River barriers to gene flow: eco-evolutionary implications of a reproductive mode shift

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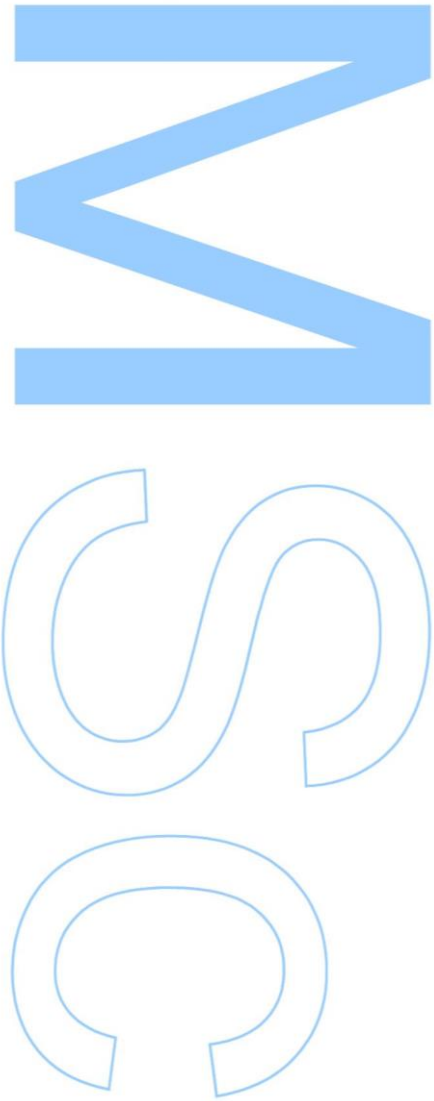

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Abstract

The fire-salamander (*Salamandra salamandra*), entails an extraordinary case of intraspecific variation in reproductive mode. A shift from larviparity (deposition of pre-metamorphic larvae in water) to viviparity (parturition of fully metamorphosed juveniles) have occurred independently within the species, comprising one of the few cases of viviparity within Urodeles. This shift implies the exclusion of the aquatic larval stage, which provides greater independence from water and affects their interaction with the surrounding environment. Nevertheless, the eco-evolutionary drivers and implications of this shift have been relatively underexplored and thus, *S. salamandra* constitutes a unique study system to address these unexplored ecological and evolutionary research questions.

Here, we studied the extent of rivers as barriers to gene flow in larviparous and viviparous salamanders and evaluated how a transition to viviparity influences the barrier effects of rivers to gene flow at both historical (mitochondrial DNA) and contemporary (microsatellites) time-scales. A total of eight larviparous and four viviparous populations were sampled across Northern Iberia. In each population, salamanders were sampled in opposite sides of the rivers and comparative fine-scale genetic analyses (assignment methods, quantification of genetic differentiation and kinship analyses) were carried out to examine the extent to which rivers hinder gene flow in both reproductive modes.

We found that rivers entail partial barriers for both larviparous and viviparous populations, yet they are more effective for the latter. Levels of pairwise genetic differentiation among individuals sampled across the river were significant in four larviparous and two viviparous populations, while assignment methods recovered a clear signal of disruption of gene flow in the largest larviparous and viviparous rivers. Fine-scale kinship analyses indicate higher relatedness along riversides than between riversides, suggesting a disruption in gene flow between riversides, although this pattern was more pronounced in pueriparous salamanders separated by larger rivers.

This comparative framework highlights the value of conducting fine-scale analyses to test the effects of specific landscape features to gene flow in comparison to studies undertaken at broader spatial scales. Moreover, it is the first study that explicitly demonstrates that a shift of reproductive mode affects patterns of genetic connectivity across rivers, contributing to a better understanding of the eco-evolutionary implications underlying a shift in reproductive mode.

Keywords

Fine-scale, genetic connectivity, genetic structure, larviparity, pueriparity, *Salamandra salamandra*.

Resumo

A salamandra-de-pintas-amarelas (*Salamandra salamandra*) constitui um caso extraordinário de variação intra-especifíca nos modos reprodutivos. A transição da larviparidade (deposição de larvas pre-metamórficas na água) para a viviparidade (nascimento de juvenis completamente metamorfoseados) ocorreu pelo menos duas vezes nesta espécie de forma independente, constituindo assim um dos poucos casos de viviparismo em urodelos. Esta alteração implica a exclusão do estágio larval aquático, portanto, a viviparidade confere uma maior independência de corpos de água e consequentemente, afeta a interação dos indivíduos com o ambiente em seu redor. Ainda assim, as causas e implicações ecológicas e evolutivas desta transição no modo reprodutivo têm sido pouco explorados, pelo que a *S. salamandra* representa um sistema de estudo único para testar hipóteses relacionadas com as implicações eco-evolutivas subjacentes à evolução da viviparidade.

Na presente tese, foi estudado o papel dos rios como barreiras ao fluxo genético em populações vivíparas e larvíparas, avaliando se a viviparidade influencia os efeitos barreira impostos pelos rios no fluxo génico a uma escala histórica (ADN mitocondrial) e contemporânea (microsatélites). No total, oito populações larvíparas e quatro vivíparas foram amostradas no norte da península Ibérica. Em cada população, as salamandras-de-pintas-amarelas foram amostradas em lados opostos dos rios e, para além disso, análises genéticas (quantificação de diferenciação genética, métodos de identificação de grupos genéticos e análises de parentesco) comparativas a uma escala fina foram feitas para testar se os rios influenciam de forma diferenciada o fluxo génico em ambos os modos reprodutivos.

Foi detetado que os rios são barreiras parciais para populações larvíparas e vivíparas, embora as últimas aparentam serem mais afetadas por estes elementos da paisagem. Os níveis de diferenciação genética entre indivíduos amostrados em ambos os lados dos rios foram significativos em quatro e duas populações larvíparas e vivíparas, respetivamente, enquanto que os métodos de deteção de estrutura genética revelaram uma clara disrupção do fluxo genético, em particular, nos rios de maior dimensão em ambos os modos reprodutivos. Análises de parentesco indicaram que o nível de parentesco é maior entre indivíduos localizados no mesmo lado de um rio em comparação com indivíduos

separados por estas barreiras, embora este padrão tenha sido mais pronunciado em salamandras vivíparas.

Este grupo de análises comparadas realça a importância de fazer análises a uma escala muito fina para perceber melhor os efeitos de elementes da paisagem especifícos quando comparado com estudos feitos a uma escala muito maior. Para além disto, este estudo demonstra explicitamente que uma mudança no modo reprodutivo afeta a maneira como os rios influenciam a conetividade genética e portanto, constitui um importante contributo para perceber melhor as implicações eco-evolutivas subjacentes à evolução de um modo reprodutivo derivado.

Palavras chave

Conectividade genética, escala local, estrutura genética, larviparidade, pueriparidade, *Salamandra salamandra.*

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AMOVA – Analysis of Molecular Variance

A_R – allelic richness

Bp – base pairs

CIs – confidence intervals

cyt-b – Cytochrome b

DAPC – Discriminant Analyses of Principal Components

DNA - Deoxyribonucleic acid

H_E – expected heterozygosity

H_O – observed heterozygosity

HWE– Hardy-Weinberg Equilibrium

IBD – Identity-by-Descent

IBD – Isolation-by-Distance

IBS – Identical-by-State

K – number of clusters inferred in DAPC and STRUCTURE analyses

Lat – latitude

LD – linkage disequilibrium

LE – linkage equilibrium

Long – longitude

NF – Number of females

Nm – Number of males

Ne – effective population size

N_A – mean number of alleles per locus

N_{PA} – number of individuals containing private alleles

mtDNA - mitochondrial DNA

P_A – number of private alleles

PCA – principal component analysis

PCR – polymerase chain reaction

PI – Parsimony Index

$PI_{(ID)}$ – Probability of Identity

PISibs – Probability of Identity accounting for the presence of relatives

PCR – Polymerase Chain Reaction

Q – membership probability to an inferred cluster

QuellerGT– Queller and Goodnight relatedness estimator

R – pairwise relatedness between individuals

RFU – Relative Fluorescence Units

SD – Standard Deviation

TrioML – Trio Maximum Likelihood relatedness estimator

µl – microliters

1. Introduction

1.1. General introduction to the study system

1.1.1. Evolution of viviparity and its eco-evolutionary implications

The reproductive mode of every species plays a crucial role in their evolution, behavior, and ecology. Reproductive mode in most vertebrate groups is characterized exclusively by oviparity (egg-laying reproduction) or viviparity (live-bearing reproduction) (Blackburn, 2015a). However, throughout their evolutionary history, many vertebrates shifted from an oviparous reproduction to a viviparous one. Specifically, viviparity has evolved independently at least 153 times (Blackburn, 2000, 2015a), although their occurrence is heterogeneous across the Tree of Life, with more than 100 origins of viviparity being recorded in squamate reptiles (Blackburn, 2015a). The shift to viviparity is of great interest as it entails - especially in females- remarkable genetic, morpho-physiological, behavioral and ecological changes. This may entail potential adaptations to the environment, involving benefits for the species that acquire them (e.g. more independence from exterior environmental conditions to lay the eggs or larvae) (Hodges, 2004; Pincheira-Donoso *et al.*, 2013; Shine, 2014; Velo-Antón *et al.*, 2015; Ma *et al.*, 2018; see Wells, (2007) and Blackburn *et al.*, 2015a for more examples).

A crucial pre-requisite for the evolution of viviparity is internal fertilization (Wells, 2007; Blackburn, 2015a). However, the acquisition of internal fertilization does not necessarily imply the emergence of viviparity, as other factors may also play an important role in the evolution of viviparity (Wells, 2007; Blackburn, 2015a). These factors are related with selective environmental pressures, that are expected to differ among and within vertebrate taxa, as different vertebrate groups and lineages exhibit different life-history traits and ecologies (Blackburn, 2015a). In squamate reptiles, viviparity has been largely associated with the “cold-climate hypothesis”, asserting that viviparity arose as an adaptation to cold and unstable climates. Under these conditions, females (which are able to thermoregulate) prolong the embryo retention within the uterus, thus avoiding to lay eggs under unsuitable cold conditions and ensuring more optimal temperatures for the development of the offspring (Hodges, 2004; Shine, 2015; Ma *et al.*, 2018). Also, it has been proposed that the transition to viviparity is due to the lack of oxygen at high elevations, which might also have prompted the longer retention of embryos to provide them a stable environment (Pincheira-Donoso *et al.*, 2017). Interestingly, transitions from viviparity back to oviparity were recently identified in a few squamate groups (Pyron and Burbrink, 2014), suggesting plasticity in reproductive modes within this group. Nevertheless, this hypothesis has been receiving some criticism (e.g. Blackburn, 2015b; Shine, 2015).

The female retention of embryos would generally provide suitable conditions for the development of the embryo, buffering unstable external environmental conditions and other harmful pressures (e.g. predation) that may be detrimental for the offspring fitness (Blackburn, 1999). Evolutionary advantages from this reproductive shift have been recorded also in aquatic animals like viviparous fishes. For instance, a recent study showed viviparity stimulated lineage diversification (Helmstetter *et al.*, 2016). These authors proposed that pregnant females carrying fertilized embryos could colonize geographically isolated watersheds and prompted speciation in isolated freshwater habitats. Nevertheless, acquisition of viviparity may also entail disadvantages to viviparous females due to the physical burden of carrying of the offspring for longer periods. For instance, it has been demonstrated that viviparous females' reptiles show lower dispersal rates and locomotor performance during pregnancy compared to their oviparous congeners (Shine, 2015). Thus, the successful fixation of this newly acquired trait (viviparity), must be linked with an increase of fitness that compensate all the inherent costs and constraints entailed by this transition (e.g. energetic costs due to prolonged retention of embryos, increased egg size or parental care).

1.1.2. Evolution of pueriparity in amphibians and its eco-evolutionary implications.

In amphibians the evolution of reproductive features is still not disentangled due to the huge diversity in reproductive modes and lifestyles, the greatest among all vertebrate groups (Wells, 2007). Nevertheless, ancestral reproductive mode in amphibians is presumably characterized by the deposition of eggs in water bodies (Duellman and Trueb, 1986; Wilkinson and Nussbaum, 1998). Despite the huge diversity in modes of reproduction most amphibians exhibit a biphasic life cycle, where aquatic larvae metamorphose into terrestrial juveniles (Wells, 2007). Nevertheless, due to their complex life cycle and numerous reproductive strategies, subcategories of oviparity and viviparity have been proposed to characterize the reproductive biology of amphibians. Hereafter, for amphibians, we are going to distinguish species that exhibit a: (1) oviparous reproduction (deposition of unfertilized or fertilized eggs); (2) larviparous reproduction (deposition of pre-metamorphic aquatic larvae); and (3) pueriparous reproduction, in which the larval aquatic stage is skipped and females give birth to metamorphosed terrestrial juveniles (*sensu* Greven, 2003; Blackburn, 2015a). The latter two reproductive modes comprise two different subcategories of viviparity. Additionally, pueriparity in amphibians imply that pueriparous amphibians exhibit a fully terrestrial life cycle (Velo-Antón *et al.*, 2015).

Unlike in Squamates, in amphibians the selective forces leading to the emergence of pueriparity, and its respective eco-evolutionary consequences, are not so well understood. One reason for this might be that in amphibians, this reproductive transition is much less common than in Squamate reptiles. Eight independent origins of pueriparity among the three extant orders have been recorded so far, entailing in total 1 % of the amphibian species (Blackburn, 2015a; see also references therein). In anurans (toads and frogs), pueriparity occurs rarely (*ca*. 0.09 %), yet, it is present in around 15 % of caecilians (Gymnophiona) (Blackburn, 2015a). Within Urodeles pueriparity is exclusively present in 11 salamander species, all within the family Salamandridae (*ca*. 1.6% of Urodel species). The scarce occurrence of pueriparity within Urodeles is puzzling, since internal fertilization -an indispensable pre-requisite for the evolution of pueriparity in vertebrates- is almost exclusive within this order (Wells, 2007), but unlike in Caecilians, the evolution of internal fertilization has not always been followed by a transition to pueriparity (Wells, 2007). Pueriparity implies the skipping of the aquatic larval stage (Wells, 2007), and so implies greater independence from water sources to survival and reproduction. Thus, terrestrial reproduction (e.g. pueriparity and direct development) entails paramount benefits related with the independence from water. For example, the ability of pueriparous individuals to persist and breed on land, increase their potential to thrive in harsher environments (García-París *et al.*, 2003; Velo-Antón *et al.*, 2007; Álvarez *et al.*, 2015; Lourenço *et al.*, 2017; Liedtke *et al.*, 2017). Terrestrial reproduction also relieves predation rates, as aquatic larvae are often subjected to intensive predations pressures (Gomez-Mestre *et al.*, 2012); additionally, it prevents from the mortality risk associated with fast-flowing floods, and with parasite transmission related to water bodies and lower larval immunity (Crump, 2015). Indeed, the evolution of terrestrial modes of reproduction, has been associated with selective pressures related to reduced availability of suitable water bodies for the development of offspring (García-París *et al.*, 2003; Velo-Antón *et al.*, 2012; Crump, 2015; Lourenço *et al.*, 2017; Liedtke *et al.*, 2017). Hence, although the exact selective pressures causing the origins of pueriparity are unknown, it has been hypothesized that historical and contemporary low availability of water could be a trigger for the evolution of pueriparity in some amphibians (García-París *et al.*, 2003; Beukema *et al.*, 2010; Velo-Antón *et al.*, 2012; Liedtke *et al.*, 2017), leading into the formulation of a “dry-climate hypothesis” (García-París *et al.*, 2003; Velo-Antón *et al.*, 2015) to explain the origin of pueriparity in fire salamanders.

The transition to pueriparity implies deep changes in morpho-physiological and behavioral traits (Buckley *et al.*, 2007; Velo-Antón *et al.*, 2015), which are accompanied by changes in the way fire salamanders interact with the surrounding environment (Lourenço *et al.*,

2019). Since dispersal is influenced by life-history traits and environmental factors (Richardson, 2012; Sánchez-Montes *et al.*, 2018), a shift to pueriparous reproduction would entail eco-evolutionary implications that could affect dispersal. While dispersal in larviparous amphibians is constrained by the availability of suitable water bodies that offer optimal breeding conditions (deposition and developing of the larvae) (Russell *et al.*, 2015), pueriparous amphibians are not constrained by the availability of water bodies (Measey *et al.*, 2007; Crump, 2015; Álvarez *et al.*, 2015; Liedtke *et al.*, 2017; Lourenço *et al.*, 2017). Given the potential difference in the interaction with the landscape, it is hypothesized that the evolution into a terrestrial reproduction would increase genetic connectivity among terrestrial-breeding amphibians since they are not tight to water for reproducing and can thrive in harsher environments, allowing them to disperse across a greater range of habitats (Measey *et al.*, 2007; Lourenço *et al.*, 2017; Sandberguer-Loua *et al.*, 2018). Nonetheless, previous studies in fire salamanders did not find significant differences in fine-scale genetic structure between the two reproductive modes, revealing no significant differences in movement patterns between reproductive modes within 1km-transects, in intact natural landscapes (Lourenço *et al.*, 2018a). Indeed, genetic connectivity has been shown to be lower among pueriparous fire salamanders' populations. Specifically, Lourenço *et al.* (2019) showed water courses (both small streams and large rivers) comprise relevant physical or behavioral barriers in pueriparous salamanders, while patterns of gene flow among larviparous populations appear relatively unaffected by these landscape features. These results seem to corroborate circumstantial evidence suggesting that lotic systems are strong barriers to gene flow, particularly in terrestrial breeding amphibians (direct-developers; Marsh *et al.*, 2007; Fouquet *et al.*, 2015). Specifically, in Fouquet *et al*. (2015), terrestrial-breeding amphibians that do not use water in any stage of their life-cycle, showed higher trans-riverine structure than aquatic-breeders. Larviparous amphibians, which deposit the larvae in water bodies and small streams and might reach big rivers by drift, could cross these water courses actively by swimming or passively by drift, enhancing gene flow between both sides.

1.2. Landscape genetics: a methodological framework to detect barriers

To understand the processes that regulate patterns of dispersal and gene flow (*i.e.* dispersal followed by successful reproduction), it is crucial to evaluate how landscape features shape dispersal and gene flow, and consequently, patterns of genetic variation across space. Landscape genetics compile a wide arrange of fields (population genetics, landscape ecology, geography and spatial statistics), thus comprising a multidisciplinary framework to assess spatial patterns of genetic variation and to identify the environmental

variables and landscape features underlying them (Manel *et al.*, 2003). Unlike classical biogeography and population genetic studies, landscape genetics aim at evaluating the role of the landscape in shaping microevolutionary processes (e.g. gene-flow, genetic drift) at multiple spatial and temporal scales (Manel *et al.*, 2003; Anderson *et al.,* 2010).

Multiple factors can affect spatial connectivity and gene flow at several spatial scales. These factors can be abiotic (e.g. topography, climate, landscape heterogeneity), biotic (biological interactions such as prey-predator, vegetation, etc), and anthropogenic features (e.g. dams, roads, urban areas). All these features may reduce connectivity, hence reducing population's viability (Cosgrove *et al.*, 2018). Disruptions in the movement of individuals have a huge impact on three essential mechanisms: resource access, demographic exchange and gene flow (Cosgrove *et al.*, 2018). Impeded gene flow changes the number of alleles exchanged among populations, directly shaping the genetic structure of natural populations (allele frequencies) (Balloux *et al.*, 2002). Hence, a barrier to dispersal prevent functional connectivity (due to crossing mortality, behavioral avoidance, or by constituting a physical obstacle to dispersal) resulting in increasing genetic differentiation between individuals or populations on opposite sides of that barrier (e.g. Hepenstrick *et al.*, 2012). In recent years, methods commonly applied within a landscape genetics framework (e.g. Bayesian assignment methods, multivariate analysis such as PCA, Isolation by distance tests, etc.), sometimes combined with other sources of data (e.g. individual-level movement information), have been increasingly used to examine more accurately the genetic effects of environmental and anthropogenic barriers. (Holderegger and Wagner, 2008, see for example Hepenstrick *et al.*, 2012; Grummer and Leaché, 2017; Lourenço *et al.*, 2017; Carvalho *et al.*, 2018; Luqman *et al.*, 2018;).

1.2.1. Sampling design

Before starting any landscape-genetics study, there are some important factors besides analytical strategies and employed statistical methods, that should be taken into account while defining study objectives and the specific research question (Hall and Beissinger, 2014). Nonetheless, the decisions made about the experimental sampling design are usually constrained by the accessibility to the study location (e.g. distance, sampling-area conditions, transport connections) and logistical conditions (e.g. budget, costs, available human and equipment resources, available time). Thus, a trade-off between inherent constraints of the study and optimal study design (sampling scheme and choice of markers), must be taken into account in order to obtain the best possible results.

Issues related with scale (both spatial and temporal) may arise when trying to assess the effect of barriers in genetic connectivity. Spatial scale and sampling design should match

the life-history (e.g. generation time), demography (e.g. population effective size) and habitat use (e.g. dispersal behavior) of the studied species (Hall and Beissinger, 2014). Specifically, dispersal behavior and generation times of the studied species are crucial when planning the scale and sampling-design: species with short dispersal ranges require data collection over short spatial scales (Anderson *et al.*, 2010; Hall and Beissinger, 2014). Additionally, the analysis of the genetic barrier effects of specific landscape features (e.g. linear barriers, such as rivers and roads) may not be evident when examining broader spatial scales of the landscape (Mullen *et al.,* 2010). In this regard, studies performed at a very fine spatial scale and, in particular, for species exhibiting low vagility, have the potential to uncover the influence on genetic connectivity of specific landscape features. This is because genetic sampling points are positioned close enough to the potential barrier, which avoids spurious results (such as genetic discontinuities) that arise from genetic isolation by distance, discrete population structure, or from the effect of other landscape features that can act as potential barriers (Anderson *et al.*, 2010). Moreover, to assess the effect of a specific potential barrier and draw conclusions at a certain taxonomic level (e.g. species level), landscape fine-scale genetic studies should contain several replicates where environmental conditions are similar among them to allow valid comparisons and more robust inferences (Anderson *et al.,* 2010).

1.2.2. Temporal scale and choice of molecular markers

Temporal scale is also crucial to understand the effect of barriers as impediment of connectivity among populations (Cushman, 2006; Zellmer and Knowles, 2009; Anderson *et al.*, 2010; Landguth *et al.*, 2010; *et al.*, 2014). This is because: (1) some generations (the number depends on the studied organism) are needed to detect the genetic signature left by the barrier (lag time) (Landguth *et al.*, 2010; Epps and Keyghobadi, 2015); Crosgrove *et al.*, 2018), (2) the response to that putative barrier may change throughout time depending on several factors (e.g. metapopulation dynamics) (Zellmer and Knowles, 2009); (3) the genetic signature left by current or recent events may be confounded by historical biogeographical processes (Titus *et al.*, 2014; Anderson *et al.*, 2010 for more examples), or conversely (4) current demographic processes may override genetic patterns resulting from historical processes. Accurate detection of the genetic effects imposed by landscape barriers depends also on the molecular markers used (Landguth *et al.*, 2010; Anderson *et al.*, 2010). Choice of markers types should consider mainly their mutation rate and polymorphism (informative content) to infer species evolutionary history at different time scales (Anderson *et al.*, 2010; Hall and Beissinger; 2014).

Microsatellites are markers of choice to investigate contemporary genetic consequences of landscape features at fine-scale (e.g. Marsh *et al.*, 2007; Clark *et al.*, 2010; Frantz *et*

al., 2012; Waraniak *et al.*, 2019). They are tandem repeats of 1-6 base pairs in length distributed with high frequency throughout the nuclear genome of most taxa. Their high mutation rate (ranging within 10^{-6} and 10^{-2} mutations per locus and generation), make these molecular markers highly polymorphic, generating the high allelic diversity that makes them suitable for genetic studies at fine-scale within a contemporary scale (ten to hundreds of generations) (Selkoe and Toonen, 2006; Wang and Santure, 2009; Hall and Beissinger, 2014). Thus, they entail effective tools to study the effect of landscape features and evolutionary processes (e.g. gene flow, genetic drift) in the genetic structure of populations (Selkoe and Toonen, 2006). Indeed, previous works employing multilocus genetic microsatellite data in *Salamandra salamandra*, have already provided satisfactory results. Specifically, in our studied species, microsatellite data was successfully used to (1) find genetic differentiation at fine-scale (*i.e.* less than 100m) (Lourenço *et al.*, 2017); (2) determine the influence of reproductive mode on patterns of dispersal (Lourenço *et al.*, 2018a); (3) provide estimates of effective population sizes (*Ne*) (Álvarez *et al.*, 2015); (4) characterize contemporary patterns of genetic diversity and population structure (Steinfartz *et al.*, 2007; Velo-Antón *et al.*, 2012; Antunes *et al.*, 2018; Dinís *et al.*, 2018); (5) paternity analysis (Caspers *et al.* 2014); and (6) to identify landscape variables governing gene flow in heterogeneous and fragmented landscapes (Antunes *et al.*, 2018; Lourenço *et al.*, 2019).

Although less widely used in landscape genetic studies, mitochondrial markers are appropriate markers when comparing population divergence at longer temporal scales (Wang, 2010). This is due to their comparatively slower mutation rates, that makes them best suited to examine deeper levels of population differentiation and infer long-term patterns of genetic divergence at large scale (Anderson *et al.*, 2010). Nonetheless, their uniparental-inheritance, makes them more prone to suffer greater effect of evolutionary processes such as genetic drift, which could lead to increased genetic variation over small spatial scales, making them informative at fine-scale (see Hall and Beissinger, 2014), although differences in sex-biased dispersal in the study species, or selective processes, can blur an accurate evaluation of the historical patterns of gene flow (Toews and Brelsford, 2012).

1.3. The Fire salamander

The fire salamander (*Salamandra salamandra*, Linnaeus 1758) belongs to the family Salamandridae (Amphibia: Caudata), comprised by 20 genera and encompassing 120 species (AmphibiaWeb; 2019). This family is divided into two major subgroups, the ‘True’ salamanders and the newts (Figure 1) (Zhang *et al*., 2008; AmphibiaWeb, 2019). This family is the only one among urodeles containing pueriparous species, with a total of 11

species being pueriparous, from which seven are *Lyciasalamandra* species (thus, comprising all the species of this genus) and four (out of six) *Salamandra* species (Veith *et al.*, 1998; Buckley, 2012; Blackburn, 2015a).

'True' salamanders clade, comprised by *Chioglossa*, *Lyciasalamandra*, *Mertensiella* and *Salamandra* genera, is found throughout the western Palearctic region (Zhang *et al.*, 2008). Within this clade, the *Salamandra* genus forms a monophyletic group (Vences *et al.*, 2014; Rodríquez *et al.*, 2017). Due to their conspicuous black and yellow coloration, four salamanders are currently recognized as fire salamanders: *Salamandra salamandra* (*S. salamandra*, Linnaeus 1758), *S. algira* (*Salamandra algira*, Bedriaga 1883), *S. corsica* (*Salamandra corsica*, Savi 1838) and *S. infrainmaculata* (*Salamandra infrainmaculata*, Mertens 1948), while *S. atra* (*Salamandra atra,* Laurenti 1768) and *S. lanzai* (*Salamandra lanzai*, Nascetti, Capula & Bullini, 1988) are black-colored species (Vences *et al.*, 2014; Rodríquez *et al.*, 2017). The genus *Salamandra* is found throughout the western Palearctic region: occupying Europe (*S. salamandra* has a widespread distribution from Southern Iberia to Eastern Europe, with a northern limit in the northernmost area of Germany, while *S. corsica* is endemic to the island of Corsica and *S. atra* and *S. lanzai* are adapted to higher elevation habitats of the Alps); North Africa (*S. algira* exhibit a patchy distribution in Morocco and Algeria); and Near and Middle East (S. *infraimmaculata*) (Kuzmin, 2009; Vences *et al.*, 2014).

Pueriparity is not a symplesiomorphic character (*i.e*. shared ancestral trait) within *Salamandra* genus (Vences *et al.*, 2014; Rodríguez *et al.*, 2017), but rather is an apomorphic character (*i.e.* phylogenetically derived) that evolved in at least four independent events (Figure 1) (Buckley, 2012). While the two alpine salamanders, *S. atra*, and *S. lanzai* are strictly pueriparous, the remaining four species (*S. salamandra, S. algira, S. corsica*, and *S. infraimmaculata*) are acknowledged as larviparous. Yet, although larviparity being predominant, both larviparity and pueriparity co-occur in *S. salamandra* and *S. algira*, in which the pueriparous populations are geographically restricted within the continuous distribution range of larviparous populations (García-París *et al.*, 2003; Velo-Antón *et al.*, 2007; 2012; 2015; Beukema *et al.*, 2010; Dinís and Velo-Antón, 2017) .This makes *S. salamandra* an striking evolutionary model, especially considering that *S. salamandra* and its sister species, *Salamandra algira,* comprise the only known examples of intraspecific variation in reproductive mode within Amphibians and one of the few examples among vertebrates (Buckley, 2012; Blackburn, 2015a).

The origin of pueriparity and the selective forces that triggered the transition to pueriparity in Urodeles are poorly understood. It has been suggested that pueriparity in the Alpine

salamanders arose as a response to harsh terrestrial environments related with high altitudes (Veith *et al.*, 1998; see Wells, 2007 for more references). However, this could not explain the origin of pueriparity in island populations. Because pueriparity implies that the aquatic larval stage is skipped, a “dry climate hypothesis” was proposed to explain the origin of pueriparity within *S. salamandra* (García-París *et al.*, 2003; Velo-Antón *et al.*, 2007, 2012; Beukema *et al.*, 2010). The origin of pueriparity, would remove the larval aquatic stage; therefore, a possible trigger for this evolutionary event would be the historical or contemporary absence of water bodies. This is a plausible scenario in the Cíes Islands where water is scarce, as well as in the karstic limestone substrate (where surface water does not accumulate, due to high permeability of the soil) dwelled by *S. s. bernardezi,* and in areas inhabited by *Salamandra algira tingitana*, which are associated to lower volume of precipitation than those inhabited by larviparous populations (García-París *et al.*, 2003; Velo-Antón *et al.*, 2007; Beukema *et al.*, 2010).

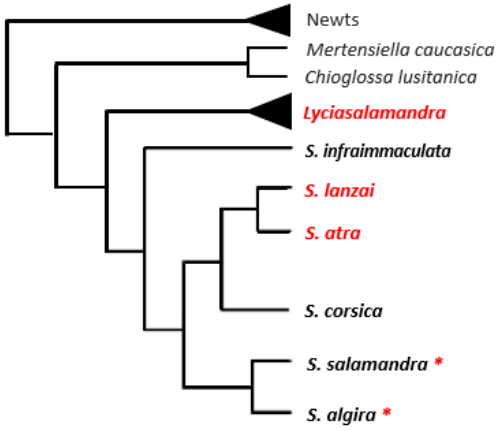


Figure 1. Simplified phylogenetic tree of the family *Salamandridae,* with emphasis in *Salamandra* genus, adapted from Zhang *et al.* (2008). Bold black and red represent larviparous and pueriparous species/genus, respectively. Red asterisks in *S. salamandra* and *S. algira* indicated that both reproductive modes co-occur within the species.

1.3.1. Evolutionary history and variation in reproductive modes in *S. salamandra*

Salamandra salamandra comprises 12 subspecies distributed throughout most of Europe, being 9 of them endemic to the Iberian Peninsula: *S. s. gallaica, S. s. bejarae, S. s. crespoi, S. s. morenica, S. s. longirostris, S. s. almanzoris, S. s. bernardezi, S. s. fastuosa* and *S. s. terrestris* (although the latter is also present in central Europe) (Figure 2). Within the Iberian Peninsula, *S. salamandra* shows high levels of intra and inter-subspecific phenotypic variability (body size, shape and coloration, reproductive modes), and genetic divergence (e.g. García-París *et al.,* 2003; Beukema *et al.,* 2016). The high levels of intraspecific phenotypic and genetic variation are likely due to the complex evolutionary history of Iberian populations, since the Iberian Peninsula acted as multiple *refugia* during

the glaciations that took place in the Quaternary (Pleistocene), which led to vicariant events and allopatric differentiation (e.g. Steinfartz *et al.,* 2000; García-París *et al.,* 2003).

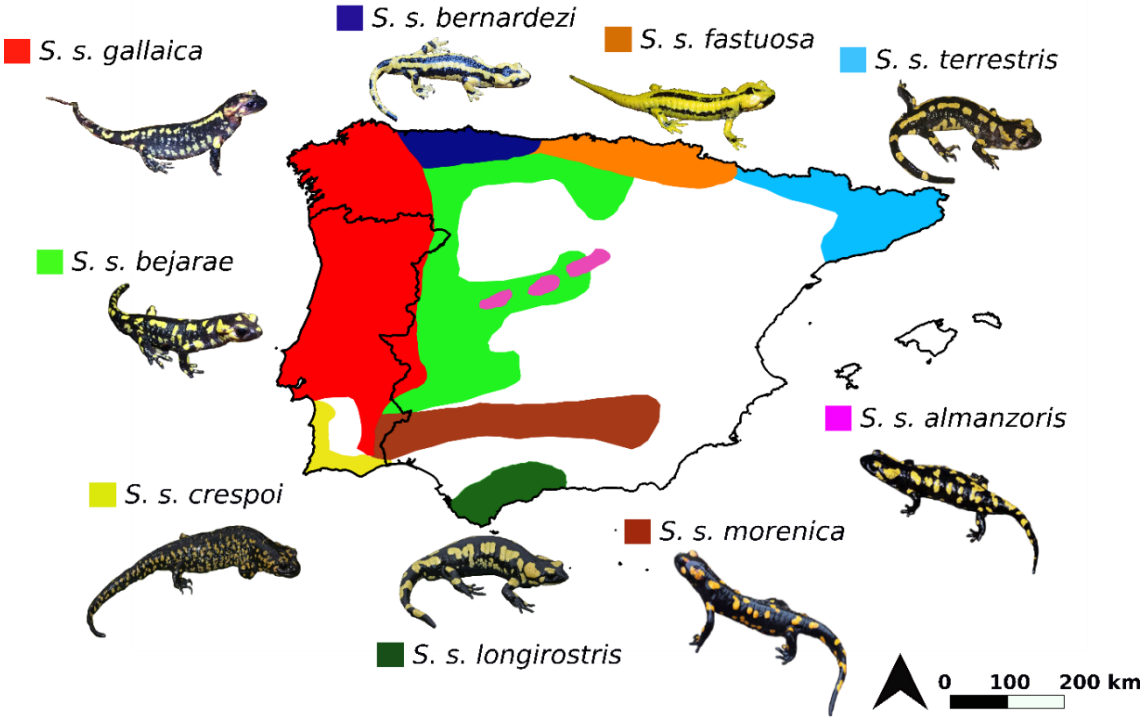


Figure 2. Approximated distribution of *Salamandra salamandra*'s subspecies within the Iberian Peninsula. The morphological variability among six subspecies is also represented. Image provided by André Lourenço.

Salamandra salamandra represents an extraordinary case of polymorphism in reproductive modes, where two live-bearing reproductive strategies co-occur: the ancestral larviparous reproduction (in which females deliver aquatic larvae), and the derived pueriparous reproduction (where females deliver fully developed terrestrial juveniles) (Velo-Antón *et al.,* 2015). Larviparity involves the intra-uterine embryonic development during a gestation period of approximately 90 days, where embryos develop synchronously and depend on the yolk masses supplied in each egg (lecitotrophy) to develop (Buckley *et al.*, 2007). After gestation, females may lay approximately up to 90 larvae (on average 30-40) in aquatic systems, including lentic environments and streams, where they will spend months before metamorphosing into terrestrial juveniles (Velo-Antón *et al.*, 2015). In contrast, in pueriparous females, not all the ovulated eggs are fertilized, and embryos hatch precociously inside the maternal oviduct. Heterochrony, *i.e.* shifts in the timing of some developmental events in a descendant organism (in this case pueriparous individuals), compared to the timing of the same events in the ancestor (in this case larviparous individuals), is considered as the trigger for pueriparity (Buckley *et al.*, 2007).Pueriparous individuals have approximately the same gestation period as larviparous ones, even though newborns show a more advanced stage of development

compared to their larviparous counterparts (Velo-Antón *et al.*, 2015). This is because heterochronic events (*i.e.* changes in the relative timing of ontogenetic events compared to the ancestor), jointly with an intrauterine cannibalistic behavior, allow for the acceleration of embryo development (Buckley *et al.,* 2007). Specifically, since pueriparous embryos develop at different rates inside the maternal uterus, some embryos rapidly consume the yolk supply, and hatch early. These embryos then feed on arrested eggs (oophagy), and other less-developed embryos (adelphopagy) and, as a result, they are able to complete their development within the maternal uterus (Buckley *et al.*, 2007). Thus, pueriparous females give birth to a reduced number (1-15) of terrestrial juveniles in post-metamorphic stages of development.

Throughout most of its range, *S. salamandra* is larviparous; however, the ancestral larviparous reproduction trait, is interrupted by some populations showing pueriparous reproduction, which evolved twice in this species: (1) in northern-Spain subspecies inhabiting the Cantabrian Mountains (*S. s. fastuosa* and *S. s. bernardezi*), and (2) in two insular populations (Ons and San Martiño populations of *S. s. gallaica*) (Figure 3). The first origin of pueriparity has occurred in the Cantabrian populations of *S. s. bernardezi* during the Pleistocene, possibly as response to past environmental pressures related with the lack of available surface water caused by the high permeability of karstic limestone substrates (García-París *et al.*, 2003). Pueriparity, along with other traits (e.g. body coloration), spread later eastwards into *S. s. fastuosa* during interglacial periods, in which the climate was more favorable (García-París *et al.*, 2003). Hence, *S. s. fastuosa,* located in the north-central part of the Iberian Peninsula, shows a mixed reproductive strategy (García-París *et al.*, 2003; Velo-Antón and Buckley, 2015). A second contact zone also occurs in northwest Iberia (northern Galicia) between *S. s. bernardezi* and larviparous *S. s. gallaica*, where individuals show both reproductive modes (*i.e.* giving birth to both larvae and fully metamorphosed individuals) were recorded (Galán, 2007). The second independent origin of pueriparity occurred more recently (*ca*. 8000 years ago) in two insular populations of *S. s. gallaica* on the Galician Atlantic Coast (Velo-Antón *et al.*, 2007). These pueriparous populations do not form a monophyletic group with mainland pueriparous populations, thus supporting the independent origin of these reproductive shift (Velo-Antón *et al.*, 2007). Moreover, these insular populations became isolated from the mainland populations when the sea level rose during the Holocene. The absence of available surface water, in which to lay the aquatic larvae, has been suggested as a

plausible scenario for the origin of this reproductive mode in these islands (Velo-Antón *et al.,* 2007; 2012).

Figure 3. Distribution range of both reproductive modes of *S. salamandra*. The distribution of larviparous populations is highlighted in blue and the distribution range of pueriparous populations, corresponding to two Atlantic continental islands and the Northern Iberian Peninsula, is depicted in reddish colour. Image adapted from Velo-Antón *et al.* (2015).

1.3.2. Phenotypic and ecological characterization of *S. s. gallaica* and *S. s. bernardezi*

Salamandra salamandra also shows high levels of intra-specific morphological variability, which also applies at the subspecies level for *S. s. gallaica* and *S. s. bernardezi* (*i.e.* high levels of inter and intra-subspecific variability, even recognizable at the population level; Velo-Antón and Buckley, 2015). Additionally, intermediate phenotypic forms might be observed in the contact zones (Velo-Antón and Buckley, 2015; Galán, 2007). *Salamandra s. bernardezi* is generally characterized by varying patterns of dorsal and lateral black stripes, over a yellow body (Beukema *et al.*, 2016), while *S. s. gallaica* is characterized by a dorsal black coloration interrupted by heterogeneous yellow spots and, besides the dominant yellow and black colors, is frequent to observe individuals with red spots (Velo-Antón and Buckley, 2015). *Salamandra s. gallaica* is larger than *S. s. bernardezi*, with individuals´ body length (from snout to cloaca) reaching up to 250 mm and 180 mm, respectively (Velo-Antón and Buckley, 2015; Velo-Antón *et al.*, 2015). Although different in body size (which might render higher dispersal capability in *S. s. gallaica*), both pueriparous and larviparous salamanders show similar dispersal tendencies across suitable habitats and at a fine-scale (Lourenço *et al.*, 2018a). Fire salamanders are relatively sedentary and present site-fidelity, with their home range sizes varying between

106 m^2 and 26788 m^2 (Schulte *et al.,* 2007; Hendrix *et al.,* 2017). Although more studies are needed, there is circumstantial evidence suggesting this species exhibits male-biased dispersal (e.g. García-París *et al.*, 2003; Lourenço *et al.*, 2018a).

The highest peak of activity for fire salamanders match with the milder and wetter months (coinciding with autumn and spring) in Iberia Peninsula, after emerging from the aestivation period to breed (Velo-Antón and Buckley, 2015). In larviparous populations, females approach water bodies (*i.e.* streams and ponds) to deliver the larvae but they are rarely seen along the bank of large rivers (Velo-Antón and Buckley, 2015). Besides one documented population showing diurnal activity (pueriparous population in San Martiño island; Velo-Antón and Cordero-Rivera, 2017), both sub-species exhibits nocturnal activity (Velo-Anón and Buckley, 2015). They have terrestrial habits, dwelling in shaded and moist environments, associated with deciduous forests, although they can also be found on shrublands, conifers, or even in eucalyptus plantations with abundant shrubs. In contrast, albeit both subspecies are generally absent from disturbed habitats, pueriparous populations can be found in water-limited and harsh environments, such as urban areas (Álvarez *et al.*, 2015; Lourenço *et al.*, 2017) and small islands (Velo-Antón *et al.*, 2007; 2012; Lourenço *et al.*, 2018b).

1.4. Study area: North-Iberian Peninsula

Our two studied subspecies are distributed across the northern Iberia, in the Eurosiberian region. The oceanic and hyper-oceanic (along the coast) bioclimatic variants of this region, characterized by wet and warm climate, makes it a suitable area for deciduous forests dominated by oak, chestnut, birch, and beech trees (Amigo *et al.*, 2017). However, since the Northwest part of the Iberian Peninsula is highly transformed by agricultural and industrial activities, the natural deciduous forest is highly intermixed with plantations of introduced eucalyptus and conifer plantations and pastures. The substrate is characterized mostly by siliceous rocks, especially in Galicia and by carbonate rich-rocks such as limestone in the Oriental part of Asturias (Amigo *et al.*, 2017). This is worth noting since, as mentioned above, the karstic limestone substrate might cause limitation in water availability, which was proposed as a trigger of pueriparity in Cantabrian populations of *S. s. bernardezi* and *S. s. gallaica* in Galician-Atlantic Islands (García-París *et al.*, 2003; Velo-Antón *et al.*, 2007; 2012). In the Asturias region and the Northern-eastern part of Galicia, the rivers are short-range and fast running, flowing relatively in parallel from the Cantabrian mountain range, draining into the Cantabric Sea (Amigo *et al.*, 2017).

1.5. Objectives and hypothesis

Due to the high intraspecific variability in reproductive strategies at intra-specific level, where we can find adjacent populations with distinct reproductive modes, or even reproductive hybrid zones of both reproductive modes, *Salamandra salamandra* entails a unique model system to assess the eco-evolutionary implications of such a macroevolutionary novelty. Specifically, we can benefit from this reproductive variation to perform robust comparisons between larviparous and pueriparous fire salamanders and thus, avoid the bias that might arise from comparing more distantly-related species (Richardson *et al.*, 2012; García *et al.*, 2017; Sánchez-Montes *et al.*, 2018).

The goals of this study are to perform a comparative fine-scale genetic analysis between both reproductive modes, in order to assess how rivers influence population genetic structure and connectivity in this species and, additionally, infer whether the potential barrier effects imposed by these landscape features are different between larviparous and pueriparous populations. To achieve so, we sampled twelve populations encompassing both pueriparous (N=4) and larviparous (N=8) reproductive modes, to assess two main objectives: (i) estimate and compare historical and contemporary patterns of genetic differentiation between individuals occurring at both sides of each river; and (ii) test for differences of gene flow between reproductive modes. Since both subspecies show terrestrial habits, and inhabit analogous landscapes with similar climatic and environmental conditions, we expect that any potential difference in genetic patterns is caused by life-history traits and reproductive modes, rather than external environmental factors (landscape composition and climate).

Although we expect that gene flow in both subspecies is negatively affected by rivers due to their low vagility, philopatry, and breeding site fidelity (Velo-Antón and Buckley, 2015), we expect a larger effect in pueriparous populations arising from their fully terrestrial life cycle (Lourenço *et al.*, 2019). Consequently, we tested one main hypothesis: Rivers act as more effective barriers to gene flow for pueriparous populations; thus, we predict (i) higher levels of genetic differentiation and (ii) lower levels of genetic relatedness between riversides for pueriparous populations. We based our hypothesis on the premise that pueriparity allows individuals to be released from an aquatic environment, and be restricted exclusively to terrestrial habitats.

2. Material and Methods

2.1. Study area and sampling collection

A fine-scale sampling was undertaken in 12 permanent rivers throughout North-Iberian Peninsula, in which, eight are located within the distribution of larviparous populations of *S. s. gallaica* and four are located within the range of pueriparous populations of *S. s. bernardezi* (Figure 4; Table 1). We chose rivers based on the habitat found on both riversides. Because habitat fragmentation may hamper dispersal and influence patterns of genetic structure in fire salamanders (Velo-Antón and Buckley, 2015; Antunes *et al.*, 2018; Lourenço *et al.*, 2019), we focused the sampling effort on rivers with continuous favorable habitat on both riversides, presenting well-preserved Atlantic deciduous forests with high availability of shelters (Velo-Antón and Buckley, 2015), and high population densities at local scales, although in few localities some disturbance was unavoidable. We chose flat transects, so that the steepness of river-margins is not a factor affecting dispersal in any study site (Lourenço et al., 2019). Moreover, we discarded transects in the high areas of the river, avoiding transects that could be partially dried during summer months. Such methodological procedure was carried out to enable not only valid comparisons between individuals sampled in the opposite sides of the river, but also among salamanders sampled in different rivers.

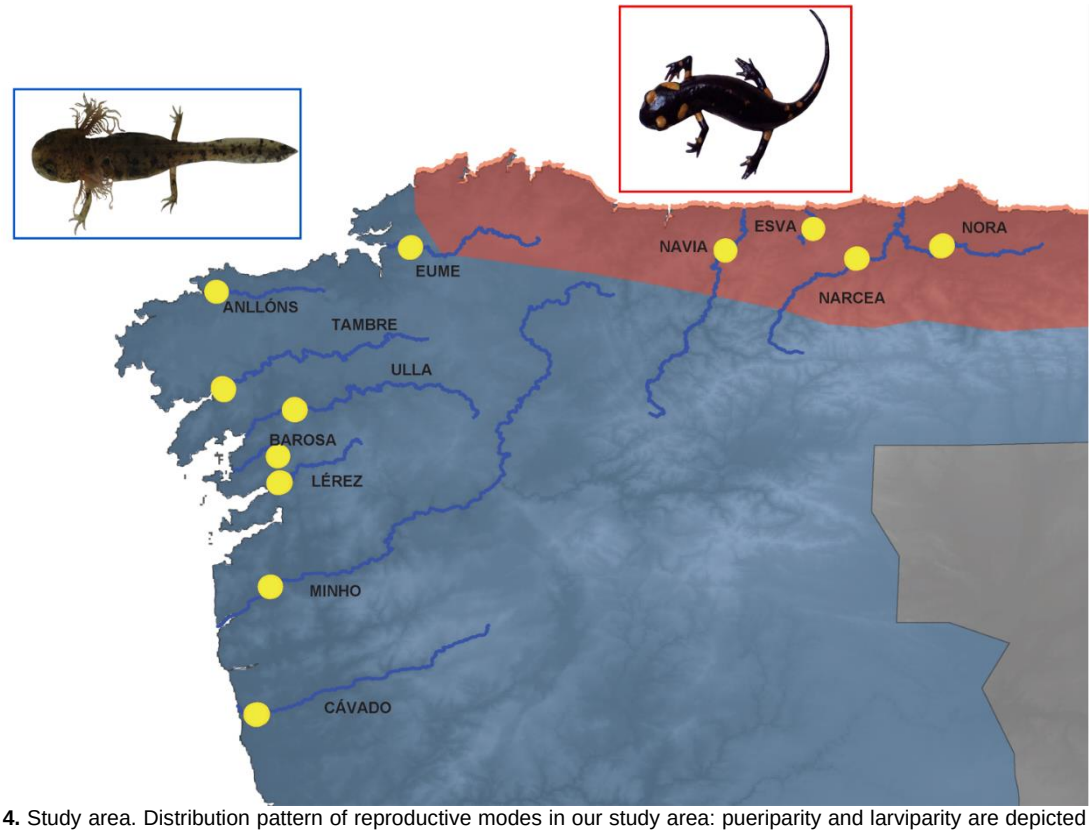


Figure 4. Study area. Distribution pattern of reproductive modes in our study area: pueriparity and larviparity are depicted in reddish and bluish colour, respectively. Sampled localities named after the studied rivers are also plotted. Photo credits: Guillermo Velo-Antón.

Fieldwork was performed matching the two activity peaks of adult fire salamanders: October and November (2018), and March-April (2019) (Velo-Antón and Buckley, 2015). We performed nocturnal transects to capture active adults along both sides of the river (e.g. Marsh *et al.*, 2007) during rainy nights, under windless conditions and with temperature ranging between 8-16ºC (Velo-Antón and Buckley, 2015). In most cases, the transects were performed avoiding any anthropogenic or natural artifact connecting both riversides (e.g. fallen tree, small bridges, rocks) that could potentially enhance migration among riversides. However, due to the high transformation of these habitats, it was impossible to avoid nearby car-traffic or suspension bridges in some rivers (Eume, Anllóns, Ulla, Lérez, Miño, Navia, Esva - see Table 1), or a small rock bridge close to Nora population. Moreover, we focused sampling effort in adults, ensuring a better identification of the sex, and preventing the excessive sampling of related individuals from the same cohort, as larvae and to a less extent juveniles are often spatially clustered, leading to spurious estimates of genetic structure and relatedness. (Wang, 2018: O'Connell *et al.*, 2019). For the same reason, we discarded individuals that were spatially clustered, leaving some space (ca. 10 m), when possible, between sampled individuals. We tried to expand the length of transects at least for 200 m on both sides of the river to ensure that the transects were considerably longer than the width of the rivers (so the differentiation between riversides is not explained just by isolation by distance). All these conditions intend to emphasize the random character of the analyses with regard to kinship (*i.e.* avoiding an overrepresentation of relatives), in order to prevent biased or inaccurate results and ensuring that the population and its properties are truly represented by the sample (Wang, 2018).

Table 1. Characterization of the rivers. Latitude (Lat), longitude (Long), an approximate measure of the width (in meters), measured in Google Earth Pro: http://earth.google.com/, and the Strahler stream order* are displayed. The presence of relatively close bridges is displayed. Rivers are sorted by reproductive modes.

Pop	Lat	Long	Width (m)	Order	Observations
Larviparous					
Eume	43°24'24.45"N	8° 5'6.15"O	17	3	Suspension bridge
Anllóns	43°13'41.94"N	8°52'33.97"O	12.5	4	Car-traffic bridge
Tambre	42°50'1.04"N	8°50'50.90"O	28	4	
Ulla	42°44'46.38"N	8°33'19.70"O	50	5	Suspension bridge
Barosa	42°33'30.04"N	8°37'34.04"O	<10	-	
Lérez	42°27'1.27"N	8°37'21.97"O	20	3	Suspension bridge
Minho	42° 1'36.40"N	8°39'29.94"O	190	7	Car-traffic bridge
Cávado	41°30'24.45"N	8°43'1.65"O	90	5	
Pueriparous					
Navia	43°23'34.74"N	6°48'6.82"O	50	6	Car-traffic bridge
Esva	43°28'48.59"N	6°26'38.28"O	10	4	Car-traffic bridge*$_1$

Narcea	43°21'32.87"N	6°16'2.93"O	30	5	
Nora	43°24'35.89"N	5°55'8.82"O	10	4	Roman bridge*[1]

*Strahler rank was derived from from the European Environment Agency Catchments and Rivers Network System v1.1 database (https://www.eea.europa.eu/data-and-maps/data/european-catchments-and-rivers-network#tab-european-data).

*1 Bridges potentially passable by salamanders.

We collected a total of 209 samples from toe tips, georeferenced, and added them to an existing database from Guillermo Velo-Antón (GVA). We added 109 samples collected in previous studies (Lourenço *et al.*, 2018a; Lourenço *et al.*, 2019) to our collected samples, comprising a final dataset of 328 individuals. Although invasive, removing a toe allow us to distinguish individuals during fieldwork, avoiding the inclusion of recaptured salamanders in downstream genetic analyses. Moreover, since salamanders are capable of regenerating the limbs, it does not prejudice the fitness of the individual (Blaustein *et al.*, 2018). The sex of each individual was recorded by visual inspection of the cloaca (Velo-Antón and Bluckley, 2015), and all tissue samples were kept in tubes with ethanol 100% to ensure DNA preservation. Because some rivers were sampled during different seasons and multiple nights and salamanders are capable of regenerating their toes, we used the option *Multilocus Matches* in GENALEX 6.5 (Peakall and Smouse, 2012), to check for genotype matches.

2.2. Laboratory procedures and genotyping

We placed tissue samples overnight in PBS buffer (phosphate-buffered saline) to clean them. We cut toe clips in small pieces to increase the surface activity of the reagents used throughout the DNA extraction protocol. DNA was extracted using Genomic DNA Tissue kit (Easy Spin), following manufacturer's protocol. Quantity and quality of the extracted DNA was evaluated on an electrophoresis 0,8 % agarose gel containing GelRed ™ (Biotum) dye. Results were assessed in the UV transilluminator Bio-Rad Universal Hood II molecular imager (Bio-Rad). Extracted DNA was kept under -20 C°, to ensure DNA preservation until further use.

A total of 14 microsatellites distributed in four optimized multiplexes (S1, S2, S3, S4; Table S1) were amplified for all samples (Steinfartz *et al.*, 2004; Hendrix *et al.*, 2010). Each multiplex had a total final volume of 100 µL, which contained a mixture of distilled water, labelled forward primers (6-FAM-blue, VIC-green, NED-yellow, PET-red) and reverse primers modified with a tail at the 5´end. The forward primer was *a priori* diluted to a concentration of 10 µM. All samples were amplified through Polymerase Chain Reactions (PCRs), following optimized and previously tested conditions (e.g. Álvárez *et al*., 2015; Lourenço *et al.*, 2018a). Each PCR reaction contained a total volume of 10 µL: 5 µL of Multiplex PCR Kit Mastermix (QUIAGEN), 3 µL of distilled water, 1 µL of the multiplex mix,

and 1-2 µL of extracted DNA (which varies depending on the intensity of the observed band in the 0,8% agarose gel). PCRs were performed following a touchdown protocol, with the same conditions for the 4 panels: the reaction started with an initial step at 95 °C for 15 min, 19 cycles at 95 °C for 30 s, 90 s of annealing at 65 °C (decreasing 0.5 °C each cycle), 72 °C for 40 s, followed by 25 cycles of 95 °C for 30 s, 56 °C for 60 s, 72 °C for 40 s, and ended with a final extension of 30 min at 60 °C. A negative control was always used to identify potential contaminations. PCR products were tested in 2% agarose gel in order to check visually their quality and quantity. To genotype samples formamide and DNA Size Standard LIZ 500 DSMO-100 (MCLAB) were added to PCR products. These were then run on an ABI3130XL capillary sequencer (Applied Biosystems). Allele scoring was performed in GENEMAPPER 4.0 (Applied Biosystems). Genotypes were assessed by two people and allele scoring was only performed when fluorescence peaks were clear and exhibited a relatively high RFU (Relative Fluorescence Units) signal (usually >100 RFU).

Four randomly chosen individuals from each riverside were selected to amplify a fragment of the mitochondrial DNA (mtDNA) gene cytochrome b (cyt b). In total, this mtDNA marker was amplified for 64 larviparous and 32 pueriparous individuals. This mtDNA marker was amplified using the primers *cytb-2* (Kocher et al., 1989) and MVZ 15 (Moritz et al., 1992). Each polymerase chain reaction had a total volume of 10-11 µL: 5 µl of MyTaqTM HS Mix 2X (Bioline), 3 µl of distilled water, 0.5 µl of each primer *a priori* diluted to a concentration of 10 µM. and 1–2 µl of DNA extract. We followed the cycling conditions described in Velo-Antón et al. (2007): initial denaturation at 94°C for 5 min, followed by 40 cycles of 1 min at 94°C, 1 min of annealing at 50°C, 72°C for 1 min, ending with a final extension of 5 min at 72°C. PCR quality and quantity were assessed in an agarose gel (2%). DNA sequencing was outsourced to Genewiz Inc. (Leipzig, Germany). All the obtained chromatograms were verified, aligned and corrected by eye using GENEIOUS PRO version 4.8.5 (http://www.geneious.com/). The aligned cyt b sequences were trimmed to avoid missing data, resulting in a consensus sequence of 750 bp.

2.3. Genetic analyses

All genetic analyses were performed independently for each river, with the samples collected in each riverside pooled and in separate, allowing us to evaluate patterns across loci for rivers and riversides independently.

2.3.1. Microsatellite quality control

Each microsatellite locus was tested for potential deviations from Hardy Weinberg Equilibrium (HWE) and Linkage Equilibrium (LE) by performing exact tests in Genepop 4.2 (Rousset, 2008; dememorization = 10000, batch number = 5000; batch length = 10000).

We applied the false discovery rate (Benjamini and Hochberg, 1995) to correct *p*-values from HWE and LE multiple exact tests. Frequencies of null alleles and inbreeding coefficients were calculated in INEST 2.0 (Chybicki and Burczyk, 2009) using the individual inbreeding model. We set a total of 200000 iterations, thinned every 200 iterations, with an initial burn-in of 20000 iterations. We used the program MICROCHECKER 2.2.3 (Van Oosterhout *et al.*, 2004) to check for large allele dropout.

To assess the informative power of our loci to discriminate between individuals, we calculated the Probability of Identity $PI_{(ID)}$ (probability of a pair of individuals sampled at random from que same population sharing identical genotype (Waits *et al.*, 2001)) and the Probability of Identity accounting for sibs ($PI_{(ID)\ sibs}$) in GENALEX 6.5 (Peakall and Smouse 2012).

2.3.2. Genetic diversity

Indices of population genetic diversity were assessed in GENALEX 6.5 (Peakall and Smouse 2012) for each river and riverside separately: mean number of alleles per marker (N_A), observed (H_O) and expected (H_E) heterozygosities, number of private alleles (P_A), number of individuals containing private alleles (N_{PA}). Allelic richness (A_R) was assessed with R package *diveRsity* (Keenan *et al.*, 2013) in R (R Development Core Team 2017) computed through 5000 bootstrap iterations.

2.3.3. Genetic structure between riversides

Three indices of genetic differentiation between riversides were also calculated and tested for significance in GenAlex (Meirmans and Hedrick, 2011). Pairwise F_{ST} (Nei, 1977) and Pairwise J'ost D_{EST} (Jost, 2008) 95% CI values' were computed through 10000 bootstrap replicates, and the probability of pairwise values were calculated based on 9999 permutations. AMOVA-F_{ST} values were also computed throughout 9999 permutations to test for statistical significance of population differentiation. We performed an Analysis of Molecular Variance (AMOVA) in GENALEX 6.5 , in order to infer the source of the genetic variation. We performed the analyses (both suppressing and including within individual variance's) by employing 9999 permutations.

To assess contemporary levels of genetic differentiation, a Bayesian model implemented in STRUCTURE 2.3.4. (Pritchard *et al.*, 2000) was used to infer the number of genetic clusters (1 or 2) and to estimate clusters' membership for each individual. Analyses were performed using an admixture model, with prior information regarding sampling site (LOCPRIOR parameter) and correlated allele frequencies. A total of 500000 iterations with a burn-in period of 50000 iterations were set for each run. Because we are interested in

determining the only possible barrier (*i.e.* the river) that might exist in each study site at such local scale, we will discuss only patterns of genetic differentiation for a number of cluster equal to two (K = 2) in each river. Ten independent runs were performed for K = 2. The admixture model enables the inclusion of admixed individuals, giving estimates of admixed proportions (Q) based on the proportion of the individual's genotype belonging to each cluster. Incorporating sampling information is advised when available data is limited and population structure is subtle (Hubisz *et al.,* 2009). To compile and graphically represent the output originated from the multiple independent runs on STRUCTURE, we used the default advanced options in software CLUMPAKK 1.1 (Kopelman *.et al.*, 2015). This newly developed method has been shown to accurately infer the correct number of genetic clusters under a variety of scenarios, such as unbalanced sampling, low numbers of loci, weak differentiation, and inbreeding (Wang, 2019).

We also inferred patterns of contemporary population genetic structure and clusters' assignments through a Discriminant Analysis of Principal Components (DAPC) (Jombart *et al.*, 2010). DAPC is a multivariate method that creates linear combinations of alleles (the discriminant functions), trying to maximize between-groups variation, while minimizing within-groups variation. DAPC first performs a Principal Component Analysis (PCA) to transform the data into principal components (PCs), followed by a discriminant analysis that identifies clusters on the retained principal components, hence allowing for the assignment of individuals to clusters (Jombart *et al.*, 2010). Unlike STRUCTURE 2.3.4., it does not rely in a Bayesian approach and do not make any assumption regarding the genetic model behind the inferred genetic structure (*i.e.* it does not make any assumption regarding HWE and LE). We performed the DAPC in the R package *Adegenet* (Jombart, 2008, 2010). To test the effect of rivers as a genetic barrier, we made *a priori* partitions of the data *i.e.* each river was previously divided into two groups consistent with the two riversides (e.g. Gummer and Leaché, 2017). We did not follow the package manual's guidelines regarding the choice of the optimum value of Principal Components (PCs). Due to our relative low sample-sizes, following the cross-validation method proposed in the manual would lead to high number of assessed PCs that could englobe any kind of variation. This could virtually overfit the discriminant functions and model any possible structure (Jombart *et al.*, 2010). To overcome this issue, we consistently chose one-third of the sample size as number of Principal Components (e.g. Grummer and Leaché, 2017; Jombart, 2008), which comprise a trade-off between choosing too many PCs or retaining very few (that would overlook some genetic variation). We chose one discriminant function for all the rivers. For each population, we calculated the memberships probabilities of each individual to each riverside, based on the retained discriminant functions. If the river is an

effective barrier, we would expect to see a high percentage of each individual's genotype assigned to its pre-defined riverside.

To examine deeper levels of population differentiation among riversides, a haplotype network was generated for each river, using TCS v. 1.21 (Clement *et al.*, 2000) with a 95% of probability of parsimony. Network layout was changed in TCSBu (Santos *et al.*, 2015).

2.3.4. Genetic relatedness analyses

We used COLONY2 (Jones and Wang, 2010) to infer pairs of related individuals and whether these were separated by the river. Using multilocus genotypes, COLONY2 implements a full-pedigree maximum likelihood method that simultaneously seek for the parentage and sibship relationships among individuals (Wang and Santure, 2009; Jones and Wang, 2010). Thus, it assigns pedigree relationships by exploring the likelihood of the entire possible genealogical relationships, and chooses the best configuration where the maximum likelihood is reported. Fire salamanders are iteroparous, which means they present overlapping generations (i.e. individuals from multiple cohorts coexist). Because, COLONY2 assumes discrete generations (*i.e.* our sampled individuals belong to the same cohort), it is important to take into account possible biases when interpreting the results. Since salamanders can live up to 20 years in the wild and have an annual or biannual reproductive cycle (Velo-Antón and Buckley, 2015), our dataset might contain parent-offspring pairs, even though we have restricted our sampling exclusively to adults. Accordingly, for each river, we divided our sampled individuals in three different subsamples according to the sex of the individual: (1) candidate mothers, (2) candidate fathers, and (3) offspring that contain the salamanders that were included in the candidate father and mother subsets (e.g. Lourenço *et al.*, 2018a). Since we do not hold specific age information and this software assumes that there is no relationship between and within candidate parents, we add the third subsample referring to the possible offspring encompassing all individuals The inclusion of each individual in one candidate parent and offspring subsamples decrease the statistical power of this method; however, even though it would be more accurate to pool each individual in just one sample group, COLONY2 has been shown to perform satisfactorily with enough marker information available *i.e.* using more than 10 microsatellites (Wang and Santure, 2009). Moreover, by including parent candidates, sibship relationships can be inferred more accurately (Wang and Santure, 2009). We analyze the data assuming polygamy for both reproductive modes and sexes. For each river, we run three different replicates with different number seeds. COLONY2 can run three replicates automatically within the same project. However, to assess if we have enough marker information to reliably infer the genetic relatedness, we run each replicate separately in different projects and, subsequently, we assess whether the

different runs converge or not. Since our replicate runs´ converge into the same configuration, we then set up single projects for each river, and let the software to run the three runs automatically. In each run we set full-likelihood method, with high likelihood precision. No priori information regarding known parents was provided and sibship scaling was deactivated. We considered a pair of individuals as relatives when inferred with a posterior probability ≥ 0.8 in at least two replicates (Wang and Santure, 2009). This threshold is lower than others used in other studies (*e.g*. ≥ 0.95: Carvalho *et al.*, 2018; Sandberguer-Loua *et al.*, 2018). However, due to the low sample size we have in some rivers, we must thoroughly consider a trade-off for choosing the probability threshold. This threshold might imply two types of errors: either identifying a pair of relatives when they are not (type I error), or ignoring true pairs of relatives when they actually are relatives (type II error). Hence, forcing a high posterior probability, could potentially decrease statistical power (Wang and Santure, 2009). We considered a crossing event when individuals comprising a pair of relatives ($p \geq 0.8$), were located in opposite sides of the river.

We used COANCESTRY 1.0.1.9 (Wang, 2011), to calculate within- river and riverside average relatedness and, thus, examine whether pairs of individuals in the same riverside are on average more related than pairs of individuals separated by the river. We calculated two estimates of pairwise relatedness (*r*) between individuals: (i) the TrioML estimator (Wang, 2007), which calculates pairwise relatedness of a dyad using a third individual as a control, avoiding wrong inferences of individuals with identical genotypes being considered Identical by State (IBS) instead of identical by descent (IBD) (Wang, 2007) and, (ii) Queller and Goodnight's point estimator (Queller and Goodnight, 1989). We did not account for genotyping errors or inbreeding and 95% CIs were computed through 1000 bootstrap resamplings. Significant differences in average pairwise relatedness values for dyads located within the same riverside and between riversides were tested using a bootstrap method implemented in COANCESTRY (see software's manual for more information). We divided dyads into groups depending on which riverside each individual belongs to, thus obtaining three groups per river (two groups for dyads with individuals belonging to the same riverside, and another group from dyads whose individuals belong to different riversides). Average relatedness for each group and significance of the differences between groups were calculated through 10000 bootstraps.

To further understand how rivers affect the spatial distribution of relatives, we pooled our individuals in three genetic relatedness intervals according to *r* (the proportion of the genome shared by descent *i.e.* from a common ancestor), as estimated by the TrioML method. For first-order relationships, the expected relatedness value is 0.5; for second-

order relatives is 0.25; and for third-order relationships is 0.125. Since we only sample a portion of the genome, the observed values of relatedness are going to deviate from the theoretical ones, in which, the estimated values may be lower or higher than the expected ones; thus, we set a mid-point threshold between theoretical values to account for this uncertainty in our estimates. Accordingly, kinship classes were organized as follows: dyads with a relatedness higher or equal than 0.375 (halfway between 0.25 and 0.5) were consider first-order relatives; pairs with relatedness $0.1875 \leq r \leq 0.375$ were considered second-order relatives; pairs with relatedness $0.09375 \leq r \leq 0.1875$ were considered third-order relatives; and those individuals with a r that falls within the range 0 to 0.0937 were considered unrelated pairs. We calculated the proportion of inter-riverside and intra-riverside dyads in each relationship order.

3. Results

3.1 Marker validation

No matching multilocus genotypes were found in any of the rivers, and all sampled individuals in a river exhibited a minimum of six to nine allele mismatches between each other (Table 2). Therefore, we concluded that none of the sampled individuals were recaptures, and thus we kept all the samples for downstream analyses. Our obtained $PI_{(ID)}$ and $PI_{(ID)\ sibs}$ values for the combinations of the 14 loci were low (Table 2), *i.e.* always lower than 0.0001 for all populations (Waits *et al.*, 2001), and the expected values were reasonably higher than the observed ones. Therefore, we can be confident that our set of 14 microsatellite loci are informative enough to discriminate individuals.

After corrections for multiple testing, we found evidence of deviations from Linkage Equilibrium in two loci (Sal3, SalE8) in four populations (Miño, Tambre, Cávado and Anllóns). Two loci (Sal E2 and Sal E8), showed substantial departures from HWE conditions due to heterozygote deficiencies although deviations from both LE and HWE were not consistent across all populations. There are many possible biological explanations for departures from HWE proportions applicable to our dataset, including non-random mating (see Caspers *et al.*, 2014), Wahlund effect, and overlapping generations, and analytical such as low sample size in some rivers. Moreover, these loci have shown to be independent and performed successfully in numerous previous studies (e.g. Steinfartz *et al.*, 2007; Velo-Antón *et al.* 2012; Caspers *et al.*, 2014; Lourenço *et al.*, 2017; 2018a; Dinis *et al.*, 2019), and thus they were retained in subsequent analyses. No microsatellite loci showed evidences for neither null alleles nor large allele dropout across all populations.

3.2. Population genetic diversity

Genetic diversity was overall high and similar between larviparous and pueriparous populations, being H_e marginally higher than H_O in all rivers (mean values for larviparous rivers in comparison with mean values for pueriparous rivers, respectively: H_O : 0.72 vs. 0.74; H_E : 0.75 vs. 0.78; Table 2). All populations showed high number of individuals containing private alleles, considering the sample sizes. Mean number of alleles were overall high (range $N_{A:}$ 4.21 – 10.79). Allele richness was overall high (average A_R across all larviparous and pueriparous rivers, respectively: 6.01 vs. 6.24), although some populations with low sample size displayed lower values (Anllóns: 4.59; Tambre: 4.95). Mean inbreeding coefficients were low in both larviparous (mean: 0.03) and pueriparous

(mean: 0.02) rivers (range of F for larviparous and pueriparous population respectively: 0.01 - 0.07; 0.01 - 0.05).

Table 2. Sampling information and genetic statistics from the studied localities. The following information is displayed for each river: sampled population (Pop), reproductive mode (mode), number of sampled individuals (n), number of males (Nm), number of females (Nf), minimum number of allele mismatches (Ma), mean number of alleles (Na), mean number of private alleles (Pa), number of individuals with private alleles (Npa), probability of identity ($PI_{(ID)}$), probability of identity between sibs ($PI_{(ID)\ sibs}$), observed heterozygosity (H_O), expected heterozygosity (H_E), allelic richness (A_R), and mean inbreeding coefficient (F).

Pop	n	Nm	Nf	Ma	Na	Pa	NPa	Ho	He	AR	$PI_{(ID)}$	$PI_{(ID)\ sibs}$	F
Pueriparous plot													
Navia	23	19	3	≥6	9.93	0.29	9	0.73	0.75	6.87	1.4E-18	6.8E-07	0.05
east	10	7	2		6.07	2.07	10	0.70	0.73	5.12	1.6E-14	3.9E-06	0.03
west	13	12	1		7.86	3.86	13	0.77	0.77	6.26	3.5E-17	1.3E-06	0.01
Esva	24	12	10	≥9	8.86	0.00		0.71	0.76	6.09	5.3E-17	1.3E-06	0.03
east	11	4	6		6.07	1.36	9	0.73	0.75	5.35	2.1E-15	2.6E-06	0.04
west	13	8	4		7.50	2.79	13	0.70	0.76	5.90	3.4E-16	1.8E-06	0.03
Narcea	19	12	6	≥8	10.14	0.79	10	0.74	0.82	7.15	2.9E-20	2.9E-07	0.02
north	7	5	2		6.50	2.21	7	0.84	0.78	5.26	1.4E-16	1.3E-06	0.01
south	12	7	4		7.93	3.64	12	0.76	0.81	5.49	1.3E-18	5.7E-07	0.02
Nora	39	20	19	≥6	11.86	0.93	25	0.75	0.81	7.04	2.4E-19	5.6E-07	0.01
north	26	9	17		10.79	3.71	25	0.74	0.80	7.59	4.1E-19	6.1E-07	0.02
south	13	11	2		8.14	1.07	13	0.76	0.78	6.80	1.9E-17	1.1E-06	0.01
mean					10.20	0.29		0.74	0.78	6.24			0.02
Larviparous plot													
Eume	50	30	20	≥7	9.00	0.29	6	0.65	0.75	5.63	1.1E-15	2.3E-06	0.03
north	21	15	6		7.71	1.42	16	0.71	0.77	6.78	3.6E-16	1.7E-06	0.04
south	29	15	14		7.57	1.29	21	0.62	0.72	6.38	1.5E-14	4.3E-06	0.02
Anllons	18	1	16	≥8	5.86	0.00		0.63	0.68	4.59	6.7E-13	1.2E-05	0.03
south	9		8		5.57	1.64	9	0.66	0.67	4.55	9.7E-13	1.3E-05	0.03
north	9	1	8		4.21	0.29	3	0.61	0.64	3.80	1.2E-11	2.5E-05	0.05
Tambre	10	1	8	≥8	5.79	0.00		0.68	0.68	4.95	2.1E-13	9.8E-06	0.02
east	6	1	5		4.29	1.50	4	0.62	0.61	3.14	3.3E-11	4.5E-05	0.02
west	4		3		4.29	1.50	6	0.77	0.67	3.46	1.7E-12	1.5E-05	0.03
Ulla	35	29	6	≥7	10.43	0.50	8	0.79	0.78	6.45	2.3E-17	1.1E-06	0.01
north	16	10	6		7.71	1.79	14	0.78	0.76	6.60	3.8E-16	1.9E-06	0.01
south	19	19			8.64	2.71	19	0.79	0.77	7.13	7.0E-17	1.4E-06	0.01
Barosa	22	9	12	≥6	8.43	0.07	1	0.69	0.76	6.03	1.1E-16	1.6E-06	0.04
north	8	3	4		6.29	0.29	8	0.65	0.70	5.01	1.6E-14	5.9E-06	**0.07**
south	14	6	8		7.07	0.52	14	0.71	0.75	5.21	5.5E-16	2.3E-06	0.03
Lerez	35	21	14	≥6	11.71	0.64	12	0.78	0.82	7.15	1.0E-19	4.2E-07	0.02
north	17	9	8		8.86	2.21	16	0.76	0.80	7.57	3.0E-18	6.8E-07	0.03
south	18	12	6		9.50	2.86	18	0.80	0.81	7.93	9.1E-19	5.9E-07	0.03
Miño	25	7	18	≥9	10.93	1.07	13	0.75	0.80	7.11	8.8E-19	6.8E-07	0.04
south	15	7	8		7.92	3.93	15	0.72	0.77	6.05	8.8E-17	1.5E-06	0.05
north	10		10		7.00	3.00	10	0.80	0.77	5.96	1.1E-16	1.5E-06	0.01

Cavado	28	20	8	≥6	12.43	1.29	18	0.76	0.85	7.71	3.7E-21	2.2E-07	0.03
south	14	8	6		8.07	2.57	14	0.79	0.81	7.01	2.8E-18	5.9E-07	0.05
north	14	12	2		9.86	4.36	14	0.73	0.83	8.03	5.9E-20	3.4E-07	0.02
mean					9.32	0.48		0.72	0.75	6.01			0.03

3.3. Genetic differentiation

Pairwise population F_{ST} values between individuals on opposite sides of the river values were low or moderate, ranging from 0.015 (Eume) to 0.075 (Tambre). D_{EST} values ranged from -0.008 (Anllóns) to 0.261 (Navia) (Table 3). Population genetic differentiation was slightly higher in pueriparous populations (mean F_{ST}: 0.039 ± 0.020; mean D_{EST}: 0.134 ± 0.138), than in larviparous ones (mean F_{ST}: 0.035 ± 0.019; mean D_{EST}: 0.071 ± 0.058). Despite the overall low values of genetic differentiation, six rivers corresponding to the widest rivers, showed significant values on pairwise genetic differentiation for both estimates: two pueriparous: Navia (F_{ST}: 0.059; D_{EST}: 0.261) and Narcea (F_{ST}: 0.055; D_{EST}: 0.247); and four larviparous: Ulla (F_{ST}: 0.021; D_{EST}: 0.040), Barosa (F_{ST}: 0.044; D_{EST}: 0.103), Cávado (F_{ST}: 0.032; D_{EST}: 0.134) and Miño (F_{ST}: 0.041; D_{EST}: 0.155).

Table 3. Pairwise genetic differentiation among riversides. F_{ST} values and D_{EST} values are displayed below and above diagonal, respectively. Statistically significant (CI= 95%) pairwise values are highlighted in bold.

Larviparous rivers					
Eume	North	South	**Anllóns**	South	North
North	0	0.013	South	0	-0.008
South	0.015	0	North	0.031	0
Tambre	East	West	**Ulla**	North	South
East	0	0.090	North	0	**0.040**
West	0.075	0	South	**0.021**	0
Barosa	North	South	**Lerez**	North	South
North	0	**0.103**	North	0	0.040
South	**0.044**	0	South	0.020	0
Minho	south	north	**Cávado**	south	north
south	0	**0.155**	south	0	**0.135**
north	**0.041**	0	north	**0.032**	0
Pueriparous rivers					
Navia	East	West	**Esva**	East	West
East	0	**0.261**	East	0	0.032
West	**0.059**	0	West	0.029	0
Narcea	North	South	**Nora**	North	South

North	0	**0.247**	North	0	-0.001
South	**0.055**	0	South	0.017	0

AMOVA analyses (Table 4), indicated that genetic variance was mostly explained by within- and among- individuals in all populations. Genetic variance explained by among-riversides variance was higher for the two pueriparous populations, Navia (6%) and Narcea (5%), which showed the highest levels in F_{ST} and D_{EST} values (Table 3). Ignoring within-individual level of genetic variance, most of the total variation was attributed to within-riverside variance (Table 5). Navia and Narcea were again the rivers were more total variance is attributed to among-riversides variance, with 6 and 5 percent, respectively. Rivers significantly affected AMOVA- F_{ST} riverside comparisons in all populations except Tambre, Anllóns, Lérez and Esva populations. Estimates of AMOVA- F_{ST} between riversides ranged from 0.003 (Anllóns) to 0.064 (Navia) (Table S2). Population genetic differentiation was again higher in Navia and Narcea populations (AMOVA-F_{ST} values: 0.064 and 0.054, respectively).

Table 4. Results of the AMOVA analyses by each river. The vertical line separate populations with different reproductive modes.

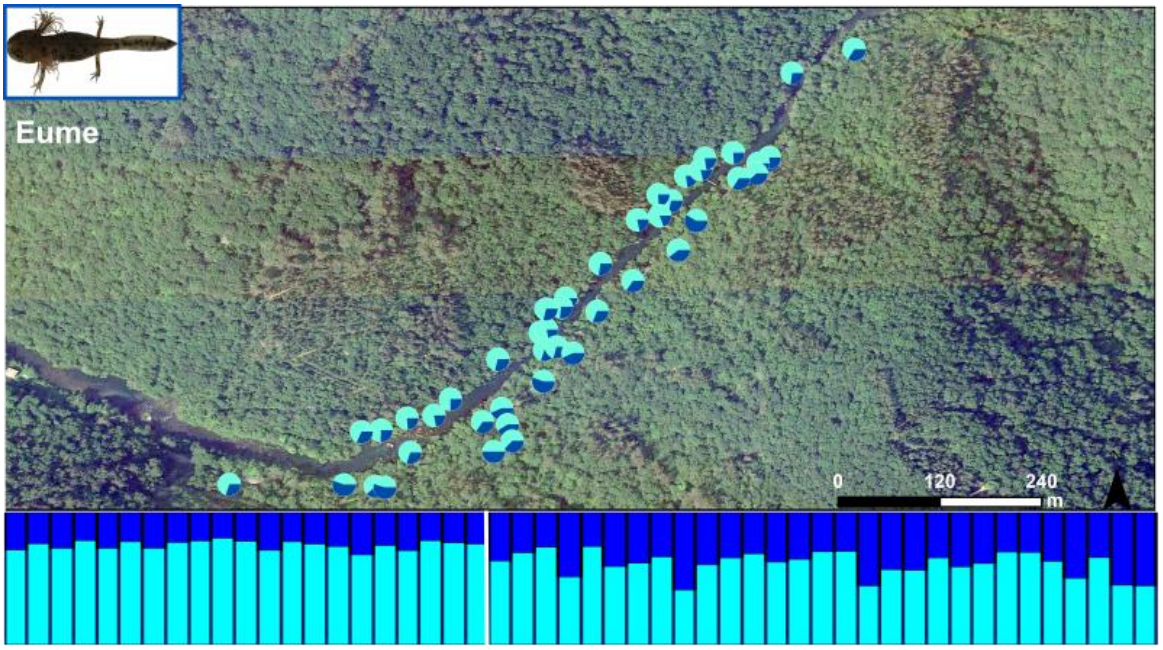
Source of variation	**Percentage of variation**											
	Eume	**Anllóns**	**Tambre**	**Ulla**	**Barosa**	**Lérez**	**Minho**	**Cávado**	**Navia**	**Esva**	**Narcea**	**Nora**
Among riversides	1%	0%	3%	1%	3%	1%	4%	2%	6%	0%	5%	1%
Among individuals	19%	13%	6%	7%	15%	11%	8%	16%	10%	14%	13%	14%
Within individuals	80%	87%	92%	92%	82%	88%	88%	83%	84%	85%	83%	85%

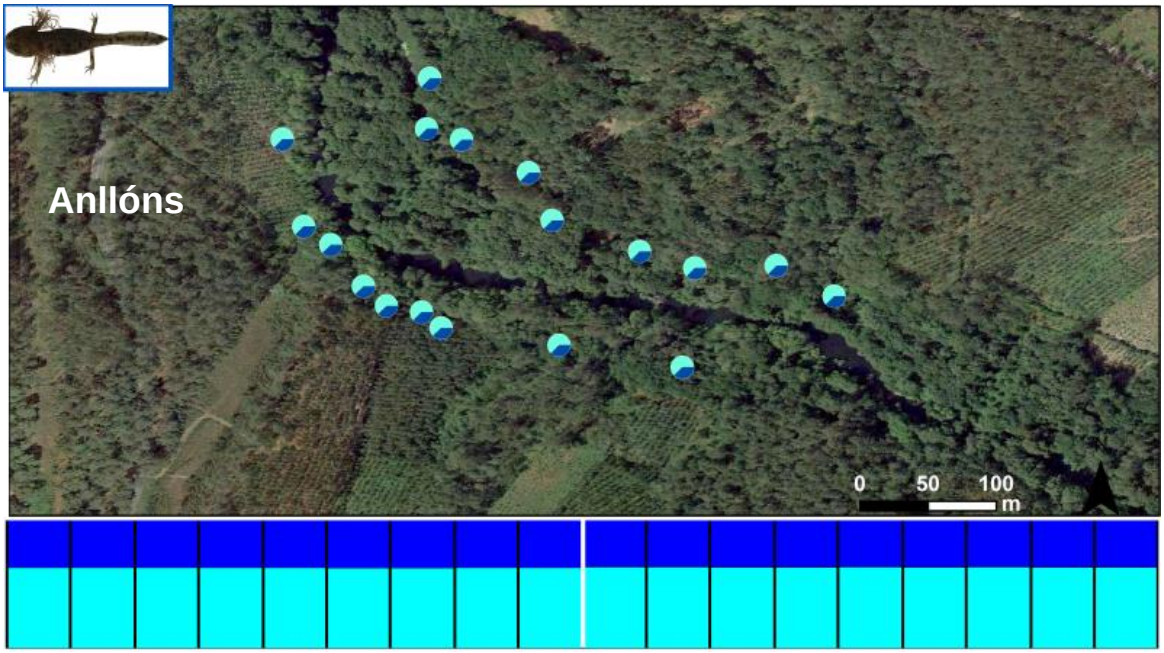
Table 5. AMOVA results when ignoring within-individual level of variance. The vertical line separate populations with different reproductive modes.

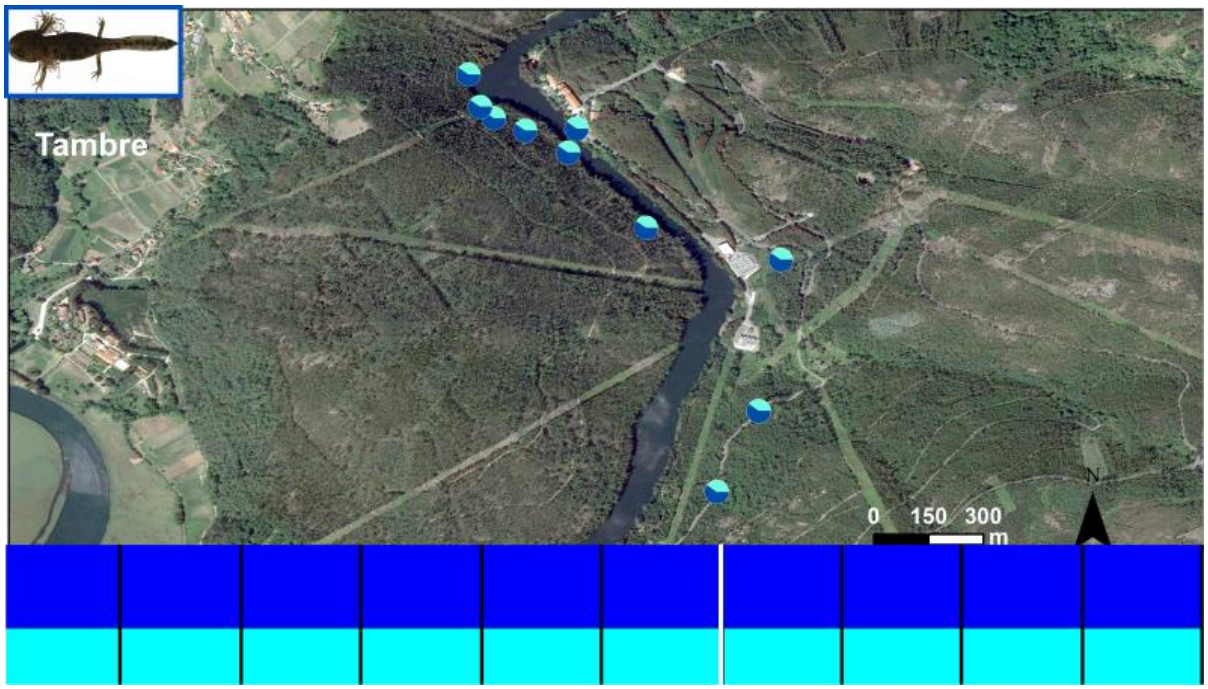
Source of variation	**Percentage of variation**											
	Eume	**Anllóns**	**Tambre**	**Ulla**	**Barosa**	**Lérez**	**Minho**	**Cávado**	**Navia**	**Esva**	**Narcea**	**Nora**
Among riversides	1%	0%	3%	2%	3%	1%	4%	2%	6%	1%	5%	1%
Among individuals	99%	100%	97%	98%	97%	99%	96%	98%	94%	99%	95%	99%

The correlated allele frequency model with LOCPRIOR, was only able to recover a barrier effect (K = 2) in Cávado, Miño and Navia populations (Figure 5), while the remainder populations show strong admixture levels between the two riversides.

Discriminant Analyses of Principal Components (DAPC) also recovered some degree of genetic differentiation for some of the studied rivers. The pueriparous Navia and Narcea populations, and the larviparous populations in Cávado and Minho, showed clear genetic differentiation and low levels of admixture among riversides (Figure S1). The larviparous Eume, Ulla, and the pueriparous Esva populations showed moderate genetic differentiation and admixture levels, while the pueriparous Nora, and the larviparous Barosa, Lérez and Anllóns populations showed high genetic overlap and admixture levels (Figure S1). Since, DAPC requires the number of observations (individuals) to be less than the number of variables (alleles), we discarded the results obtained for Tambre river (N=10).






Tambre
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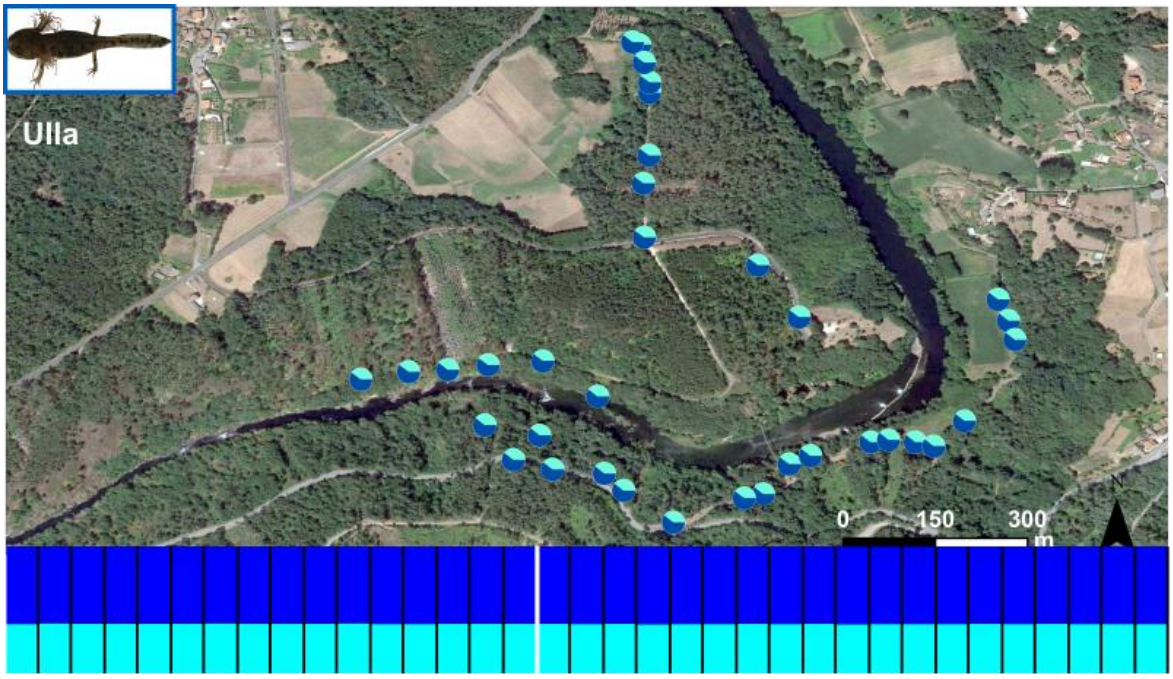
N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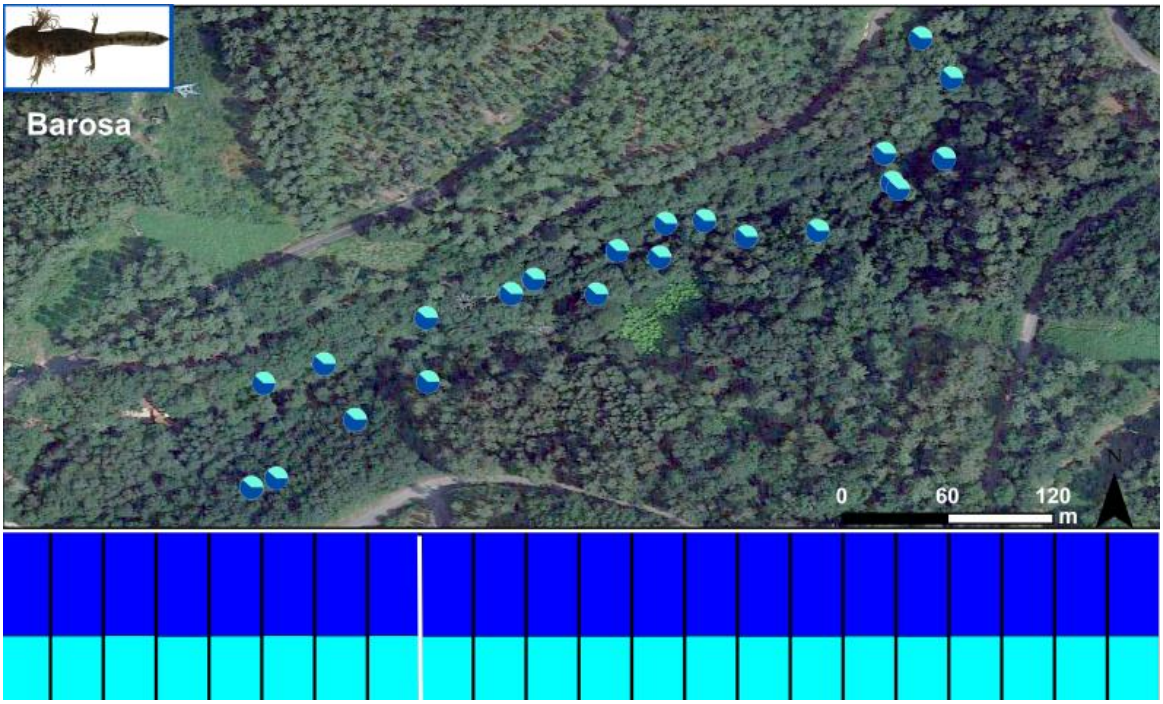
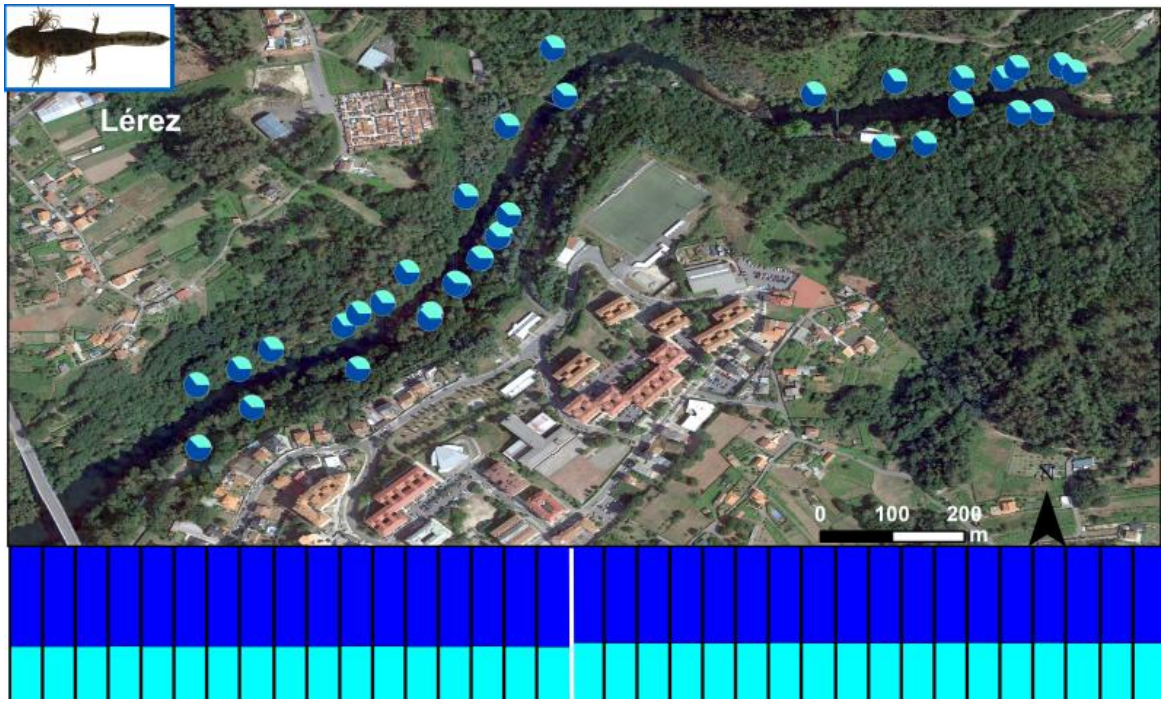
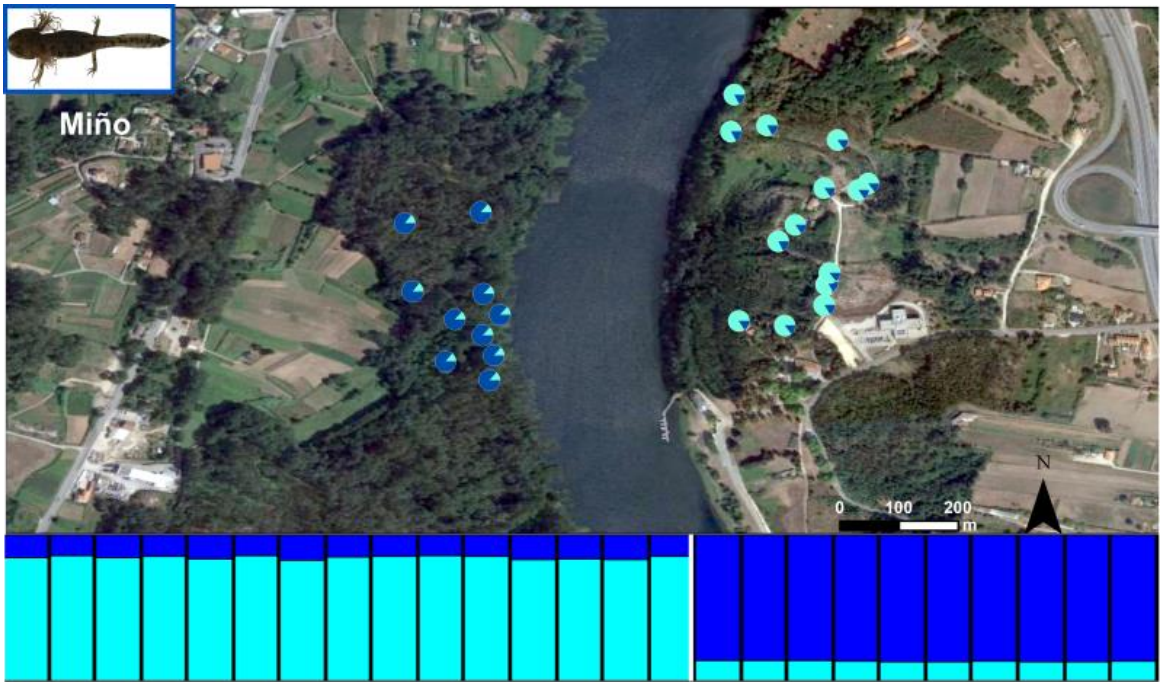
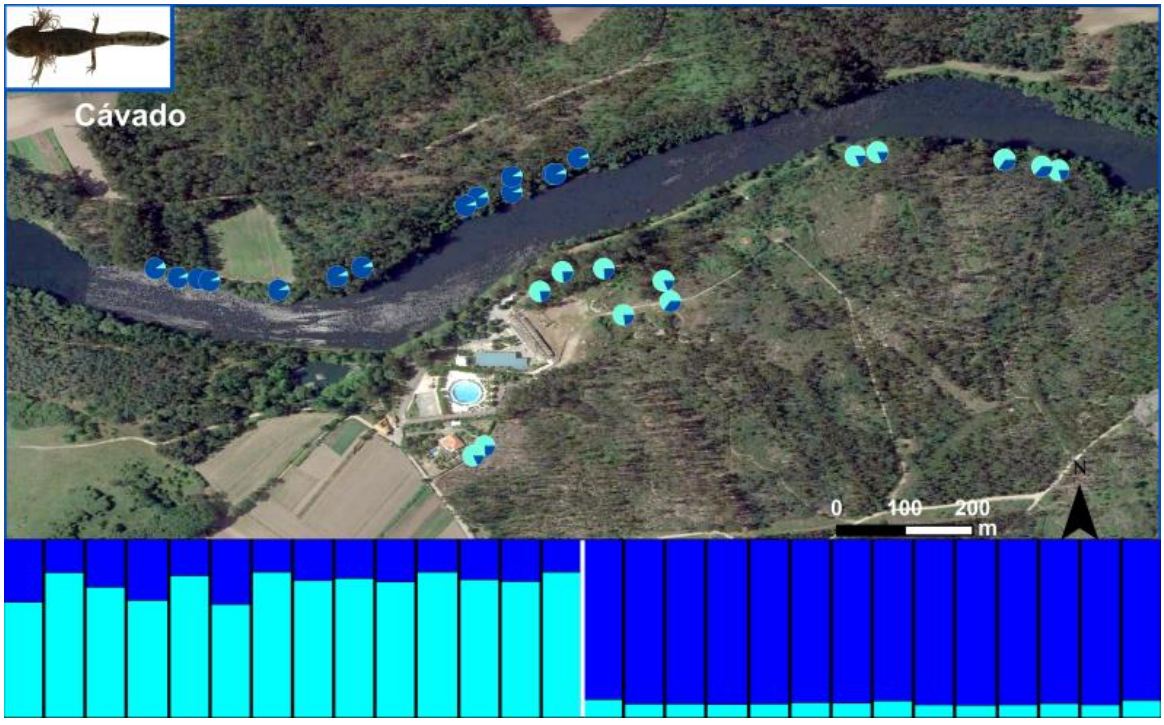
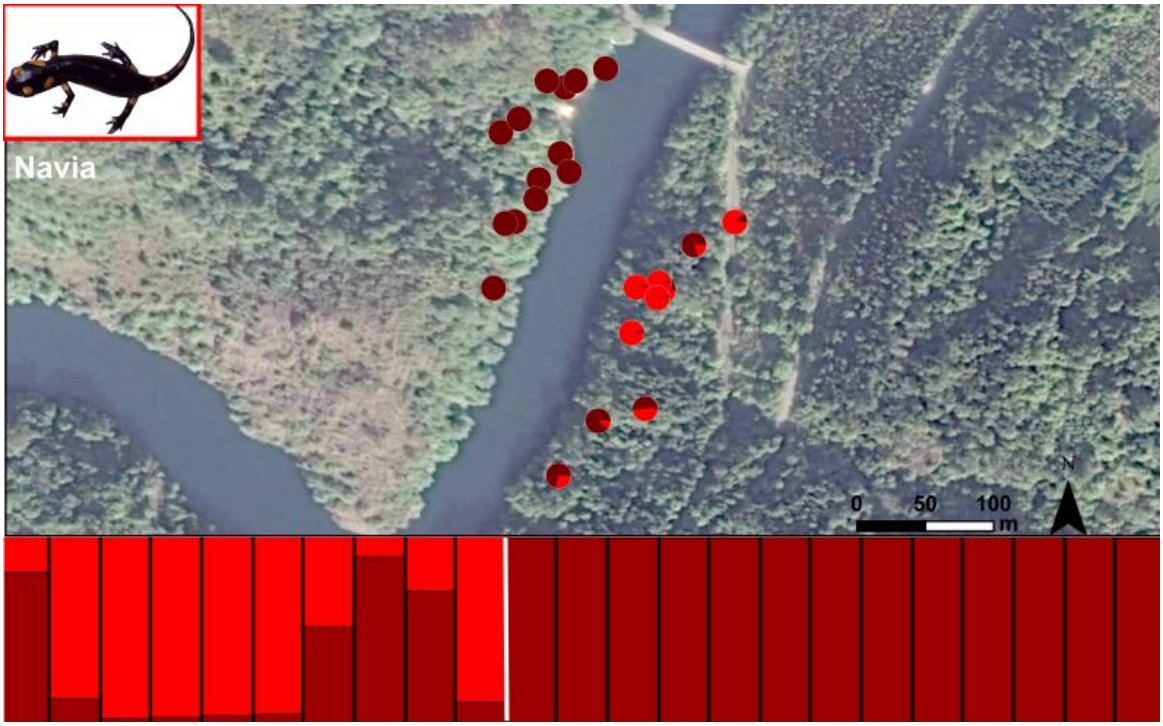
N

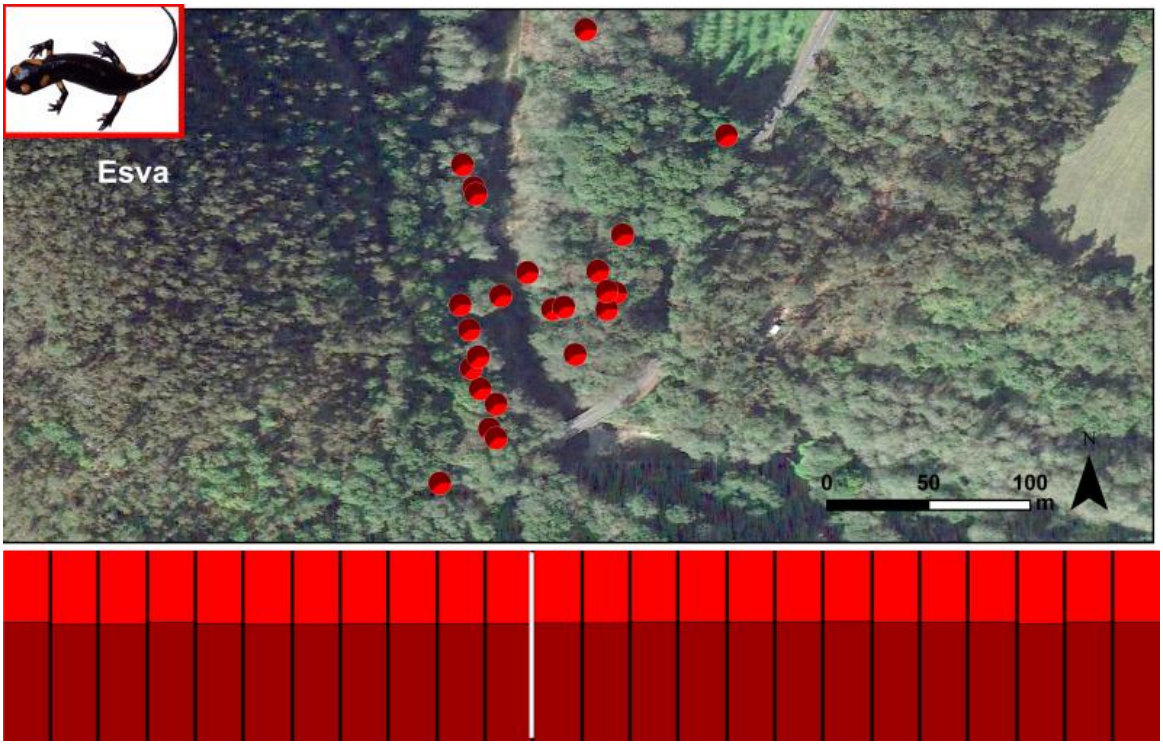

Barosa
0
60
120
m
N


Lérez
0
100
200
m
N


Miño
N
0 100 200
m


Cávado
N
0 100 200
m


Navia
0
50
100
m
N


Esva
0
50
100
m
N

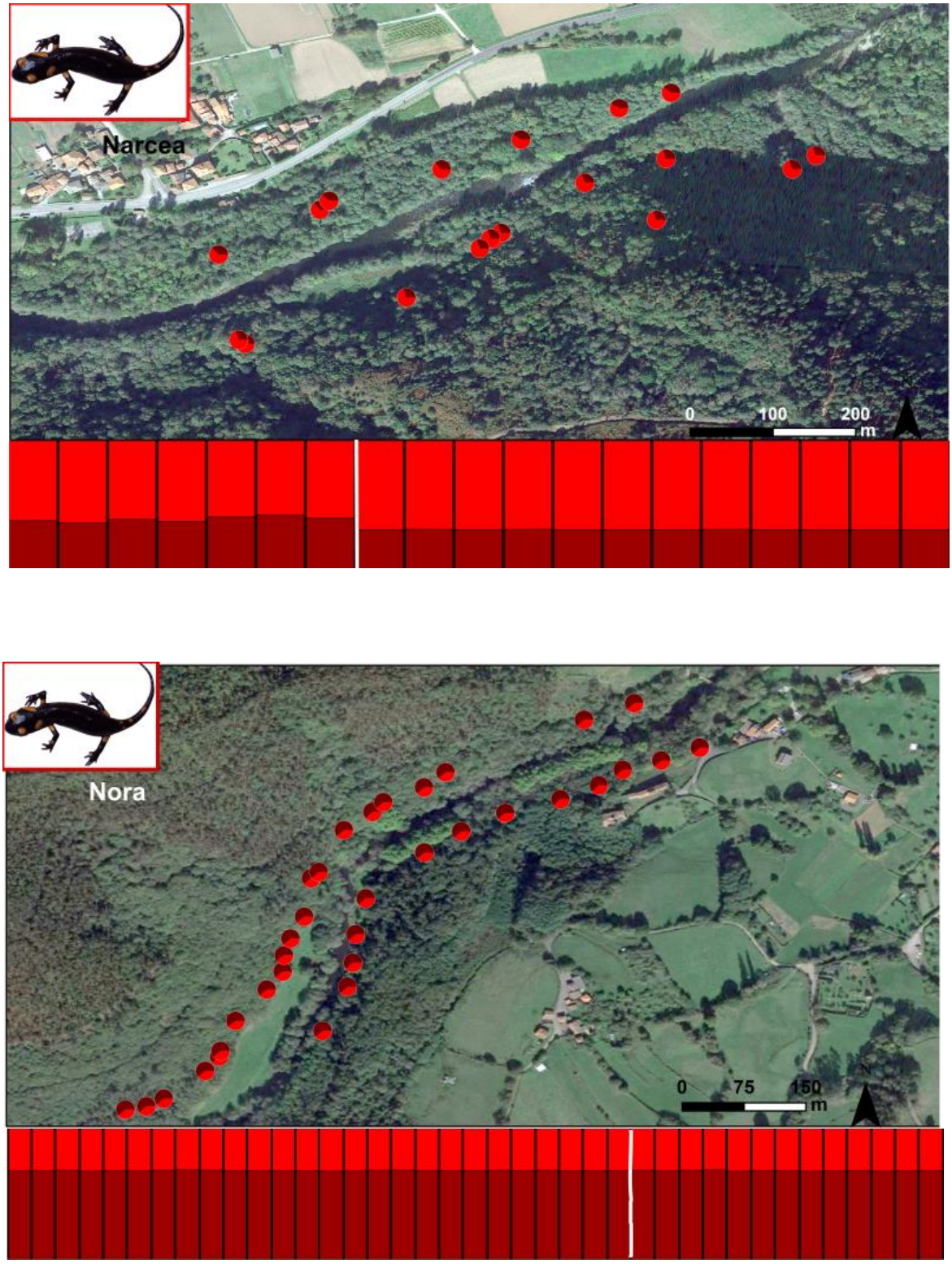
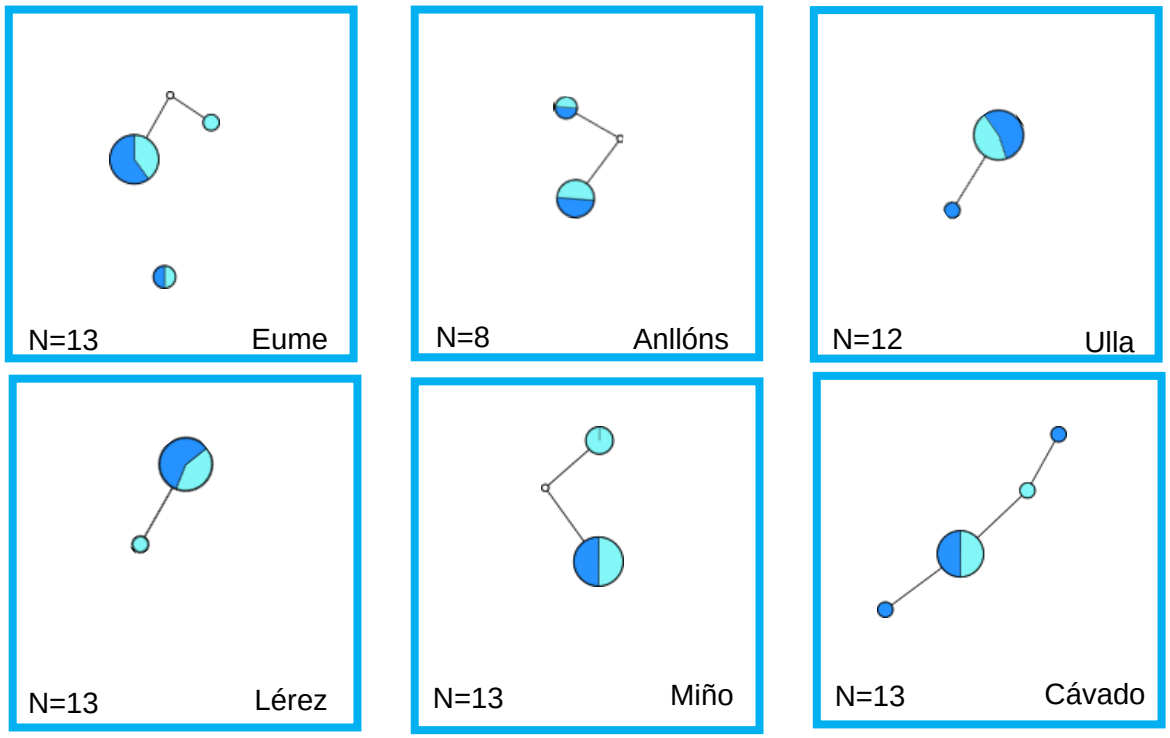


Figure 5. Maps representing the spatial distribution of cluster membership's proportions when *K* = 2. The study location is depicted in the top-left of the image. Individual pie charts represent individual cluster membership to each riverside when *K*=2. The STRUCTURE barplots below the maps represent the inferred ancestry proportions when *K*=2 for each river. Each individual is represented by a vertical bar, partitioned into 2 colours that represent its estimated membership coefficient to each riverside. Vertical white lines in the barplots separates riversides

In those rivers that show genetic signal of differentiation (Navia, Miño and Cávado), membership probabilities (Q) to the original riverside, differed slightly between analyses. In Navia all individuals were correctly assigned to their original riverside (Q > 0.8) according to DAPC analyses, while 60 (average assignment probability: 0.7) and 100 percent of the individuals were assigned by STRUCTURE to the east and west riverside, respectively. In Cávado population, DAPC correctly assigned 93 % of individuals to their original riverside, and while STRUCTURE correctly assigned 100% of the individuals to the north-side, it was only able to assign 36 % of the individuals in the south-side, although the average membership probability was 0.76, thus slightly lower than 0.80. Contrarily, STRUCTURE analyses correctly assigned 100% of individuals in Miño, but DAPC was only able to assign 94% and 80% of the individuals to the southern- and northern- riverside, respectively.

3.3.1. Genetic differentiation: mitochondrial marker

We amplified 151 samples, with a mean number of 13 samples per river. All rivers showed shared haplotypes between riversides (Figure 6). We found only one haplotype in two larviparous rivers, Barosa and Tambre.



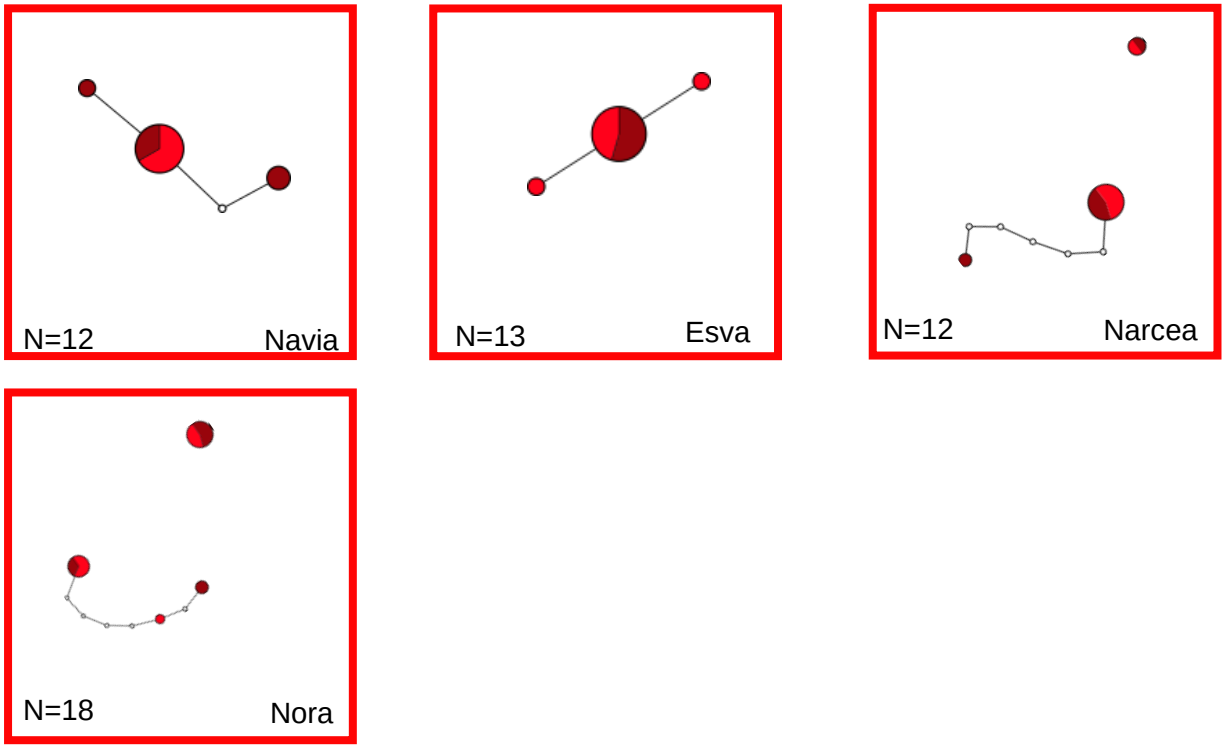


Figure 6. *Cyt b* haplotype networks inferred by TCS under 95 % parsimony probability. Each circle represents one haplotype and each colour represents a different riverside in each river. Pie chart size is proportional to the number of individuals. Number of samples (N) for each river is also represented.

3.4. Genetic relatedness

COLONY identified a very small number of relatives in the dataset, with a total of 10 pairs of relatives, two full-sibs and eight half-sibs (Table 6). Overall, the frequency of pairs of relatives was considerably higher for pueriparous (eight pairs) than larviparous populations (two pairs). Two and five pairs of relatives were found in the same riverside in two larviparous rivers (Cávado and Eume), and three pueriparous rivers (Esva, Narcea, Navia), respectively. Three pairs of relatives were encountered in opposite sides of the rivers, all corresponding to pueriparous populations (Esva and Nora).

Table 6. Kinship relationships identified in COLONY2 for each river (posterior probability > 0.8).

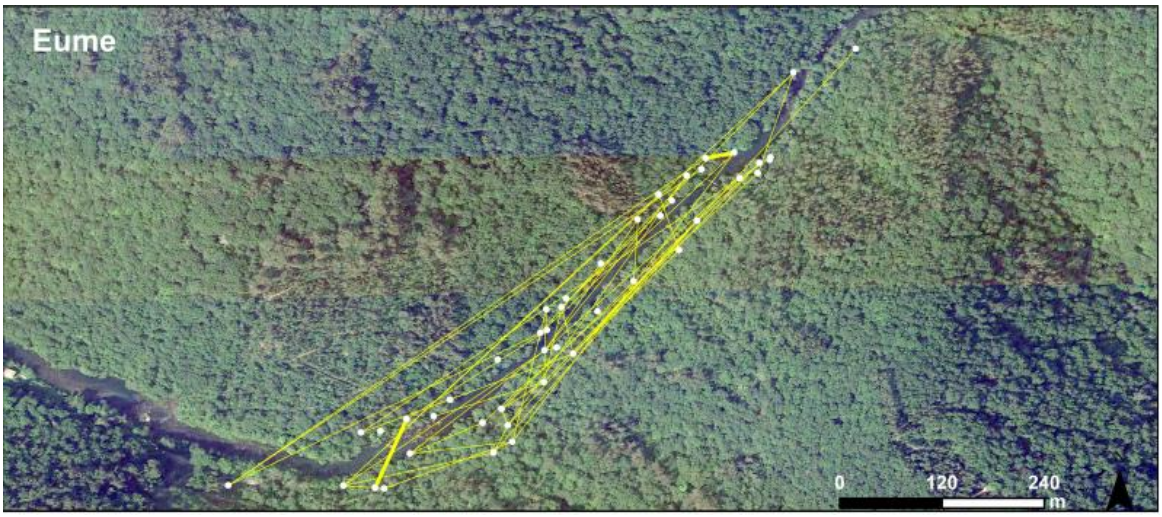
River	N_kin (same riverside)	N_kin (opposite riverside)
Pueriparous rivers		
Esva	2	1
Narcea	1	
Navia	2	
Nora		2
Larviparous rivers		
Cávado	1	
Minho		
Barosa		
Ulla		

Lérez	
Eume	1
Tambre	
Anllóns	

COANCESTRY identified a total of 506 pairs of relatives (*i.e.* $r > 0.09375$), 132 from pueriparous (out of 1405 dyads; 9 %) and 374 (out of 3522 dyads; 11%) from larviparous populations (Table 7; Figure 7). In all relatedness classes the proportion of intra-riverside dyads (*i.e.* pairs of relatives) was higher than inter-riverside dyads.

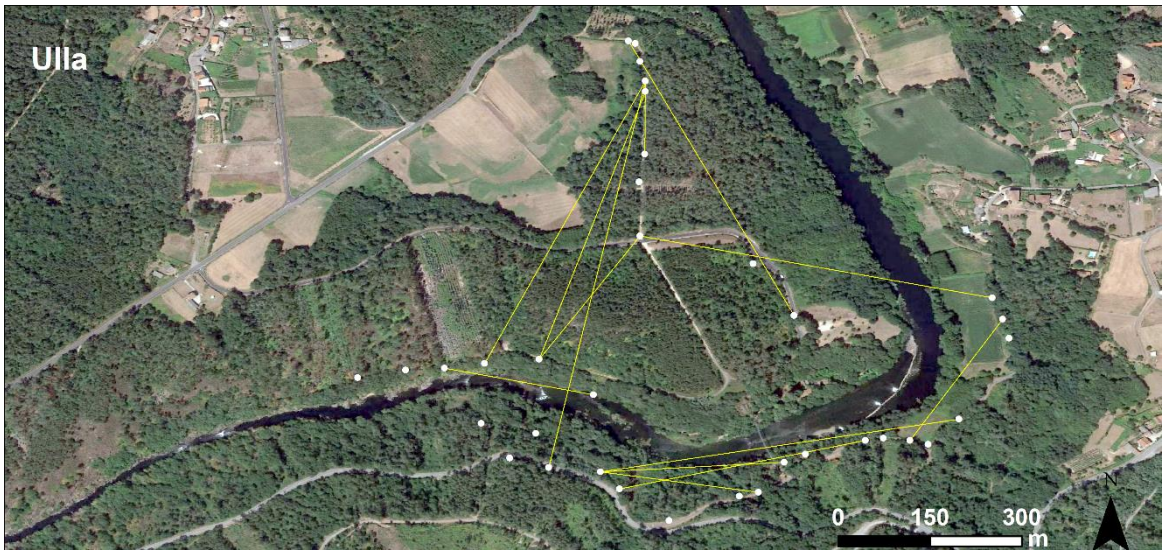
Table 7. Summary statistics of inter and intra-riverside dyads within kinship classes determined through pairwise relatedness analyses in COANCESTRY. For each reproductive mode, the number and proportion (*p:* within square brackets) of pairs of relatives found within each kinship class are displayed.

		Pueriparous		Larviparous	
Kinship class	Relatedness	INTER n [*p*]	INTRA n [*p*]	INTER n [*p*]	INTRA n [*p*]
K ≥ 0.375	First-order	0 [0]	9 [1]	1 [0.25]	3 [0.75]
0.375 > K ≥ 0.1875	Second-order	11 [0.3]	25 [0.7]	29 [0.33]	60 [0.67]
0.1875 > K ≥ 0.09375	Third-order	29 [0.33]	58 [0.67]	117 [0.42]	164 [0.58]
k < 0.09375	Distantly-related	193 [0.47]	213 [0.53]	507 [0.47]	572 [0.53]
k = 0	Unrelated	426 [0.49]	441 [0.51]	1128 [0.55]	941 [0.45]
Total		659	746	1782	1740
		1405		3522	

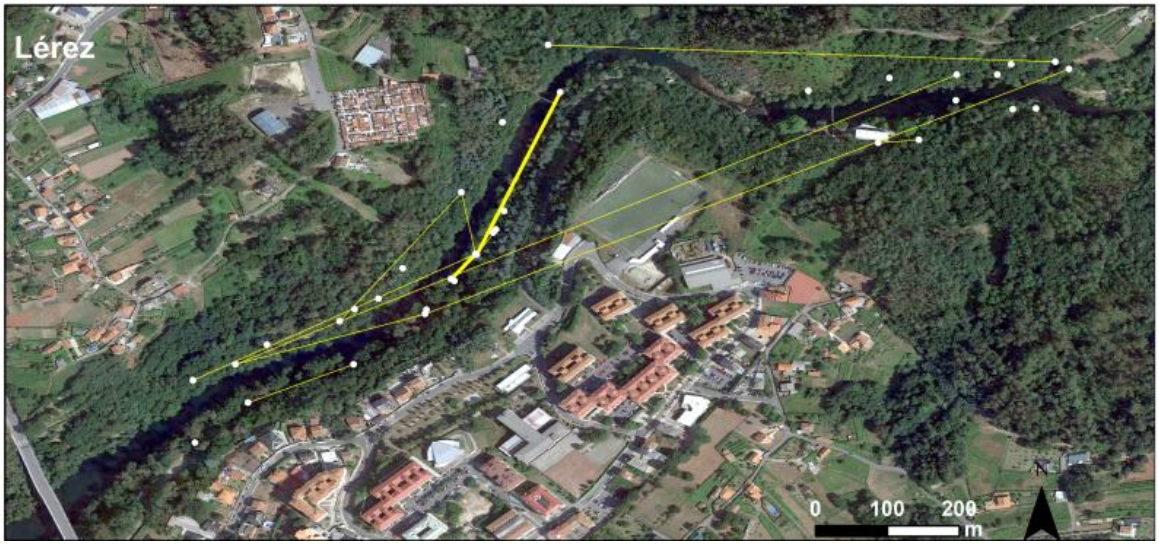



Anllóns
0 50 100
m


Tambre
0 155 310
m
N


Ulla
0 150 300
m
N


Barosa
0
60
120
m
N


Lérez
0
100
200
m
N


Minho
0
100
200
m
N


Cávado
0 100 200
m
N


Navia
0 50 100
m
N

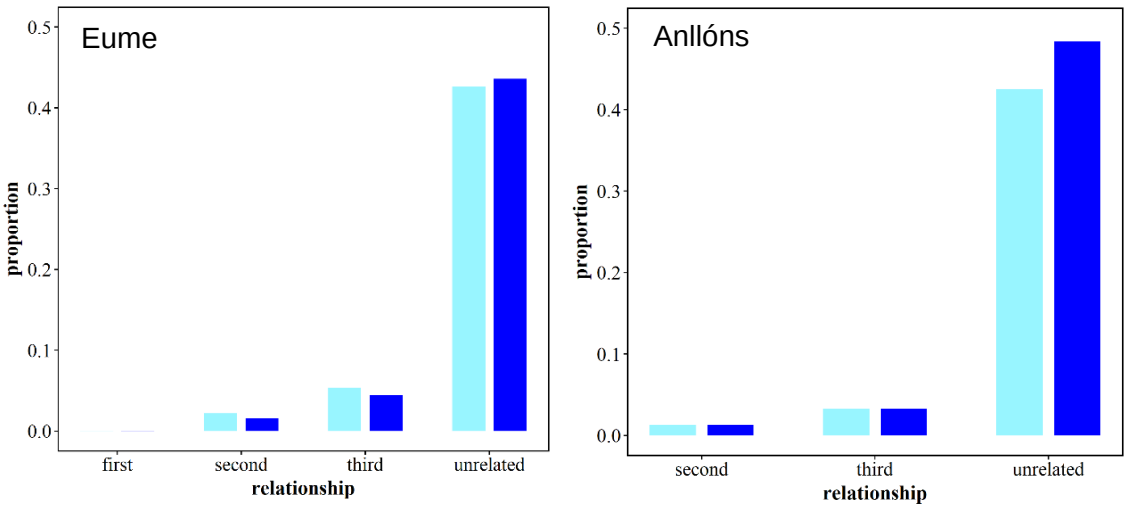

Esva
0 50 100
m
N

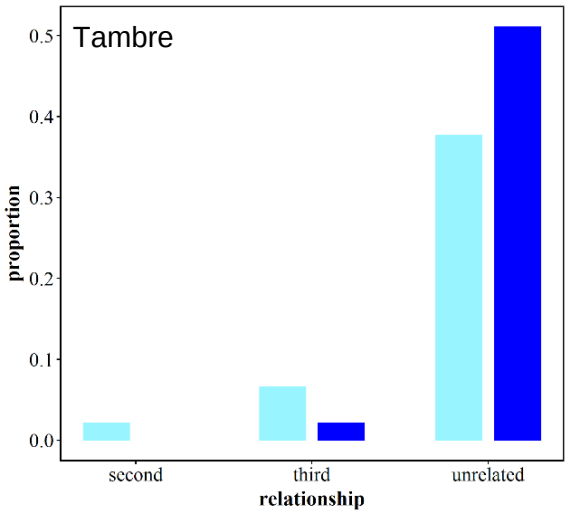
Figure 7. Aerial photographs of sampling locations. Kinship relationship identified in COANCESTRY are displayed. Thicker and thinner lines represent first- and second- order relationships, respectively.

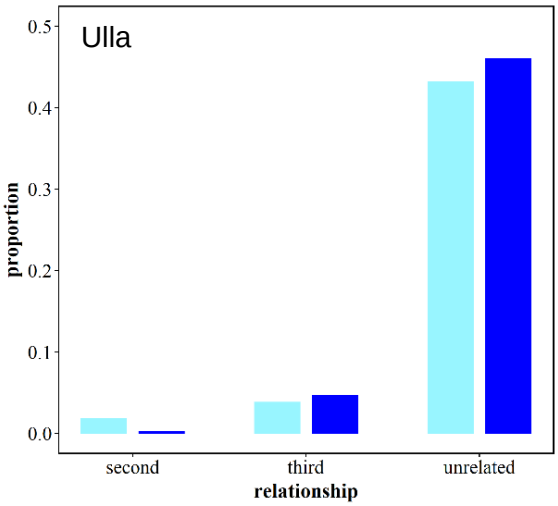
Average relatedness per river was very low for both larviparous (range TrioML: 0.023 - 0.037; QuellerGt: -0.113 - -0.022) and pueriparous (range TrioML: 0.020 - 0.032; QuellerGt: -0.059 - -0.028) populations (Table S5). Overall, genetic relatedness was marginally higher for pueriparous (mean ± SD: 0.027 ± 0.005 (TrioML); -0.045 ± 0.013 (QuellerGt)), than for larviparous (mean ± SD: 0.026 ± 0.005 (TrioML); -0.048 ± 0.029 (QuellerGt)) populations (Table S5). Average relatedness values of intra-riverside dyads were, overall, higher for pueriparous rivers (range TrioML: 0.029 (Nora) – 0.059 (Navia)) than for larviparous populations (range TrioML: 0.027 (Anllóns) – 0.040 (Eume)) (Table S6). Conversely, average values of relatedness of inter-riverside dyads were higher for larviparous (range TrioML: 0.012 (Minho) – 0.033 (Eume)) than for pueriparous (range TrioML: 0.006 (Navia) – 0.026 (Nora)) populations.

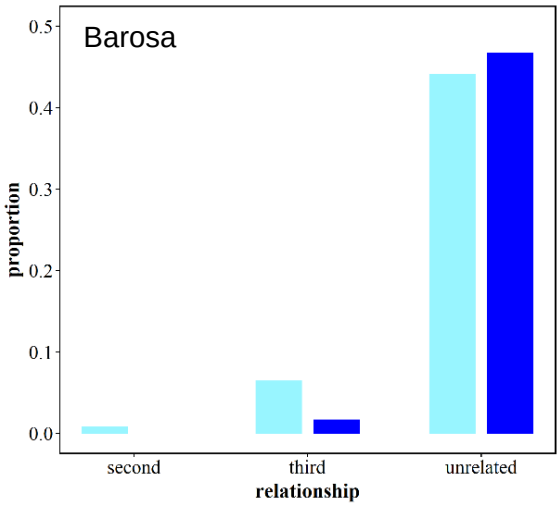
Overall, the frequency of pairs of relatives was higher for intra- than inter- riverside comparisons for first- ($r \geq 0.375$) and second- ($0.375 \geq r \geq 0.1875$) order kinship-classes in all rivers (Figures 8 and 9). No first-order relatives, both within and across riverside, were found in Anllóns, Tambre, Ulla, Barosa, Minho and Cávado (larviparous) populations, whereas pueriparous rivers only had first-order relatives belonging to the same riverside. Interestingly, Nora river show higher frequency of unrelated dyads within the same riverside, whilst all the other rivers show higher frequency of unrelated dyads across the riversides. Those rivers that seem to play a more effective role as barriers enhancing genetic differentiation (*i.e.* Navia and Narcea for pueriparous populations, and Minho and Cávado for larviparous populations) showed higher frequency of intra- than inter-riverside dyads for first, second, and third-order, while a high frequency for unrelated inter-riverside dyads was recorded. When we ignore the class unrelated ($r < 0.09375$) (Figure 9), pueriparous populations show higher proportions of intra-riverside first and second order relatives than larviparous ones. Interestingly, pueriparous populations show similar frequencies of unrelated intra and inter-riverside dyads.

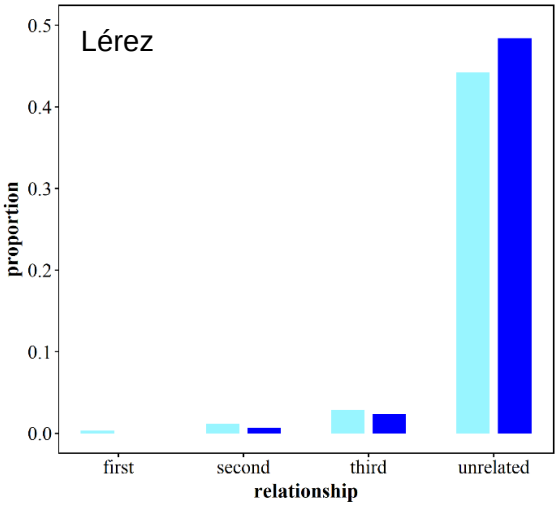
We found significant differences in mean pairwise relatedness between intra- and inter-riverside dyads in six larviparous and three pueriparous populations, and in six larviparous and two pueriparous populations for the TrioML and GuellerGt estimators, respectively (Table S7). Because both relatedness estimators did not show concordant results in Barosa, Lérez, Tambre and Esva rivers, we should interpret results with caution in these rivers. Moreover, we found significant differences between intra riverside's mean pairwise relatedness values in Esva (TrioML estimator) and Tambre (QuellerGt estimator).

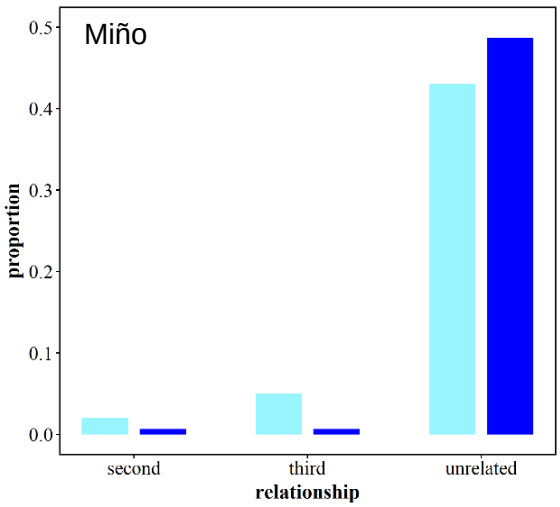


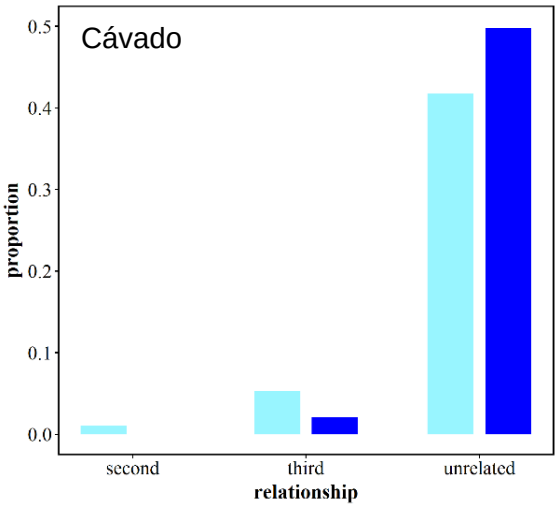

Tambre
proportion
0.0
0.1
0.2
0.3
0.4
0.5
second
third
unrelated
relationship


Ulla
proportion
0.0
0.1
0.2
0.3
0.4
0.5
second
third
unrelated
relationship


Barosa
proportion
0.0
0.1
0.2
0.3
0.4
0.5
second
third
unrelated
relationship


Lérez
proportion
0.0
0.1
0.2
0.3
0.4
0.5
first
second
third
unrelated
relationship


Miño
proportion
0.0
0.1
0.2
0.3
0.4
0.5
second
third
unrelated
relationship


Cávado
proportion
0.0
0.1
0.2
0.3
0.4
0.5
second
third
unrelated
relationship

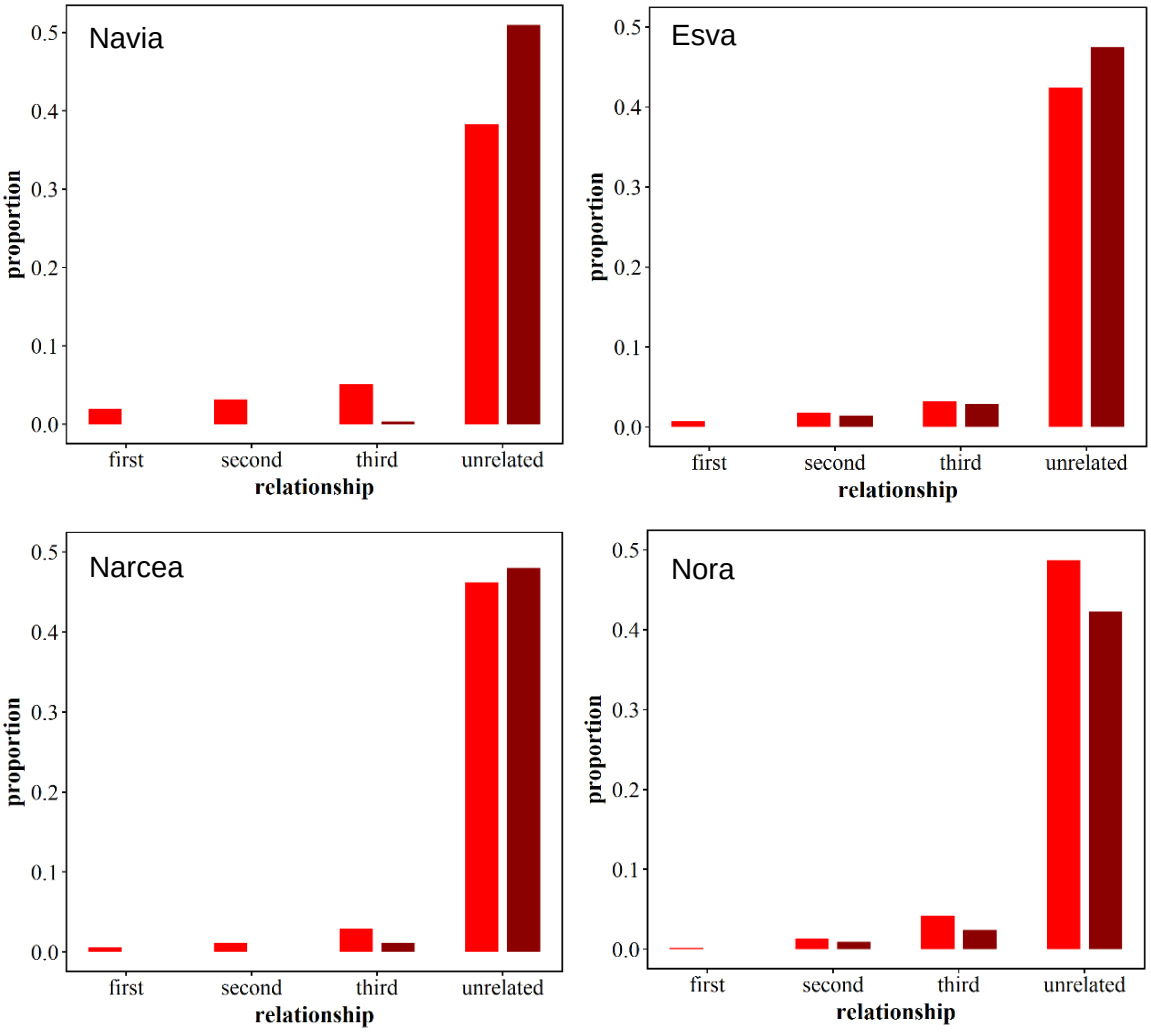
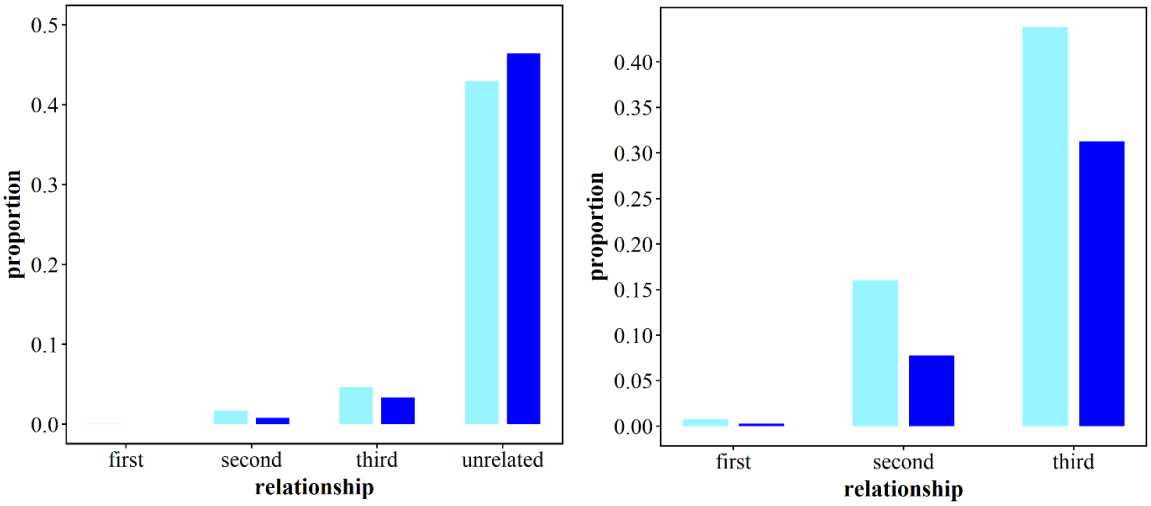


Figure 8. Frequency of pairs of relatives identified in COANCESTRY for each river. The X-axis represent the kinship-classes, while Y-axis represent the proportion of found dyads. Light and dark blue represents larviparous dyads found within the same riverside and across riversides, respectively. Light and dark red represents pueriparous dyads found within the same riverside and across riversides, respectively.



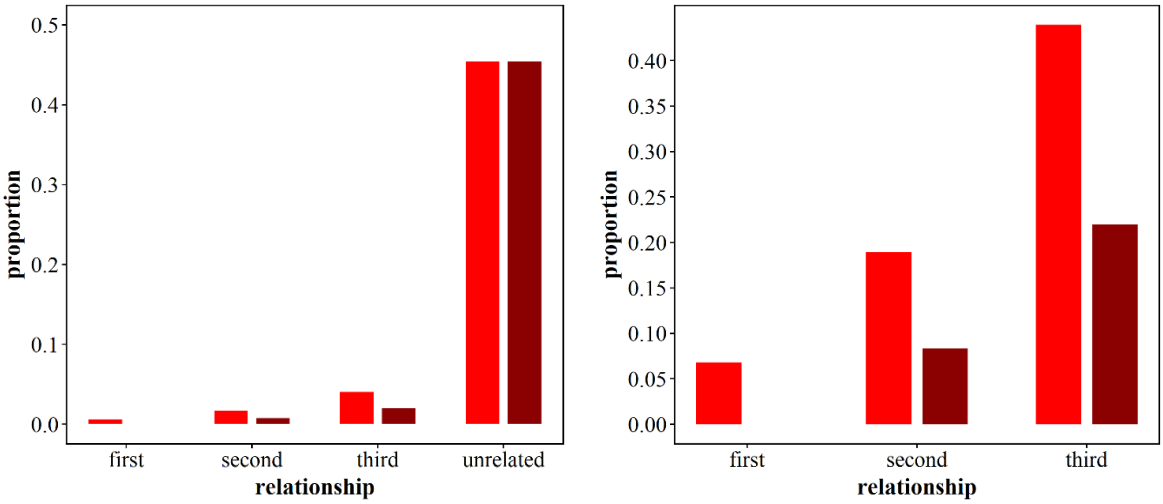


Figure 9. Histograms representing the frequency of pairs of relatives in each kinship-class for all the larviparous (blue) and pueriparous (red) rivers. Light and dark colours represent intra- and inter- riverside dyads, respectively. Left-column histograms contain the unrelated kinship-class ($r < 0.09375$).

4. Discussion

In this study we assessed the effect of rivers as barriers to gene flow in an amphibian exhibiting both aquatic and terrestrial reproduction. We took advantage of this intraspecific variability in reproduction in *S. salamandra* to make robust comparisons between both reproductive modes using a fine-scale approach comparing patterns of genetic structure and connectivity across riversides. Given that the exclusion of an aquatic stage probably results in a greater independence from water-bodies (Velo-Antón *et al.*, 2015), we hypothesized that rivers would entail more effective barriers for pueriparous than larviparous salamanders. Our results based on a combination of fine-scale assignment methods, population differentiation analyses, and kinship analyses, suggest that rivers entail partial barriers for both reproductive modes depending on the river's width (see Fouquet *et al.*, 2012), yet they appear to be more effective for pueriparous populations.

4.1. Do rivers affect gene flow in *S. salamandra*?

Fine-scale studies that explicitly test the river as putative barriers (*i.e.* experimental design is deliberately intended to find a genetic signal consistent with the barrier) are scarce in the literature (e.g. Marsh *et al.*, 2007). Nonetheless, previous large-scale studies found a significant effect of rivers as barrier to gene flow in amphibians, both presenting aquatic reproduction (e.g. Richardson *et al.*, 2012; Waraniak *et al.*, 2019) and fully-terrestrial cycles (e.g. Fouquet *et al.*, 2012, 2015). To our knowledge, the only fine-scale study with a robust sampling design that explicitly aim to demonstrate a barrier effect of streams in amphibians is Marsh *et al.* (2007). Based on genetic and mark-recapture data, Marsh *et al.* (2007) determined that low-order streams act as permeable barriers to dispersal and

gene flow in the direct-developer red-blacked salamander (*Plethodon cinereus*). In our work, results from fine-scale kinship analyses suggest that rivers, overall, comprise significant obstacles to gene flow in both reproductive modes, once: (i) we found a smaller proportion of relatives between riversides than along riversides; (ii) mean relatedness was generally higher along riversides than between riversides; and (iii) significant differences were found between mean pairwise-relatedness values between dyads located along and between riversides in most of the rivers. Nevertheless, kinship analyses also revealed that salamanders were able to disperse across the river, since we found some relatives sampled in different riversides in all rivers, suggesting that rivers act as semi-permeable obstacles for gene flow.

Our study shows a remarkable genetic differentiation among sampled riversides for F_{ST} and D_{EST} estimators in some of the sampled rivers, considering the small geographic scale of this study (Table 2). The F_{ST} values obtained are comparable to those documented by another fine-scale study showing rivers as barriers to gene flow in a terrestrial salamander species (Marsh *et al.*, 2007), but lower than others documented for fire salamanders where barriers are extremely effective to dispersal and gene flow such as seawater (Velo-Antón *et al.*, 2012) and urban environments (Lourenço *et al.*, 2017). Moreover, the F_{ST} values obtained in larviparous Miño, Cávado and Barosa and pueriparous Navia and Narcea populations are comparable to those observed for larviparous populations that are several Km apart (Lourenço *et al.* 2019). Rivers showed significant effects on population genetic structure in six localities (two pueriparous populations in rivers Navia and Narcea, and four larviparous populations in rivers Ulla, Barosa, Minho and Cávado), thus supporting the premise that these landscape features comprise semi-permeable obstacles for the dispersal of fire salamanders. AMOVA analyses suggest that little genetic variance is due to differences between riversides, while high variation is found among individuals, which can be explained by the high polymorphism shown by our microsatellite loci. Nevertheless, AMOVA results further support the results from other analyses, suggesting that Navia and Narcea populations are undergoing the strongest barrier effects.

STRUCTURE was not able to detect a genetic signal of differentiation in most rivers, even with the inclusion of LOCPRIOR parameter, which allows the detection of subtle genetic structure (Hubisz *et al.*, 2009). By setting *K*=2 (Blair *et al.*, 2012; Meirmans *et al.*, 2015), we aimed to explicitly test a disruption in gene flow, which would be reflected in a strong pattern of genetic differentiation consistent with the river. Thus, due to the fine-scale nature of our study, even though uppermost levels of genetic structuring may exist along riversides (*e.g.* caused by IBD), we would detect the barrier effect of the river before any other signal. In a previous study evaluating the performance of different methods to detect

recent linear barriers to gene flow, they found STRUCTURE as one of the methods that performed the best (Blair *et al.*, 2012). However, the application of this assignment method to our study has two main shortcomings: (i) they are highly dependent on sample size (see Manel *et al.*, 2005), and (ii) they work better at broader geographic scales (Blair *et al.*, 2012) and at higher levels of genetic differentiation (*e.g.* Fst > 0.07) (Berry *et al.*, 2004; Latch et al., 2006). Accordingly, results from STRUCTURE only detected a strong barrier effect in three rivers (two larviparous: Minho and Cávado, and one pueriparous: Navia), which also show high levels of population differentiation (Table XXX). Despite the clear signal of population subdivision, STRUCTURE was not able to recover two clusters with no admixture levels, suggesting that (i) our markers are not sufficient to grasp the genetic signal, or that (ii) there is indeed gene flow between riversides.

DAPC analyses were most sensitive to recover genetic signals of barrier effects in all rivers, which is likely due to the intrinsic nature of the analysis that maximizes genetic differences between groups, while minimizing it within groups (Jombart *et al.*, 2010). This could lead to confounding results, since it could tend to find population structure when there is weak population differentiation (*e.g.* Grummer and Leaché, 2017). Accordingly, the moderate level of population differentiation found in Ulla (larviparous) could be an analytical artifact. Additionally, DAPC has been shown to work better at broader scales, with species with long-range dispersal (e.g. 60 km; Blair *et al.*, 2012). Thus, results from this assignment method should be interpreted cautiously.

The use of mitochondrial markers allow us to infer historical patterns of gene flow and population divergence, and compare them with contemporary levels of gene flow (e.g. Lada *et al.*, 2007; Wang *et al.*, 2010). Our results suggest gene flow between riversides in all rivers at historical time. This is congruent with previous results for larviparous populations of *S. s. gallaica*, that showed shallow genetic structure at broad spatial scale (14 haplotypes found in an area of ca. 1400 km^2) (Lourenço *et al.,* 2019). Yet, the same study also reported high levels of genetic structure for pueriparous populations (21 distinct haplotypes found in an area of ca. 1800 km^2), which is incongruent with our results that suggest gene-flow between riversides. Nevertheless, this could be due to several reasons: (1) the short fragment of the sequences (320 bp) does not enable us to detect different haplotypes; (2) in case it actually exists different haplotypes between riversides, they could be distributed throughout larger areas (e.g. Lourenço *et al*., 2019), and the fine-scale sampling design of our study does not allow us to detect them; (3) the differentiation processes could not be strong enough to avoid shared haplotypes between riversides (*i.e.* rivers are semi-permeable rather than impermeable barriers to gene flow), nevertheless, the frequencies of these haplotypes could have different frequencies between riversides,

which would be consistent with a semi-permeable barrier effect exerted by rivers. Overall, haplotype networks would be more informative when differentiation processes are stronger (e.g. caused by impermeable barriers), which would allow us to detect different haplotypes between riversides, and with more data, that would allow us to assess the frequency and distribution of haplotypes in all rivers.

The absence of genetic signal from a putative barrier does not necessarily imply that such barrier does not exist. Yet, it could indicate that these rivers do not impede gene flow between riversides to a detectable extent by our employed tools. Quantify the extent of a landscape feature as a barrier is not a straightforward task. Even though an element imposes considerable constraints to gene flow, a few dispersing individuals may be able to homogenize allele frequencies and buffer any genetic signal of a potential barrier effect (Putman and Carbone, 2014). For instance, Carvalho *et al.* (2018) demonstrated that motorways strongly constrained genet movement across a motorway resorting to radio-tracking and kinship analyses; however, there was no apparent genetic signal since the movements across the motorway were enough to ensure high levels of gene flow that prevent the subsequent development of any spatial genetic structure. Moreover, a signal might be present but could not be detectable with the employed sampling design due to the low sample sizes in some of the studied rivers, which entail direct limitations in some of the employed genetic methods (COLONY2, STRUCTURE, DAPC). For instance, since COLONY2 identified putative relatives based on entire genealogical relationships within the sampled population, low sample sizes may lead to an overlook of some potential relatives, obscuring subsequent comparisons between rivers. In addition to sample size, there are other demographic processes, which we did not account for and that could influence contemporary genetic differentiation. For instance, large effective population sizes (*Ne*), can buffer the detrimental effects of genetic drift and, thus, reduce the genetic differentiation that arises from impeded gene flow and an exacerbated effect of drift, thus hampering the identification of a landscape feature shaping genetic structure (Gauffre *et al.*, 2008; Weckworth *et al.*, 2013; Lourenço *et al.*, 2017). In fact, despite the small scale of this study, the estimated low mean inbreeding coefficients, along with the low number of relatives from COLONY2, likely indicate that the study populations exhibit relatively high effective population sizes (*Ne*) , that may be counteracting the genetic barrier effects of river (e.g. Gauffre *et al.*, 2008; Luqman *et al.*, 2018; see also Álvarez *et al.*, 2015 and Lourenço *et al.*, 2017 for the positive role of demography on urban and isolated populations of *S. salamandra)*.

Moreover, if fire salamanders are indeed crossing the rivers, we cannot infer exactly how these barriers are crossed; therefore, we cannot ascertain whether the crossing events

are produced unintentionally (e.g. in top of a drifting object, drifting larvae, human-mediated), or intentionally (e.g. taking advantage of crossing structures such as bridges, falling logs). Indeed, there are records of fire salamanders crossing Nora bridge (André Lourenço, personal observation), thus the presence of bridges in some rivers (e.g. Nora and Esva) is a potential weakness of this study. Nevertheless, due to the high transformation of the habitat, finding large patches of suitable habitat facing each other, within medium or large rivers, and without nearby bridges is extremely difficult. Nevertheless, the presence of largely intact good-quality habitat in both sides of the river, together with the site-fidelity shown by *S. salamandra* (Rebelo and Leclair, 2003; Schulte *et al.*, 2007), suggest that the use of these structures is unlikely, particularly of those metal hanging and car traffic bridges, which are unsuitable for fire salamanders and most likely constitute impassible barriers. Although expensive and time-consuming, complementing our genetic data with direct observations of salamander dispersal, *e.g.* through radio-tracking, or capture-mark-recapture techniques such as PIT-tag based analyses (e.g. Schulte *et al.*, 2007; Hendrix *et al.,* 2017), would provide dispersal data that would help to disentangle the source and frequency of gene flow across the river (e.g. Marsh *et al.*, 2007).

4.2. Are rivers more effective barriers for pueriparous than larviparous populations?

Life-history traits and movement ability are known to influence species responses to different landscape features (Richardson, 2012; García *et al.*, 2017). A shift in reproductive strategies may influence genetic population connectivity (e.g. Sandberguer-Loua *et al.*, 2018; Lourenço *et al.*, 2019), and the response to potential barriers. For instance, Fouquet *et al.* (2015) found that amphibians with a fully-terrestrial cycle exhibited higher river-associated structure than amphibians with aquatic reproduction.

In our study system, pueriparous (terrestrial) populations show different levels of genetic connectivity and population differentiation possibly depending on the characteristics of the river (e.g. size), and the type of crossing structures (e.g. roman bridges, bridges paved with asphalt, car-traffic, metal hanging structures, bridges that accumulate vegetation in the margins). Consequently, Navia and Narcea rivers (the two largest rivers among the studied pueriparous populations) show strong barrier effects that are consistent among all genetic analyses. Our obtained F_{ST} values for these rivers are significant and higher than those obtained in other fine-scale study showing rivers as barriers to gene flow in other salamander species (Marsh *et al.*, 2007). Despite Navia population having an adjacent car traffic bridge (see in Figure 5 -Table 1), the strong barrier effects imposed by this river, as

revealed by our analyses, potentially indicate this bridge is rarely used by salamanders and/or that very few individuals successfully cross it (e.g. due to road mortality). Interestingly, even the less sensitive methods (the two assignment methods) suggested clear patterns of population differentiation. However, this does not hold for Narcea river, which shows considerable levels of admixture proportions in STRUCTURE, even though remaining analyses indicate this river impedes gene flow substantially.

Although these rivers have a considerable width (Navia: 50 m, Narcea: 30 m), they are similar to other studied larviparous rivers that show shallower genetic differentiation (e.g. Ulla: 50 m, Eume: 17 m). This partially support our hypothesis, suggesting that reproductive mode plays a relevant role in promoting the gene flow across rivers, hence discarding river size as the only explanatory effect causing a reduction on gene flow. Nevertheless, we cannot ascertain whether these rivers entail physical barriers e.g. they attempt to cross but fail (e.g. because they are poor swimmers), or behavioral barriers *i.e.* they do not attempt to cross the river, as their life cycle is tightly linked to terrestrial habitats (Velo-Antón and Buckley, 2015). Conversely, the other two pueriparous populations (Nora and Esva) show minor signals of barrier effects for microsatellite markers. Kinship analyses show certain constraints on gene flow between riversides (higher proportion of relatives along than across riversides for all rivers but Nora), yet there were no significant differences between mean average relatedness between dyads located along and dyads located across riversides, and assignment methods were not able to recover any differentiation signal. This holds especially for Nora river, which shows extremely low values of population differentiation (Table 3), and higher proportions of unrelated individuals ($r < 0.09375$) along than across riversides, which could possibly be explained by the age (it dates back to Roman times), size (small, ~10 m) and material (stone) of the bridge, which contains a pavement of dirt, making it suitable for occasional dispersal events (personal observation André Lourenço). Since these two rivers are the narrowest in our study, we argue that the higher levels of contemporary gene flow in these rivers could be due to the higher tendency of these salamanders to transverse the streams through the more permeable bridges (stone or small car traffic structures) or occasional objects (e.g. fallen trees) connecting both riversides.

Since larviparous salamanders are linked to water-bodies to reproduce, we hypothesized that gene flow across streams is more frequent in larviparous populations. Yet, Richardson *et al.* (2012) showed that medium and large rivers are barriers for larviparous salamanders (*Ambystoma maculatum*) breeding in ponds. Larviparous *S. salamandra* in our study populations usually deliver larvae in water-bodies such as ponds, and streams of different magnitude (preferably creeks) with slow waters, using their edges to deposit the larvae

(Velo-Antón and Buckley, 2015; Veith *et al.*, 2018). Thus, larvae may reach the river by drift from the adjacent creeks, hence encountering a possible barrier. As results from Cávado and Minho rivers suggests, large rivers comprise nearly impermeable obstacles for larviparous populations. The continuum water-flow together with the widths of the rivers (190 and 90 meters for Minho and Cávado, respectively), may have contributed to these patterns. Although these rivers are considerably large, their widths are not enough for explaining the genetic differentiation just by isolation by distance, since salamanders have shown to disperse for larger distances than the width of these rivers (up to 503, as documented by Schulte *et al.*, 2007; and up to 1.9 km in Hendrix *et al.*, 2017). Additionally, our sampling design control for isolation by distance effects across rivers since the length of the transect was always larger than the width of the river (*i.e.* there was longer distances between the most distant individuals along rivers, than between rivers). Consequently, in case isolation by distance patterns exists they would be also detected along rivers. Results from other larviparous populations show high admixture levels and high proportion of relatives belonging to different riversides, suggesting that smaller rivers may not entail physical or behavioral barriers for larviparous salamanders. This holds particularly for Eume river (17 m of width) that show no signal of barrier effect at both historical and contemporary levels. Conversely, large rivers (such as Cávado and Minho) may entail efficient physical barriers (*i.e.* they attempt to cross but fail). The fact that larvae are predated by fishes (e.g. trouts) (Velo-Antón and Buckley, 2015) which are generally absent in small ponds and creeks, likely have contributed to larvae deposition in small water-bodies. Accordingly, the high predation risk of larvae entailed by fish predators, may render rivers as potential barriers, either by highly predation risk or by predator avoidance. Moreover, large rivers (like Cávado and Miño) are associated with downstream larval drift (Veith *et al.*, 2018), that can be massive after severe rains (Reindhart *et al.*, 2018). These strong drifts may be related with high mortality risk of larvae due to physical damages caused by other drifting objects (Segev and Blaustein, 2014). Contrarily, in narrower rivers with low water-flow velocities, larvae may be able to actively resist the entrance on the current (Segev and Balustein, 1994), or to actively move against it or hold position in creeks (rheotactic behavior) rather than drifting downstream (Veith.*et al.*, 2018). Nevertheless, rivers with considerable width (e.g. Eume and Ulla with 17 and 50 meters, respectively), were permeable barriers showing low differentiation, shared haplotypes between riversides in the haplotype network, and higher number of pairs of relatives between riversides.

Overall, according to our most efficient methods to detect the barrier (fine-scale kinship analysis), pueriparous salamanders are more affected by the river even though the

assignment methods are not sensitive enough to detect the signal. A vicariant effect exerted by pueriparous rivers could be consistent with the deep phylogeographic structure demonstrated for *S. s. bernardezi* (Beukema *et al.*, 2016; Lourenço *et al.*, 2019). In Lourenço *et al.* (2019), the authors made broad-scale comparisons between patterns of genetic connectivity of pueriparous and larviparous *S. salamandra* populations, demonstrating that the density of water courses (both streams and rivers) is significantly associated with higher resistance to gene flow only in pueriparous populations, explaining most of the genetic variation in *S. s. bernardezi*, even though both pueriparous and larviparous study areas had high density of water courses. Nonetheless, in that study the effect of the density of lotic systems could not be dissociated from other factors such as the topography or climatic components (e.g. wind exposition). Our fine-scale study allows us to control for others factors (such as wind or other landscape features like agriculture fields), isolating the river effect. Nevertheless, uneven sampling of larviparous (N=8) and pueriparous (N=4) rivers could compromise comparisons between reproductive modes. Increasing the number of pueriparous rivers is currently ongoing to better understand the effects of rivers on the dispersal of these salamanders in relation with their reproductive mode.

4.3. Eco-evolutionary implications of landscape barriers in amphibians

Anthropogenic and natural landscape barriers may entail major consequences for wildlife populations, that can be observed at broad (e.g. mountain ridge for salamanders, Velo-Antón *et al.*, 2013; and anurans, Sánchez-Montes *et al.*, 2018), and finer scales (e.g. roads, Holderegger and Di Giulio, 2010; urban areas, Lourenço *et al.* 2017). These effects of landscape features might be exacerbated in amphibians due to the their relatively low vagility and great sensitivity to habitat alteration (Cushman *et al.*, 2006).

Landscape features have been proposed as factors enhancing diversification for several amphibians (e.g. rivers in Fouquet *et al.*, 2012; 2015). In our study system, the presence of barriers has already been recognized to be drivers of allopatric diversification, such as the Guadalquivir River Basin for southern Iberian lineages (García-París *et al.*, 1998; but see Antunes *et al.*, 2018), or the seawater barrier for island-mainland fire salamanders (Velo-Antón *et al.* 2012; Lourenço *et al.*, 2018b). Alike, the highest ridges along the Cantabrian mountains, where the climate is less suitable, are thought to be the main impediment of dispersal and gene flow between larviparous and pueriparous fire salamanders occurring at the south and north slopes of these mountains, respectively (Velo-Antón *et al.*, in preparation), but other landscape features such as wind exposition and topographic complexity throughout the *S. salamandra* range reduces population connectivity and contributes to population diversification (Lourenço *et al.*, 2019).

Salamandra salamandra is an excellent example of intraspecific polymorphism regarding reproductive strategies, constituting a source of adaptive differentiation and speciation. In a study comparing patterns of adaptive responses and genetic differentiation between stream- and pond- adapted salamanders, Steinfartz *et al.* (2007) showed that sympatric populations of salamanders are genetically and ecologically differentiated, likely comprising populations in early stages of adaptive differentiation (in this case to shallow ponds). In our study system, differences in genetic structure could be expected as a consequence of differences in reproductive mode, since a shift to pueriparity might bring changes in dispersal behavior. Nevertheless, Lourenço *et al.* (2018) did not find significant differences in fine-scale genetic structure between larviparous and pueriparous populations, probably due to larvae water-borne dispersal, that might obscure genetic patterns caused by adult dispersal, although other factors hampering the detection of any potential differences (e.g. type of analyses, sample size) could not be discarded. However, a shift to pueriparity could involve potential changes in dispersal behaviour, that could potentially enhance philopatry in pueriparous populations, likely because they do not need to search for optimal breeding conditions associated with water-bodies. Hence, a decrease in dispersal would complement the effect of rivers as barriers explaining the high level of genetic structure observed in *S. s. bernardezi* (Beukema *et al.*, 2016; Lourenço *et al.*, 2019). Future studies evaluating differences in behaviour between reproductive modes will help to elucidate some ecological and evolutionary implications of changes in reproductive mode in amphibians.

To the best of our knowledge, our study is one of the very few with a fine-scale sampling design that explicitly assess the effects of rivers on genetic differentiation and connectivity within amphibians' populations. Moreover, it is the first study exclusively evaluating the potential different effect of these landscape features on genetic structure between organisms with different reproductive modes.

Our results suggest current trans-riverine structure in fire salamanders is mainly affected by large rivers, although an exacerbated effect is observed in pueriparous populations. This, together with a more restricted genetic connectivity in pueriparous populations revealed by fine-scale kinship analyses, suggests that reproductive mode plays an important role in the dispersal of individuals across the rivers and thus, in populations´ genetic diversity and structure. Nevertheless, our results should be taken with caution due to the uneven and low sample sizes across the rivers, as well as the presence of bridges potentially suitable for salamander dispersal between riversides (Nora and Esva rivers), which could have eroded the otherwise pattern of genetic differentiation in these pueriparous populations. Further efforts are being conducted to increase the number of

pueriparous populations, which will give us a better picture of the historical and contemporary patterns of genetic structure and the role of viviparity prompting genetic diversification as a consequence of dispersal constraints across areas containing river networks. Additionally, long term spatial-ecology data obtained with mark-recapture approaches would help to shed light on the nature of these movements across the rivers.

References

Alexandrino J, Froufe E, Arntzen JW, Ferrand N (2000) Genetic subdivision, glacial refugia and postglacial recolonization in the golden-striped salamander, Chioglossa lusitanica (Amphibia : Urodela). *Molecular Ecology*, **9**, 771–781.

Álvarez D, Lourenço A, Oro D, Velo-Antón G (2015) Assessment of census (*N*) and effective population size (*Ne*) reveals consistency of *Ne* single-sample estimators and a high *Ne*/*N* ratio in an urban and isolated population of fire salamanders. *Conservation Genetics Resources*, **7**, 705–712.

Amigo J, Rodríguez-Guitián MA, Honrado JJP, Alves P (2017) The lowlands and midlands of northwestern Atlantic Iberia. In: *The vegetation of the Iberian Peninsula* (ed. Loidi J), **1**, 191-250. Springer, Switzerland.

AmphibiaWeb (2019) Information on amphibian biology and conservation. Berkeley, California: AmphibiaWeb. Available: https://amphibiaweb.org/ (accessed 10-05-2019).

Anderson C, Epperson BK, Fortin MJ, Holderegger R, James PMA, Rosenberg MS, Scribner KT, Spear S (2010) Considering spatial and temporal scale in landscape-genetic studies of gene flow. *Molecular Ecology*, **19**, 3565–3575.

Antunes B, Lourenço A, Caeiro-Dias G, Dinis M, Gonçalves H, Martínez-Solano I, Tarroso P, Velo-Antón G (2018) Combining phylogeography and landscape genetics to infer the evolutionary history of a short-range Mediterranean relict, *Salamandra salamandra longirostris*. *Conservation Genetics*, **19**, 1411–1424.

Balloux, F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. *Molecular ecology*, **11**, 155-165.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society B*, **57**, 289–300.

Berry O, Tocher MD, Sarre SD (2004) Can assignment tests measure dispersal? *Molecular Ecology*, **13**, 551–561.

Beukema W, De Pous P, Donaire D, Escoriza D, Gogaerts S, Toxopeus AG, de Bie CAJM, Roca J, Carranza S (2010) Biogeography and contemporary climatic differentiation

among Moroccan *Salamandra algira*. *Biological Journal of the Linnean Society*, **101**, 626–641.

Beukema W, Nicieza AG, Lourenço A, Velo-Antón G (2016) Colour polymorphism in *Salamandra salamandra* (Amphibia: Urodela), revealed by a lack of genetic and environmental differentiation between distinct phenotypes. *Journal of Zoological Systematics and Evolutionary Research*, **54**, 127–136.

Blackburn DG (1999) Viviparity and oviparity: evolution and reproductive strategies. *Encyclopedia of Reproduction*, **4**, 840–847.

Blackburn DG (2000) Reptilian viviparity: Past research, future directions, and appropriate models. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, **127**, 391–409.

Blackburn DG (2015a) Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. *Journal of Morphology*, **276**, 961–990.

Blackburn DG (2015b) Evolution of viviparity in squamate reptiles: Reversibility reconsidered. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, **324**, 473–486.

Blair C, Weigel DE, Balazik M, Keeley ATH, Walker FM, Landguth E, Cushman S, Murphy M, Waits L, Balkenhol N (2012) A simulation-based evaluation of methods for inferring linear barriers to gene flow. *Molecular Ecology Resources*, **12**, 822–833.

Blaustein L, Segev O, Rovelli V, Bar-David,S, Blank L, Polevikov A, Pezaro N, Krugman T, Showstack S, Koplovich A, Ozeri L, Templetona AR (2018) Compassionate approaches for the conservation and protection of Fire Salamanders. *Israel Journal of Ecology & Evolution,* **63, 43–51.**

Buckley D, Alcobendas M, García-París M, Wake MH (2007) Heterochrony, cannibalism, and the evolution of viviparity in *Salamandra salamandra*. *Evolution and Development*, **9**, 105–115.

Buckley D (2012) Evolution of viviparity in salamanders (Amphibia, Caudata). *Encyclopedia of Life Sciences*, 1-13.

Carr LW, Fahrig L (2001) Effect of road traffic on two amphibian species of different vagility. *Conservation Biology,* **15**,1071–1078

Carvalho F, Lourenço A, Carvalho R, Alves PC, Mira A, Beja P (2018) The effects of a motorway on movement behaviour and gene flow in a forest carnivore: joint evidence from road mortality, radio tracking and genetics. *Landscape and Urban Planning*, **178**, 217–227.

Caspers BA, Krause ET, Hendrix R, Kopp M, Rupp O, Rosentreter K, Steinfartz S (2014) The more the better - polyandry and genetic similarity are positively linked to reproductive success in a natural population of terrestrial salamanders (*Salamandra salamandra*). *Molecular Ecology*, **23**, 239–250.

Chybicki IJ, Burczyk J (2009) Simultaneous estimation of null alleles and inbreeding coefficients. *Journal of Heredity*, **100**, 106–113.

Clark RW, Brown WS, Stechert R, Zamudio KR (2010) Roads, interrupted dispersal, and genetic diversity in timber rattlesnakes. *Conservation Biology*, **24**, 1059–1069.

Clement M, Posada DCKA, Crandall KA (2000) TCS: A computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Cosgrove AJ, McWhorter TJ, Maron M (2018) Consequences of impediments to animal movements at different scales: A conceptual framework and review. *Diversity and Distributions*, **24**, 448–459.

Crump ML (2015) Anuran reproductive modes: evolving perspectives. *Journal of Herpetology*, **49**, 1–16

Cushman SA (2006) Effects of habitat loss and fragmentation on amphibians: a review and prospectus. *Biological Conservation*, **128**, 231–240.

Dinis M, Velo-Antón G (2017) How little do we know about the reproductive mode in the north African salamander, *Salamandra algira*? Pueriparity in divergent mitochondrial lineages of *S. a. tingitana*. *Amphibia-Reptilia*, **38**, 540–546.

Dinis M, Merabet K, Martínez-Freiría F, Steinfartz S, Vences M, Burgon JD, Elmer KR, Donaire D, Hinckley A, Fahd S, Joger U, Fawzi A, Slimani T, Velo-Antón G (2019) Allopatric diversification and evolutionary melting pot in a North African Parearctic relict: The biogeographic history of *Salamandra algira. Molecular Phylogenetics and Evolution,* **130***,* 81-91.

Duellman WE, Trueb L (1986) Reproductive strategies. In: *Biology of amphibians*. 13-47. McGraw Hill, New York.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology,* **14**, 2611–2620.

Epps CW, Keyghobadi (2015) Landscape genetics in a changing world: disentangling historical and contemporary influences and inferring change. *Molecular Ecology,* **24**, 6021–6040.

Fouquet A, Ledoux JB, Dubut V, Noonan BP, Scotti I (2012) The interplay of dispersal limitation, rivers, and historical events shapes the genetic structure of an Amazonian frog. *Biological Journal of the Linnean Society,* **106,** 356-373.

Fouquet A, Courtois EA, Baudain D, Lima JD, Souza SM, Noonan BP, Rodrigues M (2015). The trans-riverine genetic structure of 28 Amazonian frog species is dependent on life history. *Journal of Tropical Ecology*, **31**, 361–373.

Frantz AC, Bertouille S, Eloy MC, Licoppe A, Chamont F, Flamand MC (2012) Comparative landscape genetic analyses show a Belgian motorway to be a gene flow barrier for red deer (*Cervus elaphus*), but not wild boars (*Sus scrofa*). *Molecular Ecology,* **21,** 3445-3457.

Galán P (2007) Viviparismo y distribución de *Salamandra salamandra bernardezi* en el norte de Galicia. *Boletín de la Asociación Herpetológica Española,* **18**, 44–49.

Gao W, Sun Y-B, Zhou W-W, Xiong Z-J, Chen L, Li H, Fu T-T, Xu K, Xu W, Ma L, Chen Y-J, Xiang X-Y, Zhou L, Zeng T, Zhang S, Jin J-Q, Chen H-M, Zhang G, Hilis DM, Ji X, Zhang Y-P, Che J (2019) Genomic and transcriptomic investigations of the evolutionary transition from oviparity to viviparity. *Proceedings of the National Academy of Sciences*, **116**, 3646–3655.

Gauffre B, Estoup A, Bretagnolle V, Cosson JF (2008) Spatial genetic structure of a small rodent in a heterogeneous landscape. *Molecular Ecology*, **17**, 4619–4629.

García VOS, Ivy C, Fu J (2017) Syntopic frogs reveal different patterns of interaction with the landscape: a comparative landscape genetic study of *Pelophylax nigromaculatus* and *Fejervarya limnocharis* from central China. *Ecology and Evolution*, **7**, 9294–9306.

García-París M, Alcobendas M, Alberch P (1998) Influence of the Gualdaquivir River Basin on mitochondrial DNA evolution of Salamandra salamandra (Caudata: Salamandridae) from southern Spain. *Copeia*, **1998**, 173–176.

García-París M, Alcobendas M, Buckley D, Wake D (2003) Dispersal of viviparity across contact zones in Iberian populations of Fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. *Evolution*, **57**, 129–143.

Gomez-Mestre I, Pyron RA, Wiens JJ (2012) Phylogenetic analyses reveal unexpected patterns in the evolution of reproductive modes in frogs. *Evolution*, **66**, 3687–3700.

Gómez A, Lunt DH (2006) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: *Phylogeography of southern European refugia* (eds Weiss S, Ferrand N), 155–188. Springer, Netherlands.

Greven H (2003) Larviparity and pueriparity. In: *Reproductive Biology and Phylogeny of Urodela* (ed Sever DM), **1**, 447-475. Science Publishers Inc, Enfield.

Grummer JA, Leaché AD (2017) Do dams also stop frogs? Assessing population connectivity of coastal tailed frogs (*Ascaphus truei*) in the North Cascades National Park Service Complex. *Conservation Genetics***, 18,** 439-451.

Hall LA, Beissinger SR (2014) A practical toolbox for design and analysis of landscape genetics studies. *Landscape Ecology*, **29**, 1487–1504.

Helmstetter AJ, Papadopulos AS, Igea, J, Van Dooren TJ, Leroi AM, Savolainen V (2016) Viviparity stimulates diversification in an order of fish. *Nature communications*, **7**, 11271.

Hendrix R, Hauswaldt JS, Veith M, Steinfartz S (2010) Strong correlation between cross-amplification success and genetic distance across all members of 'True salamanders' (Amphibia: Salamandridae) revealed by *Salamandra salamandra*-specific microsatellite loci. *Molecular Ecology Resources*, **10**, 1038–1047.

Hendrix R, Schmidt BR, Schaub M, Krause ET, Steinfartz S (2017) Differentiation of movement behaviour in an adaptively diverging salamander population. *Molecular Ecology*, **26**, 6400–6413.

Hepenstrick D, Thiel D, Holderegger R, Gugerli F (2012) Genetic discontinuities in roe deer *(Capreolus capreoulus)* coincide with fenced transportation infrastructure. *Basic and Applied Ecology,* **13,** 631-638.

Hodges, WL (2004). Evolution of viviparity in horned lizards (Phrynosoma): Testing the cold-climate hypothesis. *Journal of Evolutionary Biology*, **17**, 1230–1237.

Holderegger R, Wagner HH (2008) Landscape Genetics. *BioScience,* **58**, 199-207.

Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, **9**, 1322–1332.

Jombart T, Devillard S, Dufour AB, Pontier D (2008) Revealing cryptic spatial patterns in genetic variability by a new multivariate method. *Heredity*, **101,** 92-103.

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, **11**, 94.

Jones OR, Wang J (2010) COLONY: A program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, **10**, 551–555.

Jost L (2008) GST and its relatives do not measure differentiation. *Molecular Ecology*, **17**, 4015–4026.

Keenan K, McGinnity P, Cross TF, Crozier WW, Prodöhl PA (2013) diveRsity: an R package for the estimation of population genetics parameters and their associated errors. *Methods in Ecology and Evolution*, **4**, 782–788.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial-DNA evolution in animals - amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences,* **86**, 6196–6200.

Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I (2015) Clumpak: A program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, **15**, 1179– 1191.

Kuzmin S, Papenfuss T, Sparreboom M, Ugurtas IH, Anderson S, Beebee T, Denöel M, Andreone F, Anthony B, Schmidt B, Ogrodowczyk A, Ogielska M, Bosch J, Tarkhnishvili D, Ishchenko V (2009) *Salamandra salamandra*. In: *The IUCN Red List of Threatened Species 2009*.

Lada H, Nally RM, Taylor AC (2007) Distinguishing past from present gene flow along and across a river: the case of the carnivorous marsupial (*Antechinus flavipes*) on southern Australian floodplains. *Conservation Genetics*, **9**, 569-580.

Landguth EL, Cushman SA, Schwartz MK, McKelvey KS, Murphy M, Luikart G (2010) Quantifying the lag time to detect barriers in landscape genetics. *Molecular Ecology*, **19**, 4179–4191.

Landguth EL, Fedy BC, Oyler-McCance SJ, Garey AL, Emel SL, Mumma M, Wagner HH, Fortin M-J, Cushman SA (2012) Effects of sample size, number of markers, and allelic richness on the detection of spatial genetic pattern. *Molecular Ecology Resources*, **12**, 276–284.

Latch EK, Dharmarajan G, Glaubitz JC, Rhodes OE (2006) Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. *Conservation Genetics,* **7,** 295-302.

Liedtke HC, Müller H, Hafner J, Penner J, Gower DJ, Mazuch T, Rödel M-O, Loader SP (2017) Terrestrial reproduction as an adaptation to steep terrain in African toads. *Proceedings of the Royal Society* B, **284**, 20162598.

Lourenço A, Álvarez D, Wang IJ, Velo-Antón G (2017) Trapped within the city: integrating demography, time since isolation and population-specific traits to assess the genetic effects of urbanization. *Molecular Ecology*, **26**, 1498–1514.

Lourenço A, Antunes B, Wang IJ, Velo-Antón G (2018a) Fine-scale genetic structure in a salamander with two reproductive modes: does reproductive mode affect dispersal? *Evolutionary Ecology*, **32**, 699–732.

Lourenço A, Sequeira F, Buckley D, Velo-Antón G (2018b) Role of colonization history and species-specific traits on contemporary genetic variation of two salamander species in a Holocene island-mainland system. *Journal of Biogeography*, **45**, 1054-1066.

Lourenço A, Gonçalves J, Carvalho F, Wang IJ, Velo-Antón G (2019) Comparative landscape genetics reveals the evolution of viviparity reduces genetic connectivity in fire salamanders. *Molecular Ecology,* 10.1111/mec.15249

Luqman H, Muller R, Vaupel A, Brodbeck S, Bolliger J, Gugerli F (2018) No distinct barrier effects of highways and a wide river on the genetic structure of the Alpine newt

(Ichthyosaura alpestris) in densely settled landscapes. *Conservation Genetics,* **19,** 673-685.

Ma L, Buckley LB, Du RBHW (2018) A global test of the cold-climate hypothesis for the evolution of viviparity of squamate reptiles. *Global Ecology and Biogeography*, **27**, 679–689.

Manel S, Schwartz MK, Luikart G, Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics. *Trends in Ecology and Evolution*, **18**, 189–197.

Manel S, Gaggiotti OE, Waples RS (2005) Assignment methods: matching biological questions with appropriate techniques. *Trends in Ecology and Evolution,* **20**,136–142.

Manel S, Holderegger R (2013) Ten years of landscape genetics. *Trends in Ecology and Evolution*, **28**, 614–621.

Marsh DM, Page RB, Hanlon TJ, Bareke H, Corritone R, Jetter N, Beckman NG, Gardner K, Seifert DE, Cabe PR (2007) Ecological and genetic evidence that low-order streams inhibit dispersal by red-backed salamanders (*Plethodon cinereus*). *Canadian Journal of Zoology,* **85**, 319–327.

Measey GJ, Galbusera P, Breyne P, Matthysen E (2007) Gene flow in a direct-developing, leaf litter frog between isolated mountains in the Taita Hills, Kenya. *Conservation Genetics*, **8**, 1177–1188.

Meirmans PG, Hedrick PW (2011) Assessing population structure: F_{ST} and related measures. *Molecular Ecology Resources*, **11**, 5–18.

Meirmans PG (2015) Seven common mistakes in population genetics and how to avoid them. *Molecular Ecology*, **24,** 3223-3231.

Moritz C, Schneider CJ, Wake DB (1992) Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Systematic Biology,* **41**, 273–291.

Mullen LB, Woods HA, Schwartz MK, Sepulveda AJ (2010) Scale-dependent genetic structure of the Idaho giant salamander (*Dicamptodon aterrimus*) in stream networks. *Molecular Ecology,* **19,** 898–909.

Nei M (1977) F-statistics and analysis of gene diversity in subdivided populations. *Annals*

of human genetics, **41**, 225-233.

O'Connell KA, Mulder KP, Maldonado J, Currie KL, Ferraro DM (2019) Sampling related individuals within ponds biases estimates of population structure in a pond-breeding amphibian. *Ecology and Evolution*, **9**, 3620-3636.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics*, **28**, 2537–2539.

Pincheira-Donoso D, Tregenza T, Witt MJ, Hodgson DJ (2013) The evolution of viviparity opens opportunities for lizard radiation but drives it into a climatic cul-de-sac. *Global Ecology and Biogeography*, **22**, 857–867.

Pincheira-Donoso D, Jara M, Reaney A, García-Roa R, Saldarriaga-Córdoba M, Hodgson DJ (2017) Hypoxia and hypothermia as rival agents of selection driving the evolution of viviparity in lizards. *Global Ecology and Biogeography*, **26**, 1238–1246.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Putman AI, Carbone I (2014) Challenges in analysis and interpretation of microsatellite data for population genetic studies. *Ecology and Evolution*, **4**, 4399–4428.

Pyron RA, Burbrink FT (2014) Early origin of viviparity and multiple reversions to oviparity in squamate reptiles. *Ecology Letters*, **17**, 13–21.

Queller D, Goodnight K (1989) Estimating relatedness using genetic markers. *Evolution*, **43**, 258–275.

Queller DC, Strassmann JE, Hughes CR (1993) Microsatellites and kinship. *Trends in Ecology and Evolution*, **8**, 285–288.

R Development Core Team (2019) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available: https://www.R-project.org/

Rebelo R, Leclair MH (2003) Site tenacity in the terrestrial salamandrid *Salamandra salamandra*. *Journal of Herpetology*, **37**, 440–445.

Reinhardt T, Bauldauf L, Ilić M, Fink P (2018) Cast away: drift as the main determinant for larval survival in western fire salamanders (*Salamandra salamandra*) in headwater streams. *Journal of Zoology*, **306**, 171-179.

Richardson JL (2012) Divergent landscape effects on population connectivity in two co-occurring amphibian species. *Molecular Ecology*, **21**, 4437–4451.

Rodríguez A, Burgon JD, Lyra M, Irisarri I, Baurain D, Blaustein L, Göçmen B, Künzel S, Mable BK, Nolte AW, Veith M, Steinfartz S, Elmer KR, Philippe H, Vences M (2017) Inferring the shallow phylogeny of true salamanders (Salamandra) by multiple phylogenomic approaches. *Molecular Phylogenetics and Evolution,* **115**,16–26

Rousset F (2008) GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, **8**, 103–106.

Russell AP, Bauer AM, Johnson MK (2005) Migration in amphibians and reptiles: an overview of patterns and orientation mechanisms in relation to life history strategies. In: *Migration of organisms* (ed Elewa AMT), 151-203. Springer-Verlag, Berlin Heidelberg.

Sánchez-Montes G, Wang J, Ariño AH, Martínez-Solano I (2018) Mountains as barriers to gene flow in amphibians: quantifying the differential effect of a major mountain ridge on the genetic structure of four sympatric species with different life history traits. *Journal of Biogeography*, **45**, 318–331

Sandberger-Loua L, Rödel M-O, Feldhaar H (2018) Gene flow in the clouds: landscape genetics of a viviparous, montane grassland toad in the tropics. *Conservation Genetics*, **19**, 169–180.

Santos AM, Cabezas MP, Tavares AI, Xavier R, Branco M (2015) tcsBU: a tool to extend TCS network layout and visualization. *Bioinformatics*, **32**, 627-628.

Schulte U, Küsters D, Steinfartz S (2007) A PIT tag based analysis of annual movement patterns of adult fire salamanders (*Salamandra salamandra*) in a Middle European habitat. *Amphibia-Reptilia*, **28**, 531–536.

Schwartz MK, McKelvey KS (2009) Why sampling scheme matters: the effect of sampling scheme on landscape genetic results. *Conservation Genetics*, **10**, 441–452.

Segev O, Blaustein L (2014) Influence of water velocity and predation risk on fire salamander (*Salamandra infraimmaculata*) larval drift among temporary pools in ephemeral streams. *Freshwater Science*, **33**, 950–957.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, **9**, 615–629.

Shine, R (2014) Evolution of an Evolutionary Hypothesis: A History of Changing Ideas about the Adaptive Significance of Viviparity in Reptiles. *Journal of Herpetology*, **48**, 147–161.

Shine R (2015) The evolution of oviparity in squamate reptiles: an adaptationist perspective. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, **324**, 487–492.

Slatkin M (1987) Gene Flow and the Geographic Structure of Natural Populations. *The Science*, **236**, 787–792.

Steinfartz S, Veith M, Tautz D (2000) Mitochondrial sequence analysis of *Salamandra* taxa suggests old splits of major lineages and postglacial recolonizations of Central Europe from distinct source populations of *Salamandra salamandra*. *Molecular Ecology*, **9**, 397–410.

Steinfartz S, Kuesters D, Tautz D (2004) Isolation and characterization of polymorphic tetranucleotide microsatellite loci in the Fire salamander *Salamandra salamandra* (Amphibia: Caudata). *Molecular Ecology Resources*, **4**, 626-628.

Steinfartz S, Weitere M, Tautz D (2007) Tracing the first step to speciation: ecological and genetic differentiation of a salamander population in a small forest. *Molecular Ecology*, **16**, 4550–4561.

Storfer A, Murphy MA, Spear SF, Holderegger R, Waits LP (2010) Landscape genetics: where are we now? *Molecular Ecology*, **19**, 3496–3514.

Titus VR, Bell RC, Becker CG, Zamudio KR (2014) Connectivity and gene flow among Eastern Tiger Salamander (*Ambystoma tigrinum*) populations in highly modified anthropogenic landscapes. *Conservation Genetics,* **15,** 1447-1462.

Toews DP, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology*, **21**, 3907-3930.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Veith M, Steinfartz S, Zardoya R, Seitz A, Meyer A (1998) A molecular phylogeny of “true” salamanders (family Salamandridae) and the evolution of terrestriality of reproductive modes. *Journal of Zoological Systematics and Evolutionary Research*, **36,** 7–16.

Veith M, Baubkus M, Kugel S, Kulpa C, Reifenrath T, Schafft M, Wagner N (2019) Drift compensation in larval European fire salamanders, Salamandra salamandra (Amphibia: Urodela)?. *Hydrobiologia*, **828**, 315-325.

Velo-Antón G, García-París M, Galón P, Cordero Rivera A (2007) The evolution of viviparity in holocene islands: ecological adaptation versus phylogenetic descent along the transition from aquatic to terrestrial environments. *Journal of Zoological Systematics and Evolutionary Research*, **45**, 345–352.

Velo-Antón G, Zamudio KR, Cordero-Rivera A (2012) Genetic drift and rapid evolution of viviparity in insular fire salamanders (*Salamandra salamandra*). *Heredity*, **108**, 410–418.

Velo-Antón G, Parra JL, Parra-Olea G, Zamudio KR (2013) Tracking climate change in a dispersal-limited species: reduced spatial and genetic connectivity in a montane salamander. *Molecular Ecology,* **22***,* 3261-3278.

Velo-Antón G, Buckley D (2015) Salamandra común – *Salamandra salamandra*. In: *Enciclopedia Virtual de los Vertebrados Españoles* (eds Martínez-Solano I, Salvador A). Museo Nacional de Ciencias Naturales, Madrid. Availabe: http://www.vertebradosibericos.org

Velo-Antón G, Santos X, Sanmartín-Villar I, Cordero-Rivera A, Buckley D (2015) Intraspecific variation in clutch size and maternal investment in pueriparous and larviparous *Salamandra salamandra* females. *Evolutionary Ecology*, **29**, 185–204.

Velo-Antón G, Cordero-Rivera A (2017) Ethological and phenotypic divergence in insular fire salamanders: diurnal activity mediated by predation? *Acta Ethologica*, **20**, 243-253.

Vences M, Sanchez E, Hauswaldt JS, Eikelmann D, Rodríguez A, Carranza S, Donaire D, Gehara M, Helfer V, Lötters S, Werner P, Schulz S, Steinfartz S (2014) Nuclear and mitochondrial multilocus phylogeny and survey of alkaloid content in true salamanders

of the genus *Salamandra* (Salamandridae). *Molecular Phylogenetics and Evolution*, **73**, 208–216.

Waits LP, Luikart G, Taberlet P (2001) Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Molecular Ecology,* **10***,* 249-256.

Wang IJ (2010) Recognizing the temporal distinctions between landscape genetics and phylogeography. *Molecular Ecology*, **19**, 2605-2608.

Wang J (2007) Triadic IBD coefficients and applications to estimating pairwise relatedness. *Genetic Research*, **89**, 135–153.

Wang J, Santure AW (2009) Parentage and sibship inference from multilocus genotype data under polygamy. *Genetics*, **181**, 1579–1594.

Wang J (2011) Coancestry: A program for simulating, estimating and analysing relatedness and inbreeding coefficients. *Molecular Ecology Resources*, **11**, 141–145.

Wang J (2018) Effects of sampling close relatives on some elementary population genetics analyses. *Molecular Ecology Resources*, **18**, 41–54.

Wang J (2019) A parsimony estimator of the number of populations from a STRUCTURE-like analysis. *Molecular Ecology Resources*.

Waraniak JM, Fisher JDL, Purcell K, Mushet DM, Stockwell CA (2019) Landscape genetics reveal broad a fine-scale population structure due to landscape features and climate history in the northern leopard frog (*Rana pipiens*) in North Dakota*. Ecology and Evolution,* **9,** 1041-1060.

Weckworth BV, Musiani M, DeCesare N, McDevitt AD, Hebblewhite M, Mariani S (2013) Preferred habitat and effective population size drive landscape genetic patterns in an endangered species. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20131756.

Wells KD (2007) *The ecology and behavior of amphibians*. The University of Chicago Press, Chicago,

Wilkinson M, Nussbaum RA (1998) Caecilian viviparity and amniote origins. *Journal of Natural History*, **32**, 1403-1409.

Zellmer AJ, Knowles LL (2009) Disentangling the effects of historic *vs.* contemporary landscape structure on population genetic divergence. *Molecular Ecology*, **18**, 3593–3602.

Zhang P, Papenfuss TJ, Wake MH, Qu L, Wake DB (2008) Phylogeny and biogeography of the family Salamandridae (Amphibia: Caudata) inferred from complete mitochondrial genomes. *Molecular Phylogenetics and Evolution*, **49**, 586–597.

Supplementary material

Table S1. Characteristics of the 14 microsatellites used in this study. Information regarding multiplex distribution and detailed oligonucleotide information of the primers used is displayed (Steinfartz *et al.et al.*, 2004; Hendrix *et al.et al.*, 2010). The forward and reverse primers were at concentrations of 10 µM and 100 µM respectively. The multiplexes had a final volume of 100 µl, containing distilled H_2O plus the specified volumes of forward (PF), reverse primers (PR), and labels (tail). Forward primers (PF) have an extra number of base pairs at the 5' end of the original sequence, in order to allow the specific binding of the four different labelled tails (6-FAM - TGT AAA ACG ACG GCC AGT; VIC - TAA TAC GAC TCA CTA TAG GG; NED - TTT CCC AGT CAC GAC GTT G; PET - GAT AAC AAT TTC ACA CAG G). This table is adapted from Supplementary Material 2 of Álvarez *et al.* (2015).

Locus	Multiplex	Primer forward (5´→3´)	Primer reverse (5´-3´)	Tail	PF (1:10)	PR
SST-A6-I [2]	Panel S1	TTCAGTGCTCTTGCAGGTTG	AGTCTGCAAGGATAGAAAGATCG	2.0 µl (PET)	2.0 µl	2.0 µl
SST-A6-II [2]	Panel S1	ATTCTCTCTGACAAGGATTGTGG	GGTAGACAGACATCAAGGCAGAC	2.8 µl (NED)	2.8 µl	2.8 µl
SalE14 [1]	Panel S1	GCTGCCCTCTCTGCCTACTGACCAT	GCCAAGACATGGAACACCCTCCCGC	1.6 µl (VIC)	1.6 µl	1.6 µl
SST-B11 [2]	Panel S2	TCAAACGGTGCCAAAGTTATTAG	TTAATTGGCAGTTTTCTTTCCAG	2.0 µl (PET)	2.0 µl	2.0 µl
SalE12 [1]	Panel S2	CTCAGGAACAGTGTGCCCCAAATAC	CTCATAATTTAGTCTACCCTCCCAC	0.8 µl (VIC)	0.8 µl	0.8 µl
Sal29 [1]	Panel S2	CTCTTTGACTGAACCAGAACCCC	GCCTGTCGGCTCTGTGTAACC	8.0 µl (6-FAM)	8.0 µl	8.0 µl
SalE5 [1]	Panel S3	CCACATGATGCCTACGTATGTTGTG	CTCCTGTTTACGCTTCACCTGCTCC	0.6 µl (6-FAM)	0.6 µl	0.6 µl
SST-C3 [2]	Panel S3	CCGTTTGAGTCACTTCTTTCTTG	TTGCTTTACCAACCAGTTATTGTC	1.4 µl (PET)	1.4 µl	1.4 µl
SalE7 [1]	Panel S3	TTTCAGCACCAAGATACCTCTTTTG	CTCCCTCCATATCAAGGTCACAGAC	0.8 µl (NED)	0.8 µl	0.8 µl
SalE2 [1]	Panel S3	CACGACAAAATACAGAGAGTGGATA	ATATTTGAAATTGCCCATTTGGTA	3 µl (VIC)	3 µl	3 µl
SalE06 [1]	Panel S4	GGACTCATGGTCACCCAGAGGTTCT	ATGGATTGTGTCGAAATAAGGTATC	1.2 µl (VIC)	1.2 µl	1.2 µl
Sal3 [1]	Panel S4	CTCAGACAAGAAATCCTGCTTCTTC	ATAAATCTGTCCTGTTCCTAATCAG	1.2 µl (6-FAM)	1.2 µl	1.2 µl
SalE8 [1]	Panel S4	GCAAAGTCCATGCTTTCCCTTTCTC	GACATACCAAAGACTCCAGAATGGG	0.8 µl (NED)	0.8 µl	0.8 µl
SST-G9 [2]	Panel S4	CCTCGTCAGGGGTTGTAGG	CTTTCCAGGAAGAAACTGAGATG	0.8 µl (NED)	0.8 µl	0.8 µl

[1] Steinfartz S, Kuesters D, Tautz D (2004) Isolation and characterization of polymorphic tetranucleotide microsatellite loci in the fire salamander Salamandra salamandra (Amphibia: Caudata). *Molecular Ecology*, **4**, 626–628.

[2] Hendrix R, Hauswaldt JS, Veith M, Steinfartz S (2010) Strong correlation between cross-amplification success and genetic distance across all members of 'True salamanders' (Amphibia: Salamandridae) revealed by *Salamandra salamandra*-specific microsatellite loci. *Molecular Ecology*, **10**, 1038–1047.

Table S2. AMOVA-Fst values. *P*-values for a 95% significance level are displayed above the diagonal. Significant values are highlighted in bold.

Larviparous rivers					
Eume	North	South	**Anllons**	south	north
North	0	0.007	south	0	0.359
South	**0.012**	0	north	0.003	0
Tambre	East	West	**Ulla**	North	South
East	0	0.081	North	0	0.006
West	0.032	0	South	**0.016**	0
Barosa	North	South	**Lerez**	North	South
North	0	0.001	North	0	0.033
South	**0.034**	0	South	0.009	0
Minho	south	north	**Cávado**	south	north
south	0	0.000	south	0	0.000
north	**0.039**	0	north	**0.024**	0
Pueriparous rivers					
Navia	East	West	**Esva**	East	West
East	0	0.000	East	0	0.108
West	**0.064**	0	West	0.010	0
Narcea	North	South	**Nora**	North	South
North	0	0.000	North	0	0.005
South	**0.054**	0	South	**0.015**	0

Table S3. Mean pairwise relatedness values of TrioML and Queller and Goodnight estimators identified in COANCESTRY for each river and riverside. Mean pairwise relatedness values of both estimators from dyads found across riversides are also displayed. Reproductive mode is displayed (mode).

Population	Mode	R_TRI	R- QuellerGT	R_TRI_across riversides	R_QuellerGt_across riversides
Eume	larv.	0.037	-0.022	0.033	-0.031
north		0.025	-0.053		
south		0.033	-0.036		
Anllons	larv.	0.028	-0.058	0.003	-0.055
south		0.014	-0.129		
north		0.015	-0.125		
Tambre	larv.	0.026	-0.113	0.015	-0.155
east		0.017	-0.328		
west		0.011	-0.200		
Ulla	larv.	0.030	-0.031	0.025	-0.042
north		0.020	-0.071		
south		0.026	-0.057		
Barosa	larv.	0.023	-0.048	0.015	-0.072
north		0.002	-0.147		
south		0.022	-0.077		
Lerez	larv.	0.025	-0.030	0.019	-0.040
north		0.018	-0.064		
south		0.021	-0.059		
Minho	larv.	0.025	-0.042	0.012	-0.078
south		0.019	-0.071		
north		0.016	-0.113		
Cavado	larv.	0.023	-0.038	0.015	-0.055
south		0.020	-0.084		
north		0.011	-0.077		
Navia	puer.	0.032	-0.047	0.006	-0.104
east		0.030	-0.118		
west		0.038	-0.086		
Esva	puer.	0.027	-0.045	0.023	-0.049
east		0.043	-0.102		
west		0.007	-0.087		
Narcea	puer.	0.020	-0.059	0.009	-0.103
north		0.024	-0.171		
south		0.013	-0.098		
Nora	puer.	0.028	-0.028	0.026	-0.026
north		0.025	-0.043		
south		0.016	-0.088		

Table S4. Summary statistics of pairwise relatedness values for dyads belonging to the same riverside (intra) or to different riversides (inter).

Larviparous rivers					
Eume	mean	SD	**Anllóns**	mean	SD
Inter	0.033	0.057	Inter	0.029	0.052
Intra	0.040	0.063	Intra	0.027	0.052
Total	0.037	0.060	Total	0.028	0.052
Tambre	mean	SD	**Ulla**	mean	SD
Inter	0.014	0.030	Inter	0.025	0.043
Intra	0.040	0.066	Intra	0.035	0.059
Total	0.026	0.051	Total	0.030	0.052
Barosa	mean	SD	**Lérez**	mean	SD
Inter	0.015	0.033	Inter	0.019	0.041
Intra	0.030	0.054	Intra	0.030	0.060
Total	0.023	0.046	Total	0.025	0.052
Minho	mean	SD	**Cávado**	mean	SD
Inter	0.012	0.035	Inter	0.015	0.033
Intra	0.037	0.062	Intra	0.032	0.056
Total	0.025	0.052	Total	0.023	0.046
Pueriparous rivers					
Navia	mean	SD	**Esva**	mean	SD
Inter	0.006	0.021	Inter	0.023	0.052
Intra	0.059	0.113	Intra	0.032	0.078
Total	0.032	0.084	Total	0.027	0.066
Narcea	mean	SD	**Nora**	mean	SD
Inter	0.009	0.021	Inter	0.026	0.048
Intra	0.032	0.070	Intra	0.029	0.057
Total	0.020	0.053	Total	0.028	0.053

Table S5. Test of significance of mean pairwise-relatedness differences between groups identified in COANCESTRY, at 95% confidence level: a) TrioML estimator, b) Queller and Goodnight estimator. Significant differences are marked with an X.

a)

Larviparous rivers					
Eume	North	South	**Anllóns**	North	South
North			North		
Between		**X**	Between		
Tambre	East	West	**Ulla**	North	South
East			North		
Between			Between	**X**	

Barosa	North	South	**Lérez**	North	South
North			North		
Between		**X**	Between	**X**	**X**
Minho	North	South	**Cávado**	North	South
North			North		
Between	**X**	**X**	Between		**X**

Pueriparous rivers

Navia	East	West	**Esva**	East	West
East			East		**X**
Between	**X**	**X**	Between	**X**	
Narcea	North	South	**Nora**	North	South
North			North		
Between	**X**	**X**	Between		

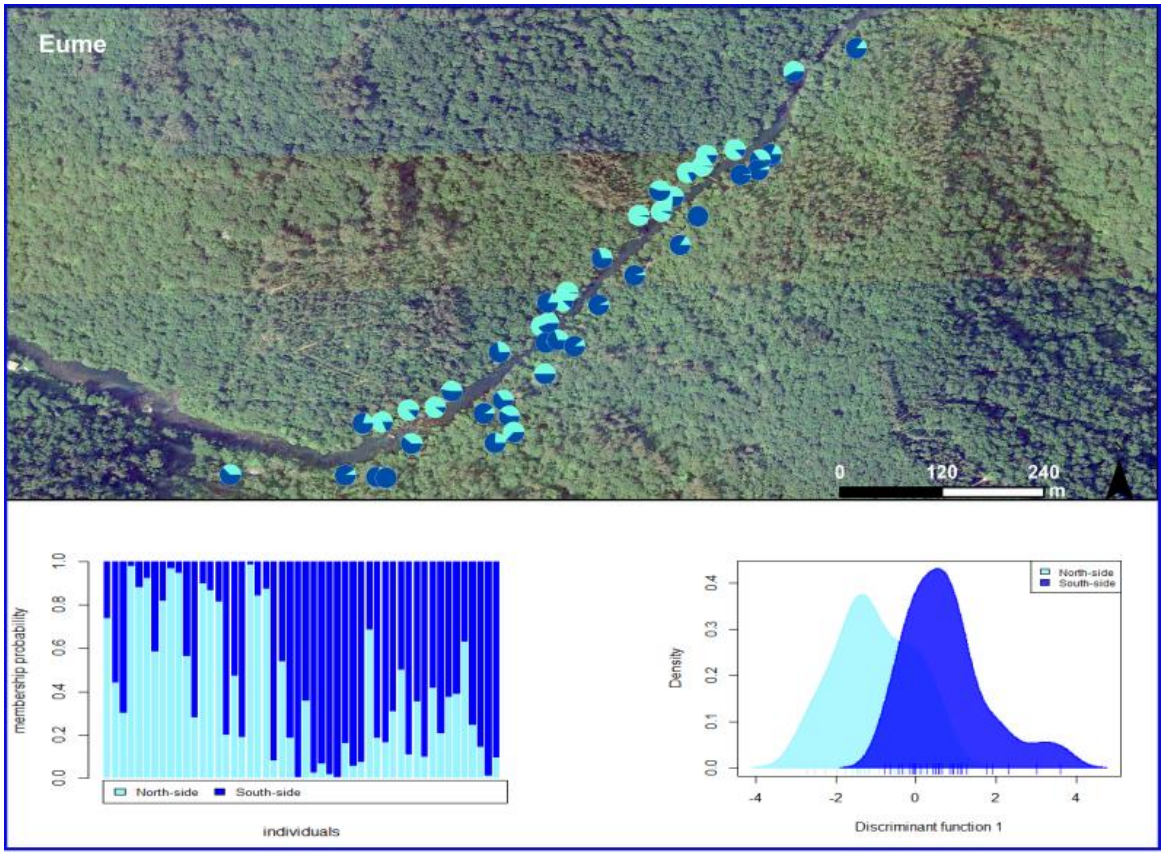
b)

Larviparous rivers

Eume	Norte	Sur	**Anllóns**	Norte	Sur
Norte			Norte		
Between		**X**	Between		
Tambre	East	West	**Ulla**	Norte	Sur
East		**X**	Norte		
Between		**X**	Between	**X**	
Barosa	Norte	Sur	**Lérez**	Norte	Sur
Norte			Norte		
Between	**X**	**X**	Between		
Minho	Norte	Sur	**Cávado**	Norte	Sur
Norte			Norte		
Between	**X**	**X**	Between		**X**

Pueriparous rivers

Navia	East	West	**Esva**	East	West
East			East		
Between	**X**	**X**	Between		
Narcea	North	South	**Nora**	Norte	Sur
North			Norte		
Between	**X**	**X**	Between		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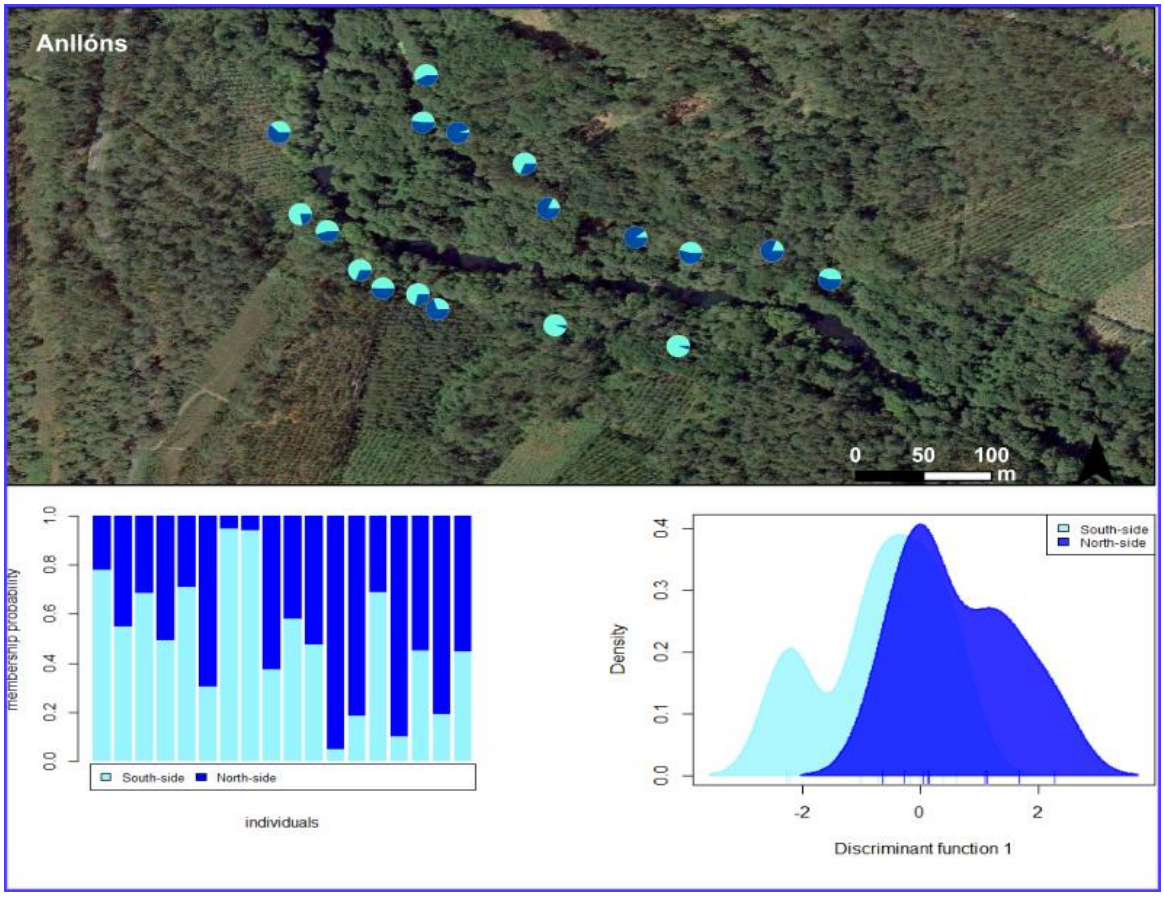
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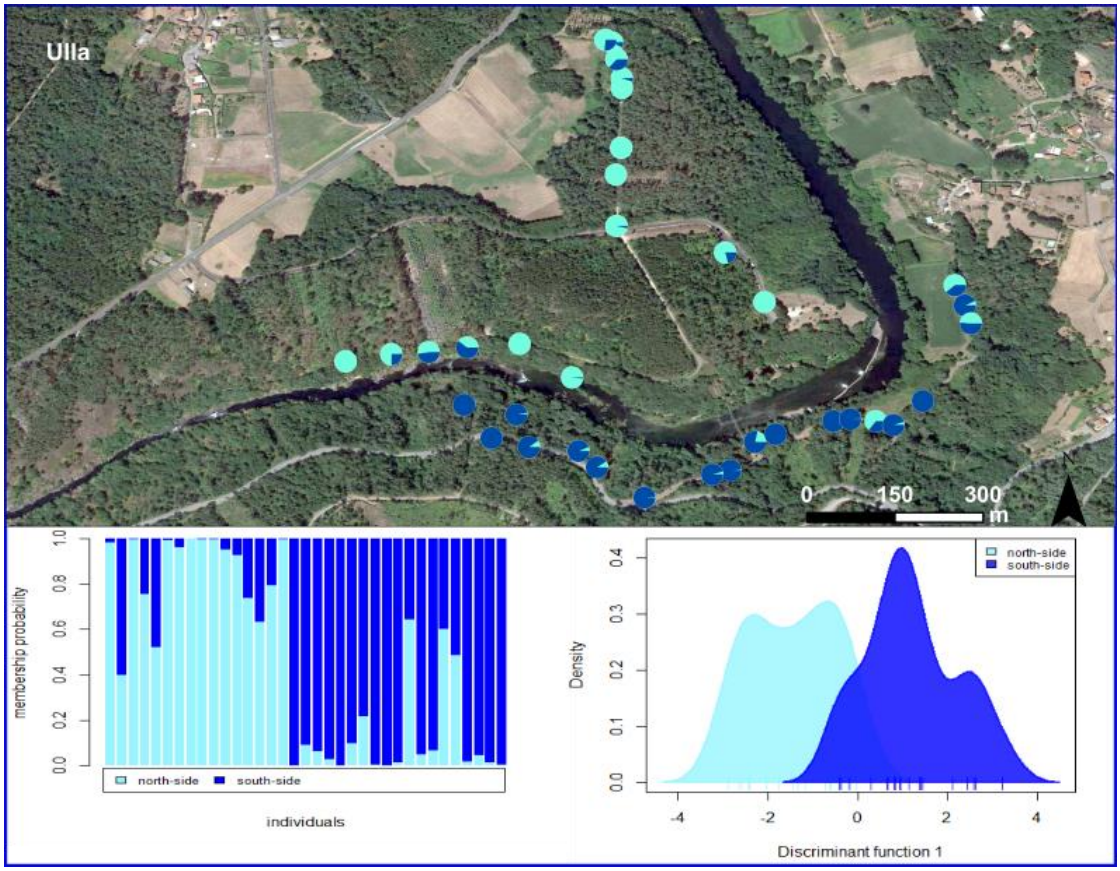
North-side
Discriminant function 1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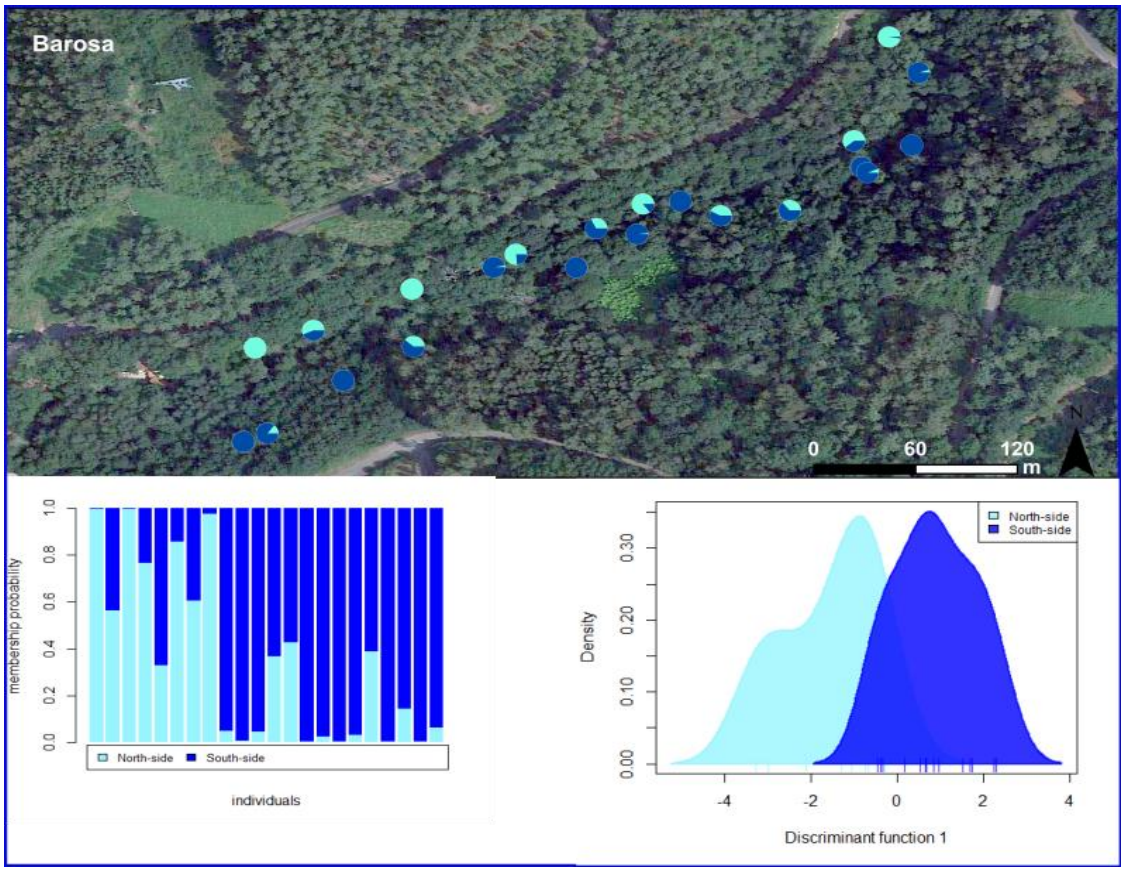
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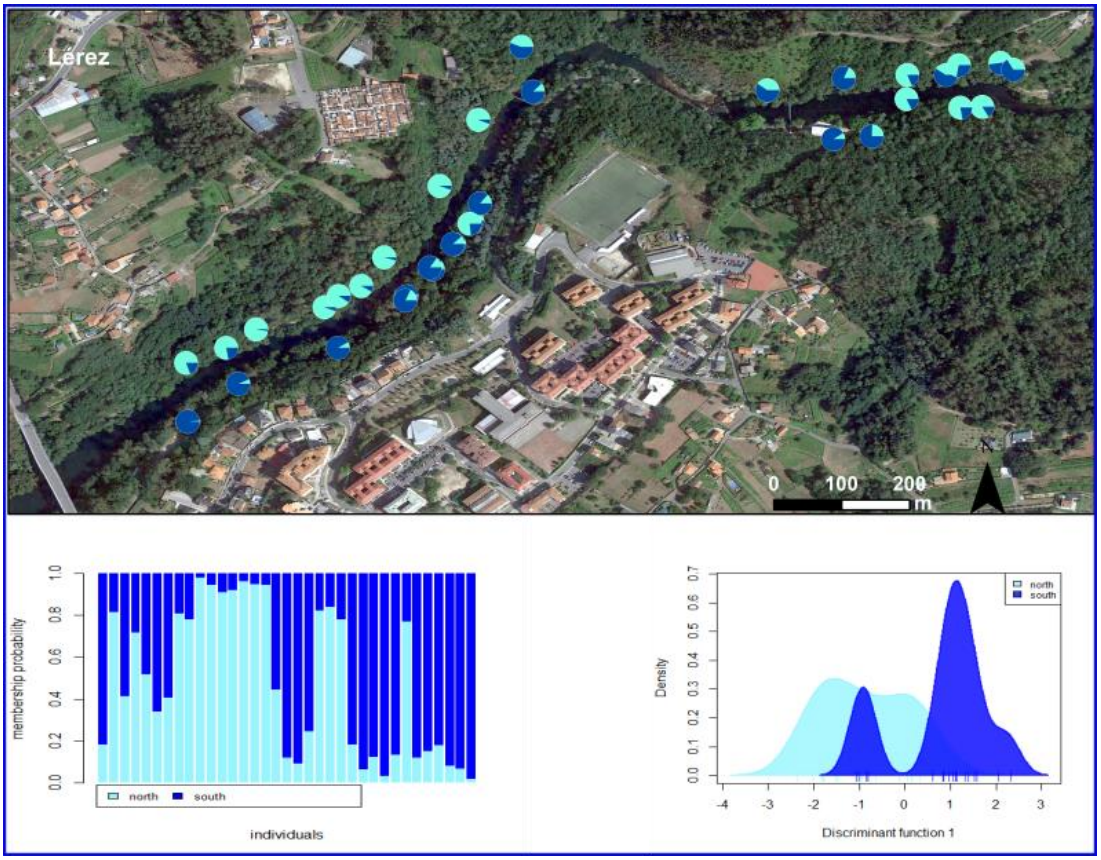
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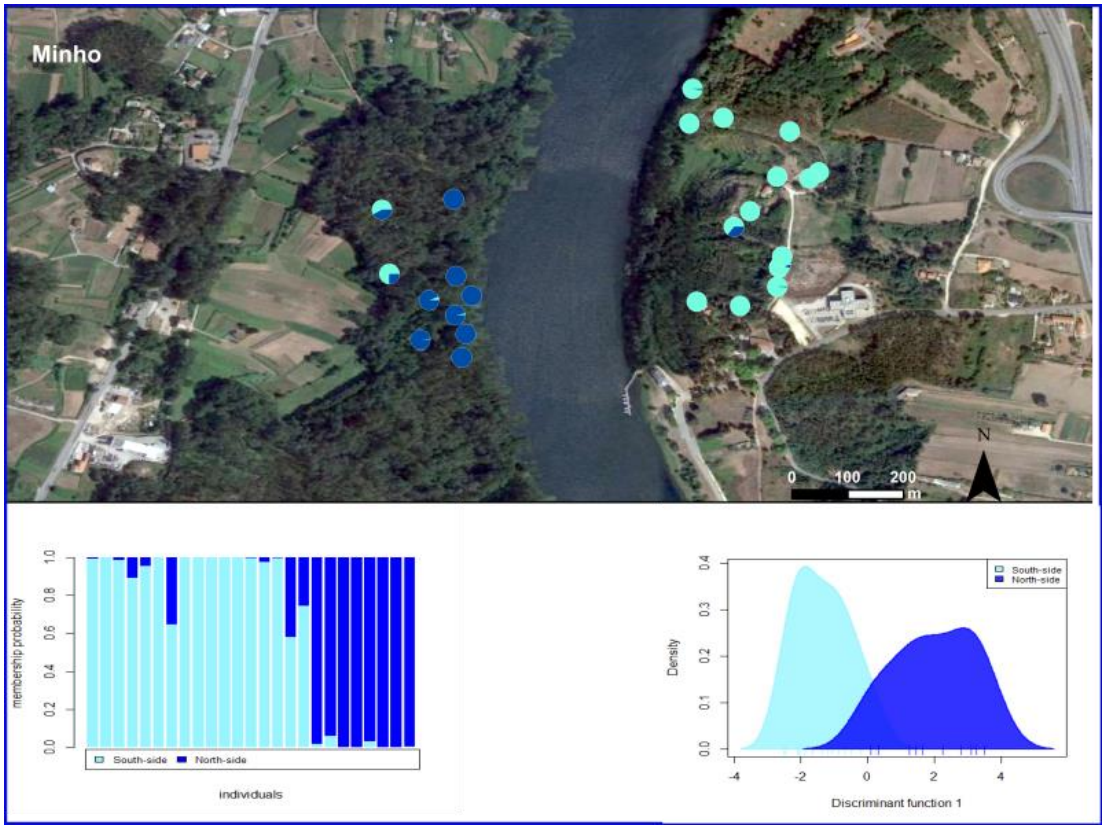
Density
Discriminant function 1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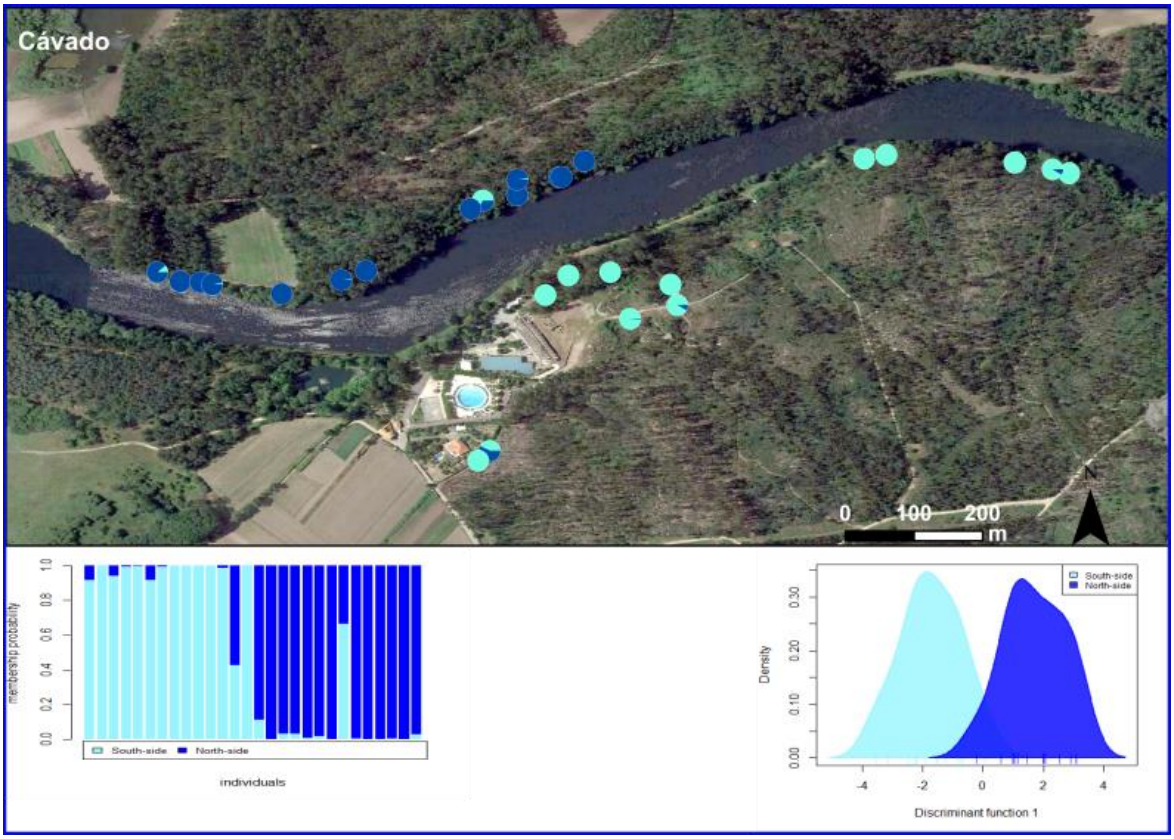
Density
Discriminant function 1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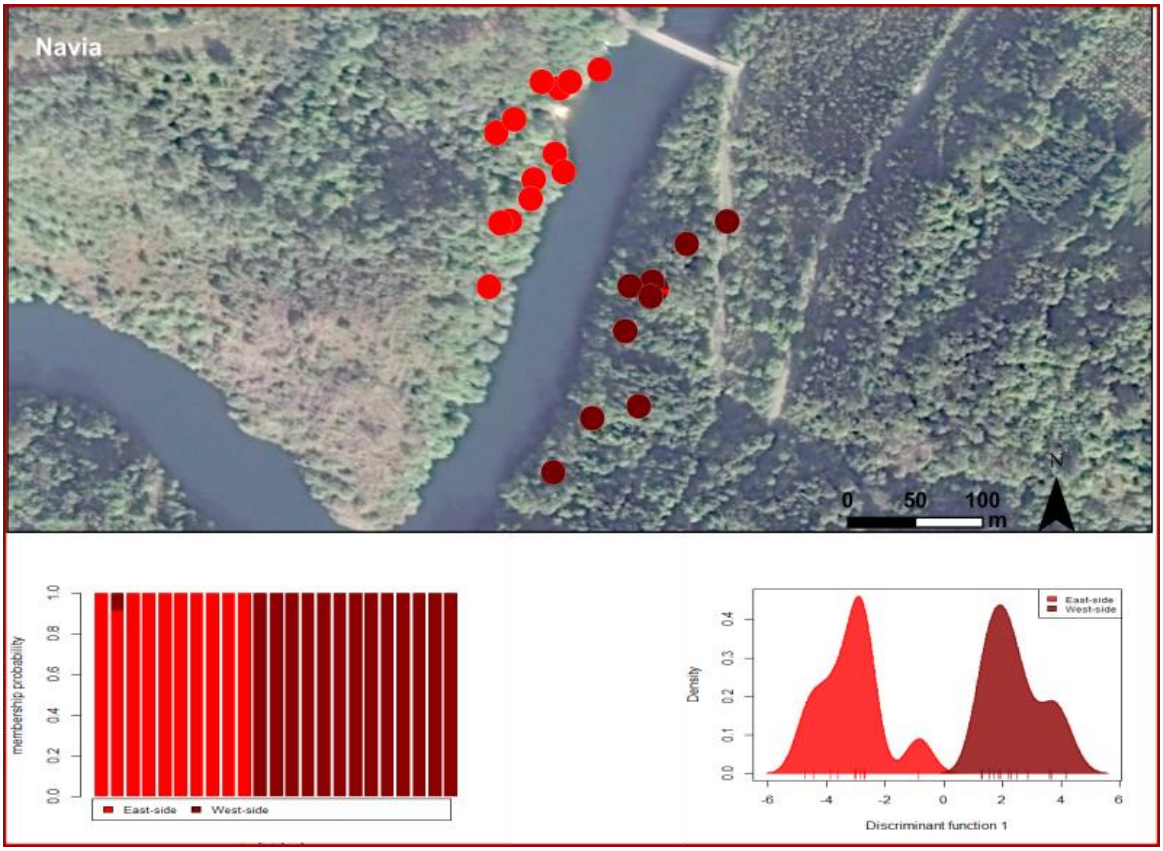
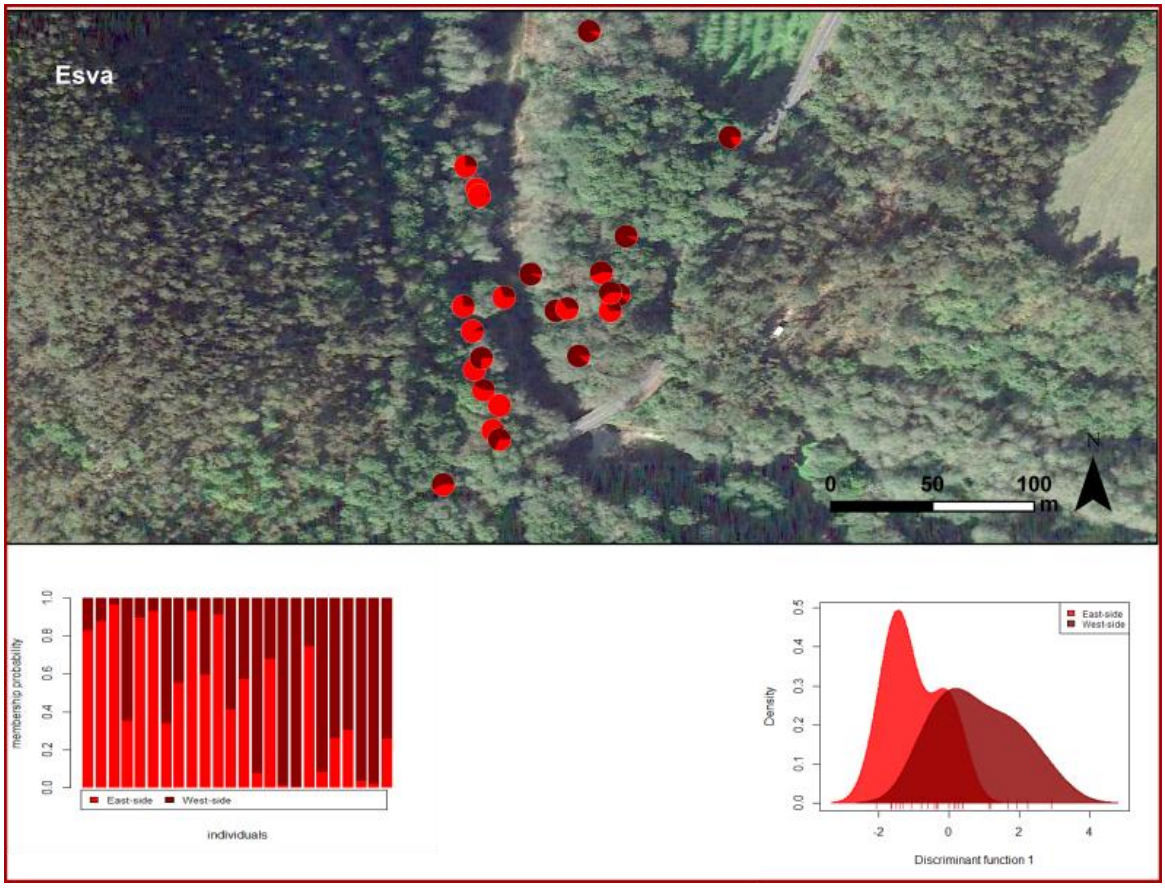
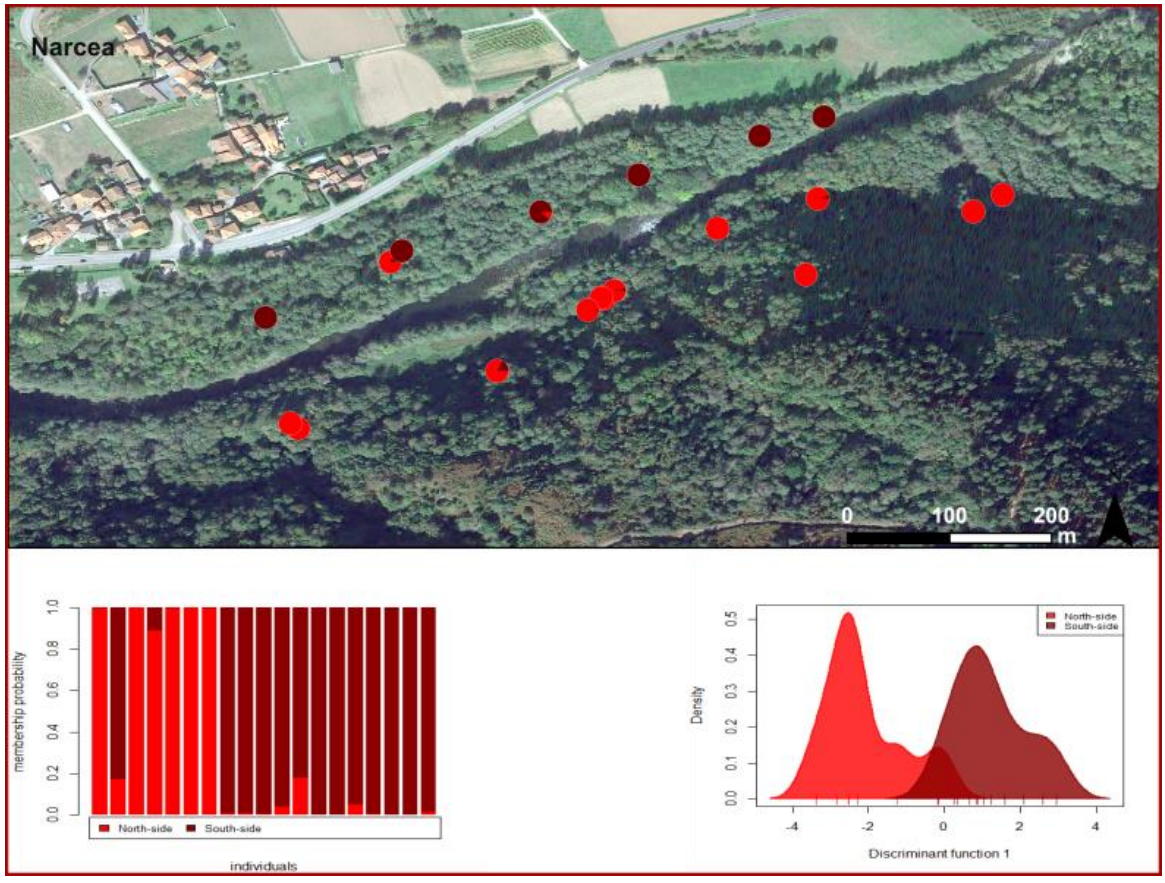
Density
Discriminant function 1


Navia
0 50 100
m
N
membership probability
East-side
West-side
Density
Discriminant function 1


Esva
0
50
100
m
N
membership probability
East-side
West-side
individuals
Density
East-side
West-side
Discriminant function 1


Narcea
0
100
200
m
membership probability
North-side
South-side
individuals
Density
North-side
South-side
Discriminant function 1

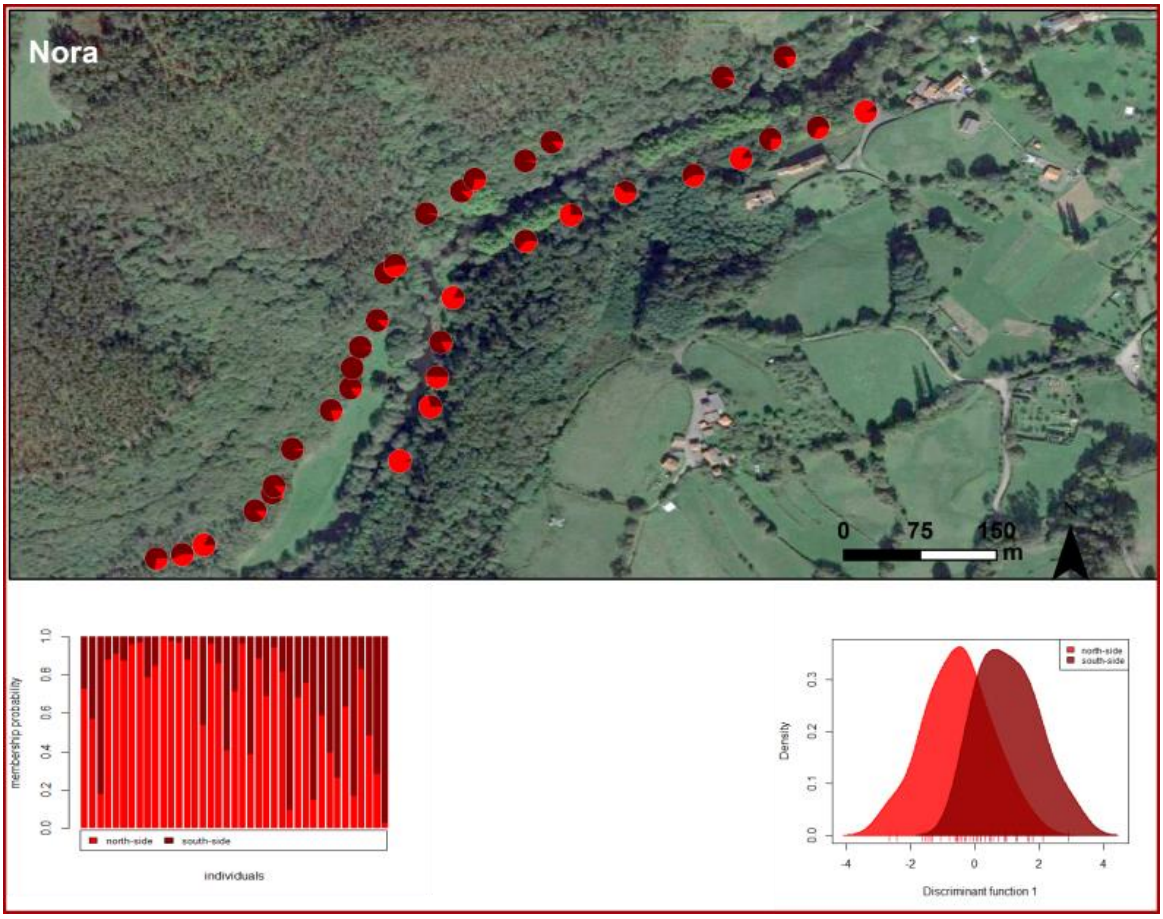


Figure S1. Results from DAPC population clustering analyses for *K* = 2. The map represents the spatial distribution of cluster memberships of each individual. Pie charts represent individual cluster membership to each riverside when *K* = 2. Bottom left figures show the population assignments of individuals to each respective cluster. Bottom right figures show the density of individuals throughout the discriminant function of the DAPC analysis. Each riverside is depicted with a different colour.