual" and "Color" to the expression levels of *ASIP*, using *ACTB* as a reference gene. "Brown" and "Individual A" are used as reference levels. Linear regression was performed with *pcr_lm()* from the R package *pcr*.

Term	Estimate	P-value	Lower CI	Upper CI
Individual B	0.20	0.78	-1.73	2.13
Individual C	1.56	0.09	-0.37	3.49
White	0.83	0.30	-1.10	2.76
Intermediate	1.14	0.18	-0.79	3.07

Chapter V

General Discussion

In this work, we studied the evolutionary mechanisms at play during rapid diversification and local adaptation by combining functional, comparative and population genomics with field-based and museum-based studies of phenotypic variation. Our comparative research reveals that ancestral hybridization can be a major contributor to large amounts of shared variation among species across rapid diverging clades (Chapter II). Furthermore, our study dissects the sources of adaptive genetic variation, not only reinforcing the important role of introgressive hybridization for adaptive phenotypic variation, but by also contributing with novel evidence on the role of ancestral polymorphisms maintained by balancing selection in local adaptation (Chapter II and III). In addition, this work brings new knowledge about molecular mechanisms behind adaptive traits, from the regulatory mechanisms of seasonal camouflage (Chapter IV), to the genes involved in the repeated evolution of winter coat color polymorphism (Chapter III). Finally, we provide an empirical example of how evolutionary genomic insights on local adaptation can be used to inform conservation of declining species (Chapter III). By establishing a link between the genetic basis of a key adaptive phenotype and the environmental factors that determine its distribution across a complex landscape, we provide a framework for determining areas where conservation efforts should be prioritized to enable evolutionary rescue in response to climate change.

5.1. The sources of adaptive genetic variation during rapid divergence

Closely related and recently diverged groups of organisms (e.g., Malinsky et al. 2018; Edelman et al. 2019; Li et al. 2019) provide outstanding examples of genetic mechanisms that underlie adaptation during rapid change in the environment and habitats. The recent diversification of hares, initiating 4.05 - 5.84 million years ago (Chapter II), resulted in the independent colonization and local adaptation to geographic regions with similar environments by distant lineages (i.e., deserts, boreal forests, grasslands, and high-elevation habitats on

different continents), thus providing an exciting opportunity to study the mechanisms and sources of adaptive genetic variation. For instance, seasonal coat color change, a trait shared by six *Lepus* species from at least two lineages that inhabit highly seasonal boreal and temperate environments, is one of these shared adaptations. In the next few sections, I discuss how the phylogenomic and population genomic studies developed in this thesis support the prominent importance of ancestral polymorphism and introgressed variation as sources of adaptive genetic variation across the rapid divergence of hares.

5.1.1. The role of hybridization as a source of adaptive variation

Introgression is an important source of genetic variation in groups of closely related species, when reproductive barriers still allow successful hybridization (Hedrick 2013; Marques et al. 2019). Our phylogenomic analyses of the *Lepus* diversification show that gene flow between species has been a pervasive phenomenon that has contributed to a significant fraction of the genetic polymorphism maintained within and across extant species (Chapter II). By studying hybridization in a phylogenetic context, we were able to control for the effect of past hybridization on levels of admixture among extant species and disentangle past versus recent events of gene flow (Chapter II). With this approach, we infer that hybridization occurred since near the divergence of *Lepus*, likely reflecting secondary contact among ancestral and extant lineages as they expanded throughout the world. For instance, the signatures of ancestral hybridization among Eurasian and African lineages may reflect the continuous movements of species that occurred through the Levant, in the Eastern Mediterranean basin (Turner and O'regan 2007). Likewise, signatures of ancestral hybridization among Eurasian and North American lineages reflect movements through the Bering Land Bridge during glacial periods (Hewitt 2004; Waltari et al. 2004). Recent geological events, such as the end of the last glaciation, also appear linked to genetic signatures of contact among species (Hewitt 2004) and correlate with some of the strongest signatures of recent gene flow in our dataset [e.g., European and mountain hares (Levänen et al. 2018) and snowshoe hares and black-tailed jackrabbits (Melo-Ferreira et al. 2014; Jones et al. 2020)]. In general, our results show that the legacy of introgression can extend far deeper than hybridization between pairs of extant species, suggesting that ancient hybridization can be an important source of genetic variation in the present gene pool of species (Malinsky et al. 2018; Edelman et al. 2019; Li et al. 2019).

This work has further linked ancestral introgression to genes that may have been involved in adaptation (Chapter II), and recent (and possibly ancestral) introgression to the evolution of coat color variation (Chapter III). For instance, hybridization among the ancestors

of the northern hare species (snowshoe hares, mountain hares, Alaskan hares, Arctic hares and white-tailed jackrabbits) resulted in introgression of genes with functions that appear related with adaptation to a seasonal boreal environment, such as associated with circadian rhythm regulation (*ARNTL2*), pigmentation (*TCF4*), thermoregulation (*EBF2*) and visual perception (*PDE6H*) (Chapter II). This event of ancestral introgression may have also resulted in the exchange of the *ASIP* white allele among winter white hare lineages, though we cannot completely exclude that the low interspecific levels of divergence result from selection in the ancestral population (Chapter III). Future studies focused on clearly linking ancestral hybridization to the origin of adaptive variation may require a better understanding of how ancestral introgression impacts patterns of haplotype divergence among daughter species (Taylor and Larson 2019). For instance, following ancestral hybridization, recombination could shorten the genomic region of sequence similarity and low divergence between hybridizing lineages, resulting in a pattern resembling shared ancestral variation (Gao et al. 2015; Racimo et al. 2015). Also, recent hybridization is expected to result in much lower divergence than expected under random drift (Joly et al. 2009) or selection in the ancestral population (Cruickshank and Hahn 2014). However, this might not be applicable to ancestral hybridization, since the relatively longer time since introgression might allow genetic differences to accumulate in the alleles shared by descendants of the donor and introgressed populations (Chapter III). So far, ancestral hybridization has only been linked to adaptation in cases where the ancestral donor species can be clearly identified, such when hybridization occurred among an extant and an extinct species [e.g., humans and Neanderthals or Denisovans (Huerta-Sánchez et al. 2014; Racimo et al. 2015)] or ancient introgression among extant allopatric species [e.g., mountain hares and Iberian hares (Seixas et al. 2018; Giska et al. 2019)].

Furthermore, our results clearly link recent introgression to local adaptation, since hybridization between white-tailed and black-tailed jackrabbits resulted in the introgression of an *ASIP* winter brown allele associated with winter coat color variation (Chapter III). Together with previous works (Jones et al. 2018; Giska et al. 2019), we find that hybridization (Chapter III) has resulted in at least three instances of introgression of an *ASIP* non-white allele linked to the evolution of polymorphic seasonal camouflage. The repeated introgression of *ASIP* variation likely underscores a consistent phenotypic effect underlying adaptation to heterogeneous snow cover across hare species. It further suggests that certain genes may be particularly prone to introgression if they have a large effect on fitness-relevant phenotypes and low pleiotropic effects. The repeated introgression of the same genes has been demonstrated for loci determining wing patterning across the *Heliconius* radiation (Pardo-Díaz et al. 2012; Edelman et al. 2019; Lewis et al. 2019; Moest et al. 2020), or the hypoxia gene

EPAS1 introgressed from Denisovans into modern humans or from Tibetan wolves into Mastiff dogs (Witt and Huerta-Sánchez 2019). Our work thus strengthens the potential for introgressive hybridization to promote and shape adaptation, extending the pool of variation on which selection can act (Hedrick 2013; Marques et al. 2019).

Future studies can now ask if the similarity in the source of variation (introgression) between the white-tailed jackrabbit, snowshoe hare and mountain hare systems extends to the selective regimes and demographic history promoting introgression of non-winter white alleles in the snowshoe and mountain hare [see for example Giska et al. (2019) and Jones et al. (2020)]. For example, was the introgressed allele immediately advantageous, as may have been the case of a winter brown introgressed allele in snowshoe hares from the Pacific Northwest (Jones et al. 2020), or did it persist as standing genetic variation that was later favored by an environmental shift, regardless of the initial driver of introgression, as was suggested for an introgressed winter gray allele in Faroese mountain hares (Giska et al. 2019). Given that continuous variation in coat color coincides with large environmental heterogeneity in white-tailed jackrabbits, directional selection may have not influenced the trait architecture as prominently as in snowshoe and mountain hares. Instead, a regime of heterogeneous selection for variation in winter coat color polymorphism experienced by white-tailed jackrabbits could have influenced the introgression of a winter dark allele at *ASIP*. Similarly, balancing selection has been shown to promote adaptive introgression of alleles controlling pistil self-incompatibility specificity in two closely related *Arabidopsis* species (Castric et al. 2008) or immune-response genes in the *Lissotriton vulgaris* newt species complex (Fijarczyk et al. 2018).

5.1.2. Old polymorphisms, balancing selection and adaptive evolution

In principle, the occurrence of deeply divergent alleles at a locus within a species may also result from the maintenance of ancestral polymorphisms across speciation events by balancing selection (Charlesworth 2006; Llaurens et al. 2017; Jamie and Meier 2020). In our work, *EDNRB* and *CORIN* alleles that we linked to winter coat color variation in white-tailed jackrabbits show deep genetic coalescence that is likely the result of long-term selection maintaining haplotypes across multiple speciation events in *Lepus* (Chapter III). Although we have not directly studied the fitness consequences of winter coat color variation in white-tailed jackrabbits (see section 5.2), winter coat color is known to be important for camouflage in closely related snowshoe hares (Zimova et al. 2016), and strongly correlates with snow cover variation across the landscape in white-tailed jackrabbits (Chapter III; Mills et al. 2018). Our results thus suggest that variable snow cover conditions across the range of ancestral

lineages leading up to the white-tailed jackrabbit may have favored the maintenance of variation for alternative winter coat colors. While many empirical examples of balancing selection are associated with heterozygous advantage or negative frequency dependent selection maintaining variation at a single locus (Charlesworth 2006; Llaurens et al. 2017; Jamie and Meier 2020), we provide an empirical example of spatial and temporal varying selection maintaining polymorphisms at several independent loci (see section 5.2.3 for further discussion). Furthermore, our comparative approach studying the evolution of adaptive alleles in the context of divergence of closely related species supports that selection can favor common variants for millions of years, likely spanning across multiple speciation events. This evidence contributes to a recent but growing body of work pointing to a more prevalent role of balancing selection throughout divergence (Jamie and Meier 2020), with comparative studies connecting local adaptation within or across species (trans-species polymorphisms) to haplotypes that predate the first speciation events in a clade (Lamichhaney et al. 2015, 2016; Andrade et al. 2019; Marques et al. 2019; Jamie and Meier 2020; Yan et al. 2020). This evidence points to the importance of studying adaptive evolution at deeper phylogenetic scales to better understand how selective pressures may act throughout generations against drift to maintain polymorphisms and shape the trajectory of adaptive genetic variation.

5.2. Molecular mechanisms of seasonal coat color change in hares

Determining the molecular underpinnings of phenotypic variation is an important component of understanding adaptive evolution and can reveal how evolution may be constrained to particular paths (Martin and Orgogozo 2013; Stern 2013; Rosenblum et al. 2014). Evolutionary constraints may be created by an historical contingency, where adaptation is often underlain by ancestral or shared genetic polymorphisms (as discussed in section 5.1). Moreover, evolution may also be constrained by interactions among genes and by functional networks limiting the possible molecular paths to build a particular phenotype (Feder 2007; Stern 2013; Rosenblum et al. 2014). Historical or genetic constraints result in evolution often converging on similar solutions to the same environmental pressures, with changes in the same developmental network, gene pathways or genes, or introgression of the same genes (Chapter III) contributing to phenotypic evolution. Ultimately, a mechanistic understanding of phenotypes may help predicting the effect of genetic variation on phenotype and fitness (Chapter III and section 5.3).

Our phylogenetic analyses of hares suggest winter white seasonal camouflage is shared among a paraphyletic group of species, as white-tailed jackrabbits, mountain, Alaskan and Arctic hares form a clade that does not include snowshoe hares (Chapter II). A simplistic

interpretation of this phylogenetic pattern would suggest independent evolution of the trait in at least two lineages. However, hares show high levels of shared polymorphism across closely related species resulting from incomplete lineage sorting, hybridization, and possibly positive selection (section 5.1). We show that the evolution of seasonal coat color change and winter coat color variation is at least partially explained by shared ancestral polymorphisms. Additional genetic constraints, like pleiotropy, may constrain adaptation to varying snow cover to the genes of the pigmentation pathway. In the next sections, I discuss how a shared evolutionary history and genetic constraints may result in repeated molecular mechanisms of adaptive evolution, using our results on the evolution and genetic basis of seasonal coat color change as an example.

5.2.1. Regulatory mechanisms underlying seasonal camouflage

The pelage molt is a broadly conserved mechanism across mammals that involves shedding and regrowth of hair at particular times of the year, allowing variation or renewal of insulation properties (Ling 1972). Seasonal coat color changing molts additionally provide varying camouflage properties across seasons (reviewed in Zimova et al. 2018). It is therefore not surprising that we found parallels across the transcriptional landscapes of color changing molts of mountain hares (Chapter IV) and snowshoe hares (Ferreira et al. 2017). The common transcriptional landscape involves differential expression of pathways that perceive environmental cues and trigger the molt process (circadian clock, nutritional cues) and pathways that are involved in hair regrowth and coloration (Chapter IV). These described expression differences parallel with known processes of the hair growth cycle in mice, humans and other model organisms (Stenn and Paus 2001; Geyfman et al. 2015). Generalizing the regulatory mechanisms underlying seasonal coat color changing molts across species is an important component of understanding population and phylogenetic analyses trying to establish genotype-phenotype associations. For instance, functional data can help inform hypotheses on how candidate ancestral introgressed genes may contribute to adaptation in seasonal coat color changing species (Chapter II). Given that we have studied two species that represent different lineages of hares with seasonally flexible camouflage, our results suggest that the color changing molt mechanism is conserved across hare species. Further comparisons will be necessary to determine if the molecular networks inferred from one genus are conserved across other more distantly related seasonally molting systems.

Our studies are some of the first to extend the known molecular mechanisms of molt regulation in model organisms to seasonal molts in non-model organisms (Schwartz and Andrews 2013; Geyfman et al. 2015; Zimova et al. 2018; Siamak Houschyar et al. 2020). The

hair growth cycle has typically been studied in organisms that exhibit asynchronous (humans) or non-seasonal molt cycles (mice, many domesticated animals) (Geyfman et al. 2015), offering a limited understanding about if and how these molecular mechanisms can be modified in natural populations that experience seasonally varying environments (Snyder-Mackler and Lea 2018). So far, the literature had described that seasonal molts in non-model organisms, including seasonal coat color changing species, are controlled by photoperiod through variations in internal levels of the hormones melatonin and prolactin [see Zimova et al (2018) for a comprehensive review]. Our results show that these varying internal cues may induce the seasonal expression of the known gene networks of the hair growth cycle and thus result in seasonal molts (Chapter IV; Ferreira et al. 2017). Although developmental studies in non-model organisms remain challenging, our approach provides an example of how recent genomic tools may be used to expand the knowledge of the regulatory mechanisms in non-model organisms that allow survival in a seasonal environment (Schwartz and Andrews 2013). Ultimately, this mechanistic understanding may be applied to model how environmental cues produce changes in the phenotype and how seasonally flexible traits may respond to predicted changes in the environment (Antoniou-Kourounioti et al. 2018).

Future functional work is necessary to describe the exact molecular cascade that allows seasonal flexibility in coat color, but the work in this thesis provides new candidate genes that may be involved in this process. The varying hormonal levels controlling molt induce rhythmic expression of gene pathways in peripheral tissues including the ones regulating the hair growth cycle (Lin et al. 2009; Schwartz and Andrews 2014; Ferreira et al. 2017) and pigment production (Slominski et al. 2015). Seasonal coat color change is tied to the hair growth cycle, so we expect that its regulation will be integrated with seasonal molts. The pigmentation pathway that underlies coat color variation is activated during the hair growth cycle and includes three processes that can affect color shade and pattern: 1) differentiation, proliferation and development of melanocytes (pigment production cells), 2) production of two different kinds of pigment (black/brown or yellow/red) in melanocytes by the melanogenesis pathway, and 3) transportation and deposition of pigment in the keratinocytes of the hair shaft (Hearing 1999; Slominski et al. 2004; Hoekstra 2006).

Given the flexible nature of the phenotype, the mechanism of coat color change must be reversible and therefore may be likely to involve the evolution of *cis*-regulatory elements that modify pigmentation after a certain hormone concentration has been reached (threshold mechanism). For instance, melatonin controls prolactin levels and both hormones induce changes in pigmentation in some models, with high levels of melatonin inhibiting melanogenesis (Logan and Weatherhead 1980; Slominski et al. 1994), and high levels of prolactin inducing dark molts (Duncan and Goldman 1984b; Gower et al. 1993). The exact

molecular connections that promote the effect of melatonin-prolactin on seasonal variation in pigmentation are not clearly established, but the literature provides interesting clues. For instance, the melanocyte stimulating hormone (α -MSH), that induces the production of dark pigment by binding the melanocortin-1-receptor (MC1R), shows circannual regulation (Altmeyer et al. 1986). MC1R is a G protein-coupled receptor in the membrane of melanocytes and has been associated with coat color variation in Arctic foxes (*Alopex lagopus*; Våge et al. 2005). Therefore, the α -MSH-MC1R axis could be thus a good candidate for regulation of seasonal coat color change. In this thesis, we associated another G protein-coupled receptor in the membrane of melanocytes, *EDNRB*, to winter coat color variation in white-tailed jackrabbits (Chapter III). *EDNRB*'s ligand Endothelin-1 (ET-1), a potent vasoconstrictor protein, is directly regulated by the circadian clock and exhibits seasonal gene expression (Kruse et al. 2002; Richards et al. 2014) and the expression of both *EDNRB* and *ET-1* is attenuated by melatonin (Park et al. 2007). *EDNRB* signaling is important for melanoblast migration during embryogenesis and results in various degrees of white spotting when disrupted in mice (Shin et al. 1999). Furthermore, binding of ET-1 to *EDNRB* during anagen promotes differentiation and proliferation of melanocytes during the post-natal hair cycle (Takeo et al. 2016). Hypothetically, a seasonally reversible phenotype may involve *cis*-regulatory evolution of *EDNRB* alleles that 1) determine the spatial pattern of response to an environmental signal early in development and/or; 2) are sensitive to seasonal variation in cyclic expression of *ET-1* (or another ligand), modifying melanocyte development during the seasonal molt. For example, the evolution of wing color plasticity connected to seasonal cues in the common buckeye butterfly (*Junonia coenia*) involves the evolution of pigmentation alleles sensitive to environmental induced changes in an endocrine signal and that are expressed during different stages of development (Van Der Burg et al. 2020). Non-sensitive pigmentation alleles are connected to the evolution of non-plastic butterfly lines. Future studies investigating the expression of these and other candidate genes across seasonal molts will be required to exactly reconstruct the molecular cascade responsible for seasonal coat color change.

5.2.2. Evolution of polymorphic seasonal camouflage through variation in the pigmentation pathway

Our work in the white-tailed jackrabbit showed that genetic variation of at least three genes with known pigmentation functions underlies continuous variation in winter coat color (Chapter III). These genes are known to act at two different steps of the pigmentation pathway, described in the previous section: *EDNRB* is directly involved in the differentiation and

proliferation of melanocytes during development and the post-natal hair cycle (Hosoda et al. 1994; Takeo et al. 2016; Li et al. 2017), whereas *ASIP* and *CORIN* have a more prominent effect on the type and deposition of pigment in the hair shaft (Enshell-Seijffers et al. 2007; Le Pape et al. 2009). How do these genes interact to produce the observed variation in winter coat color? White-tailed jackrabbits showing intermediate winter coats differ not only in overall color hue or brightness, but also in degrees of color patterning across their body (Chapter III; Figure 5.1). Variations in patterning are consistent with *EDNRB* functions, since *EDNRB* signaling governs the migration of melanoblast (melanocyte precursors) along a dorsal-lateral axis, from the rostrum to the tail, during embryogenesis (Shin et al. 1999). A disruption of this signaling can result in depigmented areas (Shin et al. 1999), which is similar to what we observed in some white-tailed jackrabbits with dark heads and dorsum but white rumps and shoulders (Figure 5.1). As hypothesized earlier, expression of a white-*EDNRB* allele earlier in development might establish the dorsal localization of environmental sensitive melanoblast populations that will differentiate melanocytes during adulthood that undergo or not some degree of seasonal color change during the molt cycle (Takeo et al. 2016).

The action of *CORIN* and *ASIP* could then be independent and complementary to *EDNRB*, as *ASIP* effects on pigmentation involve another receptor, *MC1R* (Le Pape et al. 2009). *ASIP* promotes the switch to pheomelanin production, regulates the amount of pheomelanin produced, and has an impact on melanocyte differentiation (Le Pape et al. 2009; Manceau et al. 2011; Barrett et al. 2019). We have shown that the *ASIP* ventral isoform is not expressed in the dorsum and therefore an impact on melanocyte differentiation mediated by this isoform does not seem to underlie winter white phenotypes in hares (Chapter IV; Jones et al. 2018). We can thus hypothesize that the impact of *ASIP* on the phenotype is related to higher expression of *ASIP* winter white alleles resulting in decreased pigment production, as suggested in snowshoe hares (Jones et al. 2018). *CORIN* is known to control the period of *ASIP* expression (Enshell-Seijffers et al. 2007; Avigad Laron et al. 2019) and, in agreement, *CORIN* winter white alleles seem to result in lighter pigmentation in all *ASIP* allelic backgrounds (Chapter III). This effect seems to be achieved by variation in the color of a mid-section of the hair (Figure 5.1), similar to variation of the *ASIP*-induced pheomelanin band observed in some *CORIN* mutants (Enshell-Seijffers et al. 2007; Xu et al. 2017). In addition to the *EDNRB*-induced phenotype, our results suggest that *cis*-regulatory evolution of environmentally sensitive *ASIP* and *CORIN* alleles may determine variation in pigment production during seasonal hair growth. The involvement of these genes in winter coat color variation in white-tailed jackrabbits reveals that, in addition to variation in pigment synthesis (via *ASIP*; Jones et al. 2018; Giska et al. 2019), adaptation to varying snow cover may also evolve through changes in melanocyte differentiation through the endothelin axis.

Furthermore, together with *ASIP* (Jones et al. 2018; Giska et al. 2019) and *MC1R* (Våge et al. 1997), our work expands the repertoire of pigmentation genes (*EDNRB* and *CORIN*) contributing to the evolution of seasonally flexible coat color.

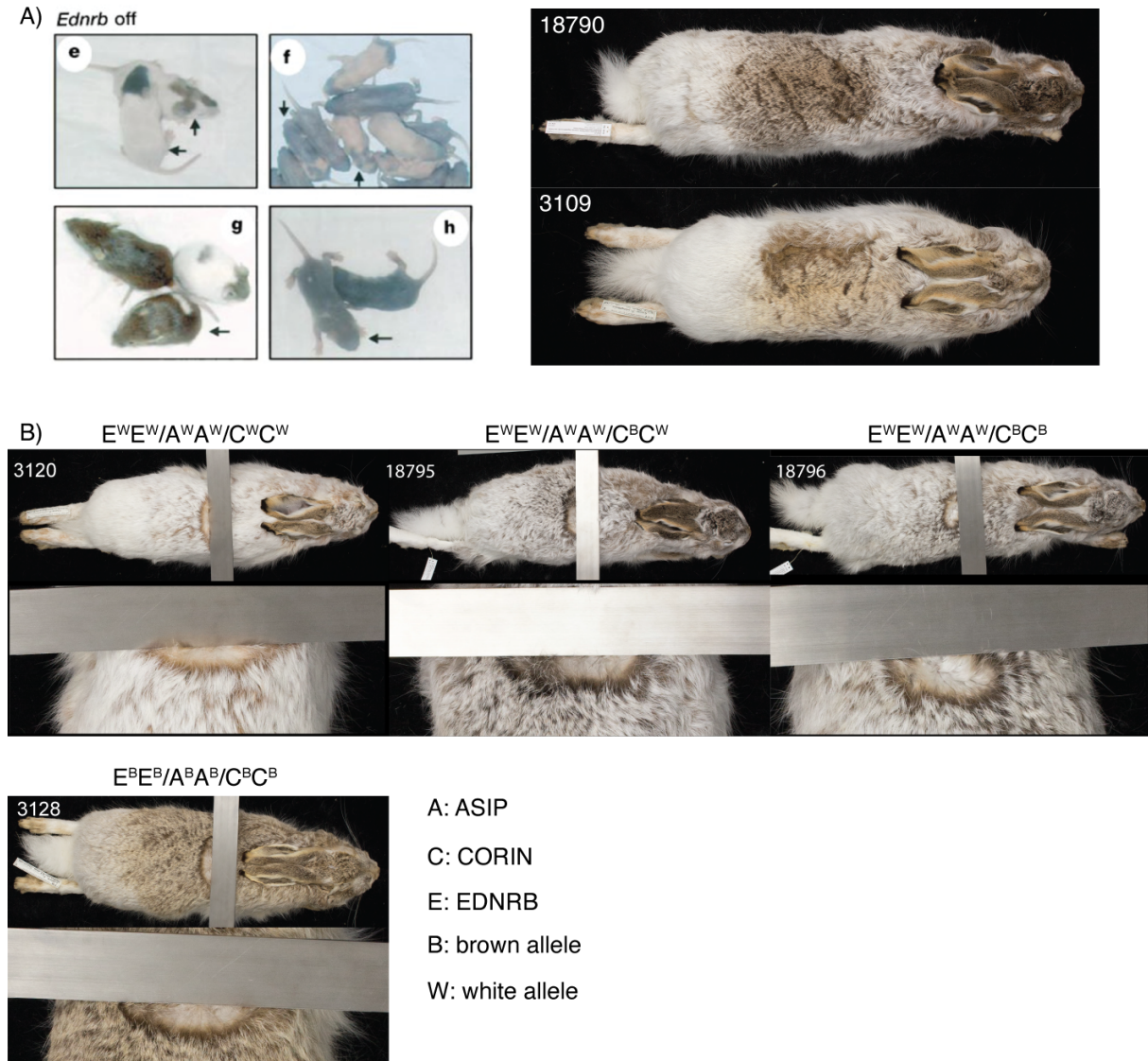


Figure 5.1 – The phenotypic effects of *EDNRB*, *CORIN* and *ASIP* on winter coat color of white-tailed jackrabbits. (A) In mice, shutting down *EDNRB* at different stages of embryogenesis (e, E10.5; f, E11.5; g E12.5; h, E13.5) results in varying degrees of white spotting (see individuals indicated with arrows). Adapted from Shin et al. 1999. We observed similar patches of pigmented areas in intermediate white-tailed jackrabbits; **(B)** The interaction between *CORIN* and *ASIP* genotypes seems to alter the coloration of a mid-section of the hair. All photographs are from white-tailed jackrabbit specimens from the Denver Museum of Nature and Science mammalogy collection and numbers refer to voucher IDs.

The involvement of the pigmentation pathway in the evolution of winter coat color variation extends to distant related seasonal coat color changing species as well. In the Arctic fox (*Alopex lagopus*), amino-acid changes in *MC1R* are linked to the evolution of alternative winter and summer coat colors (Våge et al. 2005). Given the recruitment of pigmentation genes in distant relatives, there are likely developmental constraints to altering seasonal coat

color through alternative mechanisms (Martin and Orgogozo 2013; Stern 2013; Rosenblum et al. 2014). For instance, variation in molt timing would imply alterations in the perception of environmental cues or in the translation of an external signal through hormonal pathways (Van Der Burg et al. 2020) that are important to time other fitness relevant life-cycle events, such as the growth of a thicker winter coat important for thermoregulation. Indeed, plasticity in molt timing is limited and insufficient to mitigate camouflage mismatch (Zimova et al. 2014, 2020; Kumar et al. 2020). Therefore, selection on pigmentation genes to produce variation in winter coloration may have less pleiotropic effects. The ‘hotspot-hypothesis’ argues that evolution of phenotypic diversity may target locations in networks that produce the largest effect on phenotype without affecting multiple phenotypes (i.e., less pleiotropy) (Martin and Orgogozo 2013; Stern 2013). For example, cell-specific regulator or receptor genes that integrate an upstream signal and promote a switch between alternative molecular cascades resulting in alternative phenotypes (Martin and Orgogozo 2013; Stern 2013). Consistent with this model, *EDNRB* is a receptor mediating the switch between the development of pigmented areas in the body, and *ASIP* and *CORIN* directly or indirectly regulate the switch between the production of different pigment types through the receptor *MC1R*. Furthermore, pigmentation genes appear to have limited pleiotropic effects in vertebrates and act mainly on certain cell types (e.g., melanocytes and keratinocytes in mammals) (Hoekstra 2006; Hubbard et al. 2010). *Cis*-regulatory variation in these genes further restricts their effect to particular body regions creating color patterns and dorsal-ventral variations in coat color (Wray 2007; Manceau et al. 2011; Martin and Orgogozo 2013; Mallarino et al. 2016; Van Der Burg et al. 2020). Therefore, adaptation to heterogeneous snow cover through changes in the pigmentation pathway rather than variation in molt timing supports a hypothesis that evolution may favor molecular paths with fewer changes of large effect with optimal pleiotropy (Martin and Orgogozo 2013; Rosenblum et al. 2014). This is also in line with research that points to phenotypic variation in nature being often achieved by changes in a small group of “toolkit” genes, presumably because they have large but tissue-specific effects (Martin and Orgogozo 2013; Van Belleghem et al. 2016).

5.2.3. Adaptation to snow cover variation through discrete versus quantitative winter coloration

This thesis links a multigenic architecture involving the pigmentation genes *EDNRB*, *CORIN* and *ASIP* to the quantitative variation in winter coat color of white-tailed jackrabbits observed across an environmental transition zone in Colorado, United States of America (Chapter III). This genetic architecture is more complex than has been observed in two other

hare systems, where *ASIP* is the single gene underlying a simple color polymorphism in mountain and snowshoe hares (Jones et al. 2018; Giska et al. 2019). In white-tailed jackrabbits, the quantitative trait variation in color seems to be underlined by an additive effect among *EDNRB* and *CORIN* and epistasis among *CORIN* and *ASIP*, though larger sample sizes are required to confidently determine the interactions among these genes (Mackay 2014). Human skin color is another clear example of how the combined action of different genes can create remarkable trait variation (Quillen et al. 2019).

Continuous variation in winter coloration in white-tailed jackrabbits seems to be unique among hare species (Hansen and Bear 1963), raising the question of why this more complex form of color variation has evolved in white-tailed jackrabbit and not in the other species. For instance, when comparing the predicted distribution of winter coat color probabilities across the distributions of coat color changing species (Mills et al. 2018), most color varying hares show a sharp transition zone between coat colors in comparison with the white-tailed jackrabbit. Indeed, polymorphic zones [areas where $0.2 < P(\text{white}) < 0.8$] occupy 2.1% to 14.4% of the range of hares with discrete variation in coat color, as opposed to 43% of the white-tailed jackrabbit range (Mills et al. 2018). Given the high fitness value of crypsis in seasonal coat color changing systems (Zimova et al. 2016), variation in winter coloration in white-tailed jackrabbits should be advantageous on average, or at least it should not be maladaptive. The extensive transition zone in the white-tailed jackrabbit range coincides with the temperate open-prairie system of the North American west, where heterogeneous snow cover conditions may create a variety of background colors where intermediate colorations could be better camouflaged than pure white or brown individuals (Figure 1.2; Mills et al. 2018). Contrastingly, snowshoe and mountain hares occupy both higher latitude and higher elevation coniferous forests or circumpolar tundra, where snow cover pack may be more stable throughout winter, except near coastal areas (coastal coniferous forests in snowshoe hares and British Isles and coastal Scandinavia in mountain hares), while Japanese hares occur across a sharp environmental and elevation transition in Japan (Mills et al. 2018). Therefore, less pronounced transitions in snow cover conditions may favor the evolution of a continuous coloration in white-tailed jackrabbits over the more binary variation observed in other hare species.

The specific genetic architecture of coat color variation in white-tailed jackrabbits further supports the conclusion that there has been a long history of strong natural selection for continuous trait variation. The evolution of the genetic architecture of phenotypes can be influenced by several factors, including drift, gene flow, selection, source of genetic variation, epistasis and pleiotropy (Yeaman and Whitlock 2011; Dittmar et al. 2016; Llaurens et al. 2017). Most examples of balancing selection involve a single-locus or tightly linked multilocus

architectures, such as supergenes (Charlesworth and Charlesworth 1975; Llaurens et al. 2017; Jamie and Meier 2020). However, theoretical modelling has shown that variation at multiple loci can be maintained by strong fluctuating selection imposed by environmental conditions that varies across space and time (Turelli and Barton 2004; Gulisija and Kim 2015; Wittmann et al. 2017). In this context, the inferred long-term maintenance of genetic variation at both *EDNRB* and *CORIN* (and more recently at *ASIP*) would only be expected if selection was fairly strong across a heterogeneous landscape. For instance, ephemeral conditions of snow cover that vary across short distances or time spans, could impose strong balancing selection for winter coat color polymorphisms across the range of the white-tailed jackrabbit. Winter-coloration in white-tailed jackrabbits may thus be an important empirical example of heterogeneous selection maintaining polymorphisms at least at two independent loci (Llaurens et al. 2017; Jamie and Meier 2020). Alternatively, selection does not need to be invoked if the environment is sufficiently variable that all genotypic combinations are able to survive (i.e., variation is not maladaptive). This is the case of the stick insect *T. chumash*, where recombination among multiple unlinked loci produces continuous coloration (Nosil et al. 2020). *T. chumash* is associated with a multi-colored host, the mountain mahogany, and does not experience strong selection for a particular color (Nosil et al. 2020). However, lack of selective pressures on seasonal camouflage in white-tailed jackrabbits seem unlikely given the strong negative impact on survival observed in mismatched snowshoe hares (Zimova et al. 2016). The transition zone in quantitative winter coat color of white-tailed jackrabbits is thus an interesting system to study the particular demographic and selective conditions behind the evolution of adaptive traits and their underlying genetic architecture.

5.3. Adaptive evolution and conservation: lessons from seasonal coat color change

How can the knowledge of the genetic basis and evolutionary history of an adaptive trait inform conservation? The field of conservation genetics has long sought to inform conservation actions based on patterns of genetic variation. For example, genetic tools can be used to assess the degree of population fragmentation, mitigate inbreeding depression, or to detect potentially harmful hybridization (Allendorf et al. 2010). Targeting loci underlying local adaptation and using information about their evolution has the potential to further inform management actions aimed at promoting effective adaptation to rapid environmental change (Hoffmann and Sgrò 2011; Kardos and Shafer 2018). In this work, we used genomic tools to determine the genetic basis and evolution of a fitness relevant trait in white-tailed jackrabbits

and then proposed conservation priorities to foster the survival of this species in the face of future environmental change (Chapter III).

By modelling the distribution of coat color as function of correlated climatic variables (Mills et al. 2018), we inferred the current spatial distribution of winter coat colors across the white-tailed jackrabbit range and identified at risk populations with poor conservation status (Brown et al. 2020). Applying the same modelling framework to environmental conditions forecasted by 2080, we predicted a pronounced increase in the favorability of winter brown phenotypes from 15% to 49% of the current southern range (Chapter III). How can the white-tailed jackrabbit effectively adapt to the rapid environmental shift that will occur over the next 60 years? Two of the theoretical advantages of standing genetic variation for effective local adaptation are the potential for a rapid response given its immediate availability (e.g., Lamichhaney et al. 2016; Giska et al. 2019) and the fact that such variation may have been pre-tested by past selective pressures (Barrett and Schluter 2008; Matuszewski et al. 2015). Indeed, the evolutionary history of the haplotypes underlying winter coat color variation in white-tailed jackrabbit reveals the long-term maintenance of this variation, demonstrating that it has persisted past selective pressures for millions of years (Chapter III). Moreover, if this standing adaptive variation was lost, the emergence of new adaptive mutations may be unlikely to arise within the time frame needed to respond to imminent environmental change. Thus, existing standing genetic variation for winter coat color is likely to be essential for effective adaptive responses in white-tailed jackrabbit populations confronted by climate change. However, these predicted adaptive responses may be compromised if white-tailed jackrabbit populations harboring the majority of the winter brown-associated variation continue to decline or are completely extirpated (Brown et al. 2020). These results suggest that future conservation actions should be focused towards preserving populations harboring winter brown variation. For example, local efforts to mitigate habitat loss and improve the connectivity among populations should promote the preservation and spread of adaptive alleles (Kardos and Shafer 2018; Brown et al. 2020) and are likely to yield tangible benefits in white-tailed jackrabbit conservation. Our findings may also extend to the other seasonal coat color changing systems, some of which exist at the same latitude as white-tailed jackrabbits and thus may experience similar changes in snow cover conditions (Mills et al. 2018).

The evolution and genetic basis of winter coat color variation in white-tailed jackrabbits thus provides a compelling example of how evolutionary genomic approaches may yield important tools for conservation. Prioritizing the preservation of adaptive variation that has been essential to the past survival of a threatened species may promote quick and effective responses to future climate change given that such variation has been pre-tested by selection and often exists at appreciable frequencies (Barrett and Schluter 2008; Matuszewski et al.

2015). Recent studies have similarly studied the genetic basis of fitness relevant traits in connection to restoration efforts after habitat loss (Prince et al. 2017; Thompson et al. 2020) or determining if species can adapt to pollution (Reid et al. 2016). While these studies focused on identifying loci underlying particular fitness-relevant traits, identifying ecologically relevant traits and determining their genetic basis may not be feasible for many systems and has seldom been used to guide conservation of threatened populations (Kardos and Shafer 2018). Alternatively, critical variation for species survival can be identified using genetic-environment associations (Wang and Bradburd 2014; Hoban et al. 2016). Identifying and preserving such adaptive genetic variation may thus be a fundamental tool for defining conservation priorities and promoting the evolutionary rescue of species or populations threatened by rapid environmental change.

5.4. Conclusions

This thesis contributes to the understanding of the evolutionary mechanisms that allow adaptation of rapid diverging species, specifically of the types of genetic variation that underly phenotypic diversification. First, we showed that rapidly diverging species can share large amounts of variation either due to incomplete lineage sorting or recent and ancestral hybridization (Chapter II). We underscore the importance of ancestral hybridization as a previously overlooked source of adaptive variation in extant species, as it has a significant impacted the levels of shared variation among species (Chapter II). By studying the evolution of winter coat color polymorphism in white-tailed jackrabbits, we provided an empirical example of how ancestral polymorphism that has been maintained across speciation events or how polymorphisms exchanged among diverging species can underlie local adaptation (Chapter III). In the context of adaptation of the white-tailed jackrabbit to future changes in snow cover conditions, our results imply that old standing genetic polymorphisms are the most likely to seed an effective adaptive response given they have persisted over millions of years of divergence (Chapter III). Furthermore, in the short time frame expected for anthropogenic climate change (less than 100 years), the emergence of new adaptive mutations is not expected, and it is thus vital to protect winter brown genetic variation (Mills et al. 2018). As in the Faroese mountain hare (Giska et al. 2019), the existence of adaptive standing genetic variation may allow rapid phenotypic shifts in response to varying snow cover conditions in a matter of decades.

Furthermore, our work also sheds light onto the molecular mechanisms underlying evolution of seasonal coat color change across species. By studying the relationships among

hare species, we showed that the paraphyletic evolution of seasonal camouflage and winter coat color polymorphism in at least five hare species is underlined by related genetic variation (Chapter II) that is in some instances (i.e., *ASIP*) shared among species (Chapter III). We found a common transcriptional landscape of seasonal coat color change in two hare species that represent the two lineages with varying camouflage, the snowshoe hare and the mountain hare (Chapter IV). Color varying molts in these two systems were correlated with regulatory shifts of genes involved in circadian rhythms, pigmentation and hair growth, as it would be expected given the molt mechanism is in general conserved across mammals. Regarding the evolution of winter white versus winter brown coats in response to snow cover variation, we found the involvement of three genes (*EDNRB*, *CORIN* and *ASIP*) of the pigmentation pathway across hare species (Chapter III). In the case of *ASIP*, polymorphism in winter coat color has evolved through at least three independent events of introgressive hybridization with non-color changing relatives [Chapter III, Jones et al. (2018) and Giska et al. (2019)]. Therefore, adaptation to varying snow cover appears to evolve through alterations of a few key genes within the pigmentation pathway rather than the molt phenology, likely reflecting pleiotropic constraints. While *ASIP* is involved in the evolution of seasonal camouflage in all of the hare systems studied to date, *EDNRB* and *CORIN* were linked for the first time to the evolution of winter coat color variation in white-tailed jackrabbits (Chapter III). The multilocus genetic architecture in the white-tailed jackrabbit is responsible for the continuous variation in coat color, in comparison with a single-locus architecture involved with discrete coat colors in the other systems. This genetic architecture seems to involve primarily additive effects at *EDNRB* and *CORIN* and potentially epistatic interactions between *CORIN* and *ASIP*. The unique three-locus genetic architecture in the white-tailed jackrabbit across the Colorado coat color transition zone may reflect heterogeneous selection for a highly variable winter coloration. Future studies are required to understand the interaction among these genes and the environmental conditions favoring the evolution of this quantitative genetic architecture.

5.5 Impact of this work and Future directions

The studies in this thesis contribute to a better understanding of the evolutionary mechanisms of rapid diversification and shared adaption within and across species, while raising important questions for future research. One of the most important contributions of our work includes the inferences of ancestral introgression across a species radiation, a process that has been recently described in some other closely related groups of species (Malinsky et al. 2018; Edelman et al. 2019; Li et al. 2019; Vanderpool et al. 2020). The occurrence of ancestral hybridization poses analytic challenges for the inference of relationships or levels of

gene flow among extant species. For instance, few methods are able to robustly infer species networks for datasets with more than 10 species. We show that phylogenetic methods that do not take hybridization into account will necessarily result in an oversimplified and possibly inaccurate evolutionary history. Failure to account for uncertainty in evolutionary history can also result in spurious inferences of extant introgressive hybridization. Furthermore, ancestral introgression can potentially be at the origin of adaptive variation. Current expectations to distinguish between introgression and ancestral polymorphism assume that introgression is usually more recent than the age of ancestral polymorphism maintained by random drift or selection along the genome, which might not be true in cases of ancestral introgression. Therefore, more theoretical work is required to understand the impact of this process on patterns of nucleotide diversity, genetic divergence, and genealogical relationships across the genomes of extant species.

We also contributed to a better understanding of the evolution of seasonal coat color change in *Lepus*, including the genetic basis and history of the shared winter coat color polymorphism across species. Having linked ancestral polymorphism and introgression to the evolution of winter coat color polymorphism in white-tailed jackrabbits, some questions remain unanswered. For example, does the genetic basis of seasonal color variation in other species or populations tend to involve *ASIP*, *EDNRB*, or *CORIN*? When considering hare systems, are the same haplotypes involved in color variation across species and, if so, does this shared inter-specific variation reflect long-term balancing selection or more recent introgression? Future studies in other systems with variation in winter coat color are important to answer these questions.

Despite the major progress our study made on the understanding of the transcriptional landscape of seasonal coat color changing molts, our experiments did not yield insights into the specific regulatory mechanisms underlying the seasonal production of two different coat colors. This mechanism could be independent from the mechanism that involves the evolution of winter coat color polymorphism, as there are many different hormonal pathways influencing pigmentation that may have been co-opted to allow seasonal varying pigmentation (Slominski et al. 2004). The clear reconstruction of the cascade of interactions that results in color shift across seasons would shed light onto the regulatory mechanisms that allow seasonal flexibility in general but will likely require a detailed study of gene expression differences across seasons and functional experiments that detect interactions among genes. For example, interrogation of chromatin structure through techniques such as ATAC-Seq (Assay for Transposase Accessible Chromatin with high-throughput sequencing) (Buenrostro et al. 2013), chromatin immunoprecipitation and sequencing (ChIP-seq) (Lewis et al. 2016), and chromosome conformation capture (Hi-C) (Rao et al. 2014) are promising tools to identify *cis*-

regulatory elements and interaction among regions of the genome (Lewis et al. 2019; Van Der Burg et al. 2020). These techniques could be used, for example, to identify regulatory elements of candidate genes in the pigmentation pathway, such as *ASIP*, *MC1R* and *EDNRB*, and understand if there is variation in their regulation across seasons and what potential regulators are involved.

We also provided insight into the genetic basis of continuous winter coat color in the white-tailed jackrabbits encoded by at least three loci – *EDNRB*, *CORIN* and *ASIP*. Future work is now needed to characterize the function of the molecular mechanisms underlying this quantitative phenotype. For instance, our study did not describe what possible causal mutations (structural, coding or regulatory) differentiate the winter white and winter brown alleles. The *de novo* assembled phased white-tailed jackrabbit genome produced with linked-read technology holds the potential to study the existence of structural variants among winter white and winter brown haplotypes (e.g., Meier et al. 2020). After identifying possible candidate mutations, their impact on pigment production could be further tested leveraging functional tools developed in model organisms and that have been used to understand the function of pigmentation pathway genes, including the ones mentioned in this study (Hosoda et al. 1994; Gariepy et al. 1996; Enshell-Seijffers et al. 2007; Fontanesi et al. 2010; Takeo et al. 2016).

Moreover, the white-tailed jackrabbit transition zone is a remarkable model to study the evolution of genetic architecture of adaptive traits and its connection with drift, gene flow and selection. Continuous variation in coat color is likely favored by selection, but it could also result from environmental conditions that do not exert directional selection for a particular phenotype (Nosil et al. 2020). To understand this, field studies involving translocations or tracking the survival of radio collared hares could be used to study the distribution of selective pressures across the landscape (Zimova et al. 2016; Wilson et al. 2019; Nosil et al. 2020). Given we predicted that winter brown coloration will be greatly favored by a rapid change in snow cover conditions, the transition zones like the one we study in Colorado will be a natural laboratory to study and monitor the phenotypic and genetic response to a shifting fitness optimum in the next century. For instance, these systems could provide empirical examples for theoretical predictions regarding the evolution of genetic architecture in response to selection (Charlesworth and Charlesworth 1975; Yeaman and Whitlock 2011; Llaurens et al. 2017), how the distribution of phenotypic effects varies across populations and during an adaptive bout (Orr 2005; Mackay 2014; Dittmar et al. 2016), or to study the conditions under which adaptation can allow evolutionary rescue (Matuszewski et al. 2015; Mills et al. 2018; Klausmeier et al. 2020).

Finally, given our call for action to preserve standing genetic variation for winter brown coloration in white-tailed jackrabbits, there is a need to better define the risk factors compromising the persistence of white-tailed jackrabbit populations (Brown et al. 2020) and test management actions seeking to minimize the ongoing declines and promoting connectivity among populations. There is currently limited knowledge about the population and demographic history of the white-tailed jackrabbit in general (e.g., Tapia 2010; Melo-Ferreira et al. 2014; Rosa 2015). A broad-scale inventory of population sizes, genetic structure and diversity may provide a better picture of what populations may need immediate intervention and of the degree of connectivity between them (e.g., Oh et al. 2019, but also see reviews Allendorf et al. 2010; Funk et al. 2012). Particularly, it will be necessary to localize and study additional winter brown populations and determine their degree of isolation from other populations. In this respect, the genomic resources generated in this thesis will provide valuable tools for population genomic studies of white-tailed jackrabbit populations (Hendricks et al. 2018). Furthermore, the knowledge on the genetic basis of winter coat color polymorphism can be used to monitor the response to conservation measurements (Kardos and Shafer 2018). Given the threats advanced by Brown et al. (2020), initial conservation measures may be focused on habitat restoration and limitation of hunting bags. Restoration of the habitat of the white-tailed jackrabbit may be done in synergy with ongoing conservation efforts of other threatened sage-brush species, such as the Gunnison and greater sage-grouse (Dumroese et al. 2015; Oh et al. 2019), in general benefiting the preservation of the sage-brush ecosystem of the North American west (Rowland et al. 2006; Davies et al. 2011).

In sum, our study comprises a group of integrative approaches that shed light on the evolutionary mechanisms allowing rapid divergence and adaptation among closely related species, but it also opens exciting new research lines to study these and other mechanisms, including ones related with the genetic basis of local adaptation and the potential to use evolutionary processes to inform the potential of species to survive environmental change.

5.6. References

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Appendix

The document FerreiraAMS_PhDThesis_APPENDIX.xlsx in Excel Microsoft Office 365 format contains the following tables that could not be included in the main thesis:

Table S2.13 - Windows of recent and ancient introgression between snowshoe hares (as P3) and mountain hares, Alaskan hares or white-tailed jackrabbits (as P2), detected with fraction of admixture (f_d) scans of introgression with the topology (((Iberian hare,P2),P3),Rabbit). Only significantly introgressed windows (f_d value in the top 0.5% of each distribution) in at least one species are shown. If a window is significant for all three tests it is considered to be a window of ancestral introgression.

Table S2.14 - Annotation of the genes in the 119 f_d outlier windows that putatively introgressed from the mountain hare, Alaskan hare and white-tailed jackrabbit common ancestor into snowshoe hares. For each ENSEMBL Gene ID, we provide the chromosome where it is located, the start and end positions of the gene, the external gene name, and Gene Ontology annotation.

Table S4.7 - List of Trinity genes in the transcriptome, their annotation to either Rabbit and/or Mouse ENSEMBL gene database and Associated gene name. NA = Non-Applicable

Table S4.8 - List of differentially expressed Trinity genes in at least one of the pairwise comparisons performed. For each Trinity gene, log fold change (logFC) and false discovery rate (FDR) is indicated, as well as the ENSEMBL annotation to rabbit and/or mouse, associated gene name and Gene Ontology ID. NA=Non-Applicable.

Table S4.9 – Fisher exact test of overlap between the number of unique ENSEMBL annotations for each group of genes in mountain hare and snowshoe hare molt cycle, considering a background of 9091 common genes between the skin transcriptomes of both species, calculated with *fisher.test()* in R.

Table S4.10 - Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 1 resultant from partitioning cluster analysis.

Table S4.11 - Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 2 resultant from partitioning cluster analysis.

Table S4.12 - Gene Ontology Biological Process (GO BP) terms enriched in the group of differentially expressed genes in cluster 3 resultant from partitioning cluster analysis.

Table S4.13 - Reactome pathways significantly enriched in the genes of cluster 1 resultant from the partitioning cluster analysis.

Table S4.14 - Reactome pathways significantly enriched in the genes of cluster 2 resultant from the partitioning cluster analysis.

Table S4.15 - Reactome pathways significantly enriched in the genes of cluster 3 resultant from the partitioning cluster analysis.