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Estrutura genética no limite sul da distribuição Atlântica e filogeografia na região Euroasiática

Agostinho Antunes Pereira

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Agostinho Antunes

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À memória de meus Pais

Declaração

Na elaboração desta dissertação e nos termos do nº2 do Artigo 8º do Decreto-Lei nº388/70, os resultados de trabalhos já publicados foram totalmente aproveitados e fazem parte integrante de alguns capítulos desta dissertação.

Em todos estes trabalhos, o candidato participou na obtenção, interpretação e discussão dos resultados e na elaboração das suas formas publicadas.

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Índice

Resumo

Nos últimos vinte cinco anos a caracterização genética e evolução da truta (*Salmo trutta* L.) na sua área de distribuição natural tem sido alvo de inúmeros estudos. A análise de *loci* proteicos e do DNA mitocondrial levou à descrição de padrões geográficos de diferenciação genética. A espécie consiste num mosaico de linhagens evolutivas dispersas pela sua área de distribuição, influenciadas pelo avanço e recuo dos glaciares, e subsequentes expansões e contacto secundário entre linhagens. A uma escala mais local, descreveram-se cinco grandes linhagens mitocondriais, que se diferenciaram por alopatria durante um longo período de tempo: Danúbio, Adriático, truta marmoreada, Mediterrâneo e Atlântico. A uma escala mais local, observou-se uma diferenciação genética considerável entre populações, e mesmo o isolamento total de *demes* simpátricos, que sugeriram evidências para histórias evolutivas complexas nas regiões do Sul da Europa, incluindo a Península Ibérica outrora um refúgio glaciar.

Com base nestes conhecimentos, estabeleceram-se como objectivos desta tese, aprofundar o estudo da história evolutiva da truta através da caracterização genética de populações pouco ou nada investigadas e a utilização de novas metodologias genéticas e analíticas que permitam a re-análise de cenários filogeográficos anteriormente sugeridos. A um nível microgeográfico, a caracterização genética da truta no limite sul Atlântico de distribuição foi estudada através da variação de *loci* proteicos, microssatélites e DNA mitocondrial, para inferir a história das populações e a sua importância na definição de unidades de conservação. A um nível macrogeográfico, foi determinada a genealogia de um gene nuclear e avaliada a sua utilidade para efectuar inferências filogeográficas ao longo da distribuição Euroasiática do complexo *S. trutta*.

A análise da variação genética de proteínas, sugere que as trutas do Sudoeste Atlântico (em particular, populações Ibéricas Atlânticas) são bastante diferenciadas das da vertente Norte Atlântica e Mediterrânea. O estudo mais detalhado da estrutura genética das populações Ibéricas Atlânticas, com base em proteínas, microssatélites e DNA mitocondrial, revela um padrão populacional regido por uma história evolutiva complexa. A estrutura populacional observada

para os diferentes *loci* estudados pode ser explicada com base em dois acontecimentos principais: (i) fragmentação antiga em mosaico e fluxo génico restrito na região a sul do limite de anadromia, e (ii) a existência de fluxo génico das populações Ibéricas Atlânticas do norte para o sul. O cenário histórico sugere a persistência das populações neste refúgio durante os períodos glaciares e interglaciares do Pleistoceno, causando fortes estrangulamentos populacionais e padrões de estruturação em mosaico. O fluxo génico das populações do Norte da Península Ibérica, seguida de contactos secundários com as populações mais a sul, influenciou também a estrutura genética nesta região. Adicionalmente, os padrões de diversidade genética observados não forneceram evidências para a participação do Sudoeste Atlântico (em particular, a Península Ibérica) na recolonização pós-Pleistocénica do Norte da Europa.

Os elevados níveis de substruturação observados na truta, e os padrões geográficos em mosaico das linhagens evolutivas, demonstraram a necessidade de integrar os resultados numa crítica à definição de unidades de conservação para organismos com populações muito fragmentadas. Grandes unidades, ainda que definidas por clados de DNA mitocondrial, são geralmente demasiado heterogéneas para serem consideradas uma "unidade" a conservar. Alternativamente, uma perspectiva hierarquicamente invertida dando prioridade a populações ou metapopulações é não só mais prática e eficiente para o reconhecimento mas também para a preservação da diversidade evolutiva.

A utilidade dos genes nucleares para inferências evolutivas intraespecíficas foi estudada, através da análise do gene da *transferrina* (*TF*) cujos padrões de diferenciação geográfica são consideráveis ao longo da distribuição Euroasiática do complexo *S. trutta*. A caracterização da variação nucleotídica, correspondendo a uma sequência parcial do gene *TF* levou à detecção de recombinação e conversão génica. No entanto, esses fenómenos não interferem com a observação de uma estrutura cladística evidente tendo ainda sido detectada diferenciação haplotípica posterior à recombinação/conversão génica. A análise combinada da genealogia do gene *TF* com a actual distribuição geográfica dos electromorfos *TF*, permitiu elaborar cenários sobre a origem e radiação da espécie contribuindo, assim, para complementar hipóteses sugeridas anteriormente com base em aloenzimas e DNA mitocondrial.

As populações mais antigas de truta encontram-se nos sistemas hidrográficos que desaguam nos Mares Negro, Cáspio e Aral. A expansão da espécie, a partir do Oeste Asiático para a Europa iniciou-se com uma dispersão ao longo da região Mediterrânea. Na vertente Adriática, a truta marmoreada, apesar de sugerir uma linhagem evolutiva bastante antiga, não foi a primeira a colonizar esta região. Adicionalmente, foi corroborada a hipótese da origem do *carpione*

Italiano (Lago Garda, drenagem Adriática) por hibridação entre a truta marmoreada e a truta do Mediterrâneo. A existência de habitates disponíveis nos sistemas hidrográficos da vertente Atlântica da Península Ibérica permitiram a dispersão inicial a partir da região Mediterrânea. Posteriormente, outra dispersão se seguiu, a partir dessa mesma zona, distribuindo-se amplamente ao longo da região Atlântica, sugerindo uma expansão rápida favorecida pelas oscilações climáticas do Pleistoceno.

Résumé

Dans les vingt-cinq dernières années, la structure génétique de la truite commune (*Salmo trutta* L.) a été intensivement étudiée sur l'ensemble de son aire naturelle de répartition (Eurasie et Afrique du Nord). Les patrons géographiques de différenciation ont été décrits à partir de données de polymorphisme de locus protéiques et de l'ADN mitochondrial. L'espèce apparaît comme une mosaï que des lignées évolutives dispersées sur son aire de répartition, influencée par l'avance et le recul des calottes glaciaires, suivis de phases de dispersion et de contacts secondaires entre lignées. A l'échelle macrogéographique, cinq grandes lignées de l'ADN mitochondrial, ayant probablement évolué en allopatrie sur une longue période de temps, ont été décrites: Danubien, Adriatique, truite marbrée, Méditerranéenne et Atlantique. Au niveau local, des différenciations génétiques modérées à fortes ont été observées entre populations, avec parfois des situations d'isolement reproducteur total entre dèmes sympatriques. L'hypothèse d'une histoire évolutive complexe a pu être avancée pour les populations des régions du sud de l'Europe, et en particulier, celles du refuge glaciaire de la Péninsule Ibérique.

A partir de ce cadre, le principal objectif de cette thèse a été d'entreprendre l'étude détaillée de l'histoire évolutive de la truite commune et d'évaluer des scénarios phylogéographiques déjà proposés pour expliquer la structure actuelle de l'espèce, en utilisant de données provenant de "populations-clés" peu connues et de nouvelles méthodologies génétiques et analytiques. Au niveau microgéographique, la caractérisation de la truite commune dans la limite sud de la distribution Atlantique a été réalisée avec des locus protéiques, microsatellites et l'ADN mitochondrial pour reconstruire l'histoire des populations et déterminer son importance dans la définition des unités de conservation. Au niveau macrogéographique, la généalogie d'un gène nucléaire et son intérêt pour retracer la phylogéographie sur l'ensemble du domaine Eurasien du complexe *S. trutta* ont été étudiés.

L'utilisation des locus protéiques a permis de montrer que les populations de truite commune du sud-ouest Atlantique, en particulier, celle de la Péninsule Ibérique, étaient nettement différenciées des populations nord-Atlantiques et

Méditerranéennes. Une étude plus précise de la structure génétique des protéines, microsatellites et de l'ADN mitochondrial des populations Ibériques de l'Atlantique suggère que la structure populationnelle actuelle résulte d'une histoire évolutive complexe. La structure spatiale observée pour les différents locus peut être expliquée par deux événements principaux: (i) une fragmentation historique en mosaï que et un flux génétique restreint dans l'aire située au sud de la limite de l'anadromie, et (ii) un flux génique des populations du nord de l'Ibérie vers le sud. La fragmentation historique est compatible avec la longue persistance des populations dans ce refuge pendant les périodes glaciaires et interglaciaires du Pléistocène, accompagnée d'une réduction drastique des effectifs des populations et donc de l'émergence de structurations en mosaï que. Le flux génique des populations du nord de l'Ibérie, suivi de contacts secondaires avec les populations plus au sud, a aussi influencé la structure génétique de l'espèce dans cette région. De plus, les patrons de diversité génétique observés conduisent à rejeter l'hypothèse d'une participation des peuplements du sud-ouest Atlantique (en particulier, ceux de la Péninsule Ibérique) lors de la recolonisation post-Pléistocène du nord de l'Europe.

Le fort niveau de structuration observé chez la truite commune, caractérisé par des patrons géographiques en mosaï que des lignées évolutives, souligne la nécessité d'intégrer les données obtenues à l'échelle microgéographique dans la définition des "unités" de conservation chez les organismes à forte fragmentation des populations. Les unités macrogéographiques, telles que celles qui ont été définies à partir des données mitochondriales, sont, en général, trop hétérogènes pour être considérées comme des "unités" de conservation. Par contre, une démarche inverse donnant la priorité aux populations ou métapopulations paraît beaucoup plus adaptée à la caractérisation et la préservation de la diversité évolutive.

L'utilité des gènes nucléaires pour établir des scénarios évolutifs intraspécifiques a été évaluée en sélectionnant un gène nucléaire présentant un patron de différenciation géographique très marqué dans le complexe *S. trutta*. Des séquences nucléotidiques correspondant à une portion du gène de la *transferrine* (*TF*) ont été étudiées sur toute l'aire Eurasienne de l'espèce. Malgré la détection d'événements de recombinaison et conversion génique, la structure cladistique principale attendue apparaît toujours et une structuration cladistique additionnelle postérieure à ces événements est aussi observée. Le principal signal observé dans cette généalogie nucléaire est lié avec l'actuelle distribution spatiale des électromorphes de *TF*. Il offre la possibilité de mettre en avant de nouveaux scénarios phylogéographiques et de reconsidérer les hypothèses précédemment élaborées à partir des données enzymatiques et mitochondriales.

Ainsi l'interprétation des nouvelles données nucléaires suggère que les plus anciennes populations de truite commune proviennent des bassins des Mers Noire, Caspienne et d'Aral. L'expansion de l'espèce à partir de l'ouest de l'Asie vers l'Europe se serait effectuée par dispersion à travers la région Méditerranéenne. Bien que la truite marbrée constitue un rameau évolutif très ancien, elle ne semble pas avoir été la première à envahir les bassins de l'Adriatique. L'hypothèse de l'origine par hybridation du *carpione* Italien (lac de Garde, bassin de l'Adriatique) entre la truite marbrée et la truite Méditerranéenne a été confirmée. Les habitats disponibles dans les bassins Atlantiques de la Péninsule Ibérique ont permis une première vague de dispersion à partir de la zone Méditerranéenne. Un second événement de dispersion à partir de la zone Méditerranéenne a été mis en évidence, et la distribution des haplotypes observée sur une vaste partie de la zone Atlantique suggère qu'il s'agisse d'un événement d' expansion géographique rapide favorisé par les fluctuations climatiques du Pléistocène.

Summary

Over the last twenty-five years the brown trout (*Salmo trutta* L.) has been extensively studied at the genetic level across their Eurasia and North Africa native range. Geographical patterns of genetic differentiation were largely observed by the use of protein and mtDNA loci. The species consists of a mosaic of evolutionary lineages scattered across their range, influenced by the advance and retreat of glaciers, subsequent dispersal and secondary contact between lineages. At a large geographical scale, five major mtDNA lineages, which have evolved allopatrically for a long period of time have been described: Danubian, Adriatic, Marbled trout, Mediterranean and Atlantic. At a more local scale, medium to strong genetic differentiation has been observed between populations, including the total isolation of sympatric demes, and evidence for complex evolutionary histories was suggested for southern European areas, including the once glacial refugia in the Iberian Peninsula.

Given this framework the main goals established for this thesis were to detail the study on the brown trout evolutionary history, by providing insights over less studied populations and to use new genetic and analytic tools to overcome the reliability of previous phylogeographic scenarios proposed for the species range. At a microgeographical level, the brown trout population structure at the southern limit of the Atlantic distribution was studied by using protein, microsatellites and mtDNA loci to infer population history and their relevance for the definition of conservation units. At a macrogeographical level, the genealogy of a nuclear gene and their ability for phylogeographic inferences over the Eurasian range of the *S. trutta* species complex was assessed.

The analysis of protein data, suggested that the southwestern Atlantic brown trout (in particular, Atlantic Iberian populations) is well differentiated from that in the north Atlantic and Mediterranean drainages. A more detailed study of protein, microsatellite and mtDNA genetic structure in Atlantic Iberian brown trout suggested a population pattern ruled by a complex evolutionary history. The population spatial structure observed for the different set of loci can be mainly explained by two major events: (i) an historical mosaic fragmentation and restricted gene flow in the area currently to the south of the anadromy limit, and

(ii) a relatively continuous gene flow from northern Iberian populations to the south. The historical scenario suggested a long persistence of populations in this refugial area through the Pleistocene sequential glacial and interglacial periods, causing a strong population bottlenecking and mosaic patterns of substructuring. Southwards gene flow from northern populations followed by secondary contact between allopatric groups further influenced genetic structure in this area. Additionally, the observed patterns of genetic diversity do not show evidence for a significant contribution of southwestern Atlantic populations (in particular, those from Iberian Peninsula) in the post-Pleistocene recolonization of northern Europe.

The high level of substructuring observed in the brown trout, with geographically mosaic patterns of evolutionary lineages, prompted to the integration of the results into a critique of defining "units" of conservation for organisms with highly fragmented populations. Large-scale units, even if diagnosed by mtDNA clades, are often too heterogeneous to be considered a "unit" of conservation. Alternatively, a bottom-up perspective that prioritizes populations or metapopulations is both more practical and more effective in recognizing and preserving evolutionary diversity.

The utility of nuclear genes for intraspecific evolutionary inferences was studied, by selecting a nuclear gene that exhibits considerable patterns of geographical differentiation in the *S. trutta* species complex. The nucleotide sequence, encompassing partially the *transferrin* (*TF*) gene, was surveyed over the species Eurasian range. In spite of the detection of recombination and gene conversion events, substantial cladistic structure was not disrupted, and additional structure was estimated to have emerged after words. The strong geographical signal observed in this nuclear genealogy, coupled with the current spatial distribution of *TF* electromorphs, allows the drawing of empirical phylogeographic assumptions. Accordingly, scenarios for the historical origin and radiation of the species complex were hypothesized, thus providing new insights over previous allozyme and mtDNA inferences.

The most ancient brown trout populations are in the Black, Caspian and Aral Sea drainages. Expansion of the species from western Asia to Europe started with dispersal throughout the Mediterranean region. Within the Adriatic drainages, the marbled trout, although suggesting a quite old evolutionary lineage, was not the first to arise in this region. Moreover, the hypothesis of an introgressive hybridization origin of the Italian carpione (Garda Lake, Adriatic drainage) between the marbled trout and the Mediterranean trout was confirmed. Suitable habitats in Atlantic drainages of Iberian Peninsula allowed an initial dispersal from the Mediterranean region. Latter, another dispersal event took

place, and its widespread distribution throughout the Atlantic region suggests a very fast expansion favored by the Pleistocene climatic fluctuations.

1. Introdução

A truta, *Salmo trutta* L. 1758, é uma espécie da família Salmonidae que se encontra amplamente distribuída pela Europa, estendendo-se ainda a este para a Ásia e a sul para as montanhas do Atlas, no Norte de África (MacCrimmon & Marshall 1968; Elliot 1994). Considerada como uma das espécies de vertebrados que apresenta maior estruturação genética (Ryman 1983; Allendorf & Leary 1988), a truta tem sido alvo de inúmeros estudos. Análises de polimorfismos de proteínas, DNA mitocondrial e microssatélites vieram revelar a existência de várias linhagens diferenciadas e uma complexa história evolutiva, ainda longe de ter sido totalmente esclarecida (Guyomard 1989; Bernatchez *et al.* 1992; Estoup *et al.* 1998; García-Marín *et al.* 1999a; Bernatchez 2001). Esta elevada diversidade genética parece estar relacionada com aspectos peculiares da história natural da espécie. A subdivisão da sua ampla área de distribuição em bacias hidrográficas distintas, assim como a ocorrência de múltiplas colonizações correspondentes a diferentes períodos geológicos (glaciações do Pleistoceno) terão contribuído significativamente para esta complexa estruturação populacional (Behnke 1972; Grant *et al.* 1999; Laikre *et al.* 1999).

1.1. História natural

1.1.1. Distribuição e ciclo de vida

Dentro dos peixes dulciaquícolas da região Paleártica, a truta apresenta uma das distribuições mais amplas (figura 1.1). A sua área de distribuição natural estende-se a norte, até à Noruega e às regiões do Nordeste da Rússia e, a sul, até às montanhas do Atlas no Norte de África. De oeste para este, ocorre da Islândia ao Tadjiquistão. No Norte da Europa as populações exibem, em geral, elevadas densidades e encontram-se uniformemente distribuídas. Mais para sul, devido ao aumento da temperatura, encontram-se fragmentadas, restringindo-se geralmente às regiões montanhosas (García-Marín & Pla 1996). Para além da sua distribuição nativa, a truta foi introduzida com sucesso em pelo menos vinte e quatro países, estando presente em todos os continentes com excepção da Antártida (MacCrimmon & Marshall 1968; Elliott 1994).

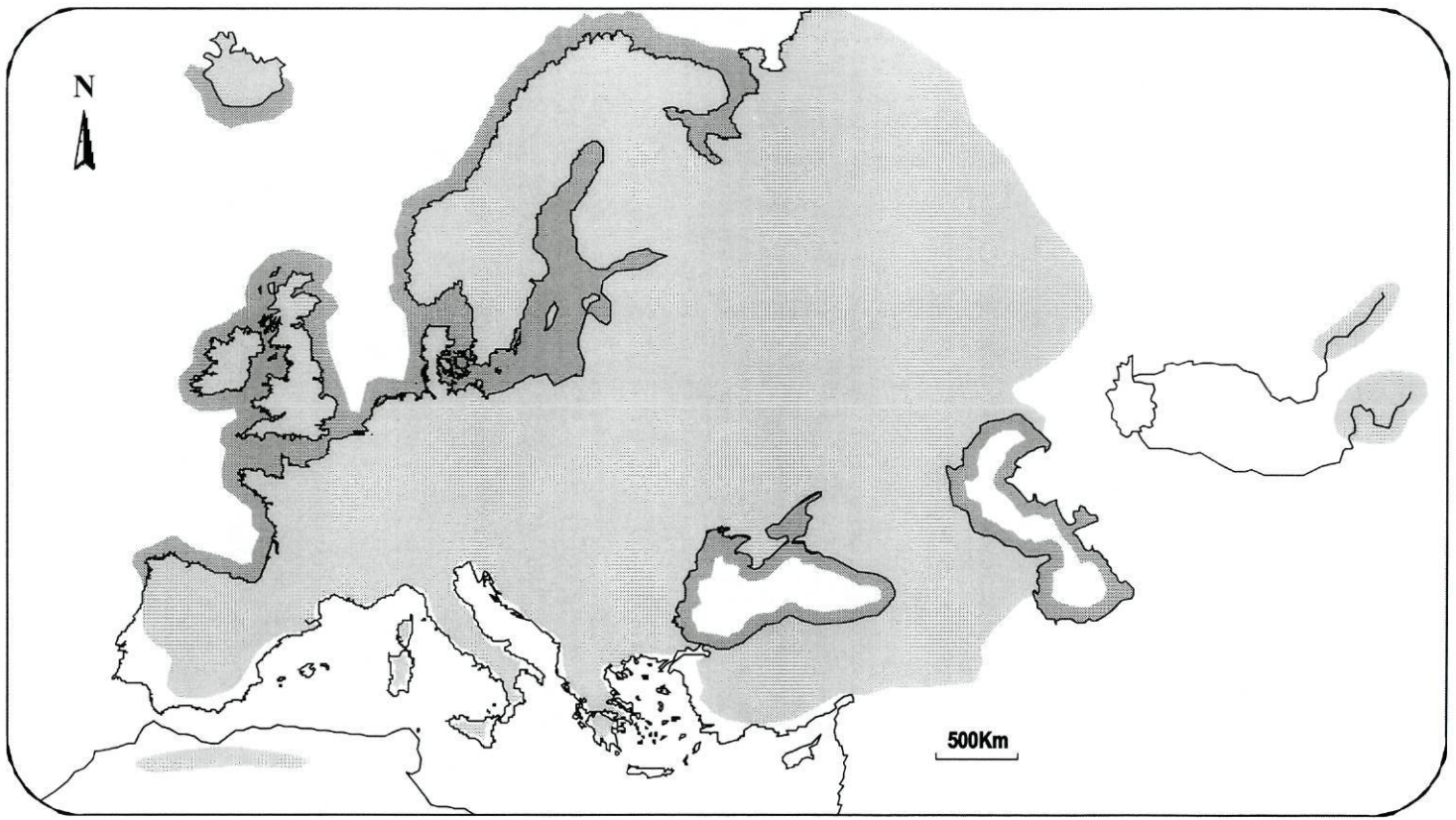

Figura 1.1. Distribuição nativa de *Salmo trutta* L. (adaptado de Guyomard 1989; Elliot 1994). A área a cinzento escuro indica a presença da forma migradora.

Em várias espécies de salmonídeos, como por exemplo *Oncorhynchus mykiss*, *O. clarki*, *Salvelinus alpinus* e *S. fontinalis*, verifica-se a existência das formas ecológicas migradora e sedentária. Em *Salmo trutta*, a forma migradora ocorre nas regiões Atlânticas de latitudes superiores a 42°N, em regiões com rios a desaguar no Mar Branco e Golfo de Cheshkaya, Mar Báltico, Mar do Norte, Mar da Irlanda, Canal da Mancha e Oceano Atlântico até ao sul do Golfo de Biscaia (figura 1.1). No Mar Mediterrâneo não existem trutas migradoras, havendo, no entanto, registos da sua presença no Mar Negro e no Mar Cáspio (Elliot 1994; Bernatchez & Osinov 1995). Através da análise electroforética da variabilidade de aloenzimas não foi detectada a existência de diferenciação genética entre indivíduos migradores e sedentários que coexistem em simpatria (Hindar *et al.* 1991; Cross *et al.* 1992). A análise de marcadores mais polimórficos, nomeadamente microssatélites e DNA mitocondrial, veio confirmar estes resultados (Pettersson *et al.* 2001).

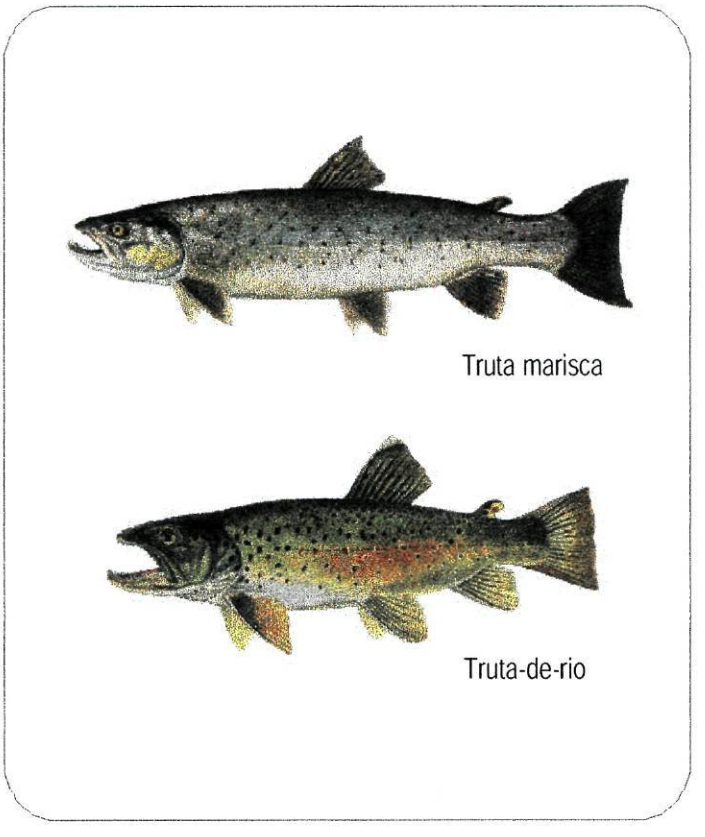

Figura 1.2. Formas migradora (truta marisca) e sedentária (truta-de-rio) de *Salmo trutta* L. (adaptado das ilustrações de Hísek em Pivnicka & Cerný 1988).

A truta-de-rio (forma sedentária; figura 1.2) torna-se sexualmente madura em água doce, sem migrar para o mar. A truta marisca (forma migradora; figura 1.2) possui um ciclo de vida dividido entre condições dulciaquícolas e marinhas. Depois da desova em água doce, os juvenis permanecem no rio natal por períodos de 1 a 4 anos. No final desse período registam-se alterações dos processos fisiológicos e a coloração castanho esverdeada sofre uma gradação para o tom prateado em consequência do processo designado por *smoltificação*. Segue-se uma migração para o mar, onde as condições aí existentes possibilitam um crescimento superior. A maturidade sexual pode ser atingida no próprio ano de migração (Krieg 1984) ou, eventualmente, prolongar-se até aos três anos de vida no meio marinho (Skaala & Nævdall 1989). Experiências com indivíduos marcados demonstraram que as migrações se podem estender por distâncias até aos 50 km da foz do rio natal (Skaala & Nævdall 1989). No entanto, um forte comportamento reprodutivo inato, designado por *homing*, assegura que os indivíduos migradores regressem ao seu rio natal para se reproduzirem. Contudo, o *homing* não se verifica com a mesma intensidade em todos os indivíduos, existindo sempre alguns errantes que migram para outros locais (Thorpe & Mitchell 1981). Morán *et al.* (1995), ao estudarem populações vizinhas de *S. trutta*, nas Astúrias (Norte de Espanha), constataram a existência de um elevado fluxo génico entre as populações da mesma bacia hidrográfica. Este facto foi igualmente detectado entre populações de diferentes bacias hidrográficas, observando-se uma relação inversa com a distância entre os rios. Esta similaridade encontrada entre rios próximos foi explicada com base na existência de indivíduos migradores que não regressam ao seu rio natal, reproduzindo-se, então, em rios das proximidades.

1.1.2. Taxionomia e evolução

A truta pertence a uma família primitiva de peixes teleósteos, Salmonidae, cujas relações com outras famílias da Ordem Salmoniformes não são ainda bem compreendidas (Lauder & Liem 1983). Devido à escassez de registos fósseis, as inferências taxonómicas nos salmonídeos baseiam-se, predominantemente, no estudo comparativo de caracteres morfológicos, bioquímicos e moleculares. A família parece ter evoluído a partir de um ancestral tetraplóide, no Cretácio, há cerca de 50 a 100 milhões de anos (Allendorf & Thorgaard 1984; Behnke 1992). Durante o Eoceno, há aproximadamente 40 a 50 milhões de anos, a família deu origem a três subfamílias que actualmente se encontram amplamente distribuídas pelo Hemisfério Norte: Coregoninae, Thymallinae e Salmoninae, esta última incluindo trutas e salmões (Stearley & Smith 1993). No Oligoceno, há 30 a 40

milhões de anos, a subfamília Salmoninae dividiu-se em dois ramos principais: um parece ter dado origem aos géneros *Hucho*, *Brachymystax* e *Salvelinus*, e o outro aos géneros *Salmo* e *Oncorhynchus* (Kendall & Behnke 1984). A separação destes últimos ocorreu provavelmente no Mioceno médio, há aproximadamente 15 milhões de anos, tendo o seu processo de especiação ocorrido possivelmente há cerca de 5 milhões de anos (Behnke 1992).

Apesar de a família Salmonidae ser um grupo de peixes bem estudado, várias questões filogenéticas, a diferentes níveis taxonómicos, permanecem por esclarecer ou estão ainda em discussão (Sanford 1990; Kottelat 1997; Oakley & Phillips 1999). Dentro do género *Salmo* reconhecem-se pelo menos duas espécies: a truta (*S. trutta*) e o salmão do Atlântico (*S. salar*). Devido a dificuldades de classificação com base na extensa diversidade morfológica e genética de *S. trutta*, a sua taxionomia continua particularmente controversa (p.ex. Elliot 1994; Kottelat 1997). Alguns autores sugerem que certas formas devam ser reconhecidas como espécies distintas, tendo sido designadas mais de 50 desde a segunda metade do século XVIII. Contudo, o reconhecimento de algumas formas identificadas sobretudo com base em diferenças morfológicas é pouco objectivo devido à reconhecida influência das condições ambientais neste tipo de caracteres, não implicando essa diferenciação, necessariamente, a existência de divergência genética (Ryman 1983; Krieg & Guyomard 1985; Bernatchez et al. 1992; Bernatchez 1995). Mesmo na presença de unidades geneticamente diferenciadas, é difícil identificar essas unidades como espécies, subespécies ou populações diferenciadas dentro da mesma espécie. Recentemente, Kottelat (1997) propôs o reconhecimento de mais de 20 espécies, sugerindo a possibilidade de esse número ser ainda incompleto. Alternativamente, outros autores têm preferido considerar a truta como uma espécie politípica, frequentemente designada por complexo *Salmo trutta* (p.ex. Elliot 1994; Bernatchez 1995). Esta confusa situação taxonómica não é exclusiva da truta, verificando-se situações idênticas noutras espécies de salmonídeos (Allendorf & Leary 1988; Bernatchez 1995).

1.2. Diversidade genética

1.2.1. Estudo da diversidade genética

Até aos anos cinquenta, o reconhecimento da extensa variabilidade das populações de *S. trutta* baseava-se essencialmente em diferenças morfológicas e ecológicas. As primeiras evidências de diferenciação genética foram obtidas com base em estudos electroforéticos de proteínas. Estes trabalhos, limitados

inicialmente a populações do noroeste da distribuição da espécie, nomeadamente da Suécia (Allendorf *et al.* 1976; Ryman *et al.* 1979; Ryman 1981), Irlanda (Ferguson & Mason 1981; Crozier & Ferguson 1986) e França (Guyomard & Krieg 1983; Krieg & Guyomard 1985), demonstraram a existência de uma elevada diferenciação genética entre populações geograficamente distintas e, por vezes, detectável também em situações de simpatria. Posteriormente, outros trabalhos realizados nomeadamente na ex-União Soviética (Osinov 1984), Noruega (Skaala & Nævdall 1989), Espanha (García-Marín *et al.* 1991; Martínez *et al.* 1993; García-Marín & Pla 1996), Suíça (Largiadèr & Scholl 1995, Largiadèr *et al.* 1996), Itália (Giuffra *et al.* 1996), Grécia (Karakousis & Triantaphyllidis 1990; Apostolidis *et al.* 1996a) e Turquia (Togan *et al.* 1995), vieram consolidar o conhecimento da estrutura genética de *S. trutta* ao longo da sua área de distribuição.

A uma escala microgeográfica, foi observada em várias regiões europeias a existência de uma elevada estruturação populacional (p.ex. Ryman *et al.* 1979; Crozier & Ferguson 1986; García-Marín & Pla 1996; Bouza *et al.* 1999, 2001, Sanz *et al.* 2000). Esta diferenciação resulta, em parte, do *homing*, que contribui para um limitado fluxo génico entre populações, podendo este ser ainda reduzido por barreiras geográficas ou ecológicas, que acentuam a diferenciação por isolamento e deriva genética. Adicionalmente, a existência de múltiplas colonizações poderão também justificar o padrão em mosaico da distribuição de diferentes linhagens evolutivas (García-Marín *et al.* 1999a; Grant *et al.* 1999; Laikre *et al.* 1999). Entre as populações estudadas não foi detectada correlação entre distâncias genéticas e geográficas (Ryman *et al.* 1983; Crozier & Ferguson 1986; Ferguson 1989). No entanto, essa correlação foi algumas vezes observada em populações com a forma migradora (Morán *et al.* 1995; Hansen & Mensberg 1998; Bouza *et al.* 1999).

Adicionalmente, a análise de variação genética ao nível do DNA mitocondrial veio introduzir novos dados sobre a história evolutiva da espécie através da identificação de várias linhagens evolutivas, corroborando e estendendo a informação já obtida com base na variação de proteínas (p.ex. Bernatchez *et al.* 1992; Bernatchez 1995, 2001; Bernatchez & Osinov 1995; Apostolidis *et al.* 1996b, 1997; Machordom *et al.* 2000). À escala macrogeográfica, foram propostas cinco linhagens mitocondriais para a distribuição Euroasiática da espécie (figura 1.3). Uma linhagem (*Ma*) designa a truta marmoreada, (*S. trutta marmoratus*), fenotipicamente distinta, e presente apenas em alguns cursos de água que desaguam no Mar Adriático (Giuffra *et al.* 1996). As restantes quatro linhagens são típicas de populações de sistemas hidrográficos geograficamente distintos: Danúbio (*Da*), Adriático (*Ad*), Mediterrâneo (*Me*) e Atlântico (*At*).

Todas estas linhagens parecem ter evoluído por isolamento geográfico durante o Pleistoceno (Bernatchez 2001), período geológico que favoreceu a formação de refúgios glaciares, nomeadamente nas penínsulas do Sul da Europa (Hewitt 1999, 2000).

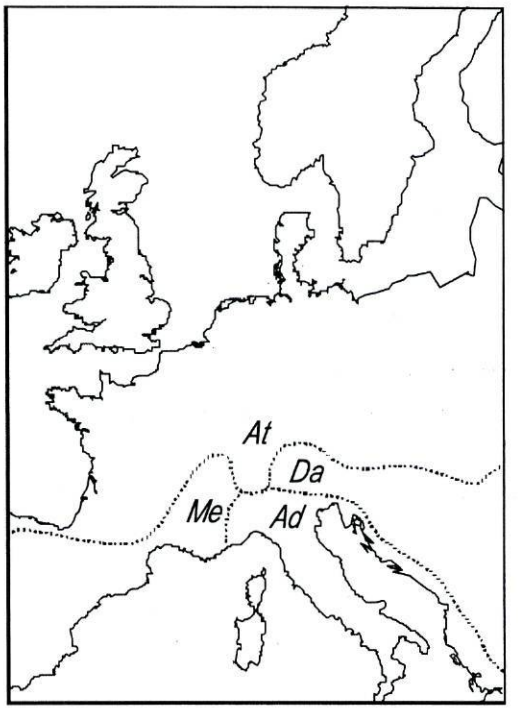

Figura 1.3. Distribuição geográfica das principais linhagens do DNA mitocondrial (adaptado de Bernatchez et al. 1992). A linha a tracejado identifica drenagens distintas, que correspondem a quatro das cinco linhagens evolutivas: Danúbio (*Da*), Adriático (*Ad*), Mediterrâneo (*Me*), e Atlântico (*At*). A linhagem da truta marmoreada (*Ma*), localizada em alguns rios que desaguam no Adriático, não está representada. De acordo com os trabalhos de Bernatchez (1995, 2001) e Bernatchez & Osinov (1995), a linhagem *Da* encontra-se também em outros sistemas hidrográficos que desaguam nos Mares Negro, Cáspio e Aral e a linhagem *At* estende-se a sul, até aos sistemas hidrográficos de drenagem Atlântica, no Grande Atlas (Norte de África).

Mais recentemente, a estrutura genética das populações de *S. trutta* tem vindo, igualmente, a ser estudada através da análise do polimorfismo de *loci* de mini e microssatélites. Estudos realizados com microssatélites (Estoup *et al.* 1993, 1998; Presa *et al.* 1994) e minissatélites (Laikre *et al.* 1995) revelaram, como é típico deste tipo de marcadores, a existência de um polimorfismo mais elevado

relativamente aos *loci* proteicos. Os microssatélites, devido à sua elevada variabilidade, revelaram-se particularmente informativos para análises a uma escala mais fina, nomeadamente em estudos de diferenciação microgeográfica (Estoup *et al.* 1998; Carlsson *et al.* 1999), dinâmica populacional (Hansen *et al.* 1997), estratégias reprodutivas (García-Vázquez *et al.* 2001) e miscigenação de populações naturais com *stocks* de piscicultura (Aurelle *et al.* 1999; Poteaux *et al.* 1999; Estoup *et al.* 2000; Hansen *et al.* 2000).

A utilização de informação relativa à variabilidade genética reflectindo diferentes partes do genoma (proteínas, microssatélites e DNA mitocondrial), tem proporcionado um conhecimento aprofundado da diferenciação e estruturação populacional em *S. trutta.* Contudo, a sua história evolutiva tem sido determinada, em particular, através de inferências genealógicas com base em sequências do DNA mitocondrial (p.ex. Bernatchez *et al.* 1992; Bernatchez 2001).

1.2.2. Diversidade na região dos Mares Negro, Cáspio e Aral

A presença da truta em regiões mais a este, nomeadamente na Rússia e em vários países do Oeste Asiático, suscitou a formulação de diversas hipóteses relativamente à origem e expansão da espécie. Dois cenários alternativos foram sugeridos. (1) Vários autores consideraram a truta como um representante da ictiofauna do Hemisfério Norte, com origem no Atlântico, que apenas recentemente, depois das últimas glaciações há cerca de 15 mil anos, teria penetrado nas bacias hidrográficas que drenam nos Mares Negro, Cáspio e Aral (p.ex. Berg 1928; Balon 1968; Kuderskiy 1974). Segundo este cenário, as colonizações teriam sido possíveis através de conexões das regiões a montante do paleo-Reno com o Danúbio, assim como em outros rios de drenagem sul e norte que, estabelecendo ligação via lagos pós-glaciares, teriam servido como corredores de migração. Foi ainda proposto que a truta poderia também ter penetrado no Mar Negro a partir do Mar Mediterrâneo (p.ex. Berg 1928). (2) Alternativamente, outros autores sugeriram que as populações de truta do Cáucaso estariam relacionadas com o ancestral a partir do qual evoluíram todas as populações dos sistemas hidrográficos que desaguam nos Mares Negro, Cáspio e Aral (Derzhavin 1934; Vladimirov 1944, 1948; Rukhkyan 1989). Estas distribuem-se por uma enorme área geográfica que corresponde a cerca de 50% da área de distribuição da espécie. Contudo, poucas populações de truta desta imensa região geográfica foram estudadas geneticamente. A maioria das populações estudadas exibem exclusivamente haplótipos mitocondriais da linhagem *Da*, apesar de alguns haplótipos *At* terem sido observados em populações a montante, no Danúbio (drenagem do Mar Negro) e no Volga (drenagem do Mar Cáspio)

(Bernatchez *et al.* 1992; Osinov & Bernatchez 1996; Weiss *et al.* 2001). Em ambos os casos, não é possível inferir com exactidão se esses haplótipos resultaram de colonizações naturais ou se foram introduzidos através de repovoamentos. Resultados semelhantes foram obtidos através do estudo da variação genética de proteínas. Os *loci LDH-C*1* e *MEP-1** são aqueles que melhor discriminam os grupos filogeográficos do Atlântico e Danúbio (Bernatchez & Osinov 1995; Riffel *et al.* 1995; Largiadèr & Scholl 1995; Osinov & Bernatchez 1996). O contacto secundário (natural e/ou artificial), seguido de miscigenação entre os dois grupos, parece ter ocorrido em regiões a montante do Danúbio e de rios da drenagem Norte do Mar Cáspio. A ocorrência de haplótipos *Da* numa população da Grécia e numa população da ex-Jugoslávia (drenagem do Adriático) (Bernatchez et al. 1992; Apostolidis et al. 1997), poderá sugerir a existência de contactos secundários adicionais noutras regiões.

Com base em variações morfológicas e ecológicas, as populações dos Mares Negro, Cáspio e Aral foram classificadas em diferentes *taxa*: as populações da drenagem do Mar Negro foram reconhecidas como *S. t. labrax*, as do Mar Cáspio como *S. t. caspius* e as do Mar Aral como *S. t. oxianus* (Berg 1948). Adicionalmente, a truta do Lago Sevan (Arménia, drenagem no Mar Cáspio), morfológica e ecologicamente distinta, foi considerada como uma espécie diferente (*S. ischchan*). Inicialmente, análises de proteínas e DNA mitocondrial não forneceram resultados conclusivos para afirmar a descontinuidade genética entre as populações destas diferentes drenagens, ou a distinção taxonómica de *S. ischchan* (Bernatchez & Osinov 1995; Osinov & Bernatchez 1996). Recentemente, uma análise mais aprofundada ao nível do DNA mitocondrial veio proporcionar elementos genéticos congruentes com a diferenciação morfo-ecológica entre populações das três drenagens (Bernatchez 2001). Contudo, a hipótese de que *S. ischchan* seria derivada do ancestral de todas as populações de truta (Behnke 1986), continua ainda a ter pouco fundamento a nível genético.

1.2.3. Diversidade na região dos Mares Mediterrâneo e Adriático

Nos sistemas hidrográficos que desaguam nos Mares Mediterrâneo e Adriático, o complexo *S. trutta* exibe a sua maior diversidade fenotípica (Behnke 1968). Várias formas com posições taxonómicas variáveis foram reconhecidas nestas áreas, em particular na região dos Balcãs e Turquia (p.ex. *S. trutta macrostigma*, *S. t. dentex*, *S. t. peristericus*, *S. marmoratus*, *S. carpio*, *S. obtusirostris*) (Behnke 1968; Banarescu *et al.* 1971; Economidis & Banarescu 1991; Kottelat 1997; Dorofeeva 1998). No entanto, a nível genético, apenas duas entidades foram claramente distinguidas: *S. marmoratus* e as populações mediterrâneas de *S. trutta*

(p.ex. Bernatchez *et al.* 1992; Giuffra *et al.* 1994, 1996; Berrebi *et al.* 2000a). Algumas das formas identificadas para a Península Balcânica não foram corroboradas por análises genéticas (Karakousis & Triantaphyllidis 1990; Apostolidis *et al.* 1996a, 1996b, 1997).

A truta marmoreada exibe características morfológicas e ecológicas únicas que a distinguem facilmente de outras populações de truta do Mediterrâneo (Behnke 1968). Em termos de classificação, é considerada como uma espécie distinta por alguns autores, *S. marmoratus* (p.ex. Kotellat 1997; Berrebi et al. 2000a), enquanto outros a referem como uma subespécie, *S. trutta marmoratus* (p. ex. Bernatchez 1995; Giuffra *et al.* 1996). A sua distribuição restringe-se a alguns rios de Itália, Eslovénia, Croácia e Albânia (drenagem do Adriático; Berrebi *et al.* 2000a). As distâncias genéticas estimadas com base na variação de proteínas e DNA mitocondrial sugeriram uma diferenciação com cerca de um a três milhões de anos (Giuffra *et al.* 1994, 1996). As populações estudadas indicaram a existência de uma reduzida estrutura populacional, observando-se uma ampla distribuição geográfica de um haplótipo mitocondrial muito frequente (Bernatchez 2001).

Nas populações de *S. trutta* do Mediterrâneo e Adriático, demonstrou-se a existência de uma elevada e complexa estrutura populacional, com base na análise de marcadores nucleares proteicos. Em áreas geográficas reduzidas observaram-se populações exibindo diferenças fixadas para um ou mais *loci*, estando muitas vezes esta heterogeneidade associada a diferenças ecológicas e morfológicas (Krieg & Guyomard 1985; Berrebi 1995; Apostolidis *et al.* 1996a; Giuffra *et al.* 1996). A análise do genoma mitocondrial revelou a existência de duas linhagens nesta região (*Me* e *Ad*; Bernatchez *et al.* 1992; Bernatchez 1995). O padrão geográfico de distribuição da diversidade genética indicou uma redução da variabilidade de este para oeste (Bernatchez 2001). A predominância de um haplótipo mitocondrial, *Me*, muito frequente e com uma ampla distribuição geográfica, é compatível com uma recente expansão demográfica desta linhagem (Bernatchez 2001) que, em alguns casos, se sobrepôs com a linhagem *Ad* em situações de parapatria (Giuffra *et al.* 1996). O padrão este-oeste de redução da variabilidade, foi também observado na linhagem *Ad*. Contrastando com as populações a este, a reduzida estruturação a oeste sugere igualmente uma história recente de expansão demográfica (Bernatchez 2001). A região dos Balcãs apresenta uma das mais elevadas diversidades fenotípicas no complexo *S. trutta*, paralelamente com uma elevada estrutura populacional, o que confirma a fragmentação histórica das populações de truta nesta região (Kottelat 1997).

1.2.4. Diversidade na região Atlântica

O grupo filogeográfico Atlântico, definido com base em análises do DNA mitocondrial (Bernatchez *et al.* 1992), inclui populações de sistemas hidrográficos com drenagem Atlântica desde as montanhas do Atlas, no Norte de África, até à Noruega, estendendo-se ainda para drenagens dos Mares Báltico e Branco. Contudo, a análise simultânea de marcadores proteicos e DNA mitocondrial sugere a existência de diferenças significativas que distinguem as populações Ibéricas Atlânticas de outras localizadas mais a norte (p.ex. García-Marín *et al.* 1991, 1999a; Martínez *et al.* 1993; Bouza et al. 1999; Machordom *et al.* 2000). Durante as glaciações do Pleistoceno, o Norte da Europa permaneceu coberto de glaciares durante longos períodos e as populações dessa região apenas aí residem desde a última glaciação, há aproximadamente 10 a 18 mil anos (Hamilton *et al.* 1989; Laikre *et al.* 1999). Regiões Atlânticas menos influenciadas pelas glaciações, nomeadamente a Península Ibérica, terão permitido a permanência prolongada de populações de truta que, segundo alguns autores, terão funcionado como refúgios a partir dos quais se terá processado, posteriormente, a colonização ou recolonização do Norte da Europa (Hamilton *et al.* 1989).

Com base na ocorrência diferencial de dois alelos do *locus* *LDH-C1**, Hamilton *et al.* (1989) sugeriram que, após as glaciações, o Noroeste da Europa foi colonizado independentemente por duas formas de truta. O primeiro colonizador terá sido a *forma ancestral*, assim designada por possuir o alelo considerado como ancestral, *LDH-C1*100*. Com o movimento de elevação isoestático dos solos e a formação de quedas de água por erosão, as áreas inicialmente colonizadas permaneceram isoladas. Posteriormente, a *forma moderna*, caracterizada pelo alelo *LDH-C1*90*, colonizou o Noroeste da Europa substituindo a *forma ancestral* nos locais de possível acesso. A presença de populações de *S. trutta* fixadas, ou com elevadas frequências do alelo *LDH-C1*100* no Norte de Espanha (García-Marín *et al.* 1991; Martínez *et al.* 1993) e na Bretanha (Krieg & Guyomard 1985; Presa *et al.* 1994) sugere que a *forma ancestral* se expandiu a partir de um refúgio na Biscaia, enquanto que a *moderna* parece ter tido origem na região do Mar do Norte, Mar Báltico ou Mar Branco (Hamilton *et al.* 1989). A análise do DNA mitocondrial efectuada por Bernatchez *et al.* (1992) não permitiu detectar variação entre indivíduos provenientes de diferentes regiões da drenagem Atlântica. Posteriormente, Hynes *et al.* (1996) sugeriram que as colonizações pós-glaciares parecem ter sido bem mais complexas do que o modelo de dupla colonização proposto com base na distribuição das frequências alélicas de *LDH-C1**.

Mais recentemente, com base na variação genética dos *loci LDH-C1** e *CK-A1**, García-Marín *et al.* (1999a) propuseram que o Noroeste da Europa foi colonizado por três linhagens distintas caracterizadas pela fixação ou predominância dos seguintes alelos proteicos: (I) *LDH-C1*90* e *CK-A1*100*; (II) *LDH-C1*100* e *CK-A1*115*; e (III) *LDH-C1*100* e *CK-A1*100*. Segundo estes autores, a colonização efectuou-se por (i) uma radiação para norte e este a partir de um refúgio localizado junto ao Canal da Mancha, (ii) uma expansão para norte a partir de drenagens Atlânticas da Península Ibérica e Sudoeste da França, e (iii) uma migração para noroeste a partir de um refúgio localizado na região do Mediterrâneo-Cáspio. As populações de truta do Noroeste da Europa parecem, assim, resultar de múltiplas colonizações, incluindo vários grupos ancestrais geneticamente diferenciados (García-Marín *et al.* 1999a).

Histórias evolutivas complexas são igualmente sugeridas pela existência de populações simpátricas, isoladas reprodutivamente, em alguns lagos no Norte da Europa. Evidências genéticas para a ocorrência de populações simpátricas foram inicialmente detectadas no Lago Bunnersjöarna, na Suécia (Allendorf *et al.* 1976; Ryman *et al.* 1979). Situações idênticas foram posteriormente identificadas no Lago Melvin (Ferguson & Mason 1981; Ferguson & Taggart 1991) e no Lago Neagh (Crozier & Ferguson 1986), na Irlanda. Desconhece-se ainda, se a ocorrência destas populações simpátricas é um fenómeno comum na truta. Estudos exaustivos, com monitorizações anuais prolongadas e incluindo centenas de indivíduos analisados em lagos na Suécia, sugerem que a existência de populações simpátricas pode ser um fenómeno mais comum do que é presentemente reconhecido (Jorde & Ryman 1996).

1.2.5. Conservação da diversidade genética

Uma grande parte da variabilidade intraespecífica de *S. trutta* foi irremediavelmente perdida devido a interferências antropogénicas e, actualmente, muita da restante variabilidade encontra-se ameaçada (Laikre *et al.* 1999). A degradação ambiental, a sobrepesca e os repovoamentos estão entre as causas mais graves que comprometem a viabilidade de muitas populações de truta (Laikre & Ryman 1996).

Os repovoamentos constituem uma grande ameaça pois são geralmente encarados como benéficos para o incremento das populações naturais mas provocam muitas vezes a extinção do património genético local (p.ex. Ryman & Utter 1987; Allendorf & Leary 1988; Leary *et al.* 1993; Hansen & Loeschcke 1994; Allendorf & Waples 1996). Largiadèr & Scholl (1995), com base em marcadores proteicos, observaram elevadas taxas de introgressão (cerca de 80 a

100 %) de *alelos Atlânticos* em algumas populações nativas do Rio Pó (Suíça, drenagem Adriática). A aparente inexistência de evidências geológicas indicando a migração natural de trutas de drenagem Atlântica para águas de drenagem Adriática e a identificação da origem dos *stocks* domésticos como exclusivos da drenagem Norte Atlântica (Krieg & Guyomard 1985; Presa *et al.* 1994), parecem explicar a elevada taxa de introgressão como resultante de acções de repovoamento nesses locais. Em concordância com esta interpretação, foram observadas taxas de introgressão idênticas em algumas populações de Espanha e França (García-Marín *et al.* 1999b; Berrebi *et al.* 2000b).

A existência de várias ameaças que afectam a integridade da extensa variabilidade intraespecífica do complexo *S. trutta* torna imperativa a criação de estratégias eficientes de gestão e conservação desta espécie politípica (Laikre *et al.* 1999). Um elevado número de estudos revelou a existência de uma grande heterogeneidade genética no *taxon* referido como *S. trutta*. Várias linhagens evolutivas foram identificadas e, dentro dessas, foi observada uma considerável estruturação populacional. Considerar a espécie *S. trutta* como a unidade básica de gestão e conservação constitui uma medida inapropriada e ineficaz. Bernatchez (1995, 2001) sugere as cinco grandes linhagens mitocondriais como as unidades básicas de conservação ou "Unidades Evolutivas Significativas" (*Evolutionary Significant Units* – ESUs; Ryder 1986; Waples 1991; Moritz 1994). Contudo, a elevada complexidade genética detectada no interior de cada uma destas unidades poderá sugerir que esta não é a melhor estratégia em termos de conservação. Por exemplo, uma única linhagem de DNA mitocondrial define as populações de truta localizadas em regiões de drenagem Atlântica (Bernatchez *et al.* 1992). Esta enorme região geográfica, uma das mais afectadas pelas glaciações do Pleistoceno, apresenta populações geneticamente heterogéneas. Uma elevada diferenciação existe entre as populações de regiões Atlânticas não glaciadas (Península Ibérica e Sul de França) relativamente às localizadas mais a norte (Krieg & Guyomard 1985; García-Marín *et al.* 1991; Martínez *et al.* 1993). Adicionalmente, uma considerável subestruturação foi ainda identificada em cada um destes grupos populacionais (p.ex. Ryman *et al.* 1979; Ferguson & Taggart 1991; Crozier & Ferguson 1986; Bouza *et al.* 1999, 2001; Machordom *et al.* 2000; Sanz *et al.* 2000). Esta elevada diversidade genética não será necessariamente preservada se a estratégia básica a adoptar se restringir a considerar grandes unidades de conservação. Laikre *et al.* (1999) consideram então, que as populações locais deverão ser as unidades básicas para gestão e conservação da espécie.

1.3. Objectivos e organização da tese

1.3.1. Enquadramento e objectivos

A presente tese pretende contribuir para o estudo da história evolutiva de *S. trutta*. A um nível microgeográfico, através da caracterização genética de populações no limite sul da distribuição Atlântica e sua relevância para a definição de estratégias de conservação. A um nível macrogeográfico, através da análise filogeográfica ao longo da distribuição Euroasiática do complexo *S. trutta* e sua implicação para a formulação de hipóteses relativas à origem e expansão da espécie.

A Península Ibérica constituiu um refúgio durante as glaciações do Pleistoceno, juntamente com outras penínsulas no Sul da Europa (Hewitt 1999, 2000; Taberlet 1998). Numa revisão recente sobre a variação genética de proteínas em populações de *S. trutta*, García-Marín *et al.* (1999a) referem que de 232 populações analisadas apenas 46 representavam áreas do sul da distribuição da espécie, nomeadamente de França, Espanha, Grécia e Rússia. Neste contexto, o estudo da variação de marcadores nucleares e citoplasmáticos no refúgio glaciar Atlântico Ibérico, onde as populações de truta puderam residir mesmo durante os períodos glaciares do Pleistoceno, será de grande interesse para abordar questões específicas sobre a história evolutiva desta espécie e a sua relevância para a conservação. A importância do estudo da complexa estrutura genética destas populações pode ser constatada pelo elevado número de publicações recentes sobre populações de truta em Espanha (p.ex. Bouza *et al.* 1999, 2001; Cagigas *et al.* 1999; García-Marín et al. 1999a, 1999b; Machordom *et al.* 1999, 2000; Sanz *et al.* 2000). No entanto, podemos considerar que a investigação da diversidade genética deste refúgio glaciar Atlântico carece ainda de detalhe, nomeadamente no que se refere à caracterização de populações de *S. trutta* de Portugal, localizadas no limite sul Atlântico da distribuição da espécie.

Ao longo das últimas duas décadas, padrões geográficos de diversidade genética em *S. trutta* foram identificados pela análise da variação de proteínas e DNA mitocondrial, permitindo determinar a existência de grandes linhagens evolutivas na sua distribuição Euroasiática. Contudo, a análise electroforética da variação de proteínas apresenta limitações para estudos filogenéticos pois não possibilita inferências seguras sobre a ancestralidade de alelos. Assim, as inferências genealógicas sobre a história evolutiva da truta resultam em particular de análises moleculares do genoma mitocondrial (p.ex. Bernatchez 2001). No entanto, as genealogias mitocondriais correspondem apenas a uma pequena

porção do registo histórico de um organismo sexuado (Avise & Wollenberg 1997). Muita da restante história deverá estar representada em genealogias de genes autossómicos. Neste contexto, o estudo da variação molecular de genes nucleares, sua análise e interpretação, poderão permitir abordar questões mais gerais sobre a história evolutiva desta espécie, tais como a sua origem e radiação.

Neste contexto definiram-se os seguintes objectivos principais deste trabalho:

- Estudo da variação de marcadores genéticos nucleares (aloenzimas, proteínas plasmáticas e microssatélites) e citoplasmáticos (região de controlo do DNA mitocondrial) em populações de *S. trutta* na vertente Atlântica da Península Ibérica.
- Interpretação dos padrões geográficos de estruturação genética, e sua importância para a definição de unidades de conservação em *S. trutta*.
- Estudo da variação do gene que codifica a *transferrina* (*TF*) no complexo *S. trutta* ao longo da região Euroasiática.
- Interpretação da genealogia do gene *TF* e sua relevância para o conhecimento da história evolutiva do complexo *S. trutta*.

1.3.2. Organização da tese

Os objectivos definidos neste trabalho são concretizados sob a forma de seis artigos científicos, que foram publicados, estão em publicação, foram submetidos ou estão em preparação.

O primeiro artigo, publicado na revista *Ecology of Freshwater Fish* (Artigo I), permitiu obter uma primeira caracterização genética de populações de truta em Portugal e estabelecer a sua relação com outras populações na Europa. Este estudo apresenta o trabalho inicial de afinação de sistemas de detecção de polimorfismos proteicos, fornecendo evidências para a potencial utilidade do *locus TF* na reconstrução da história evolutiva dessas populações.

O segundo artigo, publicado na *Biochemical Genetics* (Artigo II), resultou da investigação de novos polimorfismos proteicos, pela utilização de técnicas de focagem isoeléctrica no estudo de variação genética de proteínas plasmáticas. A detecção de um *locus* proteico altamente polimórfico (*PX*) permitiu obter uma perspectiva preliminar sobre a estruturação das populações de *S. trutta* em Portugal, num padrão de diferenciação associado com o limite sul de ocorrência da forma migradora.

O estudo da variação genética do DNA mitocondrial nas populações de truta em Portugal surgiu da colaboração estabelecida com investigadores do "Institut für Tierzucht und Genetik" na Universidade de Viena de Áustria, e deu origem à publicação de um terceiro artigo na revista *Molecular Ecology* (Artigo III). A peculiar composição haplotípica das populações estudadas permitiu reavaliar a importância do refúgio glaciar do Sudoeste Atlântico na recolonização pós-glaciar do Norte da Europa. A análise crítica de resultados publicados possibilitou igualmente questionar a possível contribuição do refúgio glaciar do Ponto-Cáspio para a referida recolonização, tal como havia sido recentemente proposto (García-Marín *et al.* 1999a). O elevado interesse dos resultados obtidos justificou um estudo posterior mais detalhado da variação do DNA mitocondrial (Artigo V).

A análise da variação do DNA mitocondrial evidenciou uma elevada substruturação das populações de truta em Portugal (Artigo III e V). Dados preliminares parecem também corroborar esse resultado ao nível nuclear (Artigo II). Os padrões de variação genética resultantes do genoma nuclear (*loci* proteicos e microssatélites) foram investigados de uma forma mais exaustiva em populações localizadas no limite sul da distribuição Atlântica (Espanha e Portugal). Este trabalho ainda em fase de preparação (Artigo IV) permitiu a formulação de hipóteses que expliquem a complexa estruturação encontrada, bem como a sugestão de algumas estratégias para a conservação dessa elevada diversidade.

A dificuldade de conservação da grande heterogeneidade genética em *S. trutta* justificou a utilização das populações de truta de Portugal como um exemplo da dificuldade de definir unidades de conservação nesta espécie politípica. Com base na variação do DNA mitocondrial, e utilizando o método de análise estatística proposto por Templeton *et al.* (1995), foi possível testar a estruturação populacional e separar a história das populações de fenómenos de dinâmica populacional contemporâneos. Os resultados obtidos, permitiram criticar a definição de unidades de conservação recentemente propostas (p.ex. Machordom *et al.* 2000; Bernatchez 2001). Este estudo está em publicação na revista *Conservation Genetics* (Artigo V).

O último artigo científico que compõe esta tese encontra-se submetido à revista *Molecular Biology and Evolution* (Artigo VI). Este trabalho é considerado fundamental para o cumprimento dos objectivos propostos por se apresentar pela primeira vez a filogeografia de um gene nuclear em *S. trutta*. A análise da variação nucleotídica de uma porção do gene que codifica a *TF*, através da aplicação de métodos analíticos recentes (Crandall & Templeton 1999; Templeton *et al.* 2000a, 2000b), permitiu não só identificar fenómenos de recombinação e conversão génica, como também construir a genealogia do gene considerando

esses eventos. A associação da informação obtida, com dados resultantes da distribuição geográfica dos variantes electromórficos da *TF* permitiu a formulação de hipóteses filogeográficas relativas à origem e expansão do complexo *S. trutta* ao longo da região Euroasiática.

2. Diversidade genética da truta no limite sul da distribuição Atlântica

Artigo I Genetic characterization of Portuguese brown trout (*Salmo trutta* L.) and comparison with other European populations (publicado em *Ecology of Freshwater Fish*).

Artigo II A highly polymorphic plasma protein locus in brown trout (*Salmo trutta* L.) populations from Portugal (publicado em *Biochemical Genetics*).

Artigo III Mitochondrial haplotype diversity among Portuguese brown trout *Salmo trutta* L. populations: relevance to the post-Pleistocene recolonization of northern Europe (publicado em *Molecular Ecology*).

Artigo IV On the southern edge of the Atlantic brown trout distribution: genetic portrait of population persistence over Pleistocene climatic cycles (em preparação).

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Genetic characterization of Portuguese brown trout (*Salmo trutta* L.) and comparison with other European populations

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Abstract – Allozyme and other protein loci were examined to study the genetic structure of Portuguese brown trout (*Salmo trutta*) populations. A total of 247 individuals from three tributaries of the Lima hydrological basin and a hatchery, all located in northern Portugal, were analyzed. Four of 22 protein coding loci were found to be polymorphic: CK-A1*, GPI-A2*, MPI-2* and TF*. A new allele at the latter locus was found in Atlantic populations. The data obtained for Portuguese brown trout were compared with published data for 14 European populations and three hatchery stocks. Six polymorphic loci (CK-A1*, GPI-A2*, GPI-B2*, LDH-C*, ME* and MPI-2*) were used in a cluster analysis. This showed the similarity of Portuguese natural populations and northern Iberian populations and that Portuguese hatchery fish have an autochthonous origin, distinct from that of other Atlantic hatchery stocks.

A. Antunes[1,2], P. Alexandrino[1,2], N. Ferrand[1,2]

[1]Departamento de Zoologia-Antropologia, Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, Porto, [2]Centro de Estudos de Ciência Animal (CECA), ICETA-U.P., Campus Agrário de Vairão, Vila do Conde, Portugal

Key words: allozyme; brown trout; genetic polymorphism; protein; *Salmo trutta*

Agostinho Antunes, Centro de Estudos de Ciência Animal (CECA), ICETA-U.P., Campus Agrário de Vairão, 4480 Vila do Conde, Portugal

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Un resumen en español se incluye detrás del texto principal de este artículo.

Introduction

Studies of protein variation revealed that the brown trout (*Salmo trutta* L.) is a highly polymorphic and geographically substructured species (Allendorf et al. 1976; Ryman et al. 1979; Ferguson & Mason 1981; Crozier & Ferguson 1986). Multiple lineages were defined from allelic variation patterns at the LDH-C* and CK-A1* loci (Hamilton et al. 1989; García-Marín & Pla 1996). Three major, geographically coherent groups appear to exist in areas that remained unglaciated during the Pleistocene (García-Marín et al. 1999): the northwestern Atlantic group characterized by the LDH-C*90 and CK-A1*100 alleles, the southwestern Atlantic group characterized by the LDH-C*100 and CK-A1*115 alleles, and the Mediterranean group characterized by the LDH-C*100 and CK-A1*100 alleles.

Iberian brown trout has been further investigated in some Spanish populations of Atlantic and Mediterranean drainages (García-Marín et al. 1991; Martinez et al. 1993; Morán et al. 1995; García-Marín & Pla 1996). This work revealed two Iberian lineages (southwestern Atlantic and Mediterranean) on the basis of the CK-A1* locus, but their clear identification requires more thorough analysis (García-Marín & Pla 1996). The TF* locus has not yet been surveyed in Iberian populations, although it has been informative in the study of the genetic differentiation within brown trout populations (Krieg & Guyomard 1985; Presa et al. 1994; Largiadèr & Scholl 1995, 1996a; Giuffra et al. 1996).

In this article, we report upon the spatial genetic structure of Portuguese brown trout based on the electrophoretic study of 22 protein loci. The analysis was extended through the inclusion of published data from other European populations. A hatchery stock often used for restocking was included in the study, and the management implications of our results are discussed.

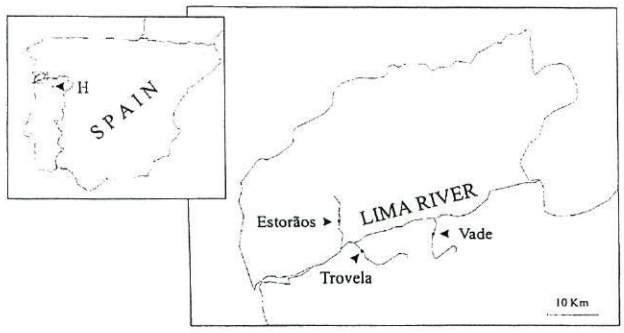


Fig. 1. Location of the *Salmo trutta* sampling sites; H=Torno hatchery.

Study sites, material and methods

Study sites, sampling and electrophoresis

Native brown trouts (n=199) were obtained from three tributaries of the Lima basin (Vade, Estorãos and Trovela) by electrofishing. The Torno hatchery located near the Ovelha river, a tributary of the Douro, was also sampled (n=48) (Fig. 1). Blood, muscle, liver and eye tissues were taken from each fish. Blood was centrifuged on site at 1500 × g at 4°C for 15 min to obtain the supernatant plasma. Samples were stored at −80°C for future use.

Homogenates obtained by grinding tissue in twice-distilled water (1:3, w/v) were centrifuged at 1500 × g for 15 min at 0°C. The supernatant was decanted and applied on either starch gels for electrophoresis or on polyacrylamide gels for isoelectric focusing, following procedures described in Antunes (1997) (see Table 1 for details). A survey of genetic variation was performed with 12 enzyme systems representing the following 22 structural loci (Enzyme Commission numbers and loci in parentheses): acid phosphatase (3.1.3.2, ACP*), adenylate kinase (2.7.4.3, AK*), creatine kinase (2.7.3.2, CK-A1*, -A2*), glucose-6-phosphate isomerase (5.3.1.9, GPI-A1*, -A2*, -B*), hemoglobin (HB*), l-lactacte dehydrogenase (1.1.1.27, LDH-A1*, -A2*, -B1*, -B2*, -C*), malic enzyme-NAD (1.1.1.39, ME*), malic enzyme-NADP (1.1.1.40, MEP-1*, -2*, -3*), mannose-6-phosphate isomerase (5.3.1.8, MPI-2*), para-albumine (PALB-1*, -2*), 6-phosphogluconate dehydrogenase (1.1.1.44, PGHD-2*) and transferrin (TF*). The nomenclature for designation of loci and alleles is that of Shaklee et al. (1990). Electromorph identity was confirmed by running samples side by side (Guyomard & Krieg 1983; Krieg & Guyomard 1985; Largiadér & Scholl 1995).

Comparison with other European populations

Portuguese brown trout populations were compared with other Iberian and non-Iberian samples for which data were available from the literature, covering 14 natural populations from Atlantic and Mediterranean drainages and three Atlantic hatchery stocks across seven polymorphic loci: CK-A1*, GPI-A2*, GPI-B2*, LDH-C*, ME*, MPI-2* and TF* (Table 2). Data on the TF* locus were not available for all populations, and analysis was performed with and without this locus.

Data analysis

Allele frequencies were estimated directly from zymograms for loci showing codominant expression. Alleles for the isoloci CK-A1, A2* were allocated to the single locus CK-A1* and their frequencies estimated by the square root of the frequency of homozygous phenotypes, i.e., under the assumption of Hardy-Weinberg equilibrium. The BIOSYS-1.7 computer program (Swofford & Selander 1989) was used to analyze the data. Phenotypic distributions of all codominantly expressed loci were tested for agreement with Hardy-Weinberg expectations using the chi-square test. Unbiased expected heterozygosities for each population were calculated from observed allele frequencies following Nei (1978). Gene diversity (G_{ST})

Table 1. Polymorphic protein systems in examined populations of *Salmo trutta*.

locus	Tissue	Electrophoretic system[1]	Staining
Creatine kinase (CK)	Muscle	IEF1-G1	Harris & Hopkinson (1976)
Glucose-6-posphate isomerase (GPI)	Muscle	IEF1-G2	Harris & Hopkinson (1976)
Mannose-6-phosphate isomerase (MPI)	Liver	SGE1	Harris & Hopkinson (1976)
Transferrin (TF)	Plasma	IEF2-G3	Coomassie Blue R-250

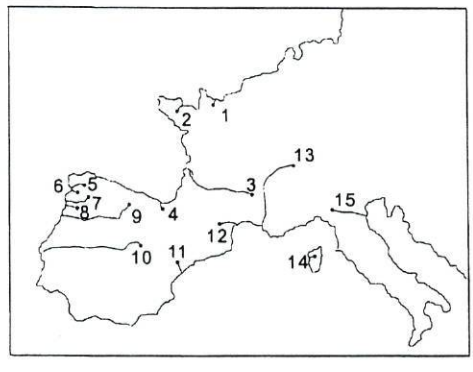
[1] System: SGE, starch gel electrophoresis; SGE1, citrate-NaOH-His/HCl, pH 6.0 (Ferrand & Amorim 1990). IEF: Isoelectric focusing performed in polyacrylamide gels; IEF1: (5% T, 3% C, 20% saccharose; 230×100×0.3 mm); IEF2: (5% T, 3% C, 6 M urea; 230×100×0.3 mm); G1, gels with 5% (v/v) of a 1:3 mixture of the ampholytes 3.5–10 Ampholine (Pharmacia) and 5–8 Pharmalyte; G2, gels with 5% (v/v) of a 1:7:2 mixture of the ampholytes 3.5–10 Ampholine (Pharmacia), 5–8 Pharmalyte and 8–10.5 Pharmalyte; G3, gels with 5% (v/v) of ampholytes 5–8 Pharmalyte.

Table 2. Allele frequencies in the populations of *Salmo trutta* examined in the present investigation and in published studies

Locality	Locus allele sample size	CK-A1*			GPI-A2*					GPI-B*			LDH-C*			ME*				MPI-2*				TF*				
		n	*100	*115	*n*	*100	*135	*140	*50	*n*	*100	*102	*n*	*100	*90	*n*	*100	*140	*150	*n*	*100	*105	*110	*n*	*100	*102	*95	*80
Iberian																												
Portugal																												
Vade		53	0.07	0.93	64	0.95	0.05			64	1.00		25	1.00		41	1.00			41	0.86	0.14		98	0.64		0.36	
Estorãos		70	0.28	0.72	58	0.85	0.25			58	1.00		25	1.00		51	1.00			51	0.72	0.28		67	0.69		0.31	
Trovela		31	0.14	0.86	26	0.85	0.17			26	1.00		25	1.00		28	1.00			28	0.79	0.21		15	0.67		0.33	
Torno Hatchery		48	0.59	0.41	48	1.00				48	1.00		25	1.00		37	1.00			37	0.65	0.35		21	1.00			
Spain																												
Santolaz[1]		23	0.17	0.83	23	0.46			0.54	23	1.00		23	1.00		23	1.00			23	0.26	0.74	0.11	–				
Arceo[2]		31	0.04	0.96	31	0.73	0.27			31	1.00		31	1.00		31	1.00			31	0.60	0.29		–				
Armenteira[2]		30	0.31	0.69	30	0.76	0.24			30	1.00		30	1.00		30	1.00			30	0.62	0.38		–				
Bubal[1]		25	0.34	0.66	25	0.78	0.22			25	1.00		25	1.00		25	1.00			25	1.00			–				
Pisuerga[1]		31		1.00	31	1.00				31	1.00		31	1.00		31	1.00			31	0.98	0.02		–				
Hoceseca[1]		19		1.00	19	1.00				19	1.00		19	1.00		19	1.00			19	1.00			–				
Villahermosa[1]		27	1.00		27	1.00				27	1.00		27	1.00		27	0.48	0.52		27	1.00			–				
H1-Hatchery[2]		32	0.55	0.45	32	1.00				32	1.00		32	0.01	0.99	32	1.00			32	0.59	0.41		–				
H2-Hatchery[2]		36	0.72	0.28	36	1.00				36	1.00		36		1.00	36	1.00			36	0.65	0.35		–				
Non-Iberian																												
Atlantic																												
Orne[3]		24	0.50	0.50	24	1.00				24	0.84	0.16	24	0.06	0.94	24	1.00			24	0.90	0.10		24	1.00			
Leffe[4]		16	0.53	0.47	16	1.00				16	1.00		16	0.03	0.97	16	1.00			16	0.56	0.44		16	1.00			
Lot[4]		20	1.00		20	1.00				20	1.00		20		1.00	20	1.00			20	0.56	0.44		20	1.00			
Etru Hatchery[4]		40	0.85	0.15	40	1.00				40	1.00		40		1.00	40	1.00			40	0.39	0.61		40	1.00			
Mediterranean																												
Maureillas[4]		18	1.00		18	1.00				18	1.00		18	1.00		18	1.00			18	1.00			18		1.00		
Chevenne[5]		11	1.00		11	1.00				11	1.00		11	1.00		11	1.00			11.	1.00			11		1.00		
Aitone[4]		20	1.00		20	1.00				20	1.00		20	1.00		20	0.76		0.24	20	1.00			20		0.66		0.34
SS-Po[6]		15	1.00		15	0.90		0.10		15	1.00		15	1.00		15	1.00			15	1.00			15	0.03	0.97		

[1] García-Marín & Pla 1996; [2] Martinez et al. 1993; [3] Krieg & Guyomard 1985; [4] Presa et al. 1994; [5] Largiadèr et al. 1996; [6] Largiadèr & Scholl 1996b.

analysis was performed to determine the components of genetic differentiation among natural populations (Nei 1973, 1975). Dendrograms were constructed with Nei's unbiased standard genetic distance (Nei 1978) using the neighbor-joining (NJ) technique in the PHYLIP 3.5 computer package (Felsenstein 1993). Bootstrap replication scores (100 replicates) were calculated to gain an impression of the strength of support from the data to the dendrogram.



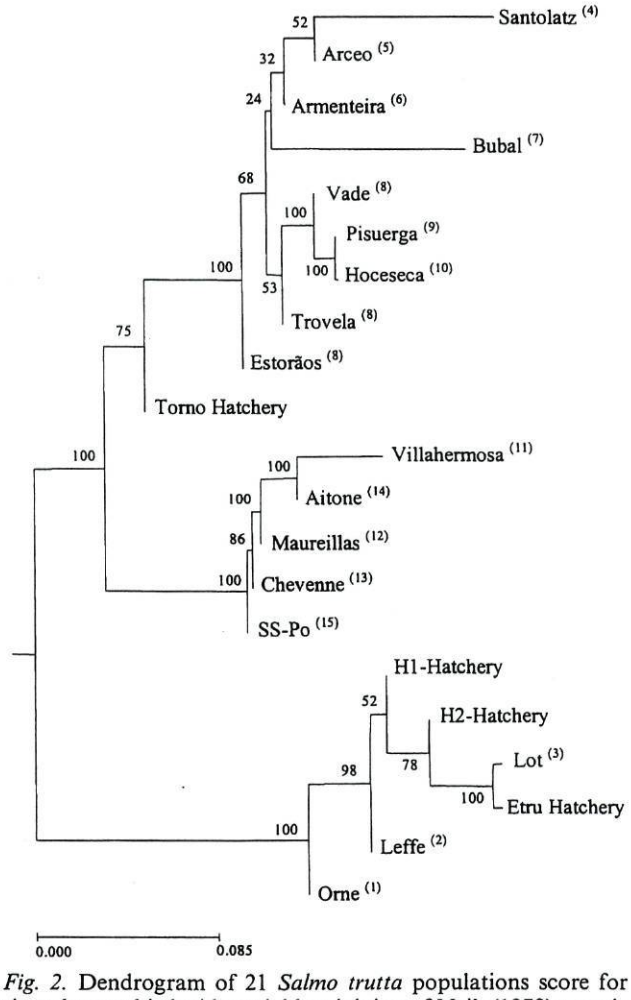


Fig. 2. Dendrogram of 21 *Salmo trutta* populations score for six polymorphic loci by neighbor joining of Nei's (1978) genetic distance. Bootstrap replication scores (%) are shown on internal branches.

Results

Genetic variation was detected at four of 22 protein coding loci: CK-A1*, GPI-A2*, MPI-2* and TF* (Table 2). The LDH-C*100 allele was fixed across the natural populations and the hatchery stock. The patterns observed for AAT-1, 2*, MDH-A1, A2* and PGM-2* were inconsistent and difficult to interprete unambiguously and were excluded from the analysis.

No significant deviations were observed when allele frequencies were tested against Hardy-Weinberg expectations. Heterozygosity was higher in Estorãos and Trovela populations (H= 0.066 and 0.061, respectively) than at Vade (H= 0.040) and the hatchery stock (H=0.043). A low level of differentiation (G_{ST}=2.7%) was observed between populations from the three Lima tributaries. A high genetic similarity was observed between Portuguese and geographically close Galician populations while some populations from Asturias, northern Spain, presented similar gene frequencies at some loci (CK-A1*, GPI-A2* and LDH-C*).

The NJ tree indicates the existence of three well differentiated groups, representing the northwestern Atlantic, the southwestern Atlantic and the Mediterranean (Fig. 2). The differentiation of the southwestern Atlantic group mainly results from the presence at high frequency (mostly higher than 0.5) of the CK-A1*115 allele and the fixation of the LDH-C*100 allele. The northwestern Atlantic and Mediterranean groups are together characterized by a high frequency of the CK-A1*100 allele and are differentiated at the LDH-C* locus with high frequencies of respectively the alleles LDH-C*90 and LDH-C*100. The Spanish and French (Etru) hatchery stocks fall within the northwestern Atlantic group. The Portuguese hatchery stock clusters with the southwestern Atlantic group with which it shares polymorphisms at the CK-A1* and MPI-2* loci and not with the Mediterranean group, which is monomorphic at these loci.

Including the TF* locus in the analysis while removing the Spanish populations did not change the topology of the NJ dendrogram (results not shown). However, the main groups are more strongly separated due to the fixation for alternate alleles in northwestern Atlantic and Mediterranean populations (TF*100 and *102, respectively) and the presence of the TF*95 and TF*100 alleles

in the Portuguese populations of the southwestern Atlantic group.

Discussion

Spatial genetic assemblages and the origin of Portuguese populations

The use of six polymorphic loci allowed the comparison of Portuguese samples with previously published data for 14 European populations and three Atlantic hatchery stocks. The three population groups detected are in agreement with those defined by García-Marín et al. (1999). The observed spatial genetic structure showed the Portuguese and northern Spanish brown trout populations to be closely related and both belong to the southwestern Atlantic group. Our results indicate that the pan-European study of spatial genetic variation at the TF* locus could form a valuable contribution to our understanding of population relationships in *Salmo trutta* and already helped to characterize northwestern Atlantic and Mediterranean populations, as well as *S. trutta marmoratus*, *S. trutta carpio* and *S. salar* (e.g. Krieg & Guyomard 1985; Presa et al. 1994; Largiadèr & Scholl 1995, 1996a; Giuffra et al. 1996).

Genetic differentiation of Portuguese populations

A low level of genetic differentiation was observed between Portuguese natural populations (G_{ST}= 2.7%) compared to the corresponding value for six populations from Galicia, Spain (G_{ST}=27.3%, Martinez et al. 1993). The simultaneous analysis of populations belonging to three distinct drainages may have inflated the latter value compared with the Portuguese populations that represent a single river system. Similarly contrasting results (7.7% versus 21.5%, expressed in F_{ST} rather than G_{ST}) were obtained by comparing Asturian brown trout populations from single and different drainages (Morán et al. 1995). The comparison of brown trout populations from streams discharging in the Atlantic and Mediterranean yielded even higher values (e.g. G_{ST}=61.5%, García-Marín et al. 1991). Based on their genetic similarity, we conclude that gene flow between the Vade, Estorãos and Trovela populations is substantial, perhaps operating through a linear composite of populations and subpopulations interchanging individuals by migration (Morán et al. 1995). The presence of the brown trout anadromous form in the Lima basin could help to increase gene flow between populations (Morán et al. 1995). A possibility that cannot be excluded is that transport from one population to the other, for example through the hatchery industry, has artificially decreased differentiation among populations.

Origin of Portuguese hatchery fish and management implications

Hatchery populations in Denmark, France, Spain and Italy all had a northern Atlantic origin (Krieg & Guyomard 1985; García-Marín et al. 1991, 1998; Presa et al. 1994). However, fish from the Torno hatchery is genetically dissimilar from the northwestern Atlantic group and is therefore unlikely to originate from the corresponding area. A purely Mediterranean origin is also unlikely because it is polymorphic at some loci that are monomorphic in populations from the Mediterranean group. It is unlikely to originate from Spanish hatchery populations because stocking incidences from those can be detected using LDH-C* as a diagnostic marker (García-Marín et al. 1991; Morán et al. 1991; Martínez et al. 1993; Arias et al. 1995; García-Marín et al. 1998). By default we assume a local, southwestern Atlantic origin. The absence of variation at two loci that are normally polymorphic in this group may be due to a small size of the original stock ("founder effect"). Alternatively, Torno fish could be not strictly autochtonous, i.e. not from the Lima basin, but originate from populations that were not yet investigated, such as those from the Douro and Mondego river basins, which regularly serve as source for stocking operations (local hatchery authorities, personal communication). Continuous recuperation is common procedure in the maintenance of hatchery populations and most hatchery stock is of mixed origin. The comparative data for the three natural populations and the Torno Hatchery warrant further consideration. Despite intensive Torno Hatchery stocking over many years and localities in the Lima basin (local hatchery authorities, personal communication), a strong influence of these activities is not apparent (Table 2, Fig. 2). Rather, the divergent allele frequencies of the hatchery sample at the four polymorphic loci suggest no displacement and minimal introgression of hatchery and wild populations. These possibilities deserve more detailed examination with regard to the effectiveness of hatchery programs, the dynamics of hatchery and wild fish interactions, and the protection and value of native populations.

The puzzling case of allele TF*95

The survey of the TF* locus in natural Portuguese populations revealed the existence of the *95 and *100 alleles. The TF*100 allele is usually fixed in

Atlantic populations (Krieg & Guyomard 1985; Presa et al. 1994), while the TF*95 allele was so far only reported in frequencies lower than 0.14 in trout from tributaries of the upper Rhone and the upper Po in Switzerland (Largiadèr & Scholl 1995; Largiadèr et al. 1996). Portuguese natural populations possess this allele at frequencies of ca. 0.35. The allele is further present at the southern limits of the brown trout distribution in Portugal where, at the upper ranges of the river Zêzere, it reaches a frequency of 0.80 (A. Antunes, unpublished results). The simultaneous presence of the TF*95 allele in Portuguese and Mediterranean trout is puzzling. Either a single TF*95 allele exists, identical in Portuguese and Mediteranean populations, or the gene product referred to as TF*95, although indistinguishable by electrophoretic mobility and isoelectric point, is distinct from the *95 allele found in Mediterranean populations. The first hypothesis would suggest common ancestry, in which case the TF* locus would be a good candidate to help uncover the trout dispersal pattern. In the absence of the anadromous form south of 42° latitude, contemporary gene flow between Iberian and Mediterranean populations is unlikely. Further investigation is required to clarify these issues. Promising strategies would include (1) the study of the TF* locus in selected populations from the Iberian Peninsula and adjacent Mediterranean areas and (2) the gene sequencing of various electrophoretic TF* variants. Similarly, the presence in high frequencies of some alleles at the LDH-A2* and MEP-3* loci in both Mediterranean populations and in populations from the river Guadalquivir in southwestern Spain may indicate historical gene flow between those areas or even the merging of Atlantic and Mediterranean groups (García-Marín & Pla 1996).

Resumen

1. En 247 individuos, procedentes de tres afluentes de la cuenca del río Lima y de una piscifactoría al N de Portugal, examinamos aloenzimas y loci proteínicos para estudiar la estructura genética de las poblaciones portuguesas de *S. trutta*.
2. En 22 loci codificadores de proteínas encontramos 4 loci polimórficos: CK-A1*, GPI-A2*, MPI-2* y TF* y en las poblaciones atlánticas, además, hallamos un nuevo alelo para el último locus.
3. Utilizamos 6 loci polimórficos (CK-A1*, GPI-A2*, GPI-B2*, LDH-C*, ME* y MPI-2*) para comparar las poblaciones portuguesas con 14 poblaciones europeas y tres de piscifactoría. Los análisis mostraron que las poblaciones portuguesas silvestres son parecidas a las del norte de la Península y que los individuos procedentes de la piscifactoría portuguesa tienen un origen autóctono, distinto de los de otras piscifactorías atlánticas.

Acknowledgments

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A Highly Polymorphic Plasma Protein Locus in Brown Trout (*Salmo trutta* L.) Populations from Portugal

A. Antunes,[1,2] N. Ferrand,[1] and P. Alexandrino[1]

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Genetic polymorphism of an unidentified plasma protein (PX) is described for the first time in Salmo trutta *(L.) by means of isoelectric focusing. The analysis of 414 individuals from different geographic origins in Portugal allowed the identification of nine alleles. Heterozygosity in natural populations is generally above 0.60, thus giving similar values to those reported for brown trout microsatellite loci. Substructuring of Portuguese brown trout is evident between northern and southern basins. Genetic affinities between the southernmost rivers and the hatchery stock were detected, suggesting the existence of recent stocking influences.*

KEY WORDS: plasma protein; polymorphism; isoelectric focusing; brown trout; *Salmo trutta*; salmonids.

INTRODUCTION

The genetic relationships of brown trout (*Salmo trutta* L.) populations across Europe have been extensively evaluated through analysis of protein polymorphism. Several lineages were defined from allelic variation patterns at the LDH-C* and CK-A1* loci (Hamilton *et al.*, 1989; García-Marín and Pla, 1996), and more recently García-Marín *et al.* (1999) proposed four major geographic

[1] Centro de Estudos de Ciência Animal (CECA), ICETA-UP, Campus Agrário de Vairão, 4480 Vila do Conde, and Departamento de Zoologia-Antropologia, Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, 4050 Porto, Portugal.

[2] To whom correspondence should be addressed at Centro de Estudos de Ciência Animal (CECA), ICETA-UP, Campus Agrário de Vairão, 4480 Vila do Conde, Portugal. Fax: +351 252 661780. e-mail: aantunes@mail.icav.up.pt.

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groups in areas that remained unglaciated during the Pleistocene: the Mediterranean, Ponto-Caspian, and northwestern and southwestern Atlantic groups. Substructuring can already be discerned (García-Marín *et al.*, 1999), but an extension of the number of genetic polymorphisms is still necessary to elucidate more clearly the genetic diversity and microgeographic structure of these populations. This is especially the case for the southern limit of the Atlantic distribution of both resident and anadromous brown trout forms (Antunes *et al.*, 1999; Bouza *et al.*, 1999; Weiss *et al.*, 2000). Advances in molecular biology techniques have led to the use of new genetic markers such as microsatellites, RFLPs, and sequencing of mtDNA in phylogenetic studies, generally confirming and extending the macrogeographic patterns obtained with allozymes (Bernatchez *et al.*, 1992; Presa *et al.*, 1994; Bernatchez and Osinov, 1995; Hynes *et al.*, 1996). Hypervariable markers, such as microsatellite loci, greatly increase the differentiation level above that observed with protein loci, which generally exhibit only one or two polymorphic alleles per population (Estoup *et al.*, 1993, 1998). However, at present the accurate use of such microsatellites to investigate genetic relationships between populations depends on a deeper evaluation of their mode of evolution and on the establishment of correct estimates of genetic distance (Takezaki and Nei, 1996; Angers and Bernatchez, 1997; Colson and Goldstein, 1999). Thus conventional protein loci are still of great importance in studies of genetic diversity and phylogeny.

In this paper we describe a new genetic polymorphism in *S. trutta* identified by means of isoelectric focusing (IEF). The as yet unidentified plasma protein (PX) shows a level of variability similar to those observed in some brown trout microsatellite loci.

MATERIALS AND METHODS

Brown trout native specimens were obtained from one tributary of the Minho basin (Manco), two tributaries of the Lima basin (Vade and Estorãos), the Ave River, the Mondego River, and one tributary of the Tejo basin (Zêzere) (Fig. 1). Individuals from the Torno hatchery located in northern Portugal were also sampled. This hatchery is the main source of brown trout for national stocking programs (Antunes *et al.*, 1999). A few samples from two fish-farm stocks of Atlantic salmon (*S. salar*) from the Gave and Athas French hatcheries were also included in the analysis. Blood samples were obtained from anaesthetized trout and collected in EDTA tubes (10% disodium EDTA). Thirty to 200 μl of blood was drawn per specimen from the caudal vein behind the anal fin, without sacrificing the fish. To test the congruence of PX* phenotypes in different tissues from the same individual, 20 specimens from the Minho and Lima rivers were sacrificed and the vitreous was extracted. To achieve the best detection of PX* after IEF it is very important to treat samples immediately after collection. Thus,

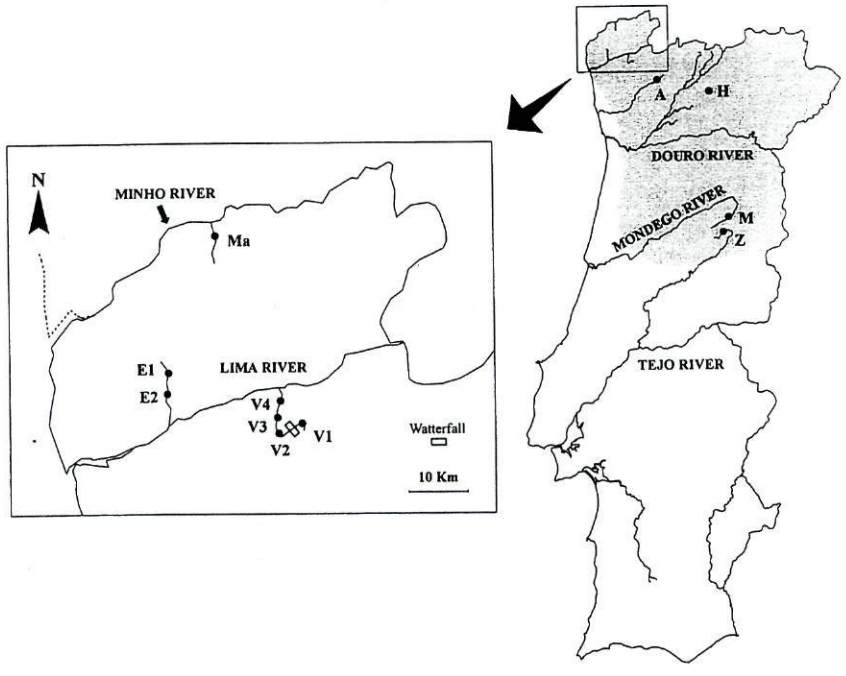

Fig. 1. Location of *Salmo trutta* sampling sites: Minho River (Ma-Manco); Lima River (E1 and E2—Estorãos; V1, V2, V3, and V4—Vade); Ave River (A); Torno hatchery (H); Mondego River (M); Tejo River (Z—Zêzere). The shaded area represents the present distribution of the species in Portugal.

both plasma and vitreous samples were centrifuged in the field at 1500g and 4°C for 15 min. The supernatant was immediately stored at −80°C until use. Plasma was diluted 1:3 or 1:10 in bidistilled water for agarose gel electrophoresis (AGE) or IEF separation, respectively. Vitreous was used undiluted for IEF separation. AGE was made following conditions described by Teisberg (1970). The best IEF separation of PX* alleles in polyacrylamide gels (T = 5%, C = 3%, saccharose = 20% (w/v); 230 × 100 × 0.3 mm) was achieved in a 1:1 mixture of pH 5–6 and pH 5–8 carrier ampholytes (Pharmacia) at a final concentration of 5% (v/v). L-Serine (60 m*M*) was also added to the gel. Glutamic acid (0.04 *M*) and NaOH (1 *M*) were used as anode and cathode solutions, respectively. Gels were prefocused at constant power setting limits at 1500 V, 25 mA, and 1 W (30 min), 2 W (15 min), and 3 W (15 min). After prefocusing, 8 μl of each sample was applied 1.5 cm from the anode in a silicone strip. Focusing was performed at constant power setting limits at 1500 V, 25 mA, and 4 W (1 h), 2000 V, 25 mA, and 5 W (1 hr), and 2500 V, 25 mA, and 6 W (1 hr). After focusing staining was done with Coomassie Blue R-250. Whenever eye samples were available from

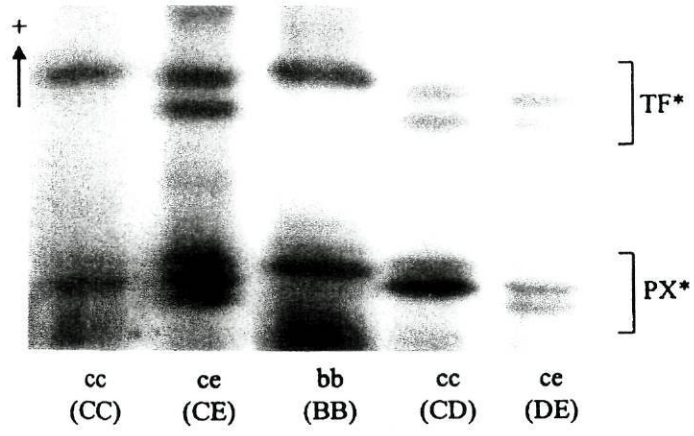

Fig. 2. Electrophoretic separation of PX* in agarose gel. Lowercase letters indicate the phenotypes recognized by AGE that discriminate three alleles. IEF phenotypes are shown in parentheses.

other populations, the LDH-C* "diagnostic" locus was screened to confirm the autochthonous (LDH-C*100/100) or allochthonous (LDH-C*90/90) origin of the fish. Allele frequencies were estimated by direct counting. The GENEPOP (version 3.1b; Raymond and Rousset, 1995) probability test option was used to determine whether populations were in Hardy–Weinberg (HW) equilibrium (Markov chain method).

RESULTS

Genetic variation was initially detected by AGE in a protein which has an abundance similar to that of transferrin (TF*) but exhibits a more cathodic position (Fig. 2). AGE separation allowed the initial identification of three alleles (Fig. 2). Yet the polymorphism was only fully revealed by IEF (Fig. 3, Table I). The numerous phenotypes found in natural populations were explained as being determined by seven codominant alleles at an autosomal locus and are compatible with a monomeric structure for this protein. The study of PX* in hatchery individuals revealed the existence of two other alleles. Patterns showing one major band and two minor anodal and cathodal repeats were interpreted as being homozygotes. Heterozygotes show a simple combination of the banding patterns of the two homozygotes corresponding alleles. Due to hatchery space limitations, no progeny testing was carried out to confirm the mode of inheritance of PX* variants. Side-by-side gel comparison of vitreous and plasma from the same individual showed the same phenotype expression in both, for the 20 specimens analyzed. The phenotypes observed were *AB, *AC, *BB, *BC, *BE, *BH,

*CC, *CD, *CE, *DE, and *EE. However, in vitreous the PX* concentration was lower. Stability in electrophoretic band position was achieved when using plasma stored for up to 4 years. Slight differences between alleles corresponding to very close-running bands could be confirmed only when 1:1 mixtures of samples revealed a clearly distinct pattern (Fig. 3b). A protein that is presumably orthologous to *S. trutta* PX* was also found in *S. salar*, displaying a more acidic isoelectric point. Two phenotypes were observed, the putative homozygote and a variant heterozygote (Fig. 3a).

Substructuring of populations is evident at different geographic levels. We found alleles that are exclusive to the northern and southern basins, leading to a strong partition in the two population groups (Table I). PX*B and *H were found only in the Minho and Lima basins (with the exception of the isolated population of Vade 1 and the Estorãos 2). In these basins we also detected the highest allelic diversity, with up to six alleles in some of the tributaries. Additionally, substructuring could also be found within the same tributary such as the Vade River, where one isolated sector upstream of a waterfall (V1) was found to be fixed for the private allele PX*A. Downstream, there is a clinal decrease in PX*A, with the remaining sectors clearly differentiated from the isolated one. Lower heterozygosity values and fewer alleles were detected in the southern basins (Mondego samples are an exception). PX*G was found only in the Mondego and Zêzere rivers and also observed at moderate frequencies in most of the hatchery stocks analyzed. Hatchery samples showed a lower level of heterozygosity and were genetically differentiated from the natural populations (e.g., presence of two private alleles and different frequencies of the shared ones). With the exception of samples from Mondego and 1997 hatchery progeny, no significant deviations from HW equilibrium were observed (F_{IS} values; Table I). In individuals from natural populations where it was possible to score the LDH-C*, fixation for the *100 allele was observed in all of them, with the exception of six and three individuals from the Mondego and Zêzere rivers, respectively, that were homozygous for the *90 allele. These nine individuals exhibited the PX* phenotypes *DD (3), *DE (1), and *DG (5). In contrast, the *90 allele was observed in several hatchery individuals, being fixed in the 1998 progeny. No heterozygous LDH-C* 100/90 have been found so far in the wild or in the hatchery.

DISCUSSION

The congruence of the phenotypes observed in vitreous and plasma from the same individuals supports a simple Mendelian inheritance mechanism at the PX* locus, as shown by Utter and Hodgins (1971) for esterase and transferrin systems in the Pacific hake (*Merluccius productus*). A simple pattern of codominance is also suggested by the observed phenotypic distributions, which were generally in good agreement with the expected distributions obtained under the assumption of

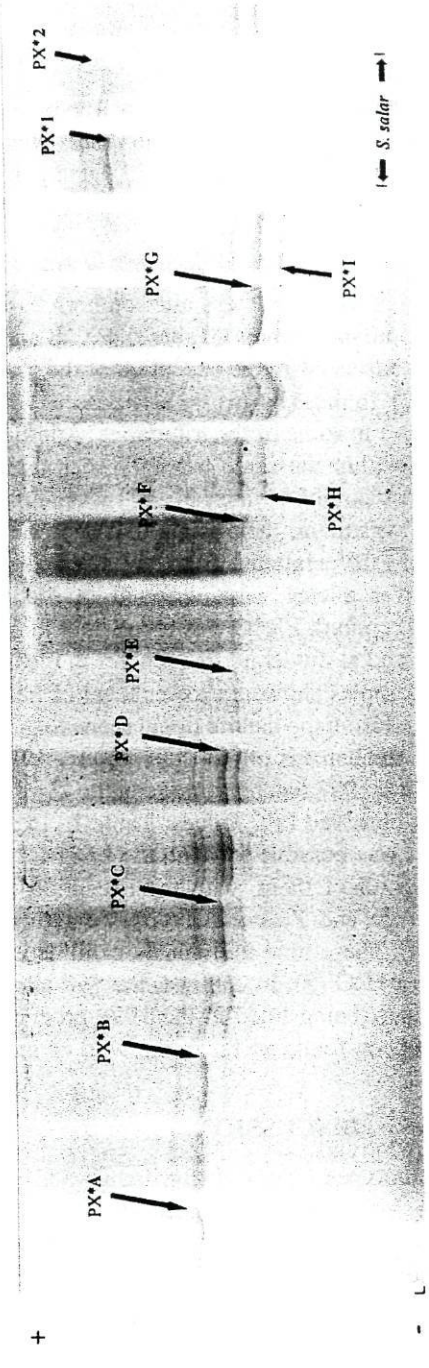

Fig. 3. (a) Brown trout and Atlantic salmon PX* phenotypic patterns observed after IEF separation in the pH gradient 5–8. (b) Detail of the slight difference between allele *H and allele *G. The mixture of samples revealed a clear distinction of the patterns and of the corresponding anodal and cathodal repeats.

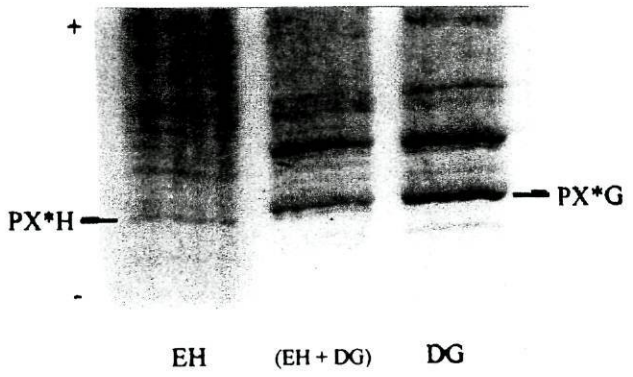

Fig. 3. (Continued)

HW equilibrium. Compared with more than 45 polymorphic allozyme loci reported to date in this species (see García-Marín *et al.*, 1999), this locus displays an unusual degree of polymorphism and heterozygosity similar to those reported for brown trout microsatellite loci (Estoup *et al.*, 1993, 1998; Presa *et al.*, 1994).

The nonuniform distribution of alleles across natural populations reveals the existence of two divergent population groups: north (Minho and Lima) and south (Ave, Mondego, and Zêzere). This genetic substructuring may result from markedly different levels of gene flow among basins, as the anadromous form has its southern limit in the Minho and Lima basins (SNPRCN, 1991) (Fig. 1). Absence of migration among basins and genetic drift effects may eventually explain the substantial decrease in allelic diversity of southern populations. A similar explanation has recently been proposed to justify important allelic distribution profile changes in brown trout populations from Galiza (northwestern Spain) close to the southern limit of the range of the anadromous form (Bouza *et al.* 1999).

Hatchery stocks were found to be genetically divergent, but the sharing of one private allele with natural populations from Mondego and Zêzere may suggest some stocking influences in these rivers. These stocking influences were confirmed by the detection of the northwestern Atlantic allele LDH-C*90 in some individuals from these rivers. Since this allele had not been detected in this hatchery previously (Antunes *et al.*, 1999), these results suggest that exogenous strains have been recently introduced (Antunes, unpublished results). As some of the allochthonous individuals detected by LDH-C* typing in Mondego and Zêzere have PX*G, we suggest that this allele was probably introduced. This fact, in combination with the observed Hardy–Weinberg disequilibrium in the Mondego River, may represent a strong indication that hatchery endogenous fish are being released into the wild. So far no evidence of introgression exists since no heterozygotes LDH-C* 100/90 have been detected.

Table I. Gene Frequencies (Standard Errors in Parentheses), Heterozygosity (Het) and Inbreeding Coefficient of Weir and Cockerham (1984) (F_{IS}) for the PX* Locus in Different *S. trutta* Populations

Population	n	Allele frequency									Het	F_{IS}
		*A	*B	*C	*D	*E	*F	*G	*H	*I		
Minho												
Manco	39	—	0.50 (±0.06)	0.22 (±0.05)	0.06 (±0.03)	0.19 (±0.04)	—	—	0.03 (±0.02)	—	0.66	−0.035
Lima												
Vade												
V1	30	1.00	—	—	—	—	—	—	—	—	—	—
V2	36	0.08 (±0.03)	0.27 (±0.05)	0.29 (±0.05)	0.22 (±0.05)	0.10 (±0.04)	—	—	0.04 (±0.02)	—	0.78	−0.057
V3	34	0.10 (±0.04)	0.26 (±0.05)	0.48 (±0.06)	0.02 (±0.02)	0.12 (±0.04)	—	—	0.02 (±0.02)	—	0.67	−0.039
V4	24	—	0.23 (±0.06)	0.42 (±0.07)	0.11 (±0.04)	0.12 (±0.05)	—	—	0.12 (±0.05)	—	0.73	−0.061
Estorãos												
E1	33	—	0.45 (±0.06)	0.29 (±0.06)	0.06 (±0.03)	0.20 (±0.05)	—	—	—	—	0.67	+0.243
E2	25	—	0.50 (±0.07)	0.12 (±0.05)	0.04 (±0.03)	0.30 (±0.07)	—	—	0.04 (±0.03)	—	0.64	−0.163
Ave	27	—	—	—	0.72 (±0.06)	0.28 (±0.06)	—	—	—	—	0.40	+0.187
Mondego	32	—	—	0.33 (±0.06)	0.28 (±0.06)	0.19 (±0.05)	—	0.20 (±0.05)	—	—	0.74	−0.002[a]
Tejo												
Zêzere	26	—	—	—	0.92 (±0.04)	0.02 (±0.02)	—	0.06 (±0.03)	—	—	0.14	−0.047
Hatchery												
Progeny 1996	20	—	—	—	—	—	0.75 (±0.07)	—	—	0.25 (±0.07)	0.38	−0.316
Progeny 1997	15	—	—	—	0.43 (±0.09)	—	—	0.50 (±0.09)	—	0.07 (±0.05)	0.56	−0.780[a]
Parents 1998	20	—	—	—	0.70 (±0.07)	0.05 (±0.04)	—	0.25 (±0.07)	—	—	0.45	+0.014
Progeny 1998	53	—	—	—	0.65 (±0.05)	0.08 (±0.03)	—	0.27 (±0.04)	—	—	0.55	−0.094

[a]Hardy–Weinberg equilibrium rejected for $P < 0.05$.

Both the allelic distribution and the high level of diversity of the PX* locus found in natural populations appear to reflect genetic substructuring within the southwestern Atlantic region. The data presented here suggest that the PX* locus can be a powerful genetic marker for the study of microgeographical diversity and of fine scale processes of adaptation and gene flow. Furthermore, it can also be very useful for selective breeding and analysis of the success of stocking programs of farmed populations.

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Mitochondrial haplotype diversity among Portuguese brown trout *Salmo trutta* L. populations: relevance to the post-Pleistocene recolonization of northern Europe

S. WEISS,*† A. ANTUNES,‡§ C. SCHLÖTTERER† and P. ALEXANDRINO‡§
*Abteilung für Hydrobiologie, Universität für Bodenkultur, Max Emanuel Straße 17, 1180 Vienna, †Institut für Tierzucht und Genetik, Veterinärmedizinische Universität, Veterinärplatz 1, 1210 Vienna, ‡Departmento de Zoologia-Antropologia, Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, 4050 Porto, Portugal, §Centro de Estudos de Ciência Animal (CECA), ICETA-U.P., Campus Agrário de Vairão, 4480 Vila do Conde, Portugal

Abstract

Mitochondrial haplotype diversity in seven Portuguese populations of brown trout, *Salmo trutta* L., was investigated by sequencing the 5′ end of the mitochondrial DNA (mtDNA) control region. Five new haplotypes were described for this species, each two to three mutational steps distant from the common north Atlantic haplotype. Significant population subdivision of mtDNA haplotypes was also apparent. Based on these results, as well as on published data describing the distribution of both mtDNA haplotypes and allozyme alleles throughout Europe, the postglacial recolonization of northern Europe was re-evaluated. It is argued that the available data do not support the contribution of two major glacial refugia (southwest Atlantic and Ponto-Caspian Basin) to this postglacial recolonization, as proposed in a recently published model. The unique genetic architecture of Portuguese brown trout within the Atlantic-basin clade of this species represents a highly valuable genetic resource that should be protected from introgression with nonendemic strains of hatchery fish.

Keywords: allozymes, glacial refugia, mtDNA, Pleistocene, Portugal, *S. trutta*

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Introduction

Investigations of mitochondrial DNA (mtDNA) sequence diversity in brown trout, *Salmo trutta* L. have been used to construct an inferred phylogenetic tree depicting four lineages corresponding to the major drainage basins (Atlantic, Mediterranean, Adriatic and Danube) and a fifth equally divergent lineage corresponding to the morphologically distinct marbled trout, *S. trutta marmoratus*, found in drainages of northern Italy (Bernatchez *et al.* 1992; Giuffra *et al.* 1994). Based on 310 bp of the 5′ end of the control region, haplotype diversity within major drainage basins was found to vary considerably. For example, a single haplotype (At1) dominated samples from Atlantic basin sites, and 10 haplotypes were detected in sites within the Caspian–Black–Aral basins (Bernatchez *et al.* 1992; Bernatchez & Osinov 1995). Allele frequencies at several diagnostic protein loci generally support a subdivision by major drainage basins and have been used to draw inferences on several regionally specific, or more broad-scale, post-Pleistocene recolonization events (Hamilton *et al.* 1989; Presa *et al.* 1994; Bernatchez & Osinov 1995; Riffle *et al.* 1995; Apostolidis *et al.* 1996; García-Marín & Pla 1996).

García-Marín *et al.* (1999) proposed a postglacial recolonization model of the north Atlantic, involving three of the four major drainage basins, based primarily on allelic distributions at two enzymatic loci (LDH-C* and CK-A1*). According to this model, following the last glacial maximum (≈ 18 000 years before present [BP]; Frenzel *et al.* 1992) the north Atlantic was first colonized by northwestwardly migrating populations from undefined passages between the North Sea and the Ponto-Caspian basin, and subsequently influenced by gene flow from unglaciated regions of both northern continental Europe as well as Atlantic drainages of the Iberian Peninsula.

Correspondence: Steven Weiss. Present address: Centro de Estudos de Ciência Animal (CECA), ICETA-U.P., Campus Agrário de Vairao, 4480 Vila do Conde, Portugal. Fax: +351 252 6611780; E-mail: sjweiss@mail.icav.up.ot

The role of a southwest Atlantic refuge in this process is described, based on frequencies of alleles that are fixed or almost fixed in rivers draining the north Iberian peninsula (LDH-C*100; CK-A1*115; García-Marín & Pla 1996) and occur at varying frequencies in Atlantic-draining rivers of France (Krieg & Guyomard 1985; Presa *et al.* 1994) as well as at some locations in Northern Ireland and Scandinavia (Allendorf *et al.* 1976; Crozier & Ferguson 1986). While studies on the north Iberian Peninsula lacked a key diagnostic locus (TF*), genetic characterization of three Portuguese populations (Antunes *et al.* 1999) revealed significant frequencies of the TF*95 allele, which had previously been seen only at low frequencies in some headwater tributaries of the Rhone (Mediterranean) and Po (Adriatic) rivers (Largiadèr & Scholl 1995; Largiadèr *et al.* 1996). The genetic distinctiveness of southwest Atlantic populations has also been described in a recent allozyme survey of northwest Spain (Bouza *et al.* 1999). Thus, a more comprehensive investigation of genetic diversity in the southwest Atlantic is required to evaluate its role in the current model of glacial refugia and postglacial gene flow.

We sequenced the 5′ end of the mtDNA control region in eight to 15 individuals from seven Portuguese river drainages. In addition, we considered published data on mtDNA haplotype diversity, as well as diagnostic allozyme alleles, to evaluate the congruency of the geographical distribution of mtDNA haplotypes with the most recent allozyme-based hypothesis of the post-Pleistocene colonization of northern Europe.

Materials and methods

Brown trout were collected by electrofishing from 12 tributaries within seven major river drainages (Fig. 1). Fish were stored at −80 °C, and frozen muscle tissue was thawed in 96% ethanol. Whole genomic DNA was isolated by using a high-salt extraction technique (Miller *et al.* 1988). The complete mtDNA control region was amplified in 76 specimens with primers H20 and L19 using conditions described in Bernatchez *et al.* (1992). The DNA from each individual was sequenced using an internal heavy-strand primer (H514) 5′-GTGGGTAACGGGCAATAAGA-3′, which binds 514 bp upstream from the 5′ end of the control region. DNA from at least two individuals of each haplotype in each population was sequenced in the opposite direction using primer L19. The polymerase chain reaction (PCR) product was purified using a QIAquick™ PCR purification kit (QIAGEN), quantified on agarose gels stained with ethidium bromide and sequenced via cycle sequencing according to the manufacturer's instructions (BigDye™; Perkin-Elmer). Cycling conditions were as follows: initial denaturation at 96 °C for 1 min; and then 25 cycles at 96 °C for 15 s, 56 °C for 5 s and 60 °C for 4 min. The cycling sequence product was electrophoresed on an ABI-377

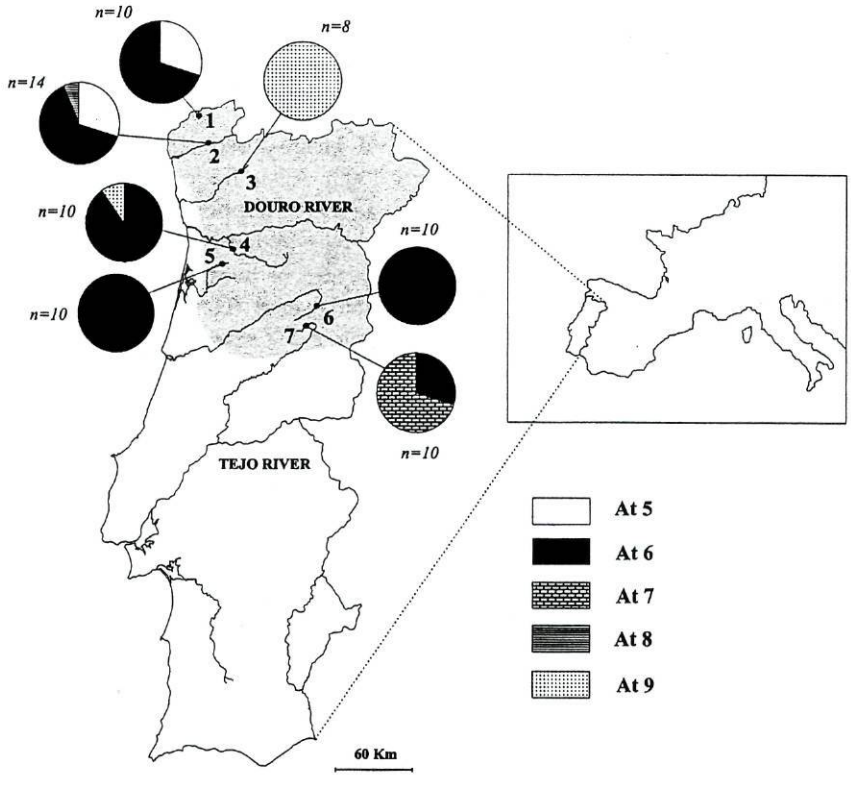

Fig. 1 Map of Portugal showing the geographical location of the seven populations sampled. The shaded pie charts display haplotype frequencies; sample sizes are shown beside each pie chart. The names of the major river basins are numbered from 1 to 7 on the figure and listed as follows (with, in parentheses, the specific tributaries sampled when different from the major river): (1) Minho (Manco); (2) Lima (Estorãos, Trovela, Vade, Tamente and Froufe); (3) Ave; (4) Douro (Ardena and Tenente); (5) Vouga (Caima River); (6) Mondego; (7) Tejo (Zêzere). The shaded area of the map represents the current distribution of brown trout in Portugal.

automated sequencer. All sequences were aligned and checked by hand using the Sequence Navigator software.

Analysis

An inferred phylogenetic tree was generated from the aligned sequences using the quartet-puzzling, maximum-likelihood (ML) procedure (Strimmer & von Haeseler 1996; Strimmer *et al.* 1997) in the program PUZZLE, version 4.0. This procedure is described in detail in Strimmer & von Haeseler (1996); its choice here is based on the fact that it provides a fast, heuristic search of ML trees, allowing the use of standard models of sequence evolution in addition to an estimation of substitution-rate heterogeneity among sites using a discrete gamma distribution. Given that the mtDNA control region displays complex mutation-rate heterogeneity (Meyer *et al.* 1999 and citations therein), and this heterogeneity cannot be accounted for by simply weighting transitions or codon position, such estimation procedures may be necessary to achieve a reasonable model of sequence evolution.

Sequence data were also converted into distance matrices (100 bootstrap replicates), calculated under a Kimura 2-parameter model, and these matrices were then used to build trees using the Neighbour-Joining (NJ) algorithm. A majority-rule consensus tree of the 100 NJ trees was visually compared to the ML tree generated with the quartet-puzzling procedure. These latter procedures were carried out using the programs SEQBOOT, DNADIST, NEIGHBOUR and CONSENSE in the PHYLIP 3.5c computer package (Felsenstein 1993), and tree graphics were produced using the program TREEVIEW (Page 1996).

Several other sequences, for both in- and outgroup comparison were used in the phylogenetic reconstruction. An Atlantic salmon *Salmo salar* sequence (GenBank acc. no. M97987) was used as an outgroup, and sequences obtained from fish collected in tributaries of both the Mur and Salzach rivers in Austria were used to represent the Danubian clade. Sequences for the common At1 haplotype (Bernatchez *et al.* 1992) were obtained from a hatchery in Wels, Austria, which imports live eggs from Denmark (material described in Weiss & Schmutz 1999) as well as samples obtained from 15 different Danubian tributaries within Austria. Although our sequence was longer (464 bp of the control region) than that originally used to define the At1 haplotype (297 bp), there was no additional variation found in the extended portion of the sequence. That is, of 500 wild fish thus far screened throughout Austria, 177 possessed the At1 haplotype with no other Atlantic clade variant being found (S. Weiss, unpublished).

For all analyses, multiple sampling locations within the Lima river basin (five), and within the Douro river basin (two) were pooled and considered as populations. Genetic variation within each population was quantified by haplotype diversity (h) and nucleotide diversity (π) (Nei 1987). To assess the geographical subdivision of haplotypes among populations, we used the test statistic Kst* described in Hudson *et al.* (1992) and available in the program PROSEQ (beta version 2.3). The significance of Kst* was calculated by permuting haplotypes (1000 times) among populations. Haplotype diversity within and among populations was additionally evaluated using analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) whereby the effects of using different molecular distance measurements were compared. The significance of the among-population variance component in the AMOVA (ϕ_{ST}) as well as pairwise F_{ST} analog values (based on a Kiwura 2-parameter model) were tested by permuting haplotypes between populations. These latter analyses were performed in the program ARLEQUIN, version 1.1 (Schneider *et al.* 1997).

Results

Table 1 shows the six variable nucleotide positions found in the 76 sequenced individuals in addition to sequence-based, Atlantic-clade haplotypes found in the literature. According to this comparison, five new Atlantic-basin haplotypes were found in Portuguese rivers, each two to three mutational steps distant from the common north Atlantic haplotype (At1) and four to five mutational steps distant from several other Atlantic clade haplotypes.

No haplotype outside the Atlantic clade was found. All fish possessed a newly described haplotype except for four individuals from the Mondego River that had the common At1 haplotype. Allozyme characterization of these populations revealed that these four fish were homozygous for the LDH-C*90 allele previously thought to be absent in Portuguese rivers (A. Antunes, unpublished; Antunes *et al.* 1999). As the remaining 72 individuals were fixed for the LDH-C*100 allele, the four with a 90/90 genotype were presumed to be directly from a nearby hatchery. (The Portugese hatchery presumed to be the source of these fish was previously screened at the LDH-C locus and no *90 alleles were found (Antunes *et al.* 1999); however, subsequent to finding the 90/90 genotype in the wild, additional monitoring revealed the *90 allele in the hatchery at low frequencies, suggesting that an allochthonous strain has been recently introduced.)

Table 2 shows haplotype frequencies among the seven Portuguese populations. One haplotype (At6) predominates in most, but not all, populations and two drainages are fixed for a single haplotype, demonstrating significant population substructure (Kst* = 0.3506, $P < 0.001$) among these rivers whose confluences with the Atlantic span a coastline of ≈ 400 km (Fig. 1). The AMOVA also showed significant population structure with ϕ_{ST} values ranging from 0.390 (Tajima-Nei distances) to 0.475 (number of pairwise differences); all ϕ_{ST} values were highly significant ($P < 0.001$).

Table 1 Variable nucleotide positions in Atlantic-basin haplotypes (found in the first 464 bp of the 5′ L-strand of the mitochondrial DNA [mtDNA] control region) and comparison with two common upper-Danubian haplotypes

	Nucleotide position												
Haplotype	2	26	111	146	226	234	235	236	262	309	388	389	390
At1	T	T	T	G	C	G	A	T	G	G	G	C	T
At4†	—	—	—	—	—	—	G	G	—	—	—	—	—
At5	—	—	C	—	—	—	—	—	T	—	—	—	—
At6	—	—	C	A	—	—	—	—	—	—	—	—	—
At7	—	—	—	A	—	—	—	—	—	A	—	—	—
At8	—	—	—	A	T	—	—	—	—	—	*	—	—
At9	—	—	—	A	—	—	—	—	—	—	*	—	—
Da2	C	A	—	—	—	—	G	G	—	—	—	T	C
Da1	C	A	—	—	—	—	—	A	—	—	—	T	C

†Haplotype At4 occurred in samples from the Garonne River, France (Apostolidis *et al.* 1997).
Three additional haplotypes with an 'At' designation are reported in Osinov & Bernatchez (1996), all differ from At1 by restriction sites outside the region analysed here.
Nucleotide positions refer to the number of bases from the first position of the control region using the complete mtDNA sequence of the rainbow trout *Oncorhynchus mykiss* (GenBank acc. no.: L29771) as a reference. Newly described haplotypes found in Portuguese rivers are marked in bold. The asterisk at position 388 for haplotype At8 and At9 refers to a single base pair deletion.

Table 2 Mitochondrial DNA (mtDNA) haplotype frequencies in seven Portuguese populations

	Haplotype						
Population	At1	At5	At6	At7	At8	At9	Total
Minho	0	3	7	0	0	0	10
Lima	0	4	9	0	1	0	14
Ave	0	0	0	0	0	8	8
Douro	0	0	9	0	0	1	10
Vouga	0	0	10	0	0	0	10
Mondego	4	0	10	0	0	0	14
Tejo	0	0	3	7	0	0	10
Total	4	7	48	7	1	9	76

The inferred phylogenetic relation of Portuguese haplotypes to others shows significant divergence from the At1 haplotype (Fig. 2). Haplotype At8 was found in only one individual; this sequence was validated by repeating the PCR-product amplification and sequencing analysis using a separate DNA extraction. As haplotypes At8 and At9 possess a common deletion at position 388 (the only indel in our data), which is not considered in the ML-based phylogenetic reconstruction, the divergence of these haplotypes from the cluster containing At5, At6 and At7 is underestimated.

While the estimated substitution-rate heterogeneity was substantial (relative rates varied from 0.0000 to 6.5239), the consequences of allowing for heterogeneity in this

Table 3 Genetic variation of the seven Portuguese populations in this study

Drainage	Minho	Lima	Ave	Douro	Vouga	Mondego	Tejo	*h*	π
Minho		NS	**	NS	NS	NS	**	0.4667	0.00187
Lima	0.0817		**	NS	NS	NS	**	0.5385	0.00146
Ave	0.6836	0.5574		**	**	**	**	0.0000	0.00000
Douro	0.1933	0.1157	0.8764		NS	NS	**	0.2000	0.00040
Vouga	0.2222	0.1376	1.0000	0.0000		NS	**	0.0000	0.00000
Mondego	0.0935	0.0506	0.4723	0.0972	0.1861		**	0.4396	0.00176
Tejo	0.4670	0.4044	0.8507	0.5851	0.6667	0.4599		0.4667	0.00093

Lower diagonal, Pairwise F_{ST} — analog values based on Kimura 2-parameter distances (gamma correction 0.09); upper diagonal, significance of these values; *h*, haplotype diversity; π, nucleotide diversity.
*Significant at the 5% level; **significant at the 1% level; NS, not significant.
An experimentwise error rate was applied using a sequential Bonferonni procedure (Sokal & Rohlf 1995, page 241).

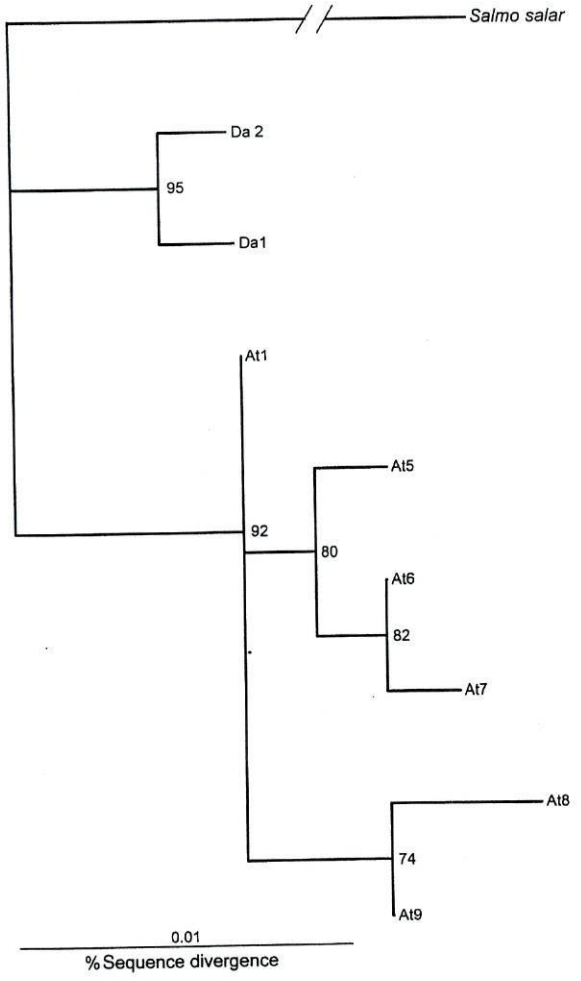

Fig. 2 Inferred phylogeny of Portugese mitochondrial DNA (mtDNA) haplotypes in relation to two common Danubian haplotypes and *Salmo salar* (GenBank acc. no. M97987). The tree was constructed using a quartet-puzzling, maximum likelihood (ML) procedure assuming an HKY model of sequence evolution (Hasegawa *et al.* 1985) and allowing for substitution-rate heterogeneity, which was estimated from the data using a discrete (eight categories) gamma distribution (alpha = 0.09). Support values for each internal branch indicate (in %) how often a cluster was obtained in the construction of 1000 intermediate trees. Both a ML tree allowing no substitution-rate heterogeneity, and a majority-rule consensus of Neighbour-Joining (NJ) trees had the same topology.

data set was not large because the identical tree topology was achieved with uniform rates, albeit with higher support for the nodes carrying haplotypes originating outside Portugal, and lower support for the topology of haplotypes found within Portugal (Fig. 2). Likewise, the majority-rule consensus tree based on a Kimura 2-parameter distance matrix produced almost identical topology to the ML tree, although bootstrap values for the internal branches carrying Portuguese haplotypes were relatively low (42–77%).

Discussion

To date, brown trout within Atlantic basin drainages have shown little variation within the mtDNA control region. This study has revealed relatively high mtDNA control-region diversity (0.22–1.07% sequence divergence), supporting the notion that the southwest Atlantic has served as a glacial refuge. While some restriction fragment length polymorphism (RFLP) analyses of Atlantic basin populations revealed a greater number of haplotypes (e.g. see Hynes *et al.* 1996), Portuguese haplotypes (varying from one to five mutational steps from each other) probably represent lineages of a greater divergence than those found thus far in north Atlantic populations. This is supported by the fact that much larger regions of mtDNA are screened in RFLP analyses and the strong evidence that the control region in brown trout is evolving much more slowly than at least some coding regions. For instance, Hansen & Loeschcke (1996) found 13 RFLP haplotypes in populations that showed no variation in the control region, and Apostolidis *et al.* (1997) reported sequence divergence of up to 4.68% among haplotypes derived from RFLP analysis of NADH 5/6 genes compared with 2.31% in the same individuals based on control-region sequences.

Despite the high divergence of mtDNA lineages in Portuguese populations relative to elsewhere in the Atlantic basin, this refuge appears to be less diverse or of more limited historical complexity than other major brown trout refugia, such as the Caspian–Black–Aral basins or the Adriatic and Aegean seas. For example, there are fewer control-region haplotypes than found in the Caspian–Black–Aral basins (Bernatchez *et al.* 1992; Osinov & Bernatchez 1996), and no evidence of admixture from divergent clades was detected, as in Mediterranean refugia where control-region sequence divergence ranged from 0.32 to 2.31% (Apostolidis *et al.* 1997). Thus, Portuguese populations have evolved, for some time, in isolation from other clades. Furthermore, the geographical heterogeneity of haplotype frequencies within Portugal suggests that populations within this refugia have themselves been isolated by physical barriers or other factors that limit gene flow and promote genetic drift. The highly significant population structuring, demonstrated by statistical analysis, is clearly obvious by the fact that two of the five newly described haplotypes (At7 and At8) are private to individual populations, and a third (At9) is fixed in one population (Ave) and found in only one other individual in the adjacent Douro drainage. While this heterogeneity is probably a result of historical factors, gene flow between these populations can presently occur only via the Atlantic ocean (samples from the Caima River were collected above a natural waterfall). Additionally, a combination of man-made barriers and, presumably, temperature conditions limit the occurrence of anadromy in this region to the two northernmost

drainages, Minho and Lima (SNPRCN 1991). Thus, although haplotype diversity in Portugal is high, the populations are isolated from each other, reflecting a unique genetic architecture even within the so-called southwest Atlantic refuge.

However, it is the divergence of Portuguese haplotypes from others in the Atlantic clade that is most revealing as it strongly suggests a long period of isolation. A minimum divergence estimate can be calculated by applying a relatively rapid substitution rate estimate for mtDNA (2% sequence divergence per 1 million years [Myr]), which is two to four times as high as that calculated for salmonid mtDNA by Martin & Palumbi (1993) and calibrated in Giuffra *et al.* (1996). Thus, haplotypes found in Portuguese rivers may have diverged from other Atlantic clade haplotypes at least 200 000 years ago (from the common At1 haplotype), in any case, long before the last glacial maximum. As such, the haplotypes found in this study should serve as markers for postglacial gene flow into the north but they have never been found in the north.

Clearly this data is in conflict with the proposition that gene flow from the southwest Atlantic (in particular, Atlantic-draining rivers of the Iberian Peninsula) has contributed to the post-Pleistocene recolonization of northern Europe. Hence, the question arises as to whether there is incongruency between the patterns of postglacial gene flow revealed by putative diagnostic allozyme alleles and the geographical distribution of mtDNA haplotypes. We argue that the incongruency is rooted only in misinterpretation, and that both allozymes and mtDNA data support a more geographically limited postglacial dispersal of brown trout into northern Europe than proposed, for example, in García-Marín *et al.* (1999).

The post-Pleistocene colonization of northern Europe

Ferguson & Fleming (1983) proposed that the northwest Atlantic was colonized independently by two races of brown trout (discussed further in Hamilton *et al.* 1989), and Hynes *et al.* (1996) revised this hypothesis (based primarily on the distribution of LDH-C* alleles) to include more than two temporally segregated colonization events. García-Marín *et al.* (1999) contributed to these multiple colonization hypotheses by proposing the glacial refugia that were involved and in what general sequence. We reiterate for clarity the putative pairs of diagnostic alleles and their corresponding glacial refugia: LDH-C*90 and CK-A1*100 for northern continental Europe; LDH-C*100 and CK-A1*115 for the southwestern Atlantic; and LDH-C*100 and CK-A1*100 for the Ponto–Caspian basin. Populations that were either fixed for, or displayed high frequencies of, these pairs of alleles were thought to have been colonized by dispersal from the corresponding refugia.

Evidence against gene flow from the Iberian Peninsula

In addition to the new mtDNA haplotypes presented in this study, Hynes *et al.* (1996) found two RFLP mtDNA haplotypes, which were private, in a north Iberian population with respect to 39 separate locations in the Atlantic basin, mostly in the northwest. Furthermore, the TF*95 allele found at intermediate frequencies in Portuguese populations is also likely to represent an older mutation as it is found in headwater populations of both Adriatic and Mediterranean rivers, basins that have been separated from the Atlantic for at least several hundred thousand years. This allele is apparently absent in populations of the north Atlantic basin (Krieg & Guyomard 1985; Presa *et al.* 1994).

The CK-A1* 115 allele is fixed or at high frequencies in six of seven Rhenian headwater populations sampled by Riffle *et al.* (1995). This data set was excluded from the analysis of García-Marín *et al.* (1999), presumably because of concerns over the stocking generally known to occur in this region. However, only two of these seven populations have records of regular stocking (three have no records of stocking) and frequencies of the *115 allele are highest (0.77–1.00) in the putatively unstocked populations. The *115 allele is also reported at high frequencies in a tributary of the Baltic sea where both the LDH-C *90 allele and Atlantic basin mtDNA haplotypes are fixed (Bernatchez & Osinov 1995). Thus, there is ample evidence that the CK-A1* 115 allele occurred in northeastern or central continental refugia during the last glacial maximum and cannot be used as a marker for postglacial gene flow from the southwest Atlantic.

Evidence against gene flow from the Danubian basin

Several lines of evidence appear to be incompatible with an early wave of postglacial dispersal from the Caspian–Black–Aral basins into the north Atlantic. First, no Atlantic basin population thus far screened has contained a mtDNA haplotype characteristic of the highly divergent Danubian clade (Bernatchez *et al.* 1992; Osinov & Bernatchez 1996). Second, 16 of the 54 allozyme alleles (across 24 loci) screened in a study that included populations from the Baltic, Barents and White seas (Osinov & Bernatchez 1996), were found exclusively in the Caspian–Black–Aral basins. Thus, postglacial gene flow from this basin would have had to occur with the complete loss of diagnostic mtDNA together with all known basin-specific allozyme alleles. Data that could be used to support northwestward dispersal appear then to be limited to the co-occurrence of the LDH-C*100 allele and CK-A1*100 in some modern north Atlantic populations, although neither of these two alleles alone can be presumed to be diagnostic of the Caspian–Black–Aral basins.

Furthermore, the presence of the LDH-C*100 allele in the north Atlantic can be based simply on the fact that it

is ancestral and must have been present in some north continental populations prior to, or during, the last glacial maximum. If the derived LDH-C*90 allele arose after the first wave of recolonization into the north, no separate refugia are required at all to explain the present patterns of allelic distribution at this locus.

Given the low number of loci with any explanatory power, it would be difficult to exclude with certainty any number of complex postglacial recolonization scenarios. This is so, not considering potential weaknesses in the diagnostic power of specific loci as a result of, for example, selection on the LDH-C* locus (Henry & Ferguson 1985), or the typically lower effective population size of mtDNA. We note that although the effective population size of mtDNA will be 25% of that of a nuclear gene assuming an equal gender ratio, polygyny, which is common in salmonid fish, will increase the variance in male reproductive success and this could lead to an effective population size of mtDNA that is as high or even higher than a nuclear gene (Hoelzer 1997).

Nonetheless, in the absence of our ability to confirm competing hypotheses, the 'simplest' model should be accepted. The geographical distribution of both mtDNA and nuclear gene markers in previously glaciated areas of northern Europe is most easily explained by postglacial dispersal from refugia located northwards of the Iberian Peninsula, as well as the Black–Caspian–Aral basins. This conclusion neither supports nor contradicts earlier hypotheses of multiple waves of recolonization (Ferguson & Fleming 1983; Hamilton *et al.* 1989; Hynes *et al.* 1996) but presently available data do not allow any further clarification of the source populations involved. Angers & Bernatchez (1998) have shown that microsatellite analysis can be used to reveal a more fine-scaled evolutionary history in salmonid fishes, and thus such an approach may aid in future attempts to identify multiple refugia within continental Europe. Regardless of the method used, however, a comprehensive and strategic sampling regimen would be required for there are many regions of central and eastern Europe that could have potentially served as distinct glacial refugia.

This study describes mtDNA diversity, which is highly unique for the Atlantic basin and presumably representative of a southwest Atlantic glacial refuge. While no attempt is made to geographically define this refuge, Portuguese rivers clearly belong to it and as such should be protected from introgression with allochthonous hatchery strains.

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S. Weiss has diverse interests in the ecology, evolution and conservation management of freshwater fishes, and is currently involved in several phylogeographical studies of salmonid fishes; his laboratory work is carried out in the population genetics group of C. Schlötterer. A. Antunes is researching the genetic structure and phylogeography of Portuguese brown trout (as part of his PhD dissertation) under the supervision of P. Alexandrino who heads the Unit of Animal Genetics and Conservation at the Centre for the Study of Animal Sciences, University of Porto.

On the southern edge of the Atlantic brown trout distribution: genetic portrait of population persistence over Pleistocene climatic cycles.

Agostinho Antunes[1,2,3], Rui Faria[1], René Guyomard [3] and Paulo Alexandrino [1,2]

[1]Centro de Estudos de Ciência Animal (CECA), ICETA-U.P., Campus Agrário de Vairão, 4485-661 Vairão, Portugal; [2]Departamento de Zoologia-Antropologia, Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, 4099-002 Porto, Portugal; [3]Laboratoire de Génétique des Poissons, INRA, 78352 Jouy-en-Josas, France.

Abstract

The Atlantic Iberian brown trout is at the edge of the species' southwestern distribution and at the southern limit of the anadromous life-history form. Twenty populations were selected from this once glacial refugia and spatial genetic structure was assessed using 11 allozyme and 4 microsatellite polymorphic loci. The patterns of genetic variation were mostly concordant among the different categories of nuclear markers, although microsatellites were much more sensitive indicators of loss of genetic variation within populations. A southwards stepwise decrease in heterozygosity was observed for both categories of markers. In populations located south of the anadromy limit, microsatellite loci showed a pattern of allele depletion indicating repeated population bottlenecking in this "southern edge". Estimates of genetic differentiation revealed a strong heterogeneity, with a clear distinction of populations located north and south of the limit of anadromy (respectively, allozyme G_{ST} = 12 and 54%; microsatellite G_{ST} = 16 and 67%). The spatial genetic structure observed both for allozyme and microsatellite loci can be explained by two major events: (i) an historical mosaic fragmentation in the area currently to the south of the anadromy limit, and (ii) a relatively continuous gene flow from northern populations to the south. The historical scenario suggested a long persistence of populations in this refuge area through the Pleistocene sequential glacial and interglacial periods, causing a strong population bottlenecking and mosaic patterns of substructuring. This picture was further influenced by southwards gene flow from northern populations followed by secondary contact between allopatric groups. Considering the present fragmented genetic structure as well as current threats to brown trout, enhancement of these populations should be viewed with caution, regarding the relative importance of both inbreeding and outbreeding depression.

Introduction

Species rarely consist of a single large population, but rather are distributed in a more or less spatial structured array of populations (McCauley 1995). Within this context, population structure is intrinsic to genetic processes, not only determined by current population dynamics, but also by historical patterns of gene flow, further shaped by genetic drift, mutation and natural selection (Slatkin 1987; Hewitt & Butlin 1997; Allendorf & Seeb 2000). In turn, biological features, such as breeding and dispersal traits, and geological, such as the effects of the last glaciations, both influence these evolutionary components (Hewitt 1999). For freshwater organisms, genetic structure is further affected by the extremely fragmented and linearly confined pattern of habitats.

Multiple combinations of these events may portray complex evolutionary histories, and an illustrative example of this complexity is the brown trout (*Salmo trutta* L.). Native to Eurasia and North Africa, the brown trout consists of a mosaic of evolutionary lineages scattered across their range, in an often-unpredictable pattern, influenced by the advance and retreat of glaciers, subsequent dispersal and secondary contact between lineages (Grant et al. 1999; Laikre et al. 1999; Antunes et al. 2001). At a large geographical scale, five major mitochondrial (mt) DNA lineages, which have evolved allopatrically for a long period of time have been described: Danubian, Adriatic, Marble trout, Mediterranean and Atlantic (Bernatchez et al. 1992; Bernatchez 2001). At a more local scale, medium to strong genetic differentiation has been observed between populations, including the total isolation of sympatric demes (e.g. Ryman et al. 1979; Ryman 1983; Crozier & Ferguson 1986; Ferguson & Taggart 1991; Estoup et al. 1998; Bouza et al. 2001). This complex genetic structure seems to have resulted from multiple factors. Among these, the hierarchical and physical characteristics of the hydrographic system, the influence of the last glaciations, and also, local differentiation without barriers to migration due to the homing behavior (Behnke 1972; Hamilton et al. 1989; Crozier & Ferguson 1986; Elliot 1994).

The Iberian Peninsula offers an excellent area to study the main factors that determine brown trout population genetic structure. As has been emphasized, the distinctive species patterns of genetic variation and subdivision across Europe seem to be linked with isolation in various Pleistocene cold-stage refugia in southern peninsulas (Hewitt 1999, 2000; Taberlet et al. 1998). Substantial allozyme divergence has been reported in Iberian brown trout populations, especially between Mediterranean and Atlantic drainages (García-Marín & Pla

1996). Further, significant differences in nuclear and mtDNA markers have distinguished Atlantic Iberian from the more northern Atlantic populations (e.g. Antunes et al. 1999; Bouza et al. 1999; García-Marín et al. 1999a; Sanz et al. 2000; Machordom et al. 2000; Weiss et al. 2000). Atlantic Iberian brown trout is at the edge of the species' southwestern distribution and at the southern limit of the anadromous life-history form (figure 1). In an area of presumed glacial refuge, substantial genetic structure has been identified, suggesting the existence of complex demographic histories in these populations (e.g. Antunes et al. 2001; Bouza et al. 2001). Thus, they represent an ideal group of populations to use as a model to investigate the relative importance of dispersal, long residency and geomorphological history on population genetic structure.

In this study, we analyze the amount and distribution of genetic diversity in coastal Atlantic Iberian brown trout populations. Our main goal is to investigate the spatial structure assessed by allozyme and microsatellite loci to: (i) examine if the effects of dispersal ability (resident *versus* anadromous life history) resulted in differences within and among population patterns of differentiation; (ii) study population structure and evolutionary history on this once glacial refuge; and (iii) evaluate implications for conservation.

Materials and methods

Study site and sampling design

The sampling scheme was drawn to investigate coastal Atlantic Iberian brown trout populations from areas scarcely or not analyzed previously. Twenty populations of brown trout were sampled by electrofishing between 1995 and 2000 (N = 847). The samples cover some basins in Spain (Asturias; Cantabric region) and most major Portuguese basins where brown trout occurs (figure 1). The southernmost limit of anadromy (SLA) currently lies in the Lima basin (SNPRCN 1991). Samples were collected to the north and south of this limit. Because we are interested in studying the native gene pool, we have assessed the impact of stocking with non-native hatchery fish. Potential introgression was assessed using a set of diagnostic and highly discriminative allozyme loci previously used for such purposes in southwest Atlantic populations. The **90* allele at the *LDH-C** locus is not native to Iberia and is usually fixed in commercially available hatchery strains (García-Marín et al. 1991, Presa et al. 1994, Arias et al. 1995). The diagnostic power of the remaining loci: *G3PDH-2**, *GPI-A1**, *IDHP-1**, *sMDH-A2** and *sMDH-B1**, is based on the assumption that

particular alleles (*G3PDH-2*50*, *GPI-A1*110*, *IDHP-1*160*, *sMDH-A2*120* and *sMDH-B1*80*) occur only in northern populations whereas southwest Atlantic populations are fixed for the **100* allele at each locus (García-Marín et al. 1991, Martínez et al. 1993, Bouza et al. 1999). The evaluation of the impact and outcome of stocking, have been previously assessed for the Portuguese populations (Antunes et al. 2001). Although some non-native hatchery fish were detected in Tenente, Mondego and Zêzere no evidence of introgression was found. The potential introgression with non-native strains was assessed for the Spanish populations only.

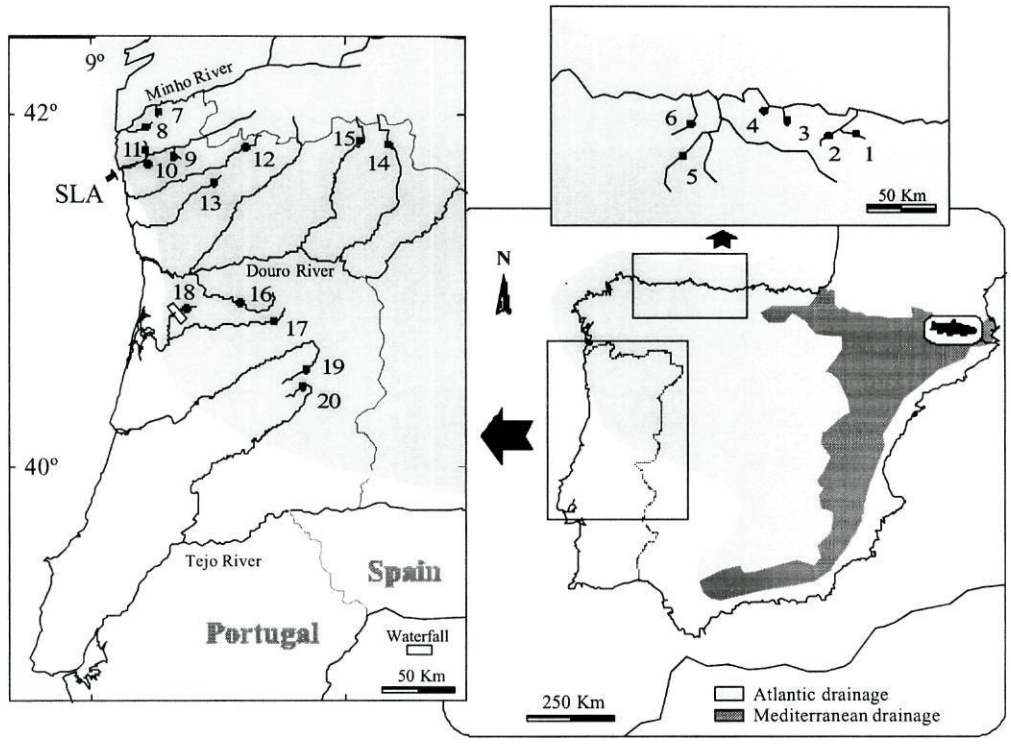

Figure 1. Geographical location of the 20 brown trout populations from the Atlantic Iberian drainage analyzed in the present study: Sella basin (1-Dobra and 2-Ponga); Cancienes basin (3); Ferrerias basin (4); Narcea basin (5-Piguena); Esva basin (6-Meras); Minho basin (7-Manco and 8-Coura); Lima basin (9-Vade, 10-Trovela and 11-Estorãos); Cávado basin (12); Ave basin (13); Douro basin (14-Sabor, 15-Tuela and 16-Tenente); Vouga basin (17-Vouga and 18-Caima); Mondego basin (19); Tejo basin (20-Zêzere). SLA denotes the current southern limit of anadromy (Lima basin). The shaded area represents squematically the current distribution of brown trout in the Iberian Peninsula. The reference population from Spain (Ter - Mediterranean drainage) is identified by a black fish.

Allozyme analysis

Extraction and storage of blood, muscle, liver and eye tissues were made in accordance with procedures described in Antunes et al. (1999). A preliminary investigation of electrophoretic variation in 34 allozyme loci and one plasma protein locus, allowed the identification of 14 polymorphic loci (including those variable for non-native hatchery stocks). The nomenclature employed followed Shaklee et al. (1990) and the harmonization proposal by the Concerted Action on brown trout (TROUT-CONCERT 1999): creatine kinase (*CK-A1**, *CK-A2**; E.C. 2.7.3.2.), glycerol-3-phosphate dehydrogenase (*G3PDH-2**; E.C. 1.1.1.8), glucose-6-phosphate isomerase (*GPI-A**, *GPI-B1**, *GPI-B2**; E.C. 5.3.1.9), isocitrate dehydrogenase (*IDHP-1**; E.C. 1.1.1.42), L-lactacte dehydrogenase (*LDH-C**; E.C. 1.1.1.27), malate dehydrogenase (*sMDH-A2*, *sMDH-B1*, *sMDH-B2*; E.C. 1.1.1.37), mannose-6-phosphate isomerase (*MPI-2**; E.C. 5.3.1.8), tripeptide aminopeptidase (*PEPB**; E.C. 3.4.11.4), Leucyl-tyrosine peptidase (*PEPLT**; E.C. 3.4._._), phosphoglucomutase (*PGM-1**; E.C. 5.4.2.2) and transferrin (*TF**). These loci were then scored for the 20 populations using starch gel electrophoresis and isoelectric focusing (IEF) procedures described by García-Marín et al. (1991), Martínez et al. (1993) and Antunes et al. (1999). The survey of the PGM-1* locus was done by IEF in polyacrylamide gels (T=5%, C=3%; saccharose 20% (w/v); 230×100×0.3 mm) in a 1:8:1 mixture of pH 3.5-10, pH 5-8 and pH 8-9.5 carrier ampholytes (Pharmacia) in a final concentration of 5% v/v. Prefocusing and focusing sets were those referred in Antunes et al. (2000).

Microsatellite analysis

Based on the heterogeneity of the allozyme variation profile observed, we selected a subset of 11 populations (four and seven, north and south of the limit of anadromy, respectively) for microsatellite analysis. To investigate evolutionary relationships in allele frequencies and because microsatellite population analyses are scarce from Iberian brown trout, we have additionally scored a reference population from the Mediterranean drainage of Spain (previously studied for allozyme variation in García-Marín & Pla 1996).

Whole genomic DNA was extracted from blood or muscle tissue following Sambrook et al. (1989). Four dinucleotide microsatellite loci were used: *BS131*, with the core repeat $(TG)_6...(TG)_{18}$; *Str-85*, core repeat $(CT)_{22}$; *Str-543*, core repeat $(CT)_{13}$; and *Str-591*, core repeat $(CT)_{11}$ (Presa & Guyomard 1996; Estoup et al. 1998). Microsatellite loci were analyzed by PCR amplification, according to the conditions described in Presa & Guyomard (1996) and Estoup et al. (1998). The resulting products were electrophoresed in a 7% denaturing polyacrylamide gel and visualized by autoradiography (after amplification with one primer end-

labeled with γ-^{33}P) or silver staining. Allele sizes were determined relatively to a standard base pair size ladder.

Statistical analyses

Allele frequencies were estimated directly from zymograms for loci showing codominant expression. The variation observed at the *sMDH-B1, B2** isoloci was attributed to both loci when more than two doses of the less common allele were observed. Alleles for the isoloci *CK-A1, A2** were allocated to the single locus *CK-A1** and their frequencies estimated by the square root of the frequency of homozygous phenotypes, under the assumption of Hardy-Weinberg (HW) equilibrium.

The GENETIX (version 4.02; Belkir et al. 1996) and the GENEPOP (version 3.1b; Raymond & Rousset 1995) were used: (i) to estimate percentage of polymorphic loci (P_{95}), mean number of alleles per locus (A), mean expected heterozygosity (H_E) and gene diversity (G_{ST}; Nei 1973, 1977); (ii) to assess deviations from HW expectations (by exact probability following Fisher's method in a Markov chain procedure) and (iii) to estimate the inbreeding coefficient of Weir & Cockerham (1984) (F_{IS}). The likelihood that each allozyme genotype belonged to the hatchery or wild stocks was determined with an assignment test using the program WHICHRUN (version 3.2; Banks and Eichert 2000). Estimates of gene flow (N_em, absolute number of migrants per generation) among populations, were obtained by using the F_{ST} based estimates following the expression $G_{ST} \approx F_{ST} = 1/(1+4N_em)$ (Wrigth 1951). Cavalli-Sforza & Edwards (1967) chord genetic distances (D_{CE}) were used to quantify genetic divergence among populations, which makes no assumption regarding constant population size or mutation rates among loci. The magnitude of the D_{CE} distance is not proportional to evolutionary time, but its use generally leads to a higher probability of depicting the correct tree topology among close related populations under either the infinite-alleles mutation model (IAM; Kimura & Crow 1964) or the stepwise mutation model (SMM; Ohta and Kimura 1973) assumptions (Nei & Takezaki 1996; Takezaki & Nei 1996). Other methods were also used to evaluate genetic distance, such as Reynolds et al.'s (1983) genetic distance (D_R) (based in the population estimator F_{ST}) and Goldstein et al.'s (1995) pairwise population distances $(\delta\mu)^2$ (takes into account deviations of allele size variance from theoretical expectations under SMM) computed using respectively, the GENDIST program included in the PHYLIP computer package (version 3.6; Felsenstein 1993) and the MICROSAT program (version 1.4; Minch 1996). The use of D_R (for allozyme and microsatellite data) and $(\delta\mu)^2$ distance (for microsatellite data)

in the Neighbor-Joining (NJ) analyses did not improve the tree structure resolution so, they were excluded from further analysis. NJ networks were constructed using the D_{CE} distance estimated with GENDIST, and applying NEIGHBOR and DRAWTREE routines in the PHYLIP computer package. Bootstrap replication values across populations were obtained to assess to what extend the NJ network topology is supported by the data.

Non-hierarchical cluster analysis was done by multidimensional scaling (MDS) with the genetic distances (Lessa 1990). The MDS was chosen to provide a perspective of the underlying structure of the D_{CE} genetic distance matrix without imposing the bifurcating evolutionary history. The MDS was generated with the software package STATISTICA (version 4.5; StatSoft 1993).

The association between genetic differentiation and proposed causal hypotheses was tested by partial Mantel tests in RT (version 2.0; Manly 1996). The D_{CE} genetic distances and heterozygosity distance (absolute pairwise difference between H_E values for populations) for allozymes and microsatellites were used as dependent variables. Geographic (waterway or riverine) distances among brown trout populations were measured following the length of the river, if necessary including the river mouth to river mouth distance across sea. The mode of life in populations was qualified as resident (south of the SLA) or anadromous/resident (north of the SLA). A regional component was also considered by splitting populations in two main regions, namely Cantabria and Portugal.

Results

Wild and hatchery fish

The multi-locus assignment test unambiguously characterized all individual fish as hatchery or wild. Some non-native hatchery fish (N = 8) were detected in Cancienes. The assignment of wild-caught individuals to the hatchery stock was primarily based on the *LDH-C*90/90* genotype. No heterozygotes (**90/100*) were observed in the wild (HW equilibrium rejected at P<0.01; F_{IS} = +1). Only individuals with the *LDH-C*90/90* genotype exhibit alleles diagnostic for the north Atlantic (*G3PDH-2*50* = 0.12, *GPI-A1*110* = 0.06, *sMDH-A2*152* = 0.25, and *sMDH-B1*80* = 0.19). All other fish in the wild were monomorphic (fixation for the **100* allele) or polymorphic for different alleles at the allozyme loci surveyed (e.g. *sMDH-A2** and *sMDH-B1**). Since our data clearly indicate a low

to non-existent level of introgression in all populations, we simply excluded all unambiguously assigned hatchery fish detected in the wild from further analysis.

Allozyme variation

Allozyme allele frequencies and measures of the genetic variability for the 20 populations analyzed are given in appendix 1. Allelic variants *sMDH-B1*79*, *sMDH-B1*83* and *PGM-1*90* do not appear to have been reported previously. The patterns observed at the *GPI-B2** locus suggested the presence of the null allele **QO* in some of the populations surveyed. However, this allele was excluded from the analysis due to the difficulty to interpret zymograms unambiguously. No significant deviation from HW expectations was found at the 5% level in all the probability tests performed. The allelic distribution showed important population differences, in particular at the *CK-A1**, *PEPLT** and *TF** loci. The frequency of *CK-A1*100* decreased in a clinal fashion southward (exception for the isolated population of Caima), a trend that is further supported by *GPI-B2*135* and *MPI-2*105*. Populations located between the Cávado and Douro basin were characterized by a moderate to fixation frequency (0.42-1) of the *PEPLT*70* allele, that was otherwise detected at a frequency below 0.25. The *TF*95*, only observed in populations from Portugal show a very heterogeneous distribution, from absence to fixation.

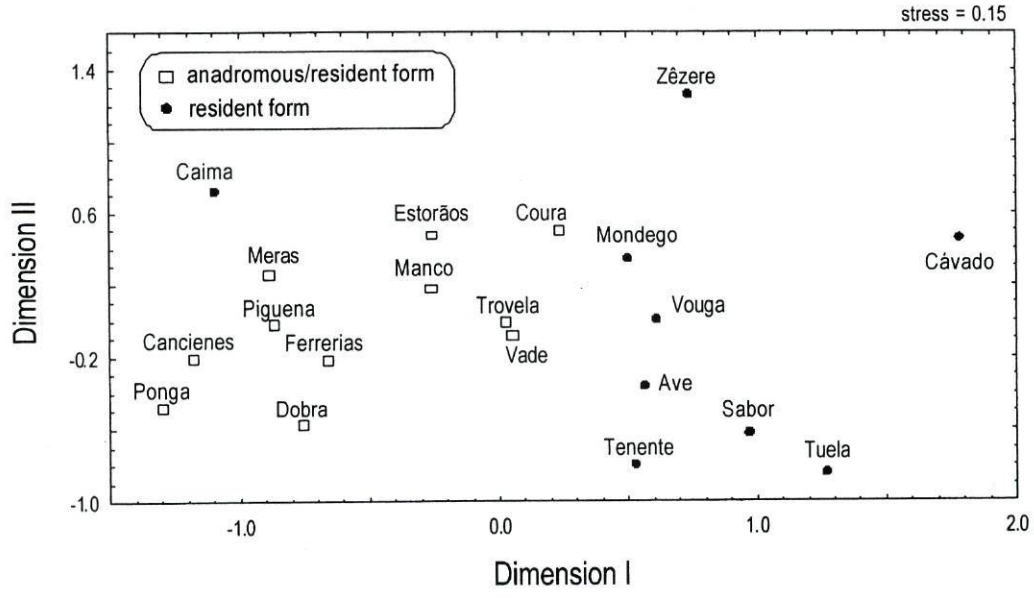

Figure 2. Multidimensional scaling of Cavalli-Sforza & Edwards (1967) chord allozyme genetic distances for the 20 brown trout populations analyzed.

Expected heterozygosity and polymorphism show a wide range of variation (H_E: 0-0.227; P_{95}: 0-0.6). This heterogeneity increases in populations to the south of the SLA by showing a lower genetic diversity and higher divergence (average H_E = 0.153 and 0.058, respectively north and south of the anadromous form occurrence). When we consider two separate groups, north and south the SLA, a great difference in the multilocus G_{ST} estimates among populations within each group is observed (12% and 54%) also contrasting with the gene flow estimates from G_{ST} (N_em = 1.9 and 0.2), respectively. The overall genetic differentiation among all natural populations studied was 35%.

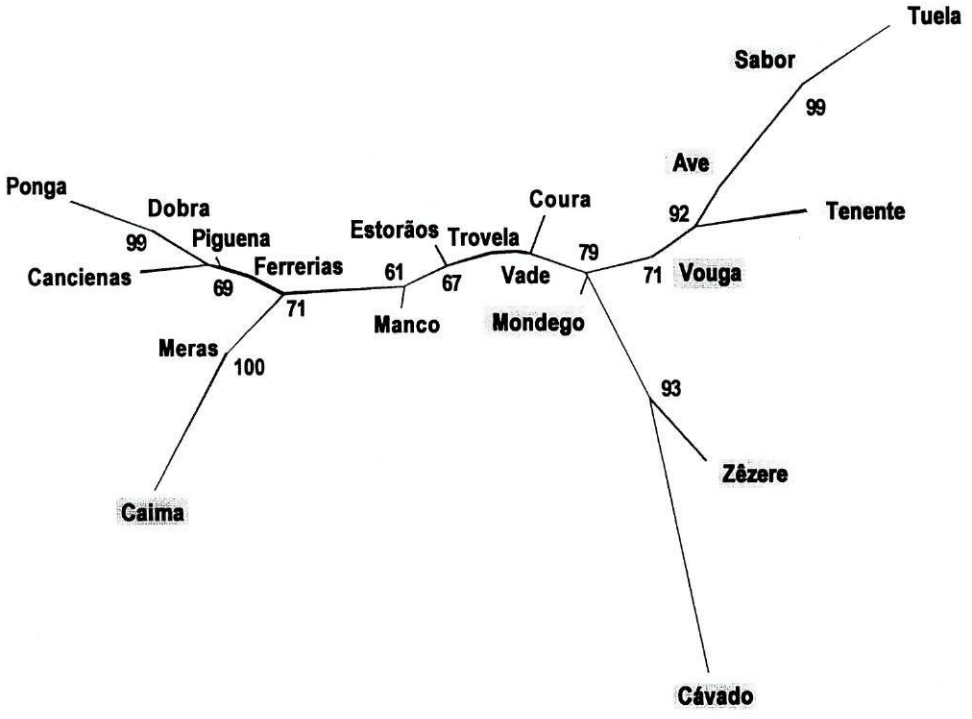

Figure 3. Neighbour-joining network from Cavalli-Sforza & Edwards (1967) chord allozyme genetic distances for the 20 brown trout populations analyzed. Numbers indicate nodes with bootstrap support higher than 50% in 100 replications. Populations southwards of the SLA are shaded grey.

Multidimensional scaling of D_{CE} genetic distances resolved the 20 brown trout populations in a coordinate system (figure 2). Dimension I reflects a north-south gradient of genetic differentiation in the Atlantic Iberian coastal region, with a stepwise decrease in genetic variability for a different set of loci, paralleled with the occurrence of anadromy. Dimension II shows the greater substructuring of populations to the south of the SLA, characterized by a mosaic pattern of genetic differentiation. Hierarchical clustering in a NJ tree (figure 3) produced an

identical picture of the population genetic differentiation. Due to the bifurcation imposed in this clustering analysis, the Caima is clustered with locations where anadromy occurs, mainly as a result of the *CK-A1**100 fixation in this isolated population. The visual inspection of the relative positions of populations in both the NJ tree and MDS plot revealed some inconsistency with their geographical location.

Table 1. Partial Mantel test for association between genetic differentiation and causal hypotheses proposed. Tests were carried out over all populations (20 analyzed with allozyme and 11 with microsatellite loci; upper panel) and over region 2 (Portugal) populations (lower panel).

Genetic differentiation (dependent variable)	Causal hypotheses (independent variables)		
	riverine distance	mode of life	region
all populations analyzed			
allozymes			
genetic distance	ns	ns	***
heterozygosity difference	***	ns	ns
microsatellites			
genetic distance	ns	ns	ns
heterozygosity difference	ns	**	ns
region 2			
allozymes			
genetic distance	ns	ns	_
heterozygosity difference	ns	ns	_
microsatellites			
genetic distance	ns	ns	_
heterozygosity difference	ns	*	_

ns – not significant; **P*<0.05; ***P*<0.01; ****P*<0.001.

Allozyme genetic distance was significantly associated with the previous defined region 1 (Cantabria) and 2 (Portugal) (table 1). Heterozygosity difference was significantly associated with riverine distances. Neither of the dependent variables was significantly associated with the mode of life. Within region 2 populations, no significant association was found between allozyme genetic differentiation and any of the independent variables.

Microsatellite variation

Microsatellite allele frequencies and measures of the genetic variability for the subset of eleven populations analyzed (plus a reference Mediterranean population) are given in appendix 2. The observed number of alleles per locus varied from 10 (*Str-85**) to 15 (*Str-543**). Significant deviation from HW expectations was found

at the 5% level in one case out of 48 probability tests performed. Homozygous excess was observed in Tenente (*BS131**; F_{IS} = 0.519). Expected heterozygosity and polymorphism showed a wide range of variation (H_E: 0.091-0.689; P_{95}: 0.25-1). The allelic distribution showed important differences in coastal Atlantic Iberian brown trout (appendix 2). Loci often exhibited disjunct allelic size distributions in which size classes were in many cases separated by several base pairs. An exception was observed at the *BS131** locus, for Manco and Vade, here the range of allele size distribution was almost continuous. Allelic size distributions with modes separated by size gaps increased southwards, followed by a decrease in the allele number and variance. Private alleles were detected at all loci. The reference population Ter was that exhibiting the highest number of private alleles (n = 6) and thus suggesting their higher differentiation (further checked by MDS and NJ analysis; data not shown).

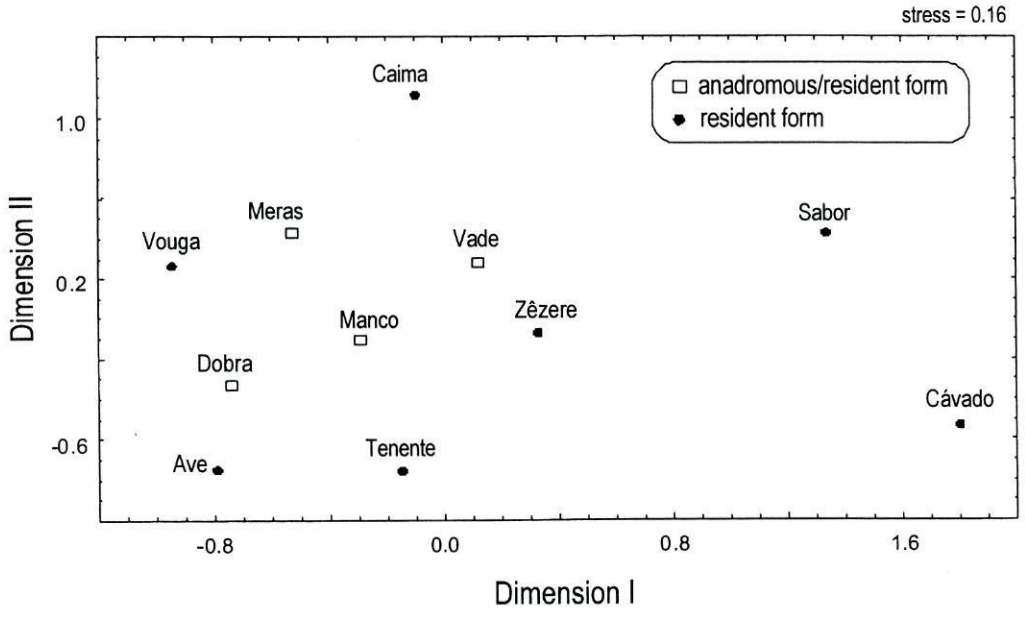

Figure 4. Multidimensional scaling of Cavalli-Sforza & Edwards (1967) chord microsatellite genetic distances for the subset of the 11 brown trout populations analyzed.

Among the Atlantic populations surveyed, Dobra was the one showing the higher number of unique alleles (n = 5). Other private alleles were also observed in Meras, Manco and Sabor (in each n = 2) and Cávado, Tenente and Zêzere (in each n = 1). Genetic diversity showed very different patterns of variation for populations with or without the anadromous form (average H_E = 0.509 and 0.252; A = 5.19 and 2.11; respectively). Genetic differentiation among the Atlantic Iberian populations indicates a strong genetic heterogeneity (G_{ST} = 54%). When

we consider separate groups, north and south of the SLA, a great difference in the multilocus G_{ST} estimates among populations within each group is observed (16% and 67%), also contrasting with the gene flow estimates from G_{ST} (N_em = 1.3 and 0.1), respectively.

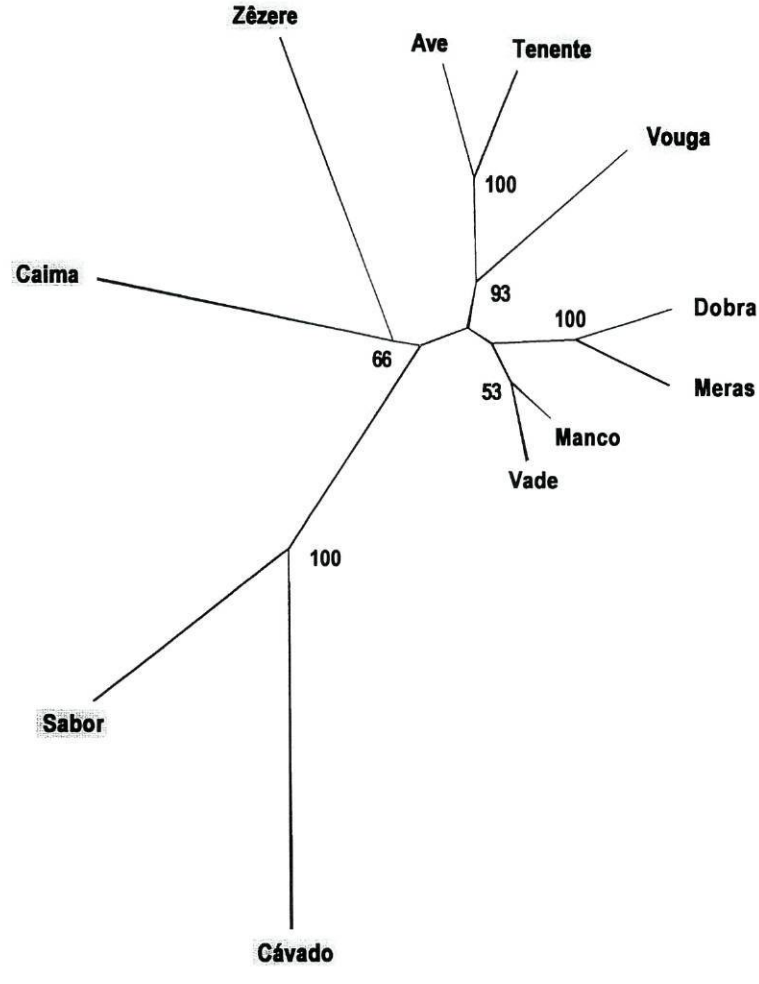

Figure 5. Neighbour-joining network from Cavalli-Sforza & Edwards (1967) chord microsatellite genetic distances for the subset of the 11 brown trout populations analyzed. Numbers indicate nodes with bootstrap support higher than 50% in 100 replications. Populations southwards of the SLA are shaded grey.

The MDS scatterplot of the D_{CE} genetic distances for the subset of 11 populations is represented in figure 4. Collections located north of the SLA appeared to be discretely differentiated. By contrast, those southwards of that limit were clearly distinct from the previous and showed a quite heterogeneous differentiation among them. The NJ tree depicting the underlying structure of the D_{CE} distance matrix illustrates the similarity of populations to the north of the

SLA and the great divergence among populations to the south of that limit (figure 5). Populations south of the SLA exhibited long branch lengths, suggesting evolutionary histories quite distinct from those inhabiting areas to the north of that limit.

The partial Mantel test identified the mode of life as significantly associated with microsatellite heterozygosity difference when considering both all populations or only those from Portugal (region 2; table 1). No significant association was found for the other independent variables and the genetic differentiation.

Discussion

Allozyme and microsatellite patterns of variation

The higher amount of among population differentiation observed at few protein loci (e.g. *CK-A1**, *PEPLT** and *TF**) could be simply explained by the effects of fragmentation followed by genetic drift (as suggested also by the microsatellite patterns of variation), rather than natural selection. Thus, the suggestion by Karl & Avise (1992) and Pogson et al. (1995) that balancing selection at protein-coding loci (allozymes) compromises the effectiveness of these markers for describing the amount and patterns of gene flow among populations is not well supported by our results. Under a selectively neutral regime, with a mutation rate much lower than the migration rate, patterns of allelic variation are affected mainly by the gene flow and genetic drift, with a certain degree of concordance among loci being expected (Allendorf & Seeb 2000). Differences in the number of alleles and heterozygosity at a locus are a source of bias for F_{ST} estimators (McDonald 1994; Hedrick 1999). Genetic variation measured as the mean number of alleles per population and overall heterozygosity was higher for microsatellites than for allozyme loci, as expected from higher mutation rates. Thus, different mutation rates between both categories of markers could affect the amount of genetic variation between populations, this effect itself depending on whether differentiation is the result of divergence under complete isolation or mutation-drift equilibrium (Allendorf & Seeb 2000). However, the two categories of loci displayed similar overall magnitude of genetic differentiation across populations and followed congruent levels of genetic substructuring. Recent studies in salmonids also have found identical patterns of differentiation at allozymes and nuclear DNA markers (e.g. Estoup et al. 1998; Scribner et al. 1998; Allendorf & Seeb 2000).

The allozymic characterization of the Atlantic Iberian brown trout is in agreement with its geographical location in the southwestern Atlantic region (high frequency of the *CK-A1*115* and the fixation of the *LDH-C*100* allele; Antunes et al. 1999; García-Marín et al. 1999a). The absence of some alleles observed in more Northern European populations (e.g. *G3PDH-2*50*, *GPI-A1*110*, *IDHP-1*160*, *LDH-C*90*, *sMDH-A2*120* and *sMDH-B1*80*; Ryman 1983; Krieg & Guyomard 1985; Guyomard 1989) also reinforces the genetic differentiation of north and south Atlantic brown trout. The genetic diversity reported for the populations analyzed lies within the range values along its natural distribution (Ferguson 1989), with higher values suggested for Atlantic than Mediterranean drainages (Krieg & Guyomard 1985; Guyomard 1989; Presa et al. 1994). Among Atlantic drainages, heterozygosity seems to be higher in northern populations (e.g. Crozier & Ferguson 1986; Presa et al. 1994) relatively to more southern ones, such as in the Iberian Peninsula (e.g. Martinez et al. 1993; García-Marín & Pla 1996). In northwestern Spain, across five river basins around and below the SLA, a significant southwards stepwise decrease of genetic diversity was observed (Bouza et al. 1999, 2001). Fluctuations in population size due to the unstable hydrology of southern areas were proposed as a possible explanation for this genetic diversity differences (Apostolidis et al. 1996). The absence of the anadromous form was added as another factor to explain the decrease in genetic variability, by precluding migration among basins and increasing the effects of drift (Bouza et al. 1999). The allozymic evidence is, however, quite distinct from the pattern of the mtDNA variation inferred for the Atlantic region.

Initial screening of mtDNA control region sequences showed complete monomorphism across several north Atlantic populations (Bernatchez et al. 1992), while Weiss et al. (2000) detected a significant southwestern Atlantic population subdivision with distinct haplotypes. The more complex pattern of diversity in the south, with a long history of fragmentation, suggests that it represents a more ancestral lineage relative to more northern populations, as would be predicted from the glacial history of the different regions (Bernatchez 2001). This is mostly congruent with the patterns described by Hewitt (1999), among 10 different taxa, characterized by a reduction in diversity from southern to northern Europe in the extent of allelic variation. This fact was attributed to rapid expansion northwards, while southern refugia allowed populations to diverge through several ice ages. The lack of congruence between allozymes and mtDNA possibly reflects population demography differences, and their influence on the spatial structure of markers with different modes of transmission and evolution. The brown trout, as a cold-water dependent organism, presently shows higher population densities and a more uniformed distribution in northern Atlantic areas. More to the south,

effective population size and migratory potential became reduced as increased temperatures restricted them to upstream regions (García-Marín & Pla 1996). Thus, the higher level of allozyme diversity in the north of Europe, though possibly reflecting a complex heritage due to multiple post-glacial colonizations (Laikre et al. 1999), was likely influenced by a more contemporary demographic history of population size expansion with a great amount of gene flow.

The spatial structure of allozyme loci in southwestern Atlantic populations revealed a southward decline of heterozygosity that is largely associated with the SLA, as previously suggested by Bouza et al. (1999). Furthermore, allele frequencies of *CK-A1*100*, *GPI-B2*135* and *MPI-2*105* show a clinal decrease to the south. These results are congruent with continuous levels of gene flow to the south, possibly occurring during Pleistocene colder climatic periods. Lower heterozygosity characterizes populations south of the SLA, which exhibited a great differentiation for a restricted area (G_{ST} = 54%) and low number of migrants per generation (N_em = 0.2). Previous studies showed in general low figures of N_em from G_{ST} estimates, although higher than 1 (e.g. Hansen et al. 1993; Riffle et al. 1995; Bouza et al. 1999), thus revealing that populations in this region were largely influenced by restricted gene flow and high genetic drift. The presence of some alleles at high frequencies in this southern edge, that were absent or much less frequent in the north (e.g. *PEPLT*70* and *TF*95*), suggests the existence of relic populations that persisted in isolation for long periods of time.

The geographical patterns of microsatellite loci variation were largely concordant with the picture showed by allozyme markers. Microsatellites, however, are much more sensitive indicators of recent loss of genetic variation within populations because of their tendency to have many more alleles than allozymes (Luikart et al. 1998). It is noteworthy that the highly polymorphic plasma protein (*PX**) showed a similar pattern of variation as well as the stepwise decrease in the number of alleles and heterozygosity, from the north (Minho and Lima basin) to the south of the SLA (Ave, Mondego and Tejo basin) (Antunes et al. 2000). The great variances in the distribution of microsatellite alleles and the higher levels of heterozygosity, in the four populations north of the SLA, suggests more stable demographic conditions over time for this area. This seems well represented in the allelic profile of the *BS131** locus, in particular for Manco and Vade populations. However, less variation was observed in Meras for the *Str-543** and *Str-591** loci, which may be related with the presence of a few physical barriers in the stream, causing a certain degree of isolation with the Esva river basin. The stepwise decrease of *Str-591*150* allele is also congruent with the pattern observed for the allozyme data. Populations located to the south of the SLA show lower levels of heterozygosity and a pattern of southward allele

depletion. An exception was, however, observed for the Tenente population, which showed higher heterozygosity and higher mean number of alleles per locus, a fact that could be related with their outlet position in Douro basin and their chance of higher gene flow promoted by the anadromous form till more recent times. Interestingly, the heterozigosity and mean number of alleles per locus in the reference population Ter (H_E = 0.402 and A = 3) were not as reduced as those detected in some of the Atlantic populations southwards of the SLA.

The substantial loss of allelic diversity in the Atlantic "southern edge" may have been caused by strong population bottlenecking (Leberg 1992) over the Pleistocene sequence of glacial and interglacial periods, causing suitable/unsuitable ecological conditions for the survivorship of the species. While random drift can lead to the loss of alleles from a population, it can also result in an increase in frequency of formerly rare alleles (Wright 1931). The increased frequency or even fixation at *Str-591** private alleles in populations south of the SLA is clearly in favor of this prediction. By contrast, populations north of that limit show private alleles always at low frequencies. The high G_{ST} value (67%) for the population group south of the SLA is more likely to reflect isolation for extensive periods of time than continuing low levels of gene flow per generation (Larson et al. 1984).

The partial Mantel test showed the existence of a significant correlation between allozyme genetic differentiation and geography (riverine distance and regional component). In contrast, the microsatellite heterozygosity difference was significantly correlated with the mode of life. It should be noted, however, that the mode of life is not independent of geography, as the presence/absence of anadromy has a strong spatial component. A model of isolation-by-distance could explain the observed genetic structure for populations north of the SLA. The anadromous form is possibly responsible for this result, a fact that has been previously suggested (e.g. Morán et al. 1995; Hansen & Mensberg 1998; Bouza et al. 1999). For populations south of the SLA, fragmentation can explain most of the observed structure, as revealed by the lack of association with geography (waterway and regional component) in the partial Mantel test for region 2 (Portugal). Within this region, the significant association between mode of life and microsatellite heterozygosity puts in evidence the great heterogeneity for populations north and south of the SLA.

The large concordance of the spatial genetic structure of allozyme and microsatellite loci is more likely a result of historical events acting on the brown trout in this once glacial refuge than from more contemporary population dynamics. Two major events are invoked to explain the observed patterns: (i) an

historical mosaic fragmentation in the area currently to the south of the SLA and (ii) a relatively continuous gene flow from northern populations to the south.

Historical scenario

The ice sheets restricted the Atlantic brown trout range to more southern regions till the end of last ice ages, which lasted until 18 000 years before present. The species was allowed to find refugia in southern areas and persisted throughout the Pleistocene climatic oscillations cycles. The concordant evidence from both allozyme and microsatellite loci suggest a scenario, for southern Atlantic coastal populations, ruled by a more stable demography north of the Lima basin (currently representing the SLA) and critical ecological conditions southwards that basin, causing unstable effective population size over time. The repeated population fragmentation and reduction of effective size in this "southern edge" throughout the Pleistocene climatic cycles would have created a pattern of genetic depauperation similar to the one observed. This is also in agreement with the mtDNA pattern detected previously for this geographical region, with a high value of ϕ_{ST} (76%) consistent with demographic events ruled by past fragmentation and restricted gene flow among populations, as revealed by the Nested Clade Analysis (NCA; Templeton et al. 1995) (Antunes et al. 2001). Moreover, the mtDNA data indicates a deep (at least 200 000 years old; Weiss et al. 2000) and complex history of isolation.

Furthermore, the history of these "southern edge" coastal populations seems to have been rather complicated by colonization events from the north. Our results clearly suggest a relatively recurrent southwards gene flow from populations located north of the Lima basin. Other studies based in allozyme (Machordom et al. 1999; Sanz et al. 2000; Bouza et al. 2001) and mtDNA (Machordom et al. 2000) further support the presence of mainland populations in Douro and Tejo basins related with Cantabric-Galician brown trout, corroborating the existence of secondary contact between allopatric groups in this region. Origins of these distinct groups may be difficult to engage. Sanz et al. (2000) proposed that the highest level of gene diversity of the Cantabrian group and the evidence for their expansion to the Douro and Tejo basins would be indicative that the group is ancestral to others in northwestern Iberian Peninsula. Nevertheless, the diversity in a region does not necessarily reflect the age of the regional population but rather could reflect the demographic history of population size expansion (e.g. Templeton 1993), as it was also suggested by our interpretation of the higher levels of allozyme heterozygosity in post-glacial recolonized areas in the North Atlantic region.

The presence of some protein alleles at high frequencies or fixed in this "southern edge" that were absent or much less frequent in the Cantabric region (e.g. *PEPLT*70* and *TF*95*; and also *sMDH-B1, B2*75* in the Douro basin and the *αMAN*90* in the Tejo basin; García-Marín & Pla 1996; Sanz et al. 2000; Bouza et al. 2001) indicates a unique genetic architecture, with the existence of relic populations that persisted in isolation for long periods of time. The Lima basin is currently at the southern limit of anadromy but, a few decades ago, several migratory individuals were observed in the Douro basin (our observations) and it is assumed that anadromy occurred further south during the last glaciation (Hamilton et al. 1989). Thus, although anadromy must have played a homogenizing role in shaping genetic structure for this region, its present occurrence may not be a good indicator of its past effects.

The detection of the *TF*95* in this region (with a tendency to increase frequency in populations located in more upstream regions; Antunes unpublished), previously seen only at low frequencies in some headwater tributaries of the Rhone (Mediterranean) and Po (Adriatic) rivers (Largiadèr & Scholl 1995; Largiadèr et al. 1996), suggests the historical merging of Mediterranean and Atlantic groups (Antunes et al. 1999). The partial nucleotide sequence of the *TF* gene indicates that *TF*95* has an old ancestry relative to *TF*100* and possibly represents the signature of an earlier colonization of the Atlantic drainages followed by dispersal from the Mediterranean region (Antunes et al. in prep.). Moreover, the RFLP-mtDNA data by Machordom et al. (2000) suggested an ancestral divergence of the Atlantic Douro group from all others in the Iberian Peninsula. Based on the high frequency of *sMDH-B1, B2*75* allele in headwater tributaries on Minho and Douro basins, Bouza et al. (1999, 2001) suggested that the Cantabric-Galician group is a more recent sublineage that was only able to colonize more accessible outlet areas of these basins, a fact that seems to be also corroborated by NOR chromosomal location data (Castro et al. 2001).

Insights for the conservation of the highly fragmented populations

Our data clearly indicates a low to non-existent level of introgression in nearly all populations, analogous to the report of "failure of a stocking policy" in northwestern Spain by Morán et al. (1991), later confirmed by Arias et al. (1995). However, it is difficult to draw general conclusions about the effects of stocking in the studied populations as introgression in Spain varies widely (0-100%; García-Marín et al. 1999b). Moreover, estimates of introgression using a small set of loci should be interpreted with caution, as recent studies indicate distinct rates of introgression among different categories of markers (e.g. proteins,

microsatellites and mtDNA) and even within the same category (e.g. proteins) (Poteaux et al. 1998, 1999). In any case, non-introgressed populations are not free from other threats such as introduced diseases, resource competition and mild negative demographic influences (Leary et al. 1993; Weiss and Schmutz 1999). Habitat improvements combined with restrictive harvest regulations are the most prudent measures to manage wild populations of salmonids (Leary et al. 1993). If enhancement is deemed necessary, "supportive breeding", where breeding fish are only chosen from the receiving population is the stocking program least likely to result in genetic changes (Ryman and Laikre 1991). Considering the present fragmented genetic structure as well as current threats to these populations, any activity involving stocking or translocations should be viewed with caution and the relative importance of both inbreeding and outbreeding depression considered (Allendorf and Waples 1996; Moritz 1999).

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Appendix 1. Allele frequency, sample size (N), expected heterozygosity (H_E), proportion of polymorphic loci at 95% (P_{95}) and average number of alleles (A) at 11 protein loci in 20 populations of brown trout. Population codes are those from figure 1.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Locus	Allele	Dob	Pon	Can	Fer	Pig	Mer	Man	Cou	Vad	Est	Tro	Cav	Ave	Sab	Tue	Ten	Vou	Cai	Mon	Zez
*CK-A1**	*100*	0.62	0.82	0.67	0.39	0.70	0.82	0.16	0.02	0.10	0.36	0.13	0.04	–	–	–	0.09	–	1	–	0.02
	115	0.38	0.18	0.33	0.61	0.30	0.18	0.84	0.98	0.90	0.64	0.87	0.96	1	1	1.000	0.91	1	–	1	0.98
	N	34	16	37	37	39	19	24	20	24	46	24	32	25	16	18	15	17	12	15	35
*GPI-B1**	*QO*	0.09	0.23	0.10	0.15	0.24	0.10	–	0.20	0.23	0.18	0.10	–	–	–	–	0.03	0.03	–	0.10	0.07
	100	0.91	0.77	0.90	0.85	0.76	0.90	1	0.80	0.77	0.82	0.90	1	1	1	1	0.97	0.97	1	0.90	0.93
	N	34	16	39	37	39	19	24	20	24	46	24	32	25	15	18	15	17	12	15	35
*GPI-B2**	*100*	0.92	0.93	0.89	0.72	0.74	0.98	0.84	0.85	0.93	0.88	0.84	1	1	0.83	1	0.98	1	1	1	1
	135	0.08	0.07	0.11	0.28	0.26	0.02	0.16	0.15	0.07	0.12	0.16	–	–	0.17	–	0.02	–	–	–	–
	N	40	30	41	40	48	30	28	26	75	60	32	32	25	15	18	25	17	12	31	40
*sMDH-A2**	*100*	1	1	0.85	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	130	–	–	0.15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	N	38	29	36	39	41	17	27	26	142	119	38	35	21	15	18	29	17	12	26	34
*sMDH-B1**	*70*	0.16	0.23	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	–
	75	0.01	0.11	–	–	–	–	0.04	–	0.02	0.03	0.05	–	–	–	–	0.18	–	–	–	–
	79	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.10	–
	83	–	–	–	–	–	–	0.08	–	–	–	–	–	0.02	–	–	–	–	–	–	–
	100	0.83	0.66	1	1	0.99	1	0.75	0.84	0.88	0.80	0.95	0.99	0.98	1	0.81	0.82	1	1	0.90	1
	125	–	–	–	–	0.01	–	0.13	0.16	0.10	0.14	–	0.01	–	–	0.19	–	–	–	–	–
	N	38	31	37	38	47	30	26	22	60	59	28	35	30	15	18	30	17	12	24	35
*sMDH-B2**	*70*	0.01	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	75	–	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–	0.03	–	–	–	–
	100	0.99	0.98	1	1	1	1	1	1	1	1	1	1	1	1	1	0.97	1	1	1	1
	N	38	31	37	38	47	30	26	22	60	59	28	35	30	15	18	30	17	12	24	35
*MPI-2**	*100*	0.59	0.63	0.62	0.58	0.63	0.62	0.88	1	0.89	0.74	0.78	1	1	1	1	1	1	1	0.93	1
	105	0.36	0.30	0.38	0.42	0.37	0.38	0.12	–	0.11	0.26	0.22	–	–	–	–	–	–	–	0.07	–
	110	0.05	0.07	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	N	21	30	39	24	42	30	21	22	56	53	29	17	20	15	18	18	17	12	15	31

*PEPB**	*100*	0.66	0.79	0.97	1	0.99	1	1	1	1	1	1	1	1	1	1	0.60	0.85	1	1	1
	120	0.34	0.21	0.03	_	0.01	_	_	_	_	_	_	_	_	_	_	0.40	0.15	_	_	_
	N	31	31	39	30	44	30	27	25	57	15	8	35	30	14	18	29	17	11	15	38
*PEPLT**	*70*	0.05	_	_	_	0.02	_	_	0.02	0.12	0.03	0.15	0.98	0.42	0.93	1	0.72	0.25	_	0.17	_
	100	0.95	1	1	1	0.98	1	1	0.98	0.88	0.97	0.85	0.02	0.58	0.07	_	0.28	0.75	1	0.83	1
	N	31	31	38	32	43	30	27	20	48	17	13	27	30	14	18	29	16	11	15	38
*PGM**	*80*	0.12	0.31	0.45	0.13	0.17	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
	90	_	_	_	_	_	_	_	_	_	0.07	_	_	_	_	_	0.05	_	_	_	_
	100	0.88	0.69	0.55	0.87	0.83	1	1	1	1	0.93	1	1	1	1	1	0.95	1	1	1	1
	N	40	31	38	19	49	29	14	19	65	70	32	35	9	15	18	10	17	12	14	31
*TF**	*95*	_	_	_	_	_	_	0.05	0.27	0.37	0.32	0.33	1	_	_	_	0.07	0.21	_	0.33	1
	100	1	1	1	1	1	1	0.95	0.73	0.63	0.68	0.67	_	1	1	1	0.93	0.79	1	0.67	_
	N	40	31	39	40	49	30	39	32	98	65	15	24	27	23	18	29	17	12	23	21
H_E		0.189	0.227	0.166	0.137	0.160	0.069	0.099	0.129	0.156	0.188	0.160	0.006	0.052	0.041	0.031	0.157	0.101	0.000	0.121	0.013
P_{95}		0.6	0.6	0.5	0.4	0.4	0.2	0.4	0.4	0.6	0.6	0.6	0.0	0.1	0.2	0.1	0.5	0.3	0.0	0.5	0.1
A		2.0	1.9	1.6	1.4	1.7	1.3	1.6	1.5	1.7	1.9	1.6	1.2	1.2	1.2	1.1	1.8	1.4	1.0	1.5	1.1

Appendix 2. Allele frequency, sample size (N), expected heterozygosity (H_E), proportion of polymorphic loci at 95% (P_{95}) and average number of alleles (A) at four microsatellite loci in 12 populations of brown trout. Population codes are those from figure 1.

		1	6	7	9	12	13	14	16	17	18	20	
Locus	Allele	Dobra	Meras	Manco	Vade	Cávado	Ave	Sabor	Tenente	Vouga	Caima	Zêzere	Ter
*Str-85**	*148*	–	–	–	–	–	–	–	0.02	–	–	0.05	–
	158	–	0.04	–	–	–	–	–	–	–	–	–	–
	162	0.02	–	–	0.02	–	–	–	–	–	–	–	–
	164	–	0.44	0.20	0.29	–	–	–	–	0.34	0.73	–	1
	166	–	–	–	0.13	1	–	1	–	0.09	–	–	–
	168	0.12	0.02	0.58	0.13	–	1	–	0.96	0.57	–	0.24	–
	170	0.02	–	0.08	0.08	–	–	–	0.02	–	–	–	–
	172	–	0.02	–	–	–	–	–	–	–	–	–	–
	174	0.69	0.48	0.14	0.35	–	–	–	–	–	–	–	–
	178	0.15	–	–	–	–	–	–	–	–	0.27	0.71	–
	N	34	23	25	26	25	28	15	21	16	22	19	19
*Str-543**	*113*	0.02	–	–	–	–	–	–	–	–	–	–	–
	115	0.04	–	–	–	–	–	–	–	–	–	–	–
	119	0.03	–	–	–	–	–	–	–	–	–	–	–
	123	–	–	0.02	0.13	–	–	0.36	–	–	–	–	–
	125	0.78	1	0.66	0.57	–	0.67	0.17	0.24	1	1	1	–
	131	0.13	–	0.04	0.06	–	0.30	–	0.50	–	–	–	–
	133	–	–	0.08	–	–	–	–	–	–	–	–	–
	137	–	–	0.04	0.20	1	–	0.17	0.17	–	–	–	–
	141	–	–	–	–	–	–	0.30	–	–	–	–	–
	147	–	–	0.02	–	–	–	–	–	–	–	–	–
	149	–	–	0.14	0.04	–	–	–	–	–	–	–	–
	151	–	–	–	–	–	–	–	0.09	–	–	–	0.64
	157	–	–	–	–	–	–	–	–	–	–	–	0.05
	161	–	–	–	–	–	–	–	–	–	–	–	0.13
	163	–	–	–	–	–	–	–	–	–	–	–	0.18
	N	34	23	25	27	25	28	15	21	16	22	19	19
*Str-591**	*140*	0.02	–	–	–	–	–	–	–	–	–	–	–
	148	–	–	0.04	0.07	–	–	–	–	–	–	–	–
	150	0.80	0.98	0.83	0.33	–	0.50	–	0.65	0.28	–	0.03	–
	152	0.03	–	–	0.02	–	–	–	–	–	–	–	–
	154	0.02	–	–	–	–	–	–	–	–	–	–	–
	156	–	–	0.13	0.58	–	–	–	0.02	–	1	–	–
	158	0.13	0.02	–	–	–	0.50	–	–	0.72	–	–	–
	162	–	–	–	–	–	–	1	–	–	–	–	–
	164	–	–	–	–	–	–	–	0.33	–	–	–	–
	166	–	–	–	–	1	–	–	–	–	–	–	–
	180	–	–	–	–	–	–	–	–	–	–	0.97	–
	186	–	–	–	–	–	–	–	–	–	–	–	0.24
	192	–	–	–	–	–	–	–	–	–	–	–	0.76
	N	34	23	24	26	25	28	15	21	16	21	18	19

*BS-131**	*148*	0.35	0.15	0.02	_	_	_	_	0.05	_	_	0.03	_
	150	_	_	0.06	0.02	_	_	_	_	_	_	_	_
	152	_	_	0.15	0.06	_	_	_	0.07	0.94	_	_	_
	154	0.06	0.65	0.21	0.27	_	_	0.77	0.02	_	_	_	_
	156	0.25	0.02	0.15	0.04	_	_	_	_	_	_	0.56	0.02
	158	0.06	_	0.10	0.09	_	_	0.23	0.26	_	1	_	0.32
	160	0.18	0.07	0.04	0.11	_	0.88	_	0.36	_	_	_	_
	162	_	_	0.19	0.09	0.24	0.12	_	0.17	_	_	_	0.02
	164	0.07	0.11	0.04	0.17	_	_	_	_	_	_	_	_
	166	_	_	_	_	_	_	_	0.07	0.06	_	_	_
	168	_	_	0.04	0.15	_	_	_	_	_	_	0.41	_
	170	0.03	_	_	_	0.76	_	_	_	_	_	_	0.32
	174	_	_	_	_	_	_	_	_	_	_	_	0.32
	N	34	23	24	27	25	25	15	21	16	22	18	19
H_E		0.486	0.289	0.570	0.689	0.091	0.284	0.269	0.497	0.270	0.099	0.252	0.402
P_{95}		1.00	0.50	1.00	1.00	0.25	0.75	0.50	0.75	0.75	0.25	0.50	0.75
A		5.50	3.25	6.00	6.00	1.25	1.75	2.00	4.25	2.00	1.25	2.25	3.00

3. Conservação da diversidade genética na truta

Artigo V Complex evolutionary history in the brown trout: insights on the recognition of conservation units (em publicação na *Conservation Genetics*).

Conservation Genetics **00:** 1–11, 2001.

Complex evolutionary history in the brown trout: Insights on the recognition of conservation units

Agostinho Antunes[1,2,*], Rui Faria[1], Steven Weiss[1] & Paulo Alexandrino[1,2]
[1]*Centro de Estudos de Ciência Animal (CECA), ICETA-U.P., Campus Agrário de Vairão, 4485-661 Vairão, Portugal;* [2]*Departamento de Zoologia-Antropologia, Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, 4099-002 Porto, Portugal (*Author for correspondence: E-mail: aantunes@mail.icav.up.pt)*

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Abstract

We screened genetic variation in a polytypic organism, whose populations are often distributed into numerous isolated habitats, and integrated the results into a critique of defining "units" of conservation for organisms with highly fragmented populations. Sixteen populations of brown trout *Salmo trutta* L. across 8 Portuguese river basins were screened for variation at 5 loci (mtDNA and allozymes). Population history based on mtDNA revealed a mosaic pattern driven by past fragmentation and restricted gene flow with little correspondence to major river drainages or recently proposed OCUs on the Iberian Peninsula. Such patterns of variation offer a challenge to conservation strategies that base themselves on defining units of conservation, particularly if such units intend to reflect a hierarchical evolutionary structure. We suggest that geographically mosaic patterns of evolutionary lineages, as well as adaptively significant traits are common characteristics of many freshwater organisms. Thus, large-scale units, even if diagnosed by mtDNA clades, are often too heterogeneous to consider a "unit" of conservation. Alternatively, a bottom-up perspective that prioritizes populations or metapopulations is both more practical and more effective in recognizing and preserving evolutionary diversity.

Introduction

Southern European peninsulas have provided refugia for temperate biota through several Quaternary cold periods (Hewitt 2000). Within such refugia, the genetic structure of populations reflects both ice-age contractions and expansions as well as more contemporary demographic processes. For freshwater organisms, genetic structure is further influenced by the extremely fragmented and linearly confined pattern of habitats, whose network may change dramatically over relatively brief geological time periods. One of the most well-studied aquatic organisms subject to all of these processes is the brown trout (*Salmo trutta* L.). Their varied life-history and natal homing behavior add another level of complexity to those factors influencing the distribution of genetic variance among groups of populations (Elliot 1994; Grant et al. 1999). One of the most genetically structured vertebrates, brown trout are considered a "polytypic" species (Behnke 1972) or alternatively, following an application of the phylogenetic species concept (PSC), a highly speciose genera (Kottelat 1997). Either perspective offers a considerable challenge to conservationists wishing to define, protect, or offer alternative management strategies for specific groups of populations (Laikre et al. 1999).

The native range of brown trout extends throughout most of Europe, into eastern Asia, with a disjunct occurrence in the Atlas Mountains of North Africa (MacCrimmon and Marshall 1968). Initial screening of mtDNA control region sequence diversity delineated five major evolutionary lineages of the species, four of which are geographically distributed according to major drainage basins (Danubian, Adriatic, Mediterranean and Atlantic) and a fifth equally divergent lineage representing the morphologically distinct marbled trout, *S. trutta marmoratus*, found in some

headwater systems of the Adriatic (Bernatchez et al. 1992). These lineages were further described as "the basic Evolutionary Significant Units (ESUs) within brown trout" (Bernatchez 1995, 2001) (Figure 1). However, other studies have consistently pointed out the genetic distinctiveness of populations in the southwest Atlantic (primarily Atlantic drainages of the Iberian Peninsula) compared to elsewhere in the Atlantic, based on protein variation (Hamilton et al. 1989; García-Marín and Pla 1996; Antunes et al. 1999; Bouza et al. 1999; García-Marín et al. 1999) and most recently mtDNA (Weiss et al. 2000; Machordom et al. 2000). Machordom et al. (2000) further proposed five conservation units (referred to as Operational Conservation Units (OCUs) or [sic] ESUs) within the Iberian Peninsula (Figure 1). Interestingly, the designation of "units" (ESUs and OCUs) has been based exclusively on mtDNA data despite a voluminous literature on protein variation as well as non-genetic sources of information. The historical definition of ESUs included both ecological and genetic data (Ryder 1986; Waples 1991), and OCUs were meant to reflect interaction with socio-economic issues (Dodsen et al. 1998).

We evaluate the genetic structure of brown trout populations within and among Portuguese basins and rivers, an area of presumed glacial refuge not analyzed in the studies proposing conservation units. These populations are at the specie's extreme southwest distribution, and at the southern limit of the anadromous life-history form. The genetic analysis is integrated into a critique of defining "units" of evolution or conservation for organisms with highly fragmented or genetically structured populations.

Methods

Samples and stocking

Sixteen populations of brown trout were sampled by electrofishing between 1995 and 2000 from eight Portuguese river basins (N = 405). Two populations were sampled from the Minho basin (Manco and Coura) and the Vouga basin (Vouga and Caima); four from the Lima basin (Froufe, Vade, Estorãos and Trovela); one each from the Cávado, Ave, and Mondego rivers; four from the Douro basin (Sabor, Tuela, Tenente and Ardena); and one from the Tejo basin (Zêzere) (Figure 1). The samples cover all major Portuguese basins that contain brown trout and provide a representative view of populations occurring just north and south of the southernmost limit of anadromy (Lima basin; SNPRCN 1991). Because we were interested in assessing the native gene pool, we also included samples from local hatcheries to insure that we could identify such stocks in the wild. Individuals from the Torno (progeny of 1999) and Fonte Santa hatcheries (parents and progeny 2000), located, respectively, in Northern and Central Portugal, were also sampled (N = 133). While the Torno hatchery is the main source of national stocking programs, the use of the Fonte Santa hatchery is more localized. All fish were stored at −80 °C until genetic analysis.

Genetic methods

Extraction and storage of muscle, liver and eye tissues were made in accordance with procedures described in Antunes et al. (1999). Potential introgression with non-native hatchery fish was assessed using a set of diagnostic or highly discriminative allozyme loci previously used for such purposes in southwest Atlantic populations. The **90* allele at the *LDH-C** (lactate dehydrogenase) locus is not native to Iberia and is usually fixed in commercially available hatchery strains (García-Marín et al. 1991; Presa et al. 1994; Arias et al. 1995). The diagnostic power of the remaining loci: *G3PDH-2** (glycerol-3-phosphate dehydrogenase); *IDHP-1** (isocitrate dehydrogenase); and *sMDH-A2** (malate dehydrogenase), is based on the assumption that particular alleles (*G3PDH-2*50*, *IDHP-1*160* and *sMDH-A2*120*) occur only in northern populations whereas southwest Atlantic populations are fixed for the **100* allele at each locus (García-Marín et al. 1991; Martínez et al. 1993; Bouza et al. 1999). Electrophoresis followed procedures described by Martínez et al. (1993) and Antunes et al. (1999).

Population structure and history were assessed by sequencing the 5′ end of the mtDNA control region (464 bp) in 52 individuals from both wild populations and hatcheries, and combining these data with published sequences from 72 different individuals (Weiss et al. 2000). Additionally, several individuals from two Mediterranean populations (Fontanaccia and Reverotte rivers in France) were sequenced. These haplotypes were included in the analysis to represent the nearest outgroup assuming the Atlantic clade split from the Mediterranean or a common ancestor following the Messinian crisis (Machordorm et al. 2000; Weiss et al. 2001).

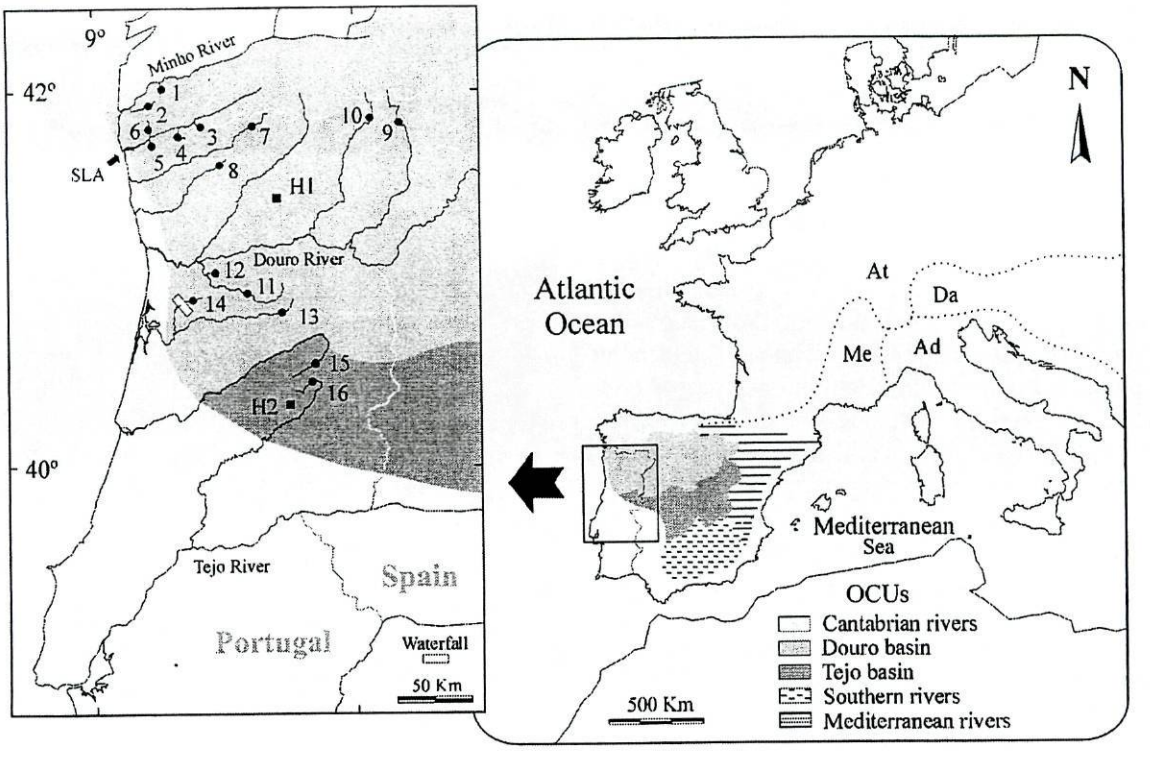

Figure 1. Geographical location of the studied populations: Minho basin (1-Manco and 2-Coura); Lima basin (3-Froufe, 4-Vade, 5-Trovela and 6-Estorãos); Cávado basin (7); Ave basin (8); Douro basin (9-Sabor, 10-Tuela, 11-Tenente and 12-Ardena); Vouga basin (13-Vouga and 14-Caima); Mondego basin (15); Tejo basin (16-Zêzere); Torno hatchery (H1); and-Fonte Santa hatchery (H2). SLA denotes the current southern limit of anadromy (Lima basin). The OCUs represented in Iberia are those proposed by Machordom et al. (2000), with some adaptation to clarify that the species is not present in some southern regions of the Peninsula. Accordingly, we have assumed that at least populations 3 through 14 in our study belong to the same OCU. Dotted lines delineate the major drainage basins that correspond to four of the five major evolutionary mtDNA lineages: Atlantic (At), Mediterranean (Me), Adriatic (Ad) and Danube (Da) (Bernatchez et al. 1992) (further described as ESUs; Bernatchez 1995, 2001). The marble trout lineage (Adriatic basin) is not represented.

Whole genomic DNA was extracted from frozen tissue following Sambrook et al. (1989) and the complete mtDNA control region was amplified via the polymerase chain reaction (PCR) with the primers H20 and L19 using conditions described in Bernatchez et al. (1992). Sequencing was accomplished with BigDye Terminater Cycle Sequencing protocols using an internal heavy-strand primer (H514) described in Weiss et al. (2000). Sequences were visualized on an ABI-310 automated sequencer (PE Applied Biosystems) and aligned and checked by hand using the Sequence Navigatorþ software (version 1.0.1; Applied Biosystems, Inc.).

Data analysis

Allozyme allele frequencies were estimated by direct counting. The GENEPOP (version 3.1b; Raymond and Rousset 1995) probability test option was used to determine if populations were in Hardy-Weinberg (HW) equilibrium (Markov chain method). The inbreeding coefficient of Weir and Cockerham (1984) (F_{IS}) was also determined. The likelihood that each allozyme genotype belonged to the hatchery or wild stocks was determined with an assignment test using the program WHICHRUN (version 3.2; Banks and Eichert 2000). Genetic variation within each population was quantified by mtDNA haplotype diversity (h) and nucleotide diversity (π) (Nei 1987). Haplotype diversity within and among populations was evaluated using an analysis of molecular variance (AMOVA; Excoffier et al. 1992) in the program Arlequin 1.1 (Schneider et al. 1997). One hierarchical AMOVA was carried out on eight populations, grouped by the Lima and Douro river basins.

A maximum parsimony network was constructed using the algorithm described by Templeton et al. (1992) that allows phylogenetic estimation with low

levels of divergence. TCS version 1.06 (Clement et al. 2000) implements the algorithm and provides a 95% plausible set for all haplotype linkages in an unrooted tree.

To discriminate between recurrent gene flow and historical events, we used nested clade analysis (NCA) (Templeton et al. 1995; Templeton 1998). NCA is a statistical method that can detect and test evolutionary processes responsible for the spatial distributions of observed genetic variation. The estimated haplotype tree was converted into a series of nested branches (clades) using the nesting rules in Templeton et al. (1987) and Templeton and Sing (1993). Clade distance (D_C), representing the geographical spread of a clade, and nested clade distance (D_N), representing the distance of a clade from the geographical center of the nested clade, were calculated. Clades were tested against their geographical locations (using actual river distances) through a permutational contingency analysis. Observed D_C and D_N values were then compared to a distribution of D_C and D_N generated by random permutations of clades against sampling locations with GeoDis version 2.0 (Posada et al. 2000). The null hypothesis tested is a random geographical distribution of all clades within a nested clade. Inferences about the population processes underlying the observed patterns of clade dispersal followed a key in Templeton (1998).

Results

Wild and hatchery fish

The multi-locus assignment test unambiguously characterized all individual fish as hatchery or wild. Forty nine fish from Tenente, Mondego and Zêzere populations were assigned to the hatchery, whereas all hatchery individuals were correctly assigned. All other fish in the wild were monomorphic for the allozyme loci surveyed (fixation for the **100* allele), while both hatchery stocks were fixed for the *LDH-C*90* allele and presented varying polymorphism at the *G3PDH-2**, *IDHP-2** and *sMDH-A2** loci (Tables 1 and 2). Congruent results were observed for mtDNA as individuals of clear hatchery origin had the common north Atlantic haplotype (At1) (Tables 3 and 4). This haplotype was also observed in one presumed native fish from the Tuela river. In contrast, wild populations revealed several Atlantic-basin, control region haplotypes (At5, At6, At7, At8, At9) first reported in Weiss et al. (2000) as well as three haplotypes (At12, At13 and At14) described here for the first time (GenBank accession nos AF330822-24).

Table 1. Genotypes at the diagnostic *LDH-C** locus in wild and hatchery fish

Locality	Year	*N*	LDH-C* genotype *90/90	*90/100	*100/100
Wild					
Minho					
Manco	1998	27	–	–	27
Coura	2000	26	–	–	26
Lima					
Froufe	1995	7	–	–	7
Vade	1996	39	–	–	39
Trovela	1996	25	–	–	25
Estorãos	1996	23	–	–	23
Cávado	2000	32	–	–	32
Ave	1998	22	–	–	22
Douro					
Sabor	2000	16	–	–	16
Tuela	2000	18	–	–	18
Tenente	1998	33	3	–	30
Ardena	1998	10	–	–	10
Vouga					
Vouga	2000	17	–	–	17
Caima	1998	12	–	–	12
Mondego					
Mondego	1998	18	6	–	12
Mondego	1999	37	32	–	5
Tejo					
Zêzere	1998	26	3	–	23
Zêzere	1999	17	5	–	12
Hatchery					
Torno	1999	59	59	–	–
Fonte Santa	2000	74	74	–	–

The assignment of wild-caught individuals to the hatchery stock was primarily based on the *LDH-C*90/90* genotype (Table 1). In 1998, the **90* allele was generally found in low to moderate frequencies (from 0.09 to 0.33). In 1999, individuals from the Mondego and Zêzere populations were sampled in a biased fashion, as the total number of fish removed for allozyme analysis was increased by adding additional individuals exhibiting pectoral fin abnormalities (similarly found in the Fonte Santa hatchery strain). This bias is reflected in the high frequency of the **90* allele (0.89) in Mondego. No heterozygotes (**90/100*) were observed in the wild (HW equilibrium rejected

Table 2. Allele frequencies at the highly discriminative loci for the individuals genotyped as *LDH-C* 90/90* in the wild and in the hatchery stocks

Locality	*N*	G3PDH-2*		IDHP-1*		MDH-A2*	
		**100*	**50*	**100*	**160*	**100*	**125*
Wild							
Tenente	3	1.0000	0.0000	1.0000	0.0000	0.8333	0.1667
Mondego[a]	38	0.9324	0.0676	0.7568	0.2432	0.8919	0.1081
Zêzere[a]	8	0.8750	0.1250	0.5000	0.5000	0.7500	0.2500
Hatchery							
Torno	52	0.8305	0.1695	0.6610	0.3390	0.7458	0.2542
Fonte Santa	56	0.7500	0.2500	0.7177	0.2823	0.8387	0.1613

[a]Pooled individuals from 1998 and 1999 collections.

Table 3. Variable nucleotide positions in Atlantic basin haplotypes (first 464 bp of the 5′ L-stand of the mtDNA control region) and comparison with two Mediterranean haplotypes

Haplotype	Nucleotide position												
	26	35	111	145	195	225	261	308	339	387	388	389	403
At1	T	C	T	G	A	C	G	G	A	G	C	T	T
At5	.	.	C	.	.	.	T	.	.	.	.	.	.
At6	.	.	C	A	.	.	.	.	.	.	.	.	.
At7	.	.	C	A	.	.	.	A	.	.	.	.	.
At8	.	.	.	A	.	T	.	.	.	*	.	.	.
At9	.	.	.	A	.	.	.	.	.	*	.	.	.
At10[a]	.	.	.	A	.	.	.	.	.	.	.	.	.
At12	.	.	.	.	.	.	.	.	.	.	T	.	.
At13	.	T	.	A	.	.	.	.	.	*	.	.	.
At14	.	.	C	A	.	.	.	.	C	.	.	.	.
Me1[b]	C	.	.	A	C	.	.	.	.	.	T	C	C
Me2[b]	.	.	.	.	C	.	.	.	.	.	T	C	C

[a]Haplotype At10 was observed in low frequencies in several small rivers from the Danube basin, Austria along with At11 which is not shown (Weiss et al. 2001).
[b]Me1 and Me2 were described in Bernatchez et al. (1992) whereby only the first 310 bp were sequenced.
*Nucleotide deletion.

at $P < 0.01$; $F_{IS} = +1$). Only individuals with the *LDH-C*90/90* genotype exhibit alleles diagnostic for the north Atlantic (*G3PDH-2*50*, *IDHP-1*160* and *sMDH-A2*120*) (Table 2).

mtDNA haplotype and natural population diversity

Nine mtDNA haplotypes were revealed in the 104 wild individuals analyzed (Tables 3 and 4). One haplotype (At6) dominated in all natural populations except Cávado, Ave and Sabor. All but two haplotypes were confined to single populations. Some haplotypes were rare, found in only one individual (At1, At8 and At14, in Tuela, Froufe and Estorãos, respectively) while others were fixed in a population (At12 and At13 in Sabor and Cávado, respectively). Six of the sixteen populations sampled were polymorphic exhibiting haplotypes differing by one to three mutational steps.

The analysis of molecular variance among all samples (excluding hatchery individuals) revealed highly significant geographical structure at the population level (ϕst = 0.76, $P < 0.0001$). The hierarchical AMOVA revealed little variation (6%) between the Lima and Douro basins but molecular variance within populations (ϕst = 0.64), and among populations within groups (ϕsc = 0.62) was highly significant ($P < 0.0001$) (Table 5).

The maximum parsimony network spanned 10 mutational steps (15 considering outgroups), revealing

Table 4. Haplotype frequency and sample size in wild/hatchery fish. Haplotype and nucleotide diversity were estimated excluding hatchery individual (*LDH-C*90/90* genotypes)

	*LDH-C** genotype												
	**90/90*	**100/100*											
		mtDNA haplotypes											
Locality	At1	At1	At5	At6	At7	At8	At9	At12	At13	At14	Total	h (±SE)	π (±SE)
Wild													
Minho													
Manco	–	–	3	7	–	–	–	–	–	–	10	0.467 (±0.132)	0.0020 (±0.0017)
Coura	–	–	–	5	–	–	–	–	–	–	5	–	–
Lima													
Froufe	–	–	–	4	–	1	–	–	–	–	5	0.400 (±0.237)	0.0026 (±0.0023)
Vade	–	–	–	5	–	–	–	–	–	–	5	–	–
Trovela	–	–	–	5	–	–	–	–	–	–	5	–	–
Estorãos	–	–	–	4	–	–	–	–	–	1	5	0.400 (±0.237)	0.0009 (±0.0011)
Cávado	–	–	–	–	–	–	–	–	6	–	6	–	–
Ave	–	–	–	–	–	–	8	–	–	–	8	–	–
Douro													
Sabor	–	–	–	–	–	–	–	5	–	–	5	–	–
Tuela	–	1	–	4	–	–	–	–	–	–	5	0.400 (±0.0237)	0.0017 (±0.0017)
Tenente	2*	–	–	5	–	–	–	–	–	–	7	–	–
Ardena	–	–	–	4	–	–	1	–	–	–	5	0.400 (±0.237)	0.0017 (±0.0017)
Vouga													
Vouga	–	–	–	5	–	–	–	–	–	–	5	–	–
Caima	–	–	–	10	–	–	–	–	–	–	10	–	–
Mondego[a]	4	–	–	10	–	–	–	–	–	–	14	–	–
Tejo													
Zêzere[a]	2	–	–	3	7	–	–	–	–	–	12	0.467 (±0.132)	0.0010 (±0.0011)
Hatchery													
Torno	7	–	–	–	–	–	–	–	–	–	7	–	–
Fonte Santa	5	–	–	–	–	–	–	–	–	–	5	–	–
Total	20	1	3	71	7	1	9	5	6	1	124		

[a]Pooled individuals from 1998 and 1999 collections.

Table 5. Results of the hierarchical AMOVA on eight wild populations grouped by the Lima and Douro river basins. Hatchery individuals were excluded from calculations

	% total variation	ϕ satistics	P
Within populations	36.04	$\phi_{ST} = 0.64$	<0.0001
Among populations within groups	57.96	$\phi_{SC} = 0.62$	<0.0001
Among groups	6.01	$\phi_{CT} = 0.06$	0.2512 ± 0.0139

two alternative connections for both At5 and Me1 (Figure 2A). Haplotypes At8, At9 and At13 share a common deletion at position 387 (the only indel in our data). Figure 2B shows the nested design, assuming haplotype At5 derives from the highly frequent (68.3%) and interior At6 haplotype (following the justification of Crandall and Templeton 1993). Using this design, the NCA revealed significant associations of clades and sampling locations (Figure 3). However, the null hypothesis of no geographical association could not be rejected for the two largest clades that jointly cover the total cladogram. The demographic inferences drawn suggest restricted gene flow with some long distance dispersal detected within clade 1-3, past fragmentation detected within clade 2-1, and restricted gene flow with isolation by distance detected within clade 2-2.

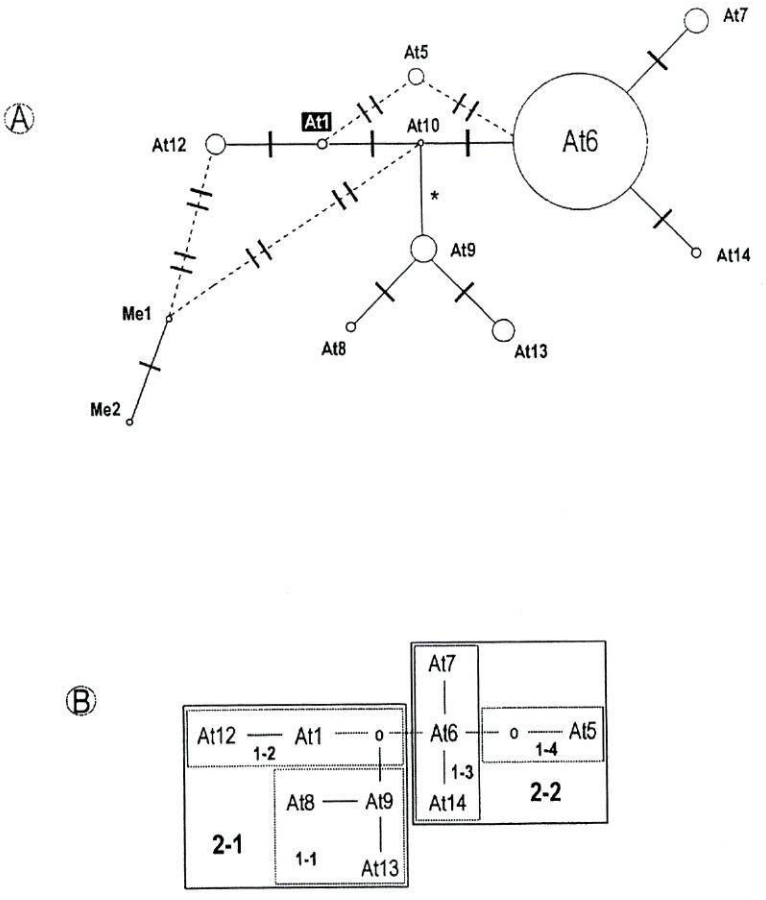

Figure 2. (A) An mtDNA haplotype network estimated with the 95% statistical limits of parsimony using the algorithm in Templeton et al. (1992). Haplotypes observed in the studied populations are represented in grey circles with their size proportional to their frequencies. The black box indicates the most common north Atlantic haplotype. Unfilled circles show other haplotypes: the At10 (Weiss et al. 2001) and Me1 and Me2 (Mediterranean outgroup). Each solid bar represents a single mutational event. The asterisk (*) represents the indel at position 387. Dashed lines indicate alternate mutational pathways in the topology of the tree. (B) Nested design for the mtDNA haplotypes detected in the studied populations. Zeros indicate undetected intermediate haplotype states. Each solid line represents one mutational step. The ambiguity of At5 was resolved as mentioned in the results. Thin-lined polygons indicate the haplotypes grouped together into 1-step clades (1-n) and thick-lined polygons enclose 2-step clades (2-n).

Discussion

Evaluation of potential introgression with non-native strains

The fixation for the *LDH-C*90* allele in the Torno hatchery stock in 1999 indicates a non-native origin. This contrasts with the fixation of the *LDH-C*100* allele in a 1995 stock (Antunes et al. 1999) when a native origin of the hatchery material was assumed. These observations corroborate the hypothesis of recent non-native introductions into Portugal (Weiss et al. 2000; Antunes et al. 2000). However, as Fonte Santa hatchery fish are also fixed for the **90* allele (including 5+ year old fish), commercial strains of north-Atlantic origin may have already been used in the past, as in other southern European countries (e.g. García-Marín et al. 1991; Presa et al. 1994). The presence of the At1 haplotype in one presumed native fish from the Tuela river suggests either introgression of hatchery strains or that this haplotype exists naturally in this population. Nevertheless, our data clearly indicate a low to non-existent level of introgression in nearly all populations, as previously reported, even for hatchery fish of presumably native origin (Antunes et al. 1999). This lack of introgression despite presence of non-native strains in the wild is analogous to the report of "failure of a stocking policy" in northwestern Spain (Morán et al. 1991).

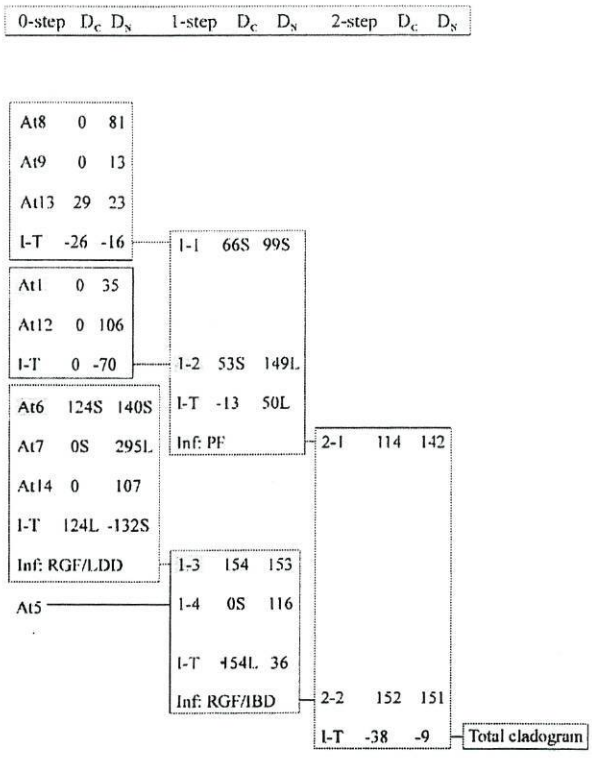

Figure 3. Nested cladistic analysis of the mtDNA haplotypes. D_C and D_N are clade and nested clade distances, respectively. An "S" means that the distance is significantly small at the 5% level, and an "L" means that it is significantly large. For nested clades, where the interior/tip status is known and both tips and interiors exist within the same nesting group, the clade named is shaded for interior clades. "I-T" gives the average difference between interior and tip clades within the nested group for clade and nested clade distance. Biological inferences are given as follows: RGF-restricted gene flow; IBD-isolation by distance; LDD-long distance dispersal; PF-past fragmentation.

Haplotype diversity and population history

The high population heterogeneity, with several haplotypes differing by one to three mutational events from the interior At6 haplotype, indicates a deep (at least 200 000 years of isolation; Weiss et al. 2000) and complex history in this small geographic region. There is strong support that the At6 lineage is ancestral and spread through the region through a common colonization process. This is based on its high frequency, interior position in the network, broad-geographic distribution, and occurrence in headwater drainages isolated by natural impassable waterfalls. The otherwise mosaic matrilineal pattern most likely results from the predominance of stochastic rather than mutational processes; i.e., restricted gene flow and highly variable drift and residency times among populations. Significant substructure in this region was also shown at the highly polymorphic plasma protein (*PX**), albeit the geographic pattern was distinctly different revealing a clinal decrease in the number of alleles (and heterozygosity) from north (Minho and Lima basin) to south (Ave, Mondego and Tejo basin) (Antunes et al. 2000).

The analysis of molecular variance showed that contemporary drainage structure serves as a poor model to explain the levels of genetic differentiation. Studies on the microgeographic structure of *S. trutta* have evidenced that the total genetic diversity among populations in this species is not correlated with geographical distances (Ryman 1983; Crozier and Ferguson 1986; Ferguson 1989). Just to the north, where anadromy is more common, Bouza et al. (1999) obtained a significant correlation between genetic (allozymes) and geographical distances, considering only non-isolated populations. Elsewhere, anadromy was also considered the responsible factor resulting in significant isolation by distance (Morán et al. 1995; Hansen and Mensberg 1998). Minho and Lima basins are currently at the southern limit of anadromy (SNPRCN 1991) but a few decades ago several migratory individuals were observed in the Douro basin (our observations) and it is assumed that anadromy occurred further south during the last glaciation (Hamilton et al. 1989). Thus, although anadromy must have played a homogenizing role in shaping genetic structure in this region its present occurrence may not be a good indicator of its past effects.

The NCA demonstrated that the present genetic structure of Portuguese brown trout populations results primarily from historical rather than contemporary processes. Past fragmentation was inferred as the predominant process shaping the structure of clade 2-1 based on the highly divergent At12 haplotype fixed in Sabor and the presence of geographically proximate haplotypes sharing a common indel in Cávado and Ave. Restricted gene flow with isolation by distance was inferred as responsible for most of the remaining genetic structure whereby some long distance dispersal is additionally inferred (clade 1-3) from the existence of two haplotypes (At7 and At14) differing one step from the interior haplotype (At6) located in geographically distant populations (Zêzere and Estorãos, respectively).

Conservation implications

Portuguese brown trout populations provide an illuminating example of the difficulty in appropriating conservation units to a large region, or even a contiguous catchment basin. Seemingly logical constructs of intraspecific genetic diversity may be misleading. Several workers assume that deep divergence between major mtDNA-defined lineages conceals substantial hidden genetic variation of adaptive significance that is undetected by the use of neutral markers (Bernatchez 1995; Moritz et al. 1995). Such mtDNA clades thus serve in identifying conservation units. While this proposition is, in general, difficult or impossible to evaluate, both empirical and theoretical considerations suggest that for brown trout this scenario may not be appropriate.

First, one of the major mtDNA lineages had been ascribed to the Atlantic basin (Bernatchez et al. 1992). This is an enormous region including glacial refuge and expansion areas both with heterogeneous networks of isolated drainages, in which a broad range of life-history patterns and phenotypic variation is recognized. However, genetic variation in the unglaciated Atlantic drainages of Iberia and southern France is highly distinct from that in the north (Krieg and Guyomard 1985; García-Marín et al. 1999; Antunes et al. 1999; Bouza et al. 1999; Núria et al. in press) with at least some basins revealing little or no gene flow to the north for at least several hundred thousand years (Weiss et al. 2000). Second, 12 of 16 populations analyzed in our study (Figure 1), located within a single OCU proposed by Machordom et al. (2000) show little genetic homogeneity with molecular variance not even corresponding to river basins. Most recently, in the Spanish region of the Douro Basin alone, a G_{ST} value of 46% was reported based on the screening of 34 allozyme loci in 62 native populations (Bouza et al. 2001). While catchment basins provide a logically functional unit for water quality management, they are not necessarily a good predictor of the genetic relationships among populations (Currens et al. 1990; McGlashan and Hughes 2000).

The potential, often cited weaknesses of mtDNA data may be one source of confusion in considering large-scale evolutionary units at intra- and interspecific boundaries (Hoelzer 1997; Paetkau 1999; Goldstein et al. 2000). However, there has been a trend toward the exclusive use of mtDNA data to define conservation units (Crandall et al. 2000). For the brown trout we suggest, *even in the unlikely event* that the major mtDNA lineages represent the common coalescence of the whole genome (i.e. the haplotype tree accurately reflects population history), they may not reflect a hierarchy of conservation priority. This is because the genealogical links between populations and metapopulations within the major mtDNA clades are too distant to correspond to any systematic selection pressures that may have uniquely shaped them. Furthermore, mutations of adaptive significance are rare and stochastically distributed through the genealogy and thus are not often shared by populations belonging to broad geographically circumscribed areas.

Given such a scenario, how can "units" of conservation be assigned? Laikre et al. (1999) stressed that the basic units for management and conservation in *Salmo trutta*, are local populations. We concur, and add that in some geographic areas populations or metapopulations are the only "units" below the species level that can be collectively effected by management actions. This population perspective conforms with our knowledge of the tremendous among population variation (phenotypic and genetic) found in brown trout (e.g. Ryman 1983; Ferguson 1989; Guyomard 1989; Laikre et al. 1999) as well as other highly fragmented salmonid fishes (e.g. the cutthroat trout; Allendorf and Leary 1988). There is little reason to suspect that major evolutionary mitochondrial lineages, as defined for brown trout, collectively harbour common traits of adaptive significance when compared to unique populations within them. Perhaps some larger-scale network of populations, such as anadromous populations of the north Atlantic, could arguably possess both common traits and a common ancestry, warranting definition as a conservation unit. However, in this same region, prominent diversity has been more easily recognized at the smallest-scale, such as for reproductively isolated sympatric demes (Ryman et al. 1979; Ferguson and Taggart 1991). Thus, practicing conservation on a population or metapopulation level makes sense from both a practical and theoretical perspective.

Conservation priority, if there indeed must be priority, should proceed from a bottom-up perspective, with the most distinct populations and metapopulations for the species, afforded the most protection. Such a perspective also conforms to the political reality in Europe that renders the consideration of a major evolutionary lineage existing across multiple provincial and international borders, a mere academic exercise with little or no conservation value. We

suspect that other highly fragmented freshwater organisms, especially those with broad as opposed to regionally endemic distributions, might also reveal mosaic patterns of evolutionary significant genetic variation. Such variation will not be necessarily conserved when conservation planning rests on recognizing narrowly defined, large-scale units.

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4. Filogeografia nuclear da truta na região Euroasiática

Artigo VI The role of nuclear genes in intraspecific evolutionary inference: genealogy of the *transferrin* gene in the brown trout (submetido à *Molecular Biology and Evolution*).

The role of nuclear genes in intraspecific evolutionary inference: genealogy of the *transferrin* gene in the brown trout.

Agostinho Antunes[1 2], Alan R. Templeton[3], René Guyomard[2] and Paulo Alexandrino[1]

[1]Centro de Estudos de Ciência Animal (CECA), ICETA-U.P., Campus Agrário de Vairão, 4485-661 Vairão, Portugal; Departamento de Zoologia-Antropologia, Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, 4099-002 Porto, Portugal. [2]Laboratoire de Génétique des Poissons, INRA, 78352 Jouy-en-Josas, France. [3]Department of Biology, Washington University, St. Louis, Missouri 63130-4899, USA.

Keywords: brown trout; gene conversion; nuclear genealogy; recombination; *Salmo trutta*; salmonids; *transferrin*.

Abstract

To date, technical and biological hurdles have precluded the retrieval of nuclear gene genealogies within most species. Among those obstacles, the possibility of intragenic recombination is one of the most demanding challenges. We studied the utility of nuclear genes for intraspecific evolutionary inferences, by selecting a nuclear gene that exhibits patterns of considerable geographical differentiation in the brown trout (*Salmo trutta*) species complex. Haplotype variation from a nucleotide sequence of ~3.7 kb encompassing a portion of the *transferrin* (*TF*) gene was surveyed in 31 brown trout individuals collected across its native Eurasian range. Statistically significant recombination and gene conversion events were detected. However, we showed that substantial cladistic structure was not disrupted by recombination or gene conversion events, and additional structure was estimated to have emerged after those events. Because loci with unusually high levels of variation might indicate the presence of selection, we tested the hypothesis of neutrality. Moreover, the strong geographical signal observed in the *TF* genealogy, coupled with the current spatial distribution of electromorphs, gave us the ability of drawing empirical phylogeographic inferences. We delineated the composition of current brown trout populations based on 3625 individuals electrophoretically scored for the *TF* locus. We hypothesized scenarios of historical radiation and dispersal events, thus providing new insights over previous allozyme and mtDNA inferences.

Introduction

The study of principles and processes governing the geographical distribution of genealogical lineages, phylogeography, has often focused on the mitochondrial (mt) genome (Avise 1998). Yet, the matrilineal pathways of ancestry registered by this molecule represent only a small portion of the total historical record of a sexual organismal pedigree (Avise and Wollenberg 1997). Much of the remainder of that history should be inferable from autosomal gene trees. However, as a result of its high complexity, few attempts have been made to estimate nuclear gene genealogies in a phylogeographic framework outside of humans (e.g. Palumbi and Baker 1994; Hare and Avise 1998). Documenting the historical incidence of intragenic recombination within a species is one of the most difficult challenges of nuclear genealogies (Avise 2000). However, recent methodologies have been proposed to overcome this difficulty, providing a tool to detect statistically significant recombination events (Crandall and Templeton 1999; Templeton et al. 2000a) and reveal the evolutionary gene structure not disrupted by recombination (Templeton et al. 2000b).

Presently, many allozyme and protein loci have been subject to DNA sequencing, offering the opportunity to understand the genealogy of classical nuclear markers. Such an understanding could be especially useful when large data sets for those markers were previously generated, allowing the choice of a locus exhibiting significant differentiation among populations at large and small geographic scales. That is the case of the *transferrin* (*TF*) locus, which is polymorphic in many vertebrate species, including fish (Kirpichnickov 1981). The locus codes an iron binding protein found in vertebrate blood serum and interstitial spaces (Loehr 1989). Among salmonids, the locus has frequently been used to differentiate populations (e.g. Payne 1974; Krieg and Guyomard 1985; Van Doornik and Milner 1996) and, more recently, it has received attention for studies of molecular adaptation (Ford et al. 1999; Ford 2000, 2001).

In this context, the brown trout (*Salmo trutta* L.) is an appropriate organism to study the utility of nuclear gene genealogies for evolutionary inferences. This species exhibits a strong genetic structuring and is considered a polytypic species with complex patterns of phenotypic diversity and life history variation, including anadromous, fluviatile and lacustrine modes of life (Behnke 1972). Over the last two decades, geographical patterns of genetic differentiation were largely observed, using both allozymes (e.g. Allendorf et al. 1976; Ryman et al. 1979; Guyomard 1989; Giuffra et al. 1996; Bouza et al. 2001) and mtDNA (e.g. Bernatchez et al. 1992; Giuffra et al. 1994; Bernatchez and Osinov 1995;

Apostolidis et al. 1997; Antunes et al. 2001). The sequencing of the mtDNA control region delineated five major evolutionary lineages (Danubian [DA], Adriatic [AD], marbled trout [MA], Mediterranean [ME] and Atlantic [AT]; Bernatchez et al. 1992; Bernatchez 2001), and the distribution of allozyme variants is partially congruent with these lineages (García-Marín et al. 1999).

Considering the *TF** locus alone, substantial geographical patterns of electrophoretic variants were described in the brown trout (e.g. Guyomard 1989; Giuffra et al. 1996; Largiadèr and Scholl 1996a) (fig. 1). The electromorph *TF*100* is generally fixed for Atlantic populations and domesticated hatchery strains. Mediterranean brown trout is often fixed for the *TF*102*, the marbled trout (*S. trutta marmoratus*) for the *TF*75* and the Italian carpione (*S. trutta carpio*) for the *TF*78*. Other electromorphs were observed in moderate to high frequencies, such as the *TF*95* in southwestern Atlantic populations from Portugal (Antunes et al. 1999) and the *TF*80* in Corsican populations (Krieg and Guyomard 1985; Presa et al. 1994; Berrebi 1995). The Atlantic salmon (*S. salar*) presents both the *TF*80* and **102* (Giuffra et al. 1996). Electrophoretic analysis of allozyme or protein variation shows however, an inherent limitation for some phylogenetic purposes in that the historical relationships of alleles remained unresolved.

In this study, we analyze sequence variation from a nuclear gene (*TF*) in the *S. trutta* species complex over most of its native Eurasian range. A nucleotide sequence of ~3.7 kb encompassing partially the *TF* gene and representing the majors electrophoretic variants is surveyed. We intend to (1) document the cladistic structure of the gene and its utility for evolutionary inferences, (2) examine the roles of both recombination/gene conversion and mutation, (3) test the hypothesis that patterns of variation among the *TF* locus can be explained by the neutral model (Kimura 1968), and (4) obtain a phylogeographic portrait for the brown trout, by overlaying the *TF* genealogy with the geographical distribution of the different electromorphs.

Material and methods

Molecular study of transferrin electromorphs

To infer the genealogical relationships of transferrin electromorphs in brown trout, we selected individuals phenotypically homozygous for the most frequent electrophoretic variants. Some individuals were scored, previously in other studies, for the *TF* locus (Berrebi 1995; Giuffra et al. 1996; Berrebi et al. 2000), while others were specifically scored for this study.

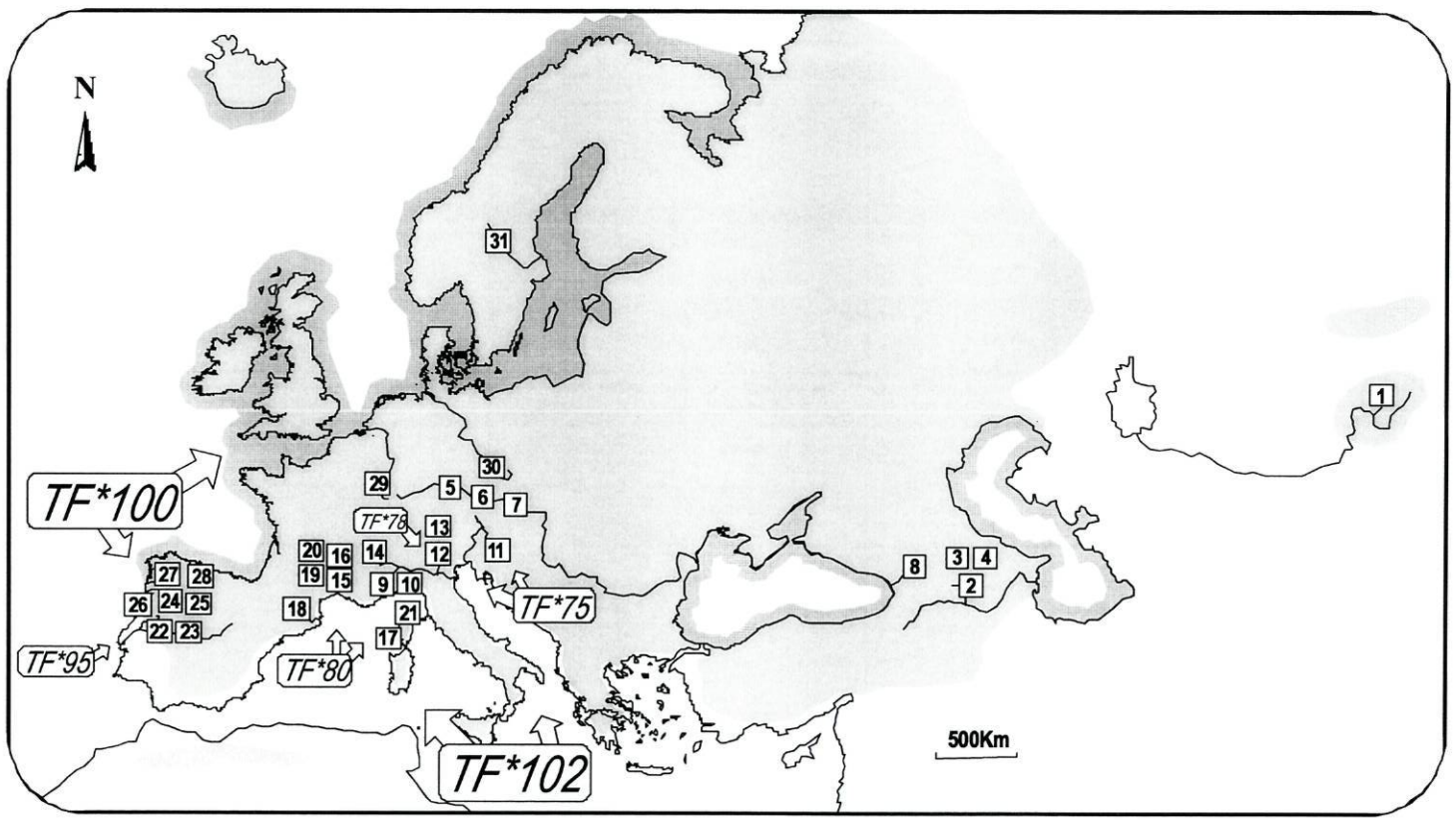

Fig. 1. Geographic locations of the brown trout specimens analyzed. Numbers correspond to individual codes from table 1. The shaded area of the map represents the current distribution of the species complex (adapted from Guyomard 1989; Elliot 1994). The dark shaded area indicates the presence of the anadromous form. The geographic distribution of the *TF* electromorphs is also shown. The sizes of the electromorph boxes intend to give a relative idea of their population frequencies. A detailed description of their distribution and frequencies is provided in the Appendix.

Table 1. Geographic origins, subspecific designation, electromorphs, haplotypes and amino acid sequences of the individual analyzed.

Species	Phenotypic identification	Country	Locality	Basin	Individual code	*TF** electromorph	Haplotypes	a.a. sequences
S. trutta								
	oxianus	Tajikistan	Sofidaron	Aral Sea	1	?	A1/A2	TFaa-A
	ischchan	Armenia	Sevan	Caspian Sea	2	?	C1/C2/C3/C4	TFaa-C-1/TFaa-C-2
	ischchan	Armenia	Sevan	Caspian Sea	3	?	C3/C5/C6/C7	TFaa-C-1/TFaa-C-2
	ischchan	Armenia	Sevan	Caspian Sea	4	?	C3/C5/C8/C9	TFaa-C-1/TFaa-C-2
	labrax	Austria	Anrasersee	Black Sea	5	?	B1/B2	TFaa-B
	labrax	Austria	Blühnbach	Black Sea	6	?	B1/B2/B3/B4	TFaa-B
	labrax	Austria	Kalkalpen National Park	Black Sea	7	?	R1/R2	TFaa100-1/TFaa100-R
	labrax	Georgia	Kodori	Black Sea	8	?	TF78-1/K1	TFaa78.80
	marmoratus	Italy	Pellice	Adriatic Sea	9	*75	TF75-1/TF75-2	TFaa75-1
	marmoratus	Italy	Pellice	Adriatic Sea	10	*75	TF75-4/TF75-5/TF75-6/TF75-7	TFaa75-1
	marmoratus	Slovenia	Trebuscica	Adriatic Sea	11	*75	TF75-1/TF75-3	TFaa75-1
	carpio	Italy	Garda	Adriatic Sea	12	*78	TF78-1	TFaa78.80
	carpio	Italy	Garda	Adriatic Sea	13	*78	TF78-2/R3	TFaa78.80/TFaa75-R
	fario	Italy	Ripa	Adriatic Sea	14	*102	TF102-1	TFaa102
	fario	France	Argens	Mediterranean Sea	15	*102	TF102-2/TF102-3	TFaa102
	fario	France	Argens	Mediterranean Sea	16	*102	TF102-1/TF102-4	TFaa102
	macrostigma	Fr (Corsica)	Pozzi	Mediterranean Sea	17	*102	TF102-5	TFaa102
	fario	Spain	Ter	Mediterranean Sea	18	*101	TF102-1/TF102-2	TFaa102
	fario	France	Argens	Mediterranean Sea	19	*80	TF80-1	TFaa78.80
	fario	France	Argens	Mediterranean Sea	20	*80	TF80-1/TF80-2	TFaa78.80
	macrostigma	Fr (Corsica)	Chiova	Mediterranean Sea	21	*80	TF80-3	TFaa78.80
	fario	Portugal	Zêzere	Atlantic Ocean	22	*95	TF95-1/R4	TFaa95/TFaa100-R
	fario	Portugal	Zêzere	Atlantic Ocean	23	*95	TF95-1/R4	TFaa95/TFaa100-R
	fario	Portugal	Cávado	Atlantic Ocean	24	*95	TF95-1/TF95-2	TFaa95
	fario	Portugal	Cávado	Atlantic Ocean	25	*95	TF95-1/TF95-3	TFaa95
	fario	Portugal	Ave	Atlantic Ocean	26	*100	TF100-6/TF100-7/TF100-8/TF100-9	TFaa100-2
	fario	Portugal	Minho	Atlantic Ocean	27	*100	TF100-1	TFaa100-1
	fario	Spain	Meras	Atlantic Ocean	28	*100	TF100-2/TF100-3	TFaa100-1
	fario	France	Vosgues	Atlantic Ovean	29	*100	TF100-4/TF100-5	TFaa100-1
	fario	Czechoslovakia	Vlatva	North Sea	30	*100	TF100-4/TF100-5	TFaa100-1
	fario	Sweden	Dalalven	Baltic Sea	31	*100	TF100-4/TF100-5	TFaa100-1
S. salar								
	_	Spain	Dobra	Atlantic Ocean	Ss1	*80	a	TFaaSs80
	_	France	Hatchery	_	Ss2	*80	a	TFaaSs80

[a]Variable heterozygous sites were observed within the Atlantic salmon specimens sequenced. However, to simplify the analysis, we have only considered one presumably shared haplotype.

The current geographical distribution of *TF* electromorphs, was also assessed by combining previously published data, with new electrophoretic screenings from several geographical areas (31 European populations corresponding to 661 individuals; see Appendix). Electromorphs were typed by agarose gel electrophoresis (AGE) (Antunes et al. 2000) and/or isoelectric focusing (IEF) (Antunes et al. 1999). A previously unknown electromorph (*TF*101*) was detected in Spanish populations from the Mediterranean drainage, as confirmed by side-by-side running of reference samples (Largiadèr and Scholl 1996a), both in AGE and in IEF separation systems. This electromorph was also included for molecular survey. A total of 31 brown trout samples was chosen, exhibiting the following electromorphs: *TF*75*, **78*, **80*, **95*, **100*, **101* and **102* (table 1; fig. 1). However, in 8 individuals coming from tributaries of the Black, Caspian and Aral Sea drainages, electromorph information was not available. We avoid samples coming from populations with genetic evidences of introgressive hybridisation resulting from stocking activities or contemporary human-induced habitat disturbance. Two Atlantic salmon (*S. salar*) specimens homozygous for *TF*80* were also examined and used as an outgroup in the phylogenetic analysis.

Sequencing was performed on diploid transferrin genotypes for the selected samples. The gene is well conserved among vertebrates and organized into 17 exons separated by 16 introns (Schaeffer et al. 1987; Mikawa et al. 1996; Ford et al. 1999). Initially, a survey was done almost in the entire transferrin gene (around 8 kb sequenced) of a brown trout and Atlantic salmon specimens followed by a preliminary screening of variable regions in several trout specimens. This procedure allowed the final selection of the gene fragments to be sequenced, avoiding portions that were often polymorphic with different allele lengths caused by tandem repeats or indels variation, making direct sequencing difficult or impossible. For each individual, exons 2, 4, 6, 7, 13 and introns 1, 3, 5, 6, 12, 13 were completely sequenced. Exons 1, 3, 5, 8, 10, 12, 14 and introns 2, 4, 7, 9 were partially sequenced (fig. 2). Genomic DNA was extracted from blood or muscle tissue following Sambrook et al. (1989). Sequences were determined via the polymerase chain reaction (PCR) amplification. The primers used were very similar to those designed from the coding region of the coho salmon (*Oncorhynchus tshawytscha*) by Ford et al. (1999), with slight adjustments based on published cDNA in brown trout and Atlantic salmon (Kvingedal et al. 1993; Lee et al. 1998): TF-ex1-F CATGAAACTGCTTCTCCTCTC, TF-ex3-R CCTCACCATAGTCCTCTGCAAT, TF-ex3-F GCCTCACTAACTACGGCCT-GCA, TF-ex5-R TGGAAGGCCCCAGCATAGTCAT, TF-ex5-F AGGTCTCAC-AAGGAGCCCTA, TF-ex7-R TTGACGGCCACCAGTTTGTTG, TF-ex7-F CG-

CAAGGACCCCGAACTGGC, TF-ex10-R ATACTGCTCCACCATGACAGGG, TF-ex12-F TACCCATGGGTCTCATCCACAA, TF-ex14-R CATCAGTGCTC-TCTGGTACAAT. Two new primers were also designed: TF-ex8-R GGCA-GCTGTACTAGTTTCTGAG and TF-ex9-F TTCAGCGCAGGCCACAGGTG. Following 5 min. of denaturation at 94°C, samples were amplified through 37 cycles of denaturing for 50 s at 92°C, annealing for 50 s at 57 to 60°C depending on the primer set, and extending for 70 s at 72°C. After the last cycle PCR was incubated a further 5 min. at 72°C. Sequencing was accomplished with BigDye Terminater Cycle Sequencing protocols. Sequences were visualized on an ABI-310 automated sequencer (PE Applied Biosystems) and checked by hand using the Sequence Navigator® software (version 1.0.1; Applied Biosystems, Inc.). Alignments were done using SeqApp (Gilbert 1996).

Haplotypes were determined by the haplotype-subtraction algorithm of Clark (1990). Confirmatory analyses were performed by molecular cloning (InsT/A Clone PCR Product Cloning Kit). Not all the haplotypes were observed directly, so some are not known with certainty. Sequence comparisons, measures of variability, and translation of exon sequences into amino acids were performed using MEGA (version 2.1; Kumar et al. 2001).

Estimation and testing of recombination/gene conversion

Detection of recombination/gene conversion events was performed using the CT algorithm of Crandall and Templeton (1999) with additions given in Templeton et al. (2000a). The algorithm starts with the estimation of a haplotype "tree" under the null hypothesis of no recombination or gene conversion. The quotation marks placed around the word "tree" is to emphasize that this "tree" may not be an accurate reflection of the evolutionary history if the null hypothesis is rejected. From the checked robustness of the different "tree" topologies in Templeton et al. (2000a) we chose the statistical parsimony (SP) method for the tree-estimation. The SP (Templeton et al. 1992; Crandall 1994; Crandall and Templeton 1996) favors the parsimonious solutions that avoid placement of homoplasies on short branches that lie within the "limit of parsimony" (Templeton et al. 1992). Maximum-parsimony trees were generated using PAUP (version 4.0; Swofford 1997). Comparison of the adjusted character distances with the corresponding patristic distances, allowed the elimination of the maximum-parsimony trees that violate the limits of parsimony. The generated trees were also compared with the correspondent networks estimated in TCS (version 1.13; Clement et al. 2000).

After the "tree" estimation, the null hypothesis of no recombination was tested by means of associations between the position of apparent homoplasies in the "tree" and the order of physical positions in the DNA sequence. This

association is used on the CT algorithm to identify putative recombinants, tracing pathways through the "tree" and matching the homoplasies clustered on a branch with their occurrences elsewhere in the "tree". The algorithm also allows the identification of crossover intervals and candidate parental types. To identify statistically significant recombinants a runs test based on a hypergeometric distribution was used. A program in MATHEMATICA (Wolfram 1996) was written to perform those tests (Templeton et al. 2000a).

The CT hypergeometric test is based on the ideal expectation of two runs under recombination: α matched homoplasious sites from one parental type, defining a run on one end of the physical sequence, followed by a run of β sites (i.e., the mutations on the pathway interconnecting the matched homoplasies) from the other parental type, on the other end of the sequence. Gene conversion is another genetic mechanism for placing a physical cluster of nucleotides states on a new haplotype background. It can place a small run of variable sites from one haplotype into the middle of a second one, thereby resulting in three runs by physical location. It can also result in only two runs, but such cases are indistinguishable from recombination. After the recombination tests were performed, an additional runs test was done to detect gene conversion events that have resulted in three or more runs. To test gene conversion with more then two runs, α apparent homoplasies on a "branch" are matched to another region of the "tree", with β other mutations lying on the pathway between the two "tree" regions (Crandall and Templeton 1999). The $\alpha + \beta$ mutations are then ordered by physical position, and the number of runs, say δ, of α and β mutations in the physically ordered sequence is recorded. Under the null hypothesis of no recombination/gene conversion, the probability for the number of runs, according to Mood and Graybill (1963), is given by:

$$\text{Prob}(\delta = d) = \frac{\binom{-1}{f-1}\binom{\beta-1}{f-1}}{\binom{\alpha-\beta}{\alpha}} \; d \text{ even}, f = \frac{d}{2},$$

$$\text{Prob}(\delta = d) = \frac{\binom{\alpha-1}{f}\binom{\beta-1}{f-1} + \binom{\alpha-1}{f-1}\binom{\beta-1}{f}}{\binom{\alpha-\beta}{\alpha}} \; d \text{ odd}, f = \frac{d-1}{2}. \qquad (1)$$

Because gene conversion is tested only after recombination (d = 2), equations (1) are used to calculate the probability of δ = 2 under the null

hypothesis, and then equations (1) are divided by [1 – Prob(δ = 2)] to obtain the conditional distribution of runs, given that $d \geq 3$, the only condition under which this gene conversion test is used. For more details, see the work of Crandall and Templeton (1999) and Templeton et al. (2000a).

Inferring the cladistic structure in the presence of recombination/gene conversion

In this study we have inferred the cladistic structure of *TF* gene based primarily on an alternative and novel method, recently proposed by Templeton et al. (2000b), for removing the effects of recombination and estimating cladistic structure in a DNA region that has experienced recombination. The method has a broader range of applicability than the method of subdiving a gene into smaller regions that show little or no internal recombination (Templeton et al. 2000b). The method starts by removing all the inferred recombination events from the SP tree estimated for the entire DNA region. This mean that the recombinant haplotype itself is removed from the SP tree, the homoplasies that were used to identify the recombinant under the CT algorithm are removed and discarded since they do not represent actual mutational events, and all haplotypes and branches that are derived from the original recombinant haplotype by subsequent events are removed. The remaining portion of the SP tree after this removal is referred as the "peeled" tree (Templeton et al. 2000b). The peeled tree reflects ideally the component of haplotype diversity that has not been affected by recombination during the coalescence of this DNA region. However, additional cladistic structure could have arisen in haplotype lineages derived from the recombinant haplotype. This postrecombinational cladistic structure is estimated by those subsets of the SP tree that consist of branches and haplotypes derived from each of the original recombinants by subsequent mutational events.

Tests of neutrality

Under neutrality, the ratio of nonsynonymous unique polymorphisms to nonsynonymous shared polymorphisms is expected to be the same as the ratio of synonymous unique polymorphism to synonymous shared polymorphisms, and this prediction was tested using a contingency table (*G*-test; Templeton 1996). From the inferred cladistic structure (peeled tree and recombinant clades) two tree topological mutational categories were used: tip and interior branches. The topological contrast of tip *vs.* interior for the most part corresponds to a contrast of young *vs.* old, thus yielding heterogeneity when selection occurs (i.e. tips are new and unproven, while interiors are older and have left descendants, so have proven evolutionary success). Further, six functional mutational categories were considered: silent and replacement substitutions in known positively selected sites

in salmonids (Ford 2001), silent and replacement substitutions flanking positively selected sites (± 3 codons), and silent and replacement substitutions in other sites. Two-by-two contingency tables were then analyzed using Fisher's exact test (FET) in STATISTICA (StatSoft 1993). An exact permutational test on the entire two-by-six matrix was performed using CHIPERM (version 1.0; Posada 1998).

Results

Sequence variation and haplotype determination

3625 individuals were electrophoretically scored for the *TF* locus (see appendix for details). Fig. 1 depicts the observed geographical distribution of the *TF* electromorphs.

Noncontiguous fragments of 3,693 bp (~3.7 kb) representing ~44% of the full length of the *TF* gene (as compared with the homologous gene in medaka fish, *Oryzias latipes*; Mikawa et al. 1996) were sequenced in 31 brown trout and two Atlantic salmon individuals (table 1; fig. 1), describing a total of ~122 kb analyzed. The aligned sequence data revealed 176 variable sites, 116 of which varied within the brown trout. Of those, 106 (including 13 indels) were used for phylogenetic inferences (table 2). The nucleotide divergence was slightly less for codon sites (table 3).

For the haplotype determination, five nucleotide positions (101, 579, 2910, 3059 and 3410) often heterozygous in most diploid individuals sequenced were excluded, as they lead to the premature termination of the cascade of inference for the haplotype-subtraction algorithm of Clark (1990). Also, the length variation at a dinucleotide repeat (position 3210 to 3217) was excluded from haplotype determination, as this variation does not fall under the same evolutionary models as the other variable sites. For the genotypically heterozygous individuals 2, 3, 4, 6, 10 and 26, the unambiguous inference of two haplotypes was not possible, and to solve the problem, two possible sets of haplotypes were estimated (table 1). This haplotype ambiguity resulted often in loops of ambiguity on the estimated SP tree (mainly in tip branches; fig. 4) and contributed to an overestimation of the total number of different haplotypes (n = 48). These ambiguities could be resolved in the future by applying a population frequency criterion proposed by Crandall and Templeton (1993) or via molecular haplotyping. For the moment, ambiguities were kept, but we represent as dashed lines the links that were nearly equally probable.

Table 2. Sequence variants used to defined haplotypes in the brown trout *TF* gene.

Site[a]	Position[b]	Domain[c]	Variant[d]	Site[a]	Position[b]	Domain[c]	Variant[d]	Site[a]	Position[b]	Domain[c]	Variant[d]
1	5	ex_1	A → C	37	1687	ex_5	T → A	72	2782	in_9	G → A
2	15	in_1	G → C	38	1754	in_5	G → A	73	2789	in_9	A → G
3	16	in_1	insATATACATA	39	1759	in_5	T → C	74	2791	in_9	C → T
4	48	in_1	delG	40	1760	in_5	T → C	75	2802	in_9	G → A
5	156	ex_2	insAGA	41	1761	in_5	T → C	76	2811	in_9	A → T
6	165	ex_2	A → C	42	1762	in_5	C → T	77	2814	in_9	G → T
7	207	ex_2	G → A	43	1770	in_5	C → A	78	2826	in_9	G → A
8	228	ex_2	G → C	44	1836	in_5	C → T	79	2855	in_9	G → T
9	365	in_2	G → T	45	1847	in_5	C → T	80	2882	in_9	T → C
10	368	in_2	delCTTGTGCGTTTT	46	1856	in_5	T → A	81	2933	in_9	delTA, delTT
11	390	in_2	C → A	47	1860	in_5	T → A	82	2934	in_9	A → T
12	475	in_2	C → T	48	1861	in_5	A → C	83	2944	in_9	C → G
13	521	in_2	T → A	49	1881	in_5	T → C	84	2968	in_9	T → C
14	667	in_3	C → T	50	1884	in_5	T → G	85	3018	in_9	T → C
15	684	in_3	C → T	51	1927	in_5	insTG	86	3048	in_9	delTTTG
16	779	in_3	T → A	52	1936	in_5	G → A	87	3055	in_9	T → G
17	805	in_3	C → T	53	1943	in_5	A → G	88	3077	in_9	C → A
18	812	in_3	A → G	54	1997	ex_6	A → G	89	3080	in_9	A → C
19	831	in_3	A → G	55	2005	in_6	T → A	90	3081	in_9	T → A
20	840	in_3	G → A	56	2021	in_6	G → C	91	3085	in_9	C → A
21	851	in_3	G → C	57	2065	in_6	A → G	92	3097	in_9	C → T
22	853	in_3	G → T	58	2068	in_6	A → T	93	3170	ex_{10}	G → A
23	908	in_3	T → G	59	2069	in_6	T → C	94	3218	in_{12}	T → C
24	955	ex_4	C → A	60	2072	in_6	C → G	95	3222	in_{12}	T → G
25	980	ex_4	A → G	61[1]	2083	in_6	A → G	96	3225	in_{12}	delTA
26	1090	ex_4	C → T	61[2]	2083	in_6	delACAACTGT	97	3273	in_{12}	delAT
27	1113	in_4	G → A	62	2159	in_6	A → G	98	3293	in_{12}	insCTT
28	1178	in_4	G → A	63	2233	ex_7	A → G	99	3345	ex_{13}	G → T
29	1220	in_4	delA–A	64	2289	ex_7	G → A	100	3354	ex_{13}	C → A
30	1256	in_4	A → T	65	2290	ex_7	G → A	101	3385	ex_{13}	A → C
31	1307	in_4	G → A	66	2293	ex_7	C → T	102	3386	ex_{13}	A → C
32	1537	in_4	T → C	67	2298	ex_7	A → C	103	3409	ex_{13}	G → T
33	1555	in_4	C → A	68	2376	in_7	G → A	104	3447	ex_{13}	A → G
34	1570	in_4	A → C	69	2611	in_7	T → G	105	3507	in_{13}	T → C
35	1594	in_4	A → G	70	2612	in_7	T → C	106	3649	in_{13}	A → G
36	1612	in_4	C → G	71	2613	in_7	delT				

[a]Site number assigned to each variable nucleotide position used to define haplotypes in order from 5' to 3'.
[b]Position in the aligned sequences studied (including the out-group).
[c]Domain location of the variable nucleotide position. Exon and intron number position are indicted by ex_n and in_n, respectively.
[d]Substitution and insertion/deletion variants are reported as the state in the baseline sequence → alternative state.

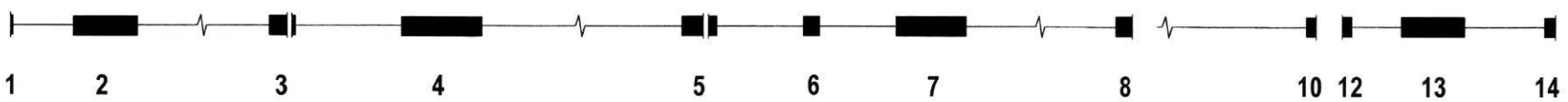

Fig. 2. Schematic diagram of the *TF* gene regions surveyed. The black boxes represent the exon domains.

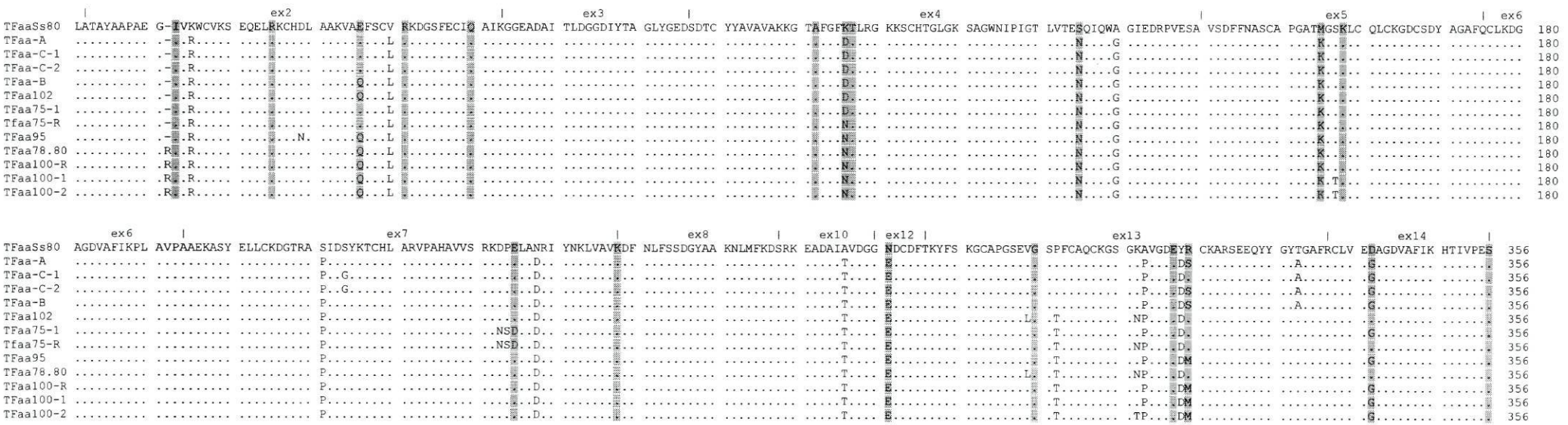

Fig. 3. Alignment of inferred partial *TF* protein sequences in the brown trout. Points indicate identity of amino acid residues with the outgroup (*S. salar*). Exon segments (ex_n) are indicated on the top of the alignment. Amino acid residues though to be positively selected in salmonids (Ford 2001) are shaded.

Table 3. Observed number of transition/transversion pairs of nucleotides, and estimation of the number of nucleotide substitutions per site (p-distance in codon and overall positions) between the DNA sequences for the brown trout *TF* gene (excluding gaps).

	Transition		Transversion					
	TC	AG	TA	TG	CA	CG	p_{coding}	$p_{overall}$
Average	7	7	3	4	5	2	0.0034	0.0072

Generally, the individuals scored as phenotypically homozygous for a *TF* electromorph were genotypically homozygous or heterozygous for a sequence differing by few nucleotides (≤ 10). Yet, the individuals 7, 13, 22 and 23 were heterozygous for alleles differing by a great number of nucleotides (23 to 40). Electromorph phenotype was not available for individual 7. The other three exhibit one haplotype corresponding to the electromorph for which they were phenotypically scored as homozygous, and another, corresponding to a distinct electromorph.

The amino acid (a.a.) sequences inferred from the translation of exons, revealed 356 sites representing ~52% of the total length of the *TF* protein in salmonids (Lee et al. 1998). The aligned sequence data for all taxa revealed 27 variable positions, with 15 of these positions varying within brown trout and describing 12 different sequences (fig. 3). With the exception of two, all brown trout a.a. polymorphisms were biallelic (position 312 and 320 had three alleles). The relation between electromorphs and their corresponding partial a.a. sequences revealed that some different electromorphs have identical partial sequences (*TF*78* and **80* = TFaa78.80; *TF*101* and **102* = TFaa102) whereas *TF*100* exhibited two different sequences (TFaa100-1 and TFaa100-2). *TF*80*, shared by Atlantic salmon and brown trout, exhibited very distinct a.a. sequences. The a.a. divergence among brown trout varied between 1.4 and 1.6%. The nucleotide divergence among haplotypes varied between 0.1 and 1.1%. Overall we detect a strong correlation between the nucleotide and the a.a. sequence, and the correspondent electromorph.

Phylogenetic inference under the null hypothesis of no recombination/gene conversion

The SP tree estimated under the null hypothesis of no recombination and gene conversion is presented in fig. 4.

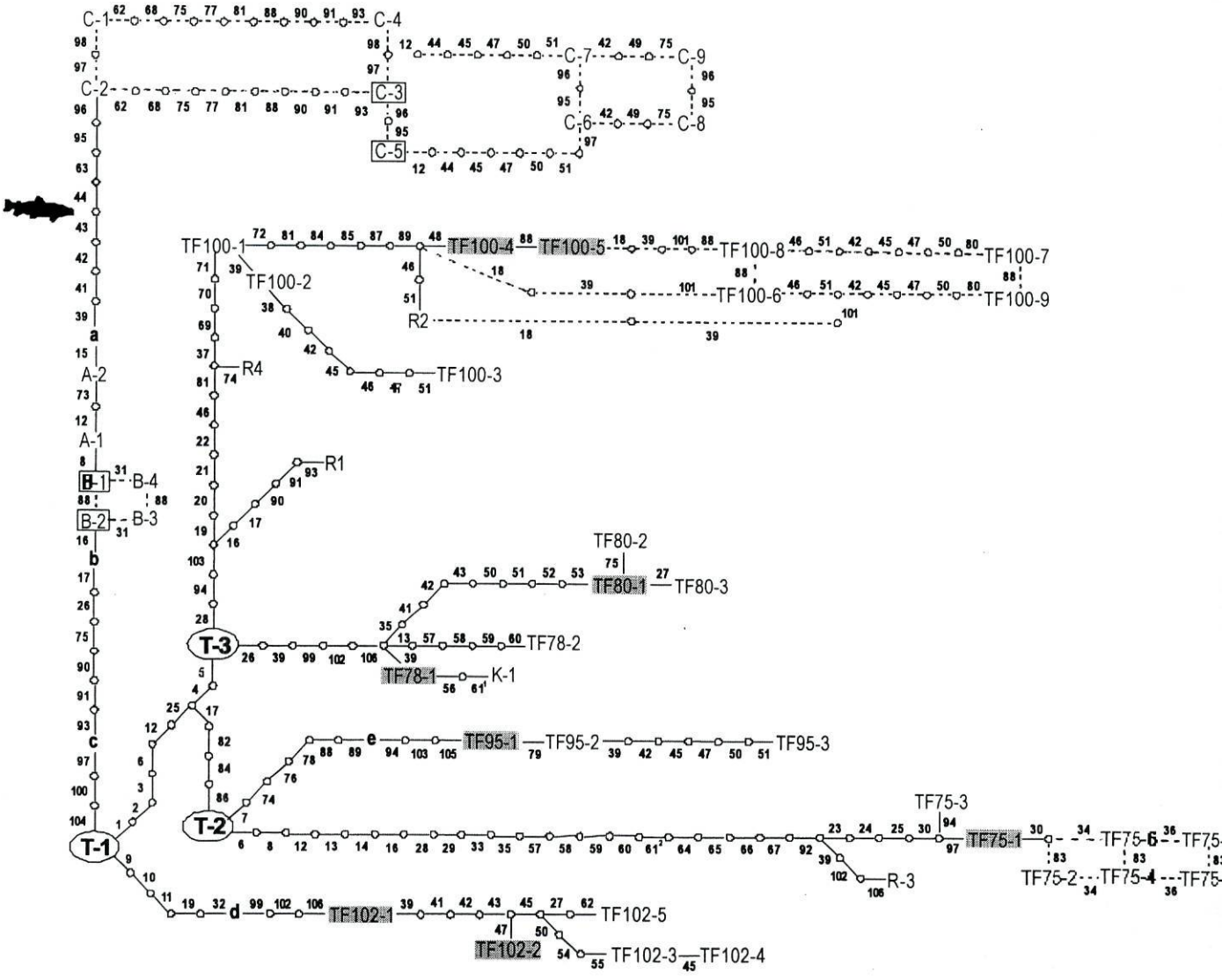

Fig. 4. The total haplotype network estimated under the null hypothesis of no recombination or gene conversation. Small circles indicate nodes in the tree that represent intermediate haplotype states not found in the sample. Each line (solid or dashed) represents a single mutational event. The site involved in the mutation is indicated near the line by a small boldface number, with the numbers corresponding to the variable site numbers given in table 2. Dashed lines indicate where loops or alternatives create ambiguity in the topology of the tree. Nodes that define three major termini are indicated by an oval containing T-i, where i can be 1, 2 or 3. Other nodes involved in recombination events are indicated by the boldface lowercase letter a-e. Haplotypes observed more than once are shaded grey. Haplotypes observed more than once, but resulting from an overestimation haplotype determination (see results), are boxed. The black salmonid fish indicates the connection with the outgroup (*S. salar*) and hence indicates the rooting of the tree.

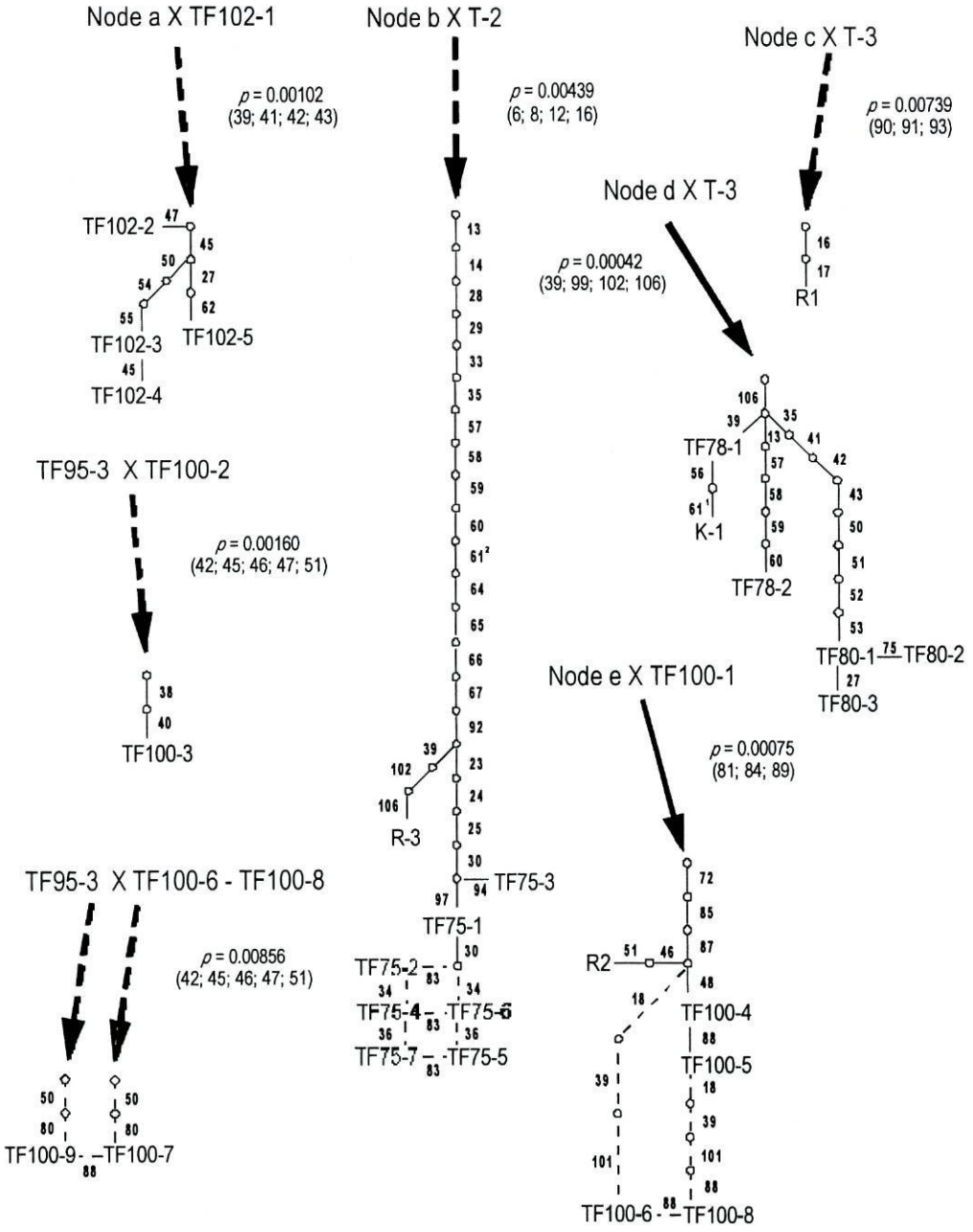

Fig. 5. The estimated recombinant clades. Solid and dashed arrows represent recombination and gene conversion events, respectively. The p value near the arrow corresponds to the tail probability from the hypergeometric test of the null hypothesis of no recombination and gene conversion, and the spanned variable sites involved in the crossover event are indicated in parentheses. The layout of the network is the same as that given in fig. 4.

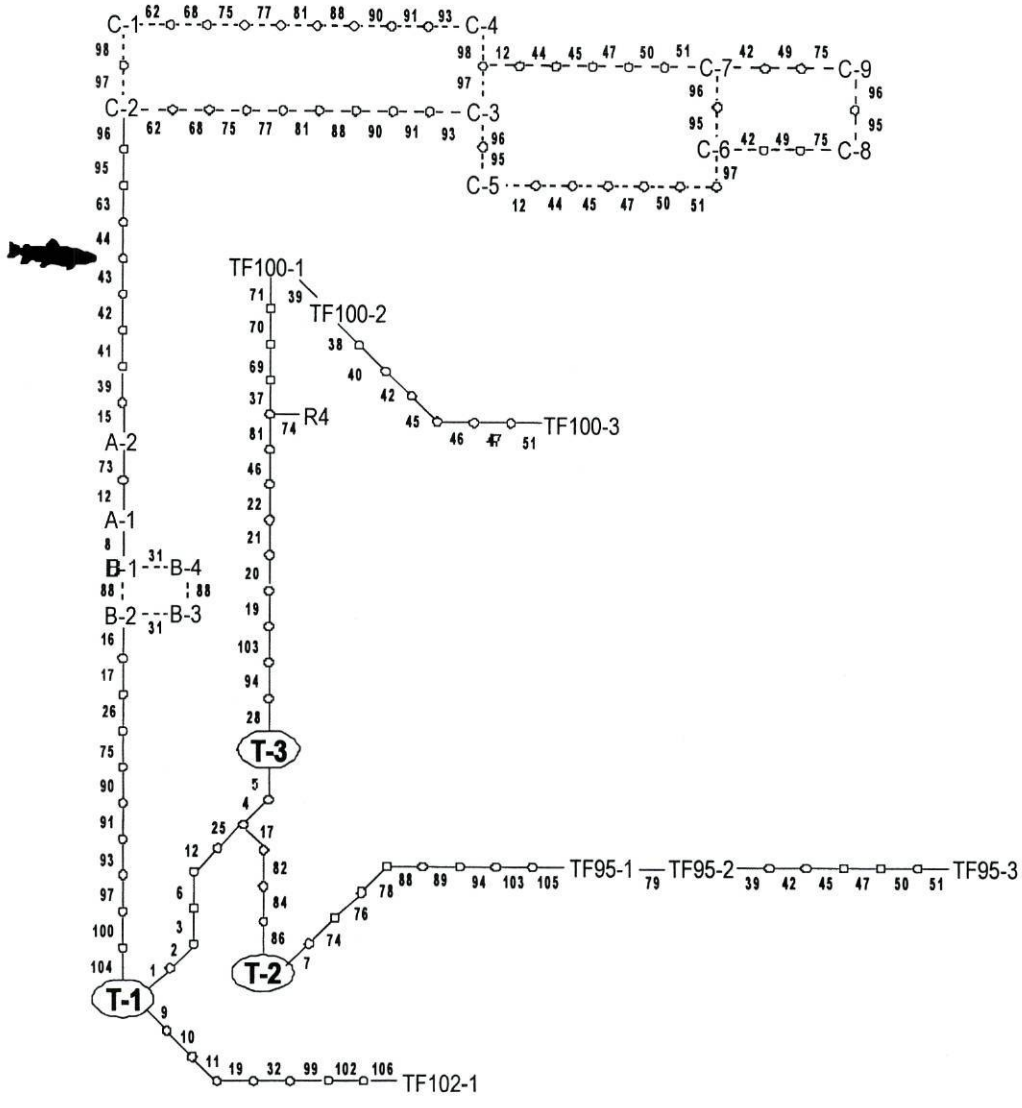

Fig. 6. The estimated haplotype tree after all recombinant and gene conversion events have been removed. The layout of the network is the same as that given in fig. 4.

Detection of statistically significant recombination/gene conversion events

Two recombination and five gene conversion events were significant at the 5% level, and were identified by applying the algorithm of Crandall and Templeton (1999) to the null hypothesis tree. Fig. 5 shows the tail probability from the

hypergeometric test of the null hypothesis of no recombination and gene conversion, and the spanned variable sites involved in the crossover event. Most of the variable sites were nucleotide substitutions, but a few involved insertions or deletions. Physical locations of recombinant sites comprise three regions in the gene: (I) a portion of exon 2 and intron 3, and intron 2; (II) a portion of intron 5; and (III) a portion of intron 9, and exon 10, 13 and 14. Recombination/gene conversion events were identified in region (I) – (II) – (III) at the following frequencies: 1, 3, and 2, respectively.

Estimation of the transferrin gene genealogy

Fig. 6 presents the cladistic structure that remains after the removal of all significant recombination or gene conversion events, the homoplasies attributable to them, and any additional structure that has evolved from the original recombinant/converted haplotypes. Fig. 5 shows the two recombination and five gene conversion events, along with the cladistic structure estimated to have arisen after the event. Since recombination/gene conversion was not concentrated in a single hotspot region, subdivision of the studied DNA sequence in smaller segments that showed little or no internal recombination would discard more than 40% of our data set. As a result, the SP trees of the subdivided DNA region, 5' region (1-164 bp), internal region (780-1758 bp), and 3' region (1929-2932 bp) were not as informative as the peeled tree and recombinant clades estimated under the methodology proposed by Templeton et al. (2000b) (data not shown). The few topological discrepancies between the subregional trees and the peeled tee could be explained by a substantial reduction of the informative variable sites within each subregion.

Tests of neutrality

Contingency tables were constructed by counting the number of mutational events in the various categories and the various types of branches for the post-recombinant clades and the peeled tree (fig. 5 and 6). From the 29 positively selected amino acid sites in salmonid *TF*, defined by Ford (2001), 20 were part of our data set (fig. 3). The contingency tables and test results are shown in table 4 and 5. For the different tests, none of the results were significant at a 5% level. However, the entire two-by-six test showed to be highly significant ($P<0.001$).

Table 4. Topological and functional mutational categories used for the contingency analysis.

	Positively selected sites[a] (PSS)		Flanking PSS[b] (FPSS)		Other sites (OS)	
	Silent	Replacement	Silent	Replacement	Silent	Replacement
Tip (T)	0	2	0	1	0	2
Interior (I)	0	5	2	5	7	4

[a]According to Ford (2001).
[b]± 3 codons.

Table 5. Two-by-two contingency analysis of the different mutational categories tested.

Mutational categories	FET probability
T/I *vs.* PSS	na
T/I *vs.* FPSS	1^{ns}
T/I *vs.* OS	0.192^{ns}
T/I *vs.* PSS+FPSS	1^{ns}
T/I *vs.* PSS+OS	0.249^{ns}
T/I *vs.* FPSS+OS	0.229^{ns}
T/I *vs.* PSS+FPSS+OS	0.144^{ns}

na – not applicable; ns – not significant.

Discussion

Electrophoretic vs *molecular variation*

Considerable molecular differentiation was found between and within the distinct *TF* electromorphs analysed. Many different haplotypes were lumped into a single electromorph class. Haplotypic differences within an electromorph were generally not related with a.a. changes. Yet, for the electromorph **100* we observed two different protein sequences differing by one a.a.. A remarkable case is the electromorph *TF*80* identified in the Atlantic salmon and brown trout exhibiting 18 a.a. differences. This provides empirical evidence for the limitation of gel

electrophoresis as a detector of genetic variation when comparisons are made at a generic level (Ramshaw et al. 1979; Barbadilla et al. 1996). Furthermore, it demonstrates the danger of inferring ancestral relationships between electromorphs based on their transpecific sharing.

On the other hand, we found cases where electrophoretic differences were not detected in the partial nucleotide sequences analyzed. The distinct electromorph *TF*101* was not differentiated at the sequence level from *TF*102*, since both have identical a.a. sequences in the portion of the gene sequenced, and also shared nucleotide haplotypes in the sequenced portion of the gene. Also, electromorph *TF*78* and **80* showed identical a.a. sequences. However, in this case we found some divergence at the haplotype level. Both cases show that the detected electrophoretic variation results from mutations in other coding regions of the gene.

Transferrin gene genealogy

One traditional method for estimating cladistic structure in a DNA region subject to recombination, consists of removing recombination events by splitting the DNA region into subsegments with little to no recombination (e.g. Templeton and Sing 1993). However, the method would be difficult to implement under uniform recombination, unless recombination was either rare and/or concentrated into hotspots. The methodology of Templeton et al. (2000b) provides a way of analyzing these data, in which recombination/gene conversion events were identified in three distinct spots and no single hotspot.

In spite of the recombination/gene conversion events detected in the portion of the *TF* gene analyzed, substantial cladistic structure was still discriminated. The peeled tree (fig. 6) provides an estimate of the cladistic structure contained within the entire 3.7 kb region sequenced that has not been altered by any detected recombination or gene conversion events. This peeled tree contains the branches leading to the three major termini (T-1, T-2 and T-3) that were defined primarily by sites 5' to the recombinational region (I). Moreover, the fact that branches among the 3 major termini resulted in part from rare genomic changes (RGCs; Rokas and Holland 2000) implies that this evolutionary structure is quite old.

Substantial cladistic structure has also arisen after recombination/gene conversion events (fig. 5). Interestingly, those events provide evidence for secondary intergradation between distinct sub-clades. Some of these currently occur in the same geographical region, such as the TF95 and TF100 (Atlantic drainage), and the TF102 and TF78.80 (Mediterranean drainage). Gene conversion between sub-clades TF-BCA X TF102, and TF-BCA X TF75,

currently occupying distinct geographical areas (Black, Caspian and Aral Sea, as opposed to Adriatic and Mediterranean drainages) could represent a signature of past intergradations between those sub-clades. Another evidence for secondary contact, followed by introgressive hybridization is represented by the R1 and R2 haplotypes in individual 7, from the upper Danube. Those haplotypes are related to the TF100 sub-clade but they evolved by gene conversion and recombination events with other sub-clades (fig. 5). This result is supported by previous studies which showed that some unstocked populations from the Danubian drainages or other Black Sea basins may exhibit mtDNA and allozyme alleles specific for Atlantic brown trout (Bernatchez et al. 1992; Bernatchez and Osinov 1995). They have proposed intergradation of ancient Danubian and Atlantic populations after secondary contact in postglacial times as the most likely explanation for this occurrence, a fact that was also corroborated by subsequent studies (e.g. Largiadèr and Scholl 1995; Weiss et al. 2001).

Tests of neutrality

The results from the two-by-two contingency test do not provide evidence for directional selection. This is because the absence of silent substitutions on positively selected sites (PSS) precludes the application of the two-by-two test just to these sites. However, results upon the entire two-by-six matrix test do reject the null hypothesis of neutrality. Much of the significance of this test is due to the PSS and the total absence of silent substitutions. When compared to the other sites, this would imply strong directional selection on the PSS. Ford et al. (1999) suggested that positive natural selection for new alleles has played an important role in the evolution of the protein in salmonids. The selected sites generally fall on the outside of the molecule within and near areas that are bound by *TF*-binding proteins from human pathogenic bacteria, thus supporting the hypothesis that competition for iron could be a source of positive selection (Ford 2001). Ford (2000) showed that unlike patterns of variation within species, there was no evidence of greater differentiation among chinook salmon (*O. tshawytscha*) populations at nonsynonymous compared to synonymous sites. Nevertheless, we do find some evidence for directional selection at the intraspecific level in the patterns of *TF* coding variation within brown trout and thereby differences in the coalescence patterns among *TF* lineages may be expected (Takahata 1990; Stephan and Mitchell 1992).

Phylogeographic assemblages of the transferrin *gene*

Based on inferences from *TF* genealogy and the current spatial distribution of electromorphs, we hypothesize a framework for the evolution of the *S. trutta*

species complex (fig. 7). Accordingly, the most ancestral group of populations is characterized by the TF-BCA sub-clade (present in Black, Caspian and Aral Sea drainages). This conclusion is further supported by the fossil records. The oldest fossils of the brown trout were found in the Caucasus and dated from early Pleistocene, 2 million years ago (discussed in Osinov and Bernatchez 1996). The radiation of the species could have started around this period, or even earlier. Some heterogeneity was observed among this haplogroup, with haplotypes from the Sevan Lake trouts (Caspian drainage) differing by at least nine substitutions from the others. Brown trout from Sevan Lake was initially hypothesized as a species (*S. ischchan*) derived from the primitive ancestor of all brown trout populations (Benhke 1986). Later, this has been refuted based on allozyme and mtDNA analyses, suggesting that the differentiation of this population occurred recently, most likely in late glacial or postglacial times (Bernatchez and Osinov 1995; Osinov and Bernatchez 1996). However, the observed divergence in the *TF* gene supports a more ancient origin than postglacial times for this population.

The phylogenetic position and accumulated variation of TF-BCA haplogroup, coupled with their relationships with *TF*102*, is suggestive of an early radiation of the species and dispersal from Western Asia to Europe, and an early widespread distribution throughout the Mediterranean region (fig. 7*A*). The route of dispersal, could have included movements of individuals through the upper reaches of the Danube system, as has been suggested for the presence of a few DA mtDNA haplotypes in populations from Adriatic drainages (Bernatchez et al. 1992; Apostolidis et al. 1997), or by seaway, from the Black Sea to the Mediterranean. The latter was suggested by allozyme analyses of Greek (Karakousis and Triantaphyllidis 1990; Apostolidis et al. 1996) and Turkish (Togan et al. 1995) brown trout populations. Population divergence has been achieved later by allopatric fragmentation between the two major drainages.

TF75 post-recombinant clade was found in the marbled trout (*S. trutta marmoratus*) which is a phenotypically and ecologically distinct brown trout currently present in Italy, Slovenia, Croatia and Albania (Adriatic drainages), and typically fixed for *TF*75* (Giuffra et al. 1996; Berrebi et al. 2000). Based in allozyme data, Giuffra et al. (1996) proposed two alternative hypotheses for the colonization of the Pô basin, depending on the order of arrival of the two natural forms that currently occur (marbled and Mediterranean trout). Further allozyme evidence (Berrebi et al. 2000) suggests that it was the marbled trout that was the last to arrive to the Adriatic region, and our data provides the molecular support to corroborate this hypothesis. The high divergence of this post-recombinant clade and unique *TF* electromorph exhibited, suggests however, that the marbled trout has arrived very early to this region. The presence of mtDNA haplotypes from

MA lineage in three Greek populations from south Adriatic-Ionian Sea drainage (Apostolidis et al. 1997), and the detected gene conversion with the haplogroup BCA, suggest that the origin of post-recombinant clade TF75 could have been to the south of it current occurrence (fig. 7*B*). The other sub-clade from the major termini T-2, TF95, corresponding to the *TF*95* electromorph, exhibits a very distinct range distribution relative to the previous one. *TF*95* is currently present mainly in relic populations of headwater systems. The electromorph is observed, at low frequencies, in the upper Rhône (Mediterranean) and Po (Adriatic) in Switzerland (Largiadèr and Scholl 1995; Largiadèr et al. 1996), and, at middle to high frequencies, in some southwestern Atlantic upper stream populations from Portugal and Spain (Antunes et al. 1999; this study). Antunes et al. (1999) reported this puzzling trend, and suggested that if common ancestry existed between the *TF*95* from those distinct geographical regions, it would indicate the ancestral admixture of distinct geographical groups. Our data indicates that this electromorph has a quite old ancestry, further corroborated by their occurrence mainly in headwater drainages. The phylogenetic relationship of *TF*95* with *TF*75*, together with their current distribution, suggest that the **95* electromorph went through a long distance dispersal from the Adriatic region to the southernmost Atlantic regions (fig. 7*B*). Since the Adriatic and Mediterranean drainages were already colonized habitats, dispersal was not so successful. By contrast the Atlantic drainages of the Iberian Peninsula have offered the opportunity to colonize new habitats, and continuing expansion of the species range to the west.

The major termini T-3 is characterized by a codon insertion at exon 2. This is a derived brown trout character over other salmonid cDNAs (Kvingedal et al. 1993; Lee et al. 1995, 1998; Tange et al. 1997; Ford et al. 1999; Ford 2000). The mutation represents the signature of recent successful brown trout dispersal across most of their current distribution range (fig. 7*C*). Post-recombinant clade TF78.80 was observed among populations from Black Sea through the Mediterranean, while TF100 was present among the majority of the Atlantic populations. Divergence between these sub-clades reflects a recombination event and a second major dispersal event from Mediterranean to Atlantic drainages. Routes of dispersal do not seem to have been used very frequently, but rather in particular windows of time when climate and geography allowed. *TF*78* is fixed for the Italian carpione (*S. trutta carpio*), an endemic form of Garda Lake (Po basin), considered to have a recent postglacial origin, issued from a recent hybridization between the marbled trout and Mediterranean trout (Giuffra et al. 1994, 1996). Our results also support the introgressive hybridization origin of this form, as we found one Italian carpione exhibiting a sequence related with the marbled trout

post-recombinant clade (R3). The similarity of the *TF* sequences between the two individuals from Garda Lake (Adriatic Sea drainage) and one individual from the very remote Kodori River (Black Sea drainage) is puzzling, as it suggests recent gene flow between these two remotes areas. By contrast, we detected some sequence divergence in related haplotypes, corresponding to the electromorph *TF*80*, found in the vicinities of the Adriatic drainage. *TF*80* is currently observed in a few Corsican and Mediterranean French and Spanish populations (Presa et al. 1994; Berrebi 1995; this study). Noteworthy, both the distribution of *TF*78* and **80* suggests a low level of penetration in the previous established populations of the Adriatic and Mediterranean drainages. In contrast, the widespread distribution of sub-clade TF100 (corresponding to the electromorph *TF*100*) throughout the Atlantic drainage, suggests a rapid expansion. The Atlantic basin was the one most directly affected by Pleistocene glaciations in terms of habitat loss and later expansions occurring probably over the range of earlier ones (Hewitt 2000). The northern part of the Atlantic region was ice covered during the last glaciation and thus many populations have existed only since postglacial times (i.e. during the last 10,000 to 18,000 years). The extinction of populations was increased within this early structure, but the formation of discrete refugia in southwestern Atlantic regions would have allowed the long residency of some populations (e.g. Weiss et al. 2000; Sanz et al. 2000; Antunes et al. 2001; Bouza et al. 2001). This would explain part of the complexity found in the southernmost Atlantic populations, with the maintenance of some relic populations carrying the sub-clade TF95 and some structure within sub-clade TF100.

Additional complexity in the brown trout evolutionary history was then added by intergradation of differentiated sub-clades and post-recombination clades, as detected by some of the recombination/gene conversion events. Previous studies, based on allozymes and mtDNA data, indicate introgressive hybridization between different lineages in glacial or postglacial times (e.g. Bernatchez et al. 1992; Largiadèr and Scholl 1995; Osinov and Bernatchez 1996). Yet, secondary intergradation (excluding that due to stocking) seems to have been relatively limited, as suggested by allozyme studies (e.g. Bernatchez and Osinov 1995; Giuffra et al. 1996). Biological factors, in addition to physical isolation, have probably limited dispersal and introgressive hybridization, as inferred by the existence of five mtDNA lineages that evolved in geographic isolation during the Pleistocene and have remained largely allopatric since then (Bernatchez 2001).

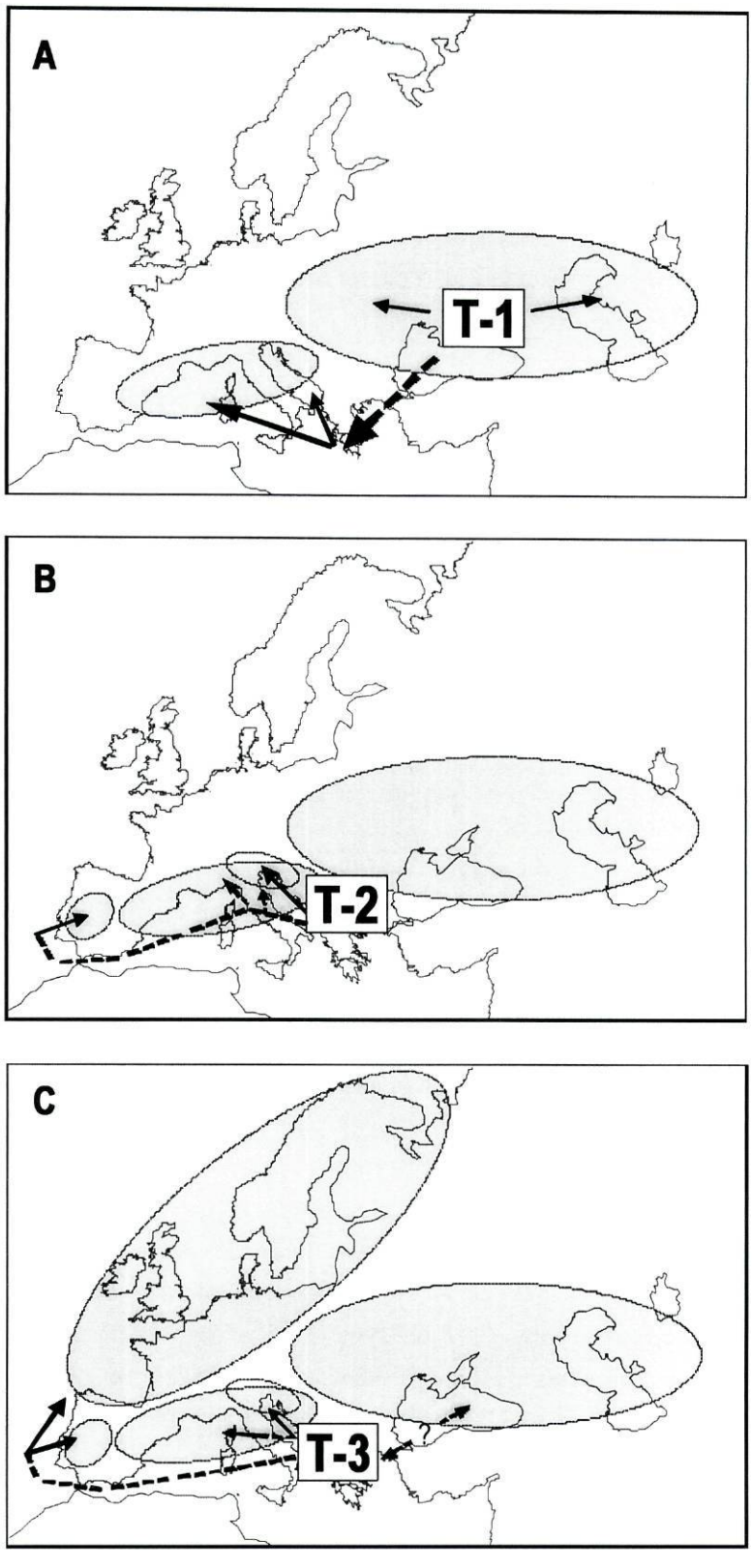

Fig. 7 (*A*, *B* and *C*). Scenarios of the origin and migration pathways of the brown trout species complex inferred from the *TF* gene genealogy.

Levels of congruence with mtDNA variation

The *TF* genealogy was able to phylogenetically recover some of the lineages recognized with the mtDNA. Brown trout from the BCA region (comprising populations geographically corresponding to the DA mtDNA lineage) seems to be mainly characterized by the TF-BCA haplogroup. The marbled trout (MA mtDNA lineage) is also characterized by just one post-recombinant clade (TF75). However, brown trout from other geographical areas exhibited *TF* haplotypes from different sub-clades. This is the case of brown trout from the Mediterranean and Adriatic basin (ME and AD mtDNA lineage, respectively) exhibiting the TF102 and TF78.80 sub-clades. Also the populations from Atlantic basin (AT mtDNA lineage) exhibited the TF95 and TF100 sub-clades. The phylogeographic scenario inferred from the *TF* genealogy seems to pre-date that of the mtDNA. While the mtDNA alleles have had sufficient time to "sort" to reciprocal monophyly, alleles at the *TF* locus have not. Because mtDNA is haploid and maternally transmitted, it is expected to have a fourfold lower N_e than autosomal nuclear loci (Birky et al. 1989). Under neutral models, genetic drift governs the process of "lineage sorting" and smaller effective population sizes (N_e) lead, on average, to higher rates of stochastic lineage extinction and fixation (Hoelzer 1997). This could also be a reason for the low mtDNA haplotype diversity found in ME and MA lineages (3 and 4 distinct haplotypes, respectively in 104 and 205 individuals analyzed; Bernatchez 2001). Contrastingly, our results focusing in a small number of individuals show a considerable number of distinct *TF* haplotypes corresponding to the major electromorphs that characterize these population groups.

Concerning the phylogenetic relationships of the *TF* gene, we found that haplotypes from the BCA region were those, among brown trout, exhibiting the highest similarity with the outgroup (fig. 6). Some of this homology resulted from RGCs, which are character states that arise rarely, and are not subjected to extensive convergent or parallel evolution, contributing to a low level of homoplasy (Rokas and Holland 2000). At the mtDNA level, the ancestral divergence of the AT lineage from all others was suggested when considering the Atlantic salmon as the outgroup (Giuffra et al. 1994; further discussed in Bernatchez 2001). The distinction of the AT group was determined by the identity at four nucleotide positions with *S. salar* that differed in all other *S. trutta* genotypes, based on pooled sequences (1.25 kb) of both control region and coding genes (cytochrome *b* and ATPase subunit VI). Interestingly, by assuming the AT clade split from the ME or a common ancestor following the Messinian crisis (Machordom et al. 2000), Antunes et al. (2001) using a control region fragment of 464 bp, observed two statistically parsimonious solutions for the connecting

branch of the two lineages. Thus suggesting that due to the higher mutation level of mtDNA, even more homoplasy could be expected when comparisons are made at the generic level. According to our data, the TF100 sub-clade corresponding to the *TF*100* electromorph, that characterizes currently the majority of Atlantic populations, was the most divergent from the outgroup.

Conclusions

In summary, the *TF* gene genealogy here presented clearly provided new phylogeographic insights over previous allozyme and mtDNA ones, showing the potential utility of nuclear genes for intraspecific evolutionary inferences. Furthermore, intragenic recombination events provided evidence of past secondary intergradations, improving the utility of nuclear markers as a tool for recovering complex evolutionary histories. Incomplete congruence between nuclear and mitochondrial phylogeographic patterns is reliable with different modes of evolution and transmission. Moreover, a variety of reasons involving demography, history, selection, and independent DNA sequences within the same organismal pedigree may result in quite different phylogeographic patterns (Hare and Avise 1997). Thus, accurate estimations of the evolutionary history of an organism should use different loci, avoiding inferences confounded by the inherent differences between maternally and biparentally inherited genes.

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APPENDIX

Frequency range of brown trout *TF** electromorphs. Entries in parentheses for maximum and minimum electromorph frequencies identify number of populations where that value occurs more than once. Frequencies are given in bold when their presence likely results from anthropogenic activities. References: (1) Krieg and Guyomard (1985); (2) Presa et al. (1994); (3) Berrebi (1995); (4) Largiadèr (1995); (5) Largiadèr and Scholl (1995); (6) Giuffra et al. (1996); (7) Largiadèr and Scholl (1996b); (8) Largiadèr et al. (1996); (9) Antunes et al. (1999); (10) Berrebi et al. (2000); (11) This study (population names and geographical locations are indicated in foot notes).

Drainage	Country	N_{pops}	N_{inds}	Electromorph	Maximum	Minimum	Reference
Black Sea	Switzerland/Germany[a]	5	91	**102*	0.700	0.100	5, 11
				****100***	**0.900**	**0.250**	
				**75*	**0.143**	**0.023**	
Adriatic	Switzerland	17	393	**102*	0.369	0.000 (8)	5
				****100***	**1.000 (3)**	**0.388**	
				**95*	0.024	0.000 (13)	
				**75*	0.369	0.000 (5)	
Adriatic	Italy	7	85	**102*	1.000	0.003	6
				****100***	**0.750**	**0.000**	
				**78*	0.320	0.000 (5)	
				**75*	0.100	0.000 (5)	
Adriatic	Italy (*S. t. marmoratus*)	8	98	**102*	0.040	0.000 (6)	6
				****100***	**0.500**	**0.000 (2)**	
				**78*	0.030	0.000 (7)	
				**75*	1.000 (2)	0.500	
Adriatic	Italy (*S. t. carpio*)	1	15	**78*	1.000	-	6
Adriatic	Slovenia (*S. t. marmoratus*)[b]	7	183	**102*	0.700	0.000 (4)	10
				****100***	**0.330**	**0.000 (4)**	
				**98*	0.040	0.000 (6)	
				**75*	1.000 (4)	0.000	
Mediterranean	Switzerland	13	269	**102*	1.000 (2)	0.013	7, 8
				****100***	**0.964**	**0.000 (2)**	
				**95*	0.141	0.000 (12)	
Mediterranean	Corsica	28	695	**102*	1.000 (8)	0.013	2, 3
				****100***	**0.840**	**0.000 (10)**	
				**80*	1.000	0.000 (20)	
				**70*	0.003	0.000 (27)	
Mediterranean	France	4	91	**102*	1.000 (4)	-	2, 10
Mediterranean	France[c]/Spain[d]	3	81	**102*	1.000	0.600	11
				****100***	**0.080**	**0.000**	
				**80*	0.350	0.000	
Mediterranean	Spain[e]	2	29	**101*	1.000 (2)	-	11
Atlantic	Portugal	3	180	**100*	0.670	0.640	9
				**95*	0.360	0.310	
Atlantic	Portugal[f]/ Spain[g]	19	446	**100*	1.000 (11)	0.000 (3)	11
				**95*	1.000 (3)	0.000 (9)	
Atlantic	France	11	264	**100*	1.000 (11)	-	1
North Sea	Switzerland	26	632	****102***	**0.217**	**0.000 (17)**	4
				**100*	1.000 (15)	0.783	
				**75*	**0.075**	**0.000 (24)**	
North Sea	France[h]/Switzerland[i]/ Czechoslovakia[j]/Norway[l]	6	64	**105*	0.100	-	11
				**100*	1.000 (5)	0.900	
Baltic Sea	Sweden[m]	1	9	**100*	1.000	-	11

[a]Krumbach (48°20'N, 11°00'E). [b]Pure and hybrid populations of marbled trout. [c]Chevennes (46°05'N, 06°20'E); Argens (43°32'N, 06°02E). [d]Artesiaga (42°55'N, 02°20'W). [e]Ter (42°05'N, 02°30'E); Jucar (39°20'N, 02°05'W). [f]Zêzere (40°25'N, 07°30'W); Mondego (40°28'N, 07°31'W); Vouga (40°48'N, 07°37'W); Caima (40°53'N, 08°05'W); Tenente (40°55'N, 08°05'W); Tuela (41°51'N; 06°53'W); Sabor (41°52'N; 06°43'W); Ave (41°35'N; 08°5'W); Cávado (41°50'N; 07°49'W); Coura (41°55'N, 08°33'W); and Minho (42°3'N, 08°35'W). [g]Tagus (39°55'N, 04°30'W); Meras (43°30'N, 07°10'W); Pigüena (43°20'N, 07°45'W); Ferrerias (43°30'N, 07°55'W); Cancienes (43°27'N, 06°10'W); Trubia (43°15'N, 06°00'W); Ponga (42°50'N, 04°10'W); Dobra (43°5'N, 04°40'W); and Arizakun (43°15'N, 02°40'W). [h]Moselie (48°30'N, 07°12'E); Ill (48°18'N, 07°10'E). [i]Saar (47°35'N, 09°28'E). [j]Vlatva (49°20'N, 14°10'E). [l]Jebersborg (60°10'N, 11°20'E). [m]Dalalven (60°30'N, 15°10'E).

5. Discussão

5.1. Diversidade genética da truta no limite sul da distribuição Atlântica

5.1.1. Distribuição geográfica dos polimorfismos proteicos

O primeiro trabalho de caracterização genética de populações de truta no limite sul da distribuição Atlântica (Portugal) foi baseado na análise de seis polimorfismos proteicos. A análise comparativa dos resultados obtidos com dados publicados de 14 populações Europeias da drenagem Atlântica e Mediterrânea (Artigo I) veio revelar a existência de três grupos bem diferenciados, que são o Noroeste Atlântico, o Sudoeste Atlântico e o Mediterrâneo (figura 5.1), tal como já fora evidenciado por García-Marín *et al.* (1999a).

A diferenciação do grupo Sudoeste Atlântico, que inclui as populações de Portugal (estendendo-se para norte sensivelmente até à Bretanha), resulta em particular da elevada frequência (geralmente superior a 0,5) do alelo *CK-A1* 115* e da fixação do alelo *LDH-C1*100*. Os grupos Noroeste Atlântico e Mediterrâneo caracterizam-se pela elevada frequência de *CK-A1*100*, diferenciando-se ao nível do *locus LDH-C1** por apresentarem, respectivamente, elevadas frequências dos alelos **90* e **100*. A inclusão do *locus TF** (e consequente remoção das populações de Espanha, não analisadas para esse marcador) contribuiu para uma maior diferenciação dos três grupos populacionais. Este resultado reflecte a fixação de alelos distintos nas populações dos grupos noroeste Atlântico (*TF*100*) e Mediterrâneo (*TF*102*), e a presença dos alelos *TF*95* e **100* nas populações de Portugal. Adicionalmente, a ausência no grupo Sudoeste Atlântico de outros alelos enzimáticos observados mais a norte (p.ex. *G3PDH-2*50*, *GPI-A1*110*, *IDHP-1*160*, *sMDH-A2*120* e *sMDH-B1*80*; Ryman 1983; Krieg & Guyomard 1985; Guyomard 1989) aumenta a diferenciação entre populações do norte e do sul da região Atlântica.

A pesquisa de variabilidade genética ao nível de 11 *loci* proteicos em 20 populações de *S. trutta* do sudoeste Atlântico (Artigo IV) veio revelar a existência de uma considerável diferenciação populacional (G_{ST} = 35%), bem como uma significativa variação regional de alguns alelos. As frequências dos alelos *CK-*

*A1*100*, *GPI-B2*135* e *MPI-2*105* apresentam um decréscimo clinal para sul, sugerindo um fluxo génico contínuo, nessa direcção, a partir das populações Cantábricas. Por outro lado, a presença em elevadas frequências dos alelos *PEPLT*70* e *TF*95* mais para sul, estando estes ausentes ou em reduzidas frequências no Norte da Península Ibérica, indica a persistência de "relíquias" populacionais que permaneceram isoladas durante longos períodos de tempo.

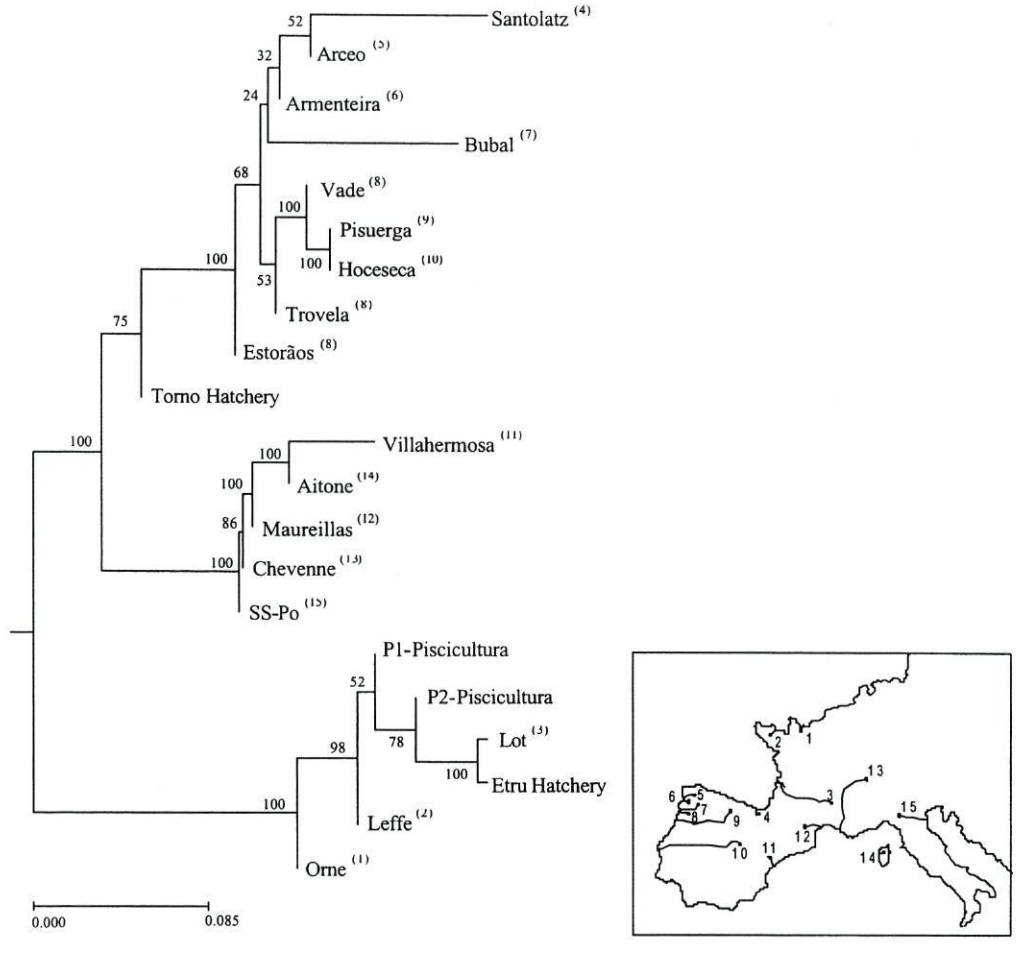

Figura 5.1. Dendrograma construído segundo o método de agrupamento *NeighborJoining* (NJ) a partir da matriz de distâncias genéticas de Nei (1978) para seis *loci* proteicos, em 17 populações naturais de *S. trutta* na Europa e quatro *stocks* de piscicultura. Os valores de *bootstrap* encontram-se representados nos ramos internos do agrupamento.

A análise dos valores de heterozigotia esperada e taxa de polimorfismo revelou a existência de heterogeneidade que parece intensificar-se nas populações localizadas a sul do actual limite de distribuição da forma migradora (LSA – limite sul de anadromia). De facto, quando se consideram separadamente dois

grupos de populações Ibéricas a norte e a sul desse limite, pode ser constatada a existência de diferentes níveis de subestruturação populacional traduzidos por distintos valores de G_{ST} (12 e 54%, respectivamente). O padrão de diferenciação populacional evidenciado pela análise de ordenação multidimensional ("MDS") aplicado à matriz de distâncias genéticas de Cavalli-Sforza & Edwards (1967), foi igualmente corroborado pelo método de agrupamento NJ. A análise de ordenação multidimensional (figura 5.2), sugere, por um lado, a existência de um gradiente norte-sul (dimensão I), e por outro, uma elevada diferenciação das populações localizadas a sul do LSA (dimensão II).

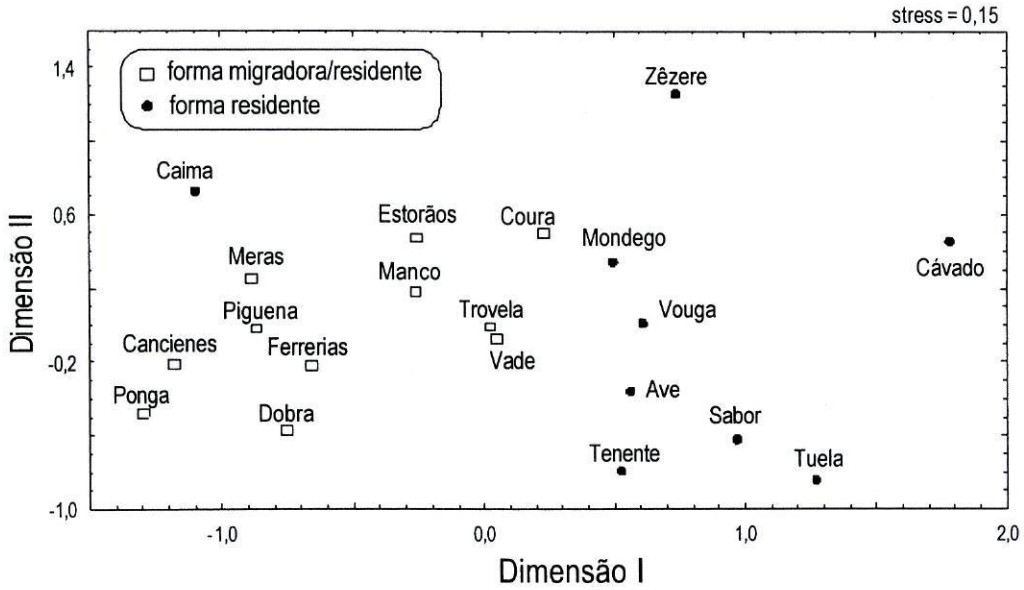

Figura 5.2. Ordenação bidimensional de 20 populações de *S. trutta* da região Atlântica da Península Ibérica após análise multidimensional, utilizando a matriz de distâncias genéticas de Cavalli-Sforza & Edwards (1967) resultante da análise de 11 *loci* proteicos polimórficos.

A referida estrutura populacional, resultou da análise de *loci* que exibem geralmente variação bi-alélica intra-populacional. Contudo, *loci* mais polimórficos poderão indicar com maior sensibilidade a perda recente de variabilidade genética (Luikart *et al.* 1998). De facto, a análise de uma proteína plasmática não identificada (*PX*), muito polimórfica, veio revelar a existência de uma diminuição clinal da diversidade genética que se traduz numa redução considerável da heterozigotia e do número de alelos em populações a sul do LSA (rio Lima) (Artigo II).

A estrutura populacional inferida com base na variação genética de *loci* proteicos sugere a existência de dois acontecimentos principais nas populações Atlânticas da Península Ibérica: (i) uma fragmentação histórica em mosaico a sul do LSA, e (ii) um fluxo génico contínuo das populações do Norte da Península Ibérica para sul, associado à presença da forma migradora.

5.1.2. Distribuição geográfica dos polimorfismos de microssatélites

A variação genética observada ao nível de quatro microssatélites em 11 populações de *S. trutta* do Sudoeste Atlântico (Artigo IV) veio confirmar a existência de uma elevada diferenciação populacional (G_{ST} = 54%). A distribuição alélica evidencia um padrão norte-sul caracterizado pela redução do número de alelos, variância e heterozigotia, intensificando-se este padrão nas populações localizadas a sul do LSA. A substancial perda de diversidade nesta região poderá ser explicada como resultante da existência de efectivos populacionais instáveis (Leberg 1992), nomeadamente por acção de constrangimentos ecológicos severos durante os períodos glaciares e interglaciares no Pleistoceno.

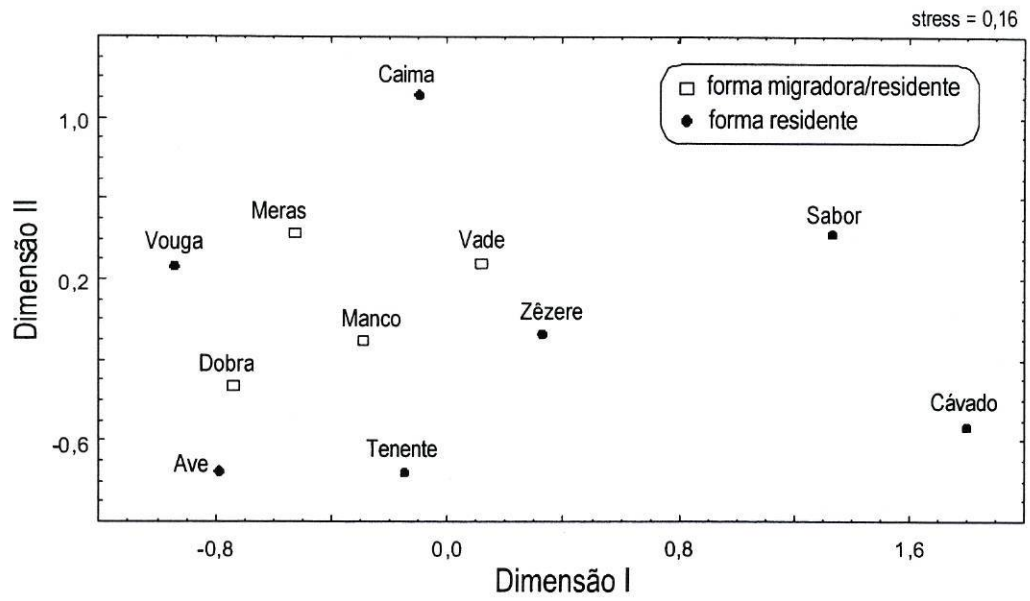

Figura 5.3. Ordenação bidimensional das 11 populações de *S. trutta* da região Atlântica da Península Ibérica após análise multidimensional, utilizando a matriz de distâncias genéticas de Cavalli-Sforza & Edwards (1967) resultante da análise de quatro microssatélites.

Se a deriva génica pode determinar a perda de alelos numa população, pode igualmente incrementar a frequência de alelos raros (Wright 1931). O aumento da frequência ou mesmo fixação de alelos privativos no *locus Str-591** em populações a sul do LSA, corrobora essa ideia. De facto, quando se considera a existência de dois grupos de populações a norte e a sul desse limite, observa-se uma elevada diferença nos valores de G_{ST} (16 e 67%, respectivamente) que traduzem distintas estimativas de fluxo génico. O elevado valor de G_{ST} estimado para populações a sul da bacia hidrográfica do rio Lima pode ser explicado como sendo uma consequência de fragmentação, e não de fluxo génico restrito recorrente (Larson *et al.* 1984).

O padrão de diferenciação populacional, evidenciado pela análise de ordenação multidimensional (figura 5.3) foi igualmente corroborado através do método de agrupamento NJ, sugerindo a existência de uma elevada diferenciação em mosaico a sul do LSA (dimensão I), enquanto as populações a norte desse limite se apresentam discretamente diferenciadas (dimensão II). O padrão de estruturação populacional observado para os microssatélites corrobora os resultados da análise de *loci* proteicos, reforçando as interpretações efectuadas de uma história complexa de fragmentação no limite sul da distribuição Atlântica.

5.1.3. Distribuição geográfica dos polimorfismos do DNA mitocondrial

Os padrões geográficos de variação do DNA mitocondrial em populações de *S. trutta* em Portugal sugerem a existência de uma elevada heterogeneidade, provavelmente resultante de fragmentação geográfica e/ou ecológica que promoveu a divergência dos haplótipos durante um período superior a 200 mil anos (Artigo III). Se por um lado a elevada estrutura genética sugere uma história evolutiva marcada por uma fragmentação antiga, o recurso a métodos analíticos que permitam testar estatisticamente as hipóteses é fundamental (Artigo V). O conhecimento das relações genealógicas entre os vários haplótipos de DNA mitocondrial, para além da sua frequência e distribuição geográfica, permitiu utilizar uma metodologia analítica objectiva, a "análise cladística hierárquica" (*nested clade analysis* – NCA; Templeton *et al.* 1995). Esta metodologia testa a associação entre a posição filogenética de um variante haplotípico e a sua distribuição geográfica, possibilitando ainda inferir sobre os processos evolutivos que terão determinado os padrões geográficos observados (p.ex. fluxo génico restrito, expansão geográfica, fragmentação).

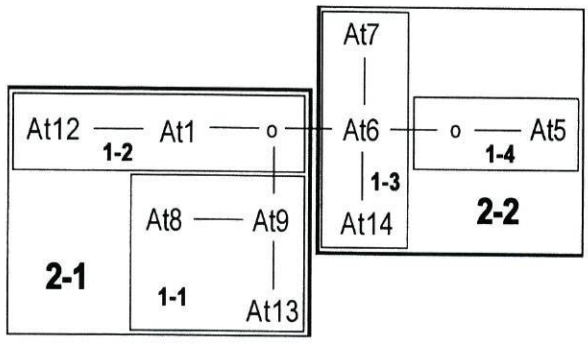

Figura 5.4. Agrupamento hierárquico para os haplótipos da região de controlo do DNA mitocondrial observados em 16 populações de *S. trutta* em Portugal. Os zeros indicam estados haplotípicos intermédios não detectados. Cada linha representa um passo mutacional. Os rectângulos representam os níveis hierárquicos 1 (1-n; traço fino) e 2 (2-n; traço grosso).

A aplicação da "análise cladística hierárquica" revelou que os padrões geográficos de variação do DNA mitocondrial parecem ter sido originados principalmente por processos históricos (figura 5.4 e 5.5). A fragmentação histórica foi determinante para a estrutura do clado 2-1, devido à fixação do haplótipo *At12* na população do Sabor e à fixação de haplótipos relacionados (partilha de uma delecção) nas populações do Cávado e Ave. O fluxo génico restrito, com isolamento pela distância, parece ter determinado a restante estrutura genética. Adicionalmente, há evidência da existência de alguma dispersão de longa distância (clado 1-3) devido à presença de dois haplótipos (*At7* and *At14*) que diferem apenas em uma mutação do haplótipo interior mais frequente (*At6*) em populações geograficamente distantes (Zêzere e Estorãos, respectivamente).

A análise de variância molecular (Excoffier *et al.* 1992) das 16 populações de *S. trutta* revelou a existência de uma elevada estruturação genética (ϕ_{ST} = 0,76; P < 0,0001). A análise hierárquica de variância molecular, considerando apenas os grupos populacionais da bacia hidrográfica dos rios Lima e Douro, revelou uma reduzida diferenciação entre ambos (6%). Contudo os valores de variância são muito significativos (P < 0,0001) considerando a percentagem de variação devido a diferenças entre indivíduos de cada população (36,04%; ϕ_{ST} = 0,64) e entre populações de cada grupo (57,96%; ϕ_{SC} = 0,62). Estes resultados corroboram a existência de um padrão de estruturação em mosaico.

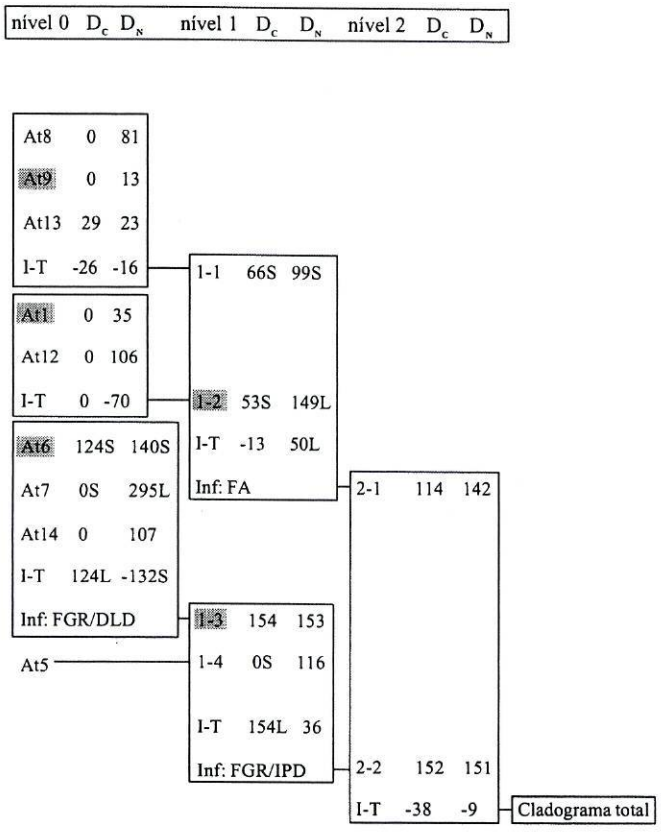

Figura 5.5. Análise das distâncias geográficas do agrupamento hierárquico de nove haplótipos mitocondriais observados em 16 populações de *S. trutta* em Portugal. D_C e D_N representam respectivamente a distância média do grupo e a distância hierárquica. Um "S" indica que a distância é significativamente pequena e um "L" que é significativamente grande. Para os grupos em que se sabe que os haplótipos são interiores ou exteriores, o nome do grupo ou haplótipo interior está a sombreado. "I-T" representa a diferença média dentro do grupo para as distâncias do grupo e distâncias hierárquicas entre grupos ou haplótipos interiores e exteriores. As inferências biológicas (Inf) correspondem a: FGR – fluxo génico restrito; IPD – isolamento pela distância; DLD – dispersão de longa distância; FA – fragmentação antiga.

5.1.4. Interpretação dos padrões de diversidade genética

5.1.4.1. História evolutiva da truta no limite sul de distribuição Atlântica

As populações do grupo norte Atlântico exibem, em geral, valores de heterozigotia superiores (estimados com base na variação de *loci* aloenzimáticos), relativamente aos encontrados mais a sul, nomeadamente em populações da Península Ibérica (p.ex. Crozier & Ferguson 1986; Presa *et al.* 1994; Martínez *et al.* 1993; García-Marín & Pla 1996). De facto, parece verificar-se a existência de

uma redução clinal dos valores de heterozigotia de norte para sul. No noroeste de Espanha, o estudo efectuado por Bouza *et al.* (1999, 2001), revelou um padrão de decréscimo da diversidade genética para sul, ao longo de cinco sistemas hidrográficos localizados junto do LSA. Propôs-se como explicação possível para essas diferenças as flutuações do efectivo populacional devidas à instabilidade dos sistemas hidrográficos das regiões mais meridionais (Apostolidis *et al.* 1996a). Bouza *et al.* (1999) sugerem a ausência da forma migradora em latitudes inferiores aos 42°N como um factor adicional para esse decréscimo de heterozigotia, devido a uma redução do fluxo génico, e ao incremento dos efeitos da deriva génica.

Contudo, na região Atlântica, as evidências fornecidas pelos polimorfismos aloenzimáticos são consideravelmente distintas das fornecidas pelo padrão de variação do DNA mitocondrial. Inicialmente, o estudo da variação da região de controlo do DNA mitocondrial evidenciou a existência de um só haplótipo em várias populações da região Atlântica a norte da Bretanha (Bernatchez *et al.* 1992). Este facto contrasta com a elevada diversidade haplotípica das populações de truta de Portugal, sugerindo uma elevada estruturação genética nesta região (Artigo III e V). Este padrão de diversidade, na região do sudoeste Atlântico, parece indicar uma longa história de fragmentação e uma maior ancestralidade das suas populações relativamente a outras localizadas mais a norte (Bernatchez 2001). Este resultado está de acordo com os padrões observados por Hewitt (1999), em 10 *taxa* diferentes, caracterizados por uma redução da diversidade genética do sul para o norte da Europa, facto atribuído a fenómenos de expansão depois da última glaciação, há cerca de 18 mil anos.

A falta de congruência entre os padrões de variação aloenzimáticos e do DNA mitocondrial resulta, provavelmente, de diferenças demográficas, tais como o efectivo populacional e a taxa de migração, e da sua influência na estimativa da estrutura genética de populações, com base em marcadores com distintos modos de transmissão e evolução. A truta, sendo uma espécie típica de águas frias, exibe actualmente elevadas densidades, bem como uma distribuição mais uniforme, na região Norte Atlântica. Mais para sul, a elevação da temperatura restringe a sobrevivência da espécie a regiões montanhosas onde as águas são frias e cristalinas, mas o efectivo populacional e o potencial migrador é consideravelmente reduzido (García-Marín & Pla 1996). Assim, a elevada heterozigotia aloenzimática no Norte da Europa parece resultar em especial de uma história demográfica mais recente de expansão populacional.

O Hemisfério Norte começou a ser influenciado pelas glaciações há sensivelmente 2,5 milhões de anos. Contudo, as grandes alterações climáticas com períodos de 100 mil anos iniciaram-se apenas há cerca de 0,7 milhões de anos,

gerando sequências de períodos glaciares e interglaciares (Webb & Bartlein 1992). Os glaciares confinaram a distribuição de *S. trutta* no Atlântico às regiões mais a sul até ao fim do último máximo glaciar, há cerca de 18 mil anos. Os resultados das análises da variação genética de *loci* proteicos e de microssatélites sugerem para o Sudoeste Atlântico um cenário histórico caracterizado por condições de maior estabilidade demográfica a norte da bacia hidrográfica do rio Lima e de condições ecológicas limitantes a sul desse rio, responsáveis por flutuações profundas e recorrentes dos efectivos populacionais (Artigo II e IV). O padrão de empobrecimento genético observado pode ser explicado pelos sucessivos estrangulamentos populacionais nesta região ocorridos ao longo dos ciclos de oscilação climática do Pleistoceno. Estas observações estão em concordância com a elevada heterogeneidade detectada ao nível do DNA mitocondrial (ϕ_{ST} = 76%; Artigo V).

A história evolutiva destas populações parece ter sido ainda influenciada por colonizações resultantes de um fluxo génico contínuo para sul provocado pela forma migradora. Outros estudos com base em aloenzimas (Machordom *et al.* 1999; Sanz *et al.* 2000; Bouza *et al.* 2001) e DNA mitocondrial (Machordom *et al.* 2000) revelaram populações no rio Douro e Tejo, em Espanha, relacionadas com as existentes na Cantábria e Galiza, sugerindo a existência de possíveis contactos secundários entre grupos alopátricos. A origem destes grupos distintos pode ser difícil de inferir. Sanz *et al.* (2000) sugeriram que a elevada diversidade genética do grupo Cantábrico, bem como as evidências da sua expansão para o rio Douro e Tejo, indicam que o grupo seria ancestral relativamente a outros no noroeste da Península Ibérica. Contudo, a diversidade genética não reflecte necessariamente a ancestralidade de uma população. Alternativamente, poderá sugerir uma história demográfica de expansão geográfica (p.ex. Templeton 1993), à semelhança da interpretação dos elevados valores de heterozigotia determinada com base em *loci* aloenzimáticos, nas regiões do Norte da Europa colonizadas depois do último máximo glaciar.

A presença de alguns alelos proteicos, em frequências elevadas ou fixados, nas populações Atlânticas mais meridionais, que se encontram ausentes ou em frequências reduzidas na região Cantábrica (p.ex. *PEPLT*70* e *TF*95*; e também *sMDH-B1, B2*75* no rio Douro e *αMAN*90* no rio Tejo; García-Marín & Pla 1996; Sanz *et al.* 2000; Bouza *et al.* 2001), sugere a existência de "relíquias" populacionais que persistiram em isolamento durante longos períodos de tempo. O sistema hidrográfico do rio Lima encontra-se actualmente no limite sul da distribuição da forma migradora. Contudo, há algumas décadas atrás, encontravam-se ainda no rio Douro trutas migradoras e é provável que esta forma tenha ocorrido ainda mais para sul durante o último evento glaciar (Hamilton *et*

al. 1989). Se a forma migradora, promovendo o fluxo génico, parece contribuir para a redução da diferenciação entre populações, nesta região a sua existência não parece ter sido determinante para impedir uma elevada estruturação populacional.

A detecção do alelo *TF*95* em algumas populações Portuguesas, apenas observado previamente em populações a montante de alguns afluentes dos rios Ródano (Mediterrâneo) e Pó (Adriático) (Largiadèr & Scholl 1995; Largiadèr *et al.* 1996), sugere a conexão histórica entre *S. trutta* do Mediterrâneo e Atlântico (Artigo I). A sequência parcial do gene TF indicou que *TF*95* parece ser mais ancestral do que o alelo *TF*100*, representando possivelmente a assinatura de uma colonização antiga das drenagens Atlânticas após uma expansão a partir da região Mediterrânea (Artigo VI). Adicionalmente, com base em dados provenientes da análise de polimorfismos de restrição ("RFLP") do DNA mitocondrial, Machordom *et al.* (2000) sugerem a divergência ancestral das populações do rio Douro. A elevada frequência dos alelos *sMDH-B1* e *B2*75* nas zonas a montante dos rios Minho e Douro, indica que o grupo da Cantábria-Galiza terá tido uma origem mais recente, pois apenas foi capaz de colonizar as áreas mais acessíveis desses sistemas hidrográficos (Bouza *et al.* 1999, 2001).

5.1.4.2. Relevância das populações Ibéricas da vertente Atlântica para a recolonização do Norte da Europa

Ferguson & Fleming (1983), com base na distribuição dos alelos *LDH-C*90* e **100* propuseram que o Noroeste da Europa foi colonizado por duas linhagens distintas de truta (discutido detalhadamente em Hamilton *et al.* 1989). Hynes *et al.* (1996) reviram essa hipótese sugerindo uma maior complexidade e a existência de múltiplos eventos de colonização. Posteriormente, García-Marín *et al.* (1999a) proposeram refúgios glaciares e uma ordem geral de acontecimentos para explicar a recolonização do Norte da Europa. A fixação ou elevadas frequências de alelos diagnósticos são características dos seguintes refúgios glaciares: (I) no noroeste Atlântico, *LDH-C*90* e *CK-A1*100*; (II) no sudoeste Atlântico, *LDH-C*100* e *CK-A1*115*; e (III) na região do Ponto-Cáspio, *LDH-C*100* e *CK-A1*100* (figura 5.6*A*). As populações actuais do Norte da Europa que ocupam regiões anteriormente cobertas por glaciares e que exibam aqueles pares de alelos são consideradas como tendo sido colonizadas pelo correspondente refúgio.

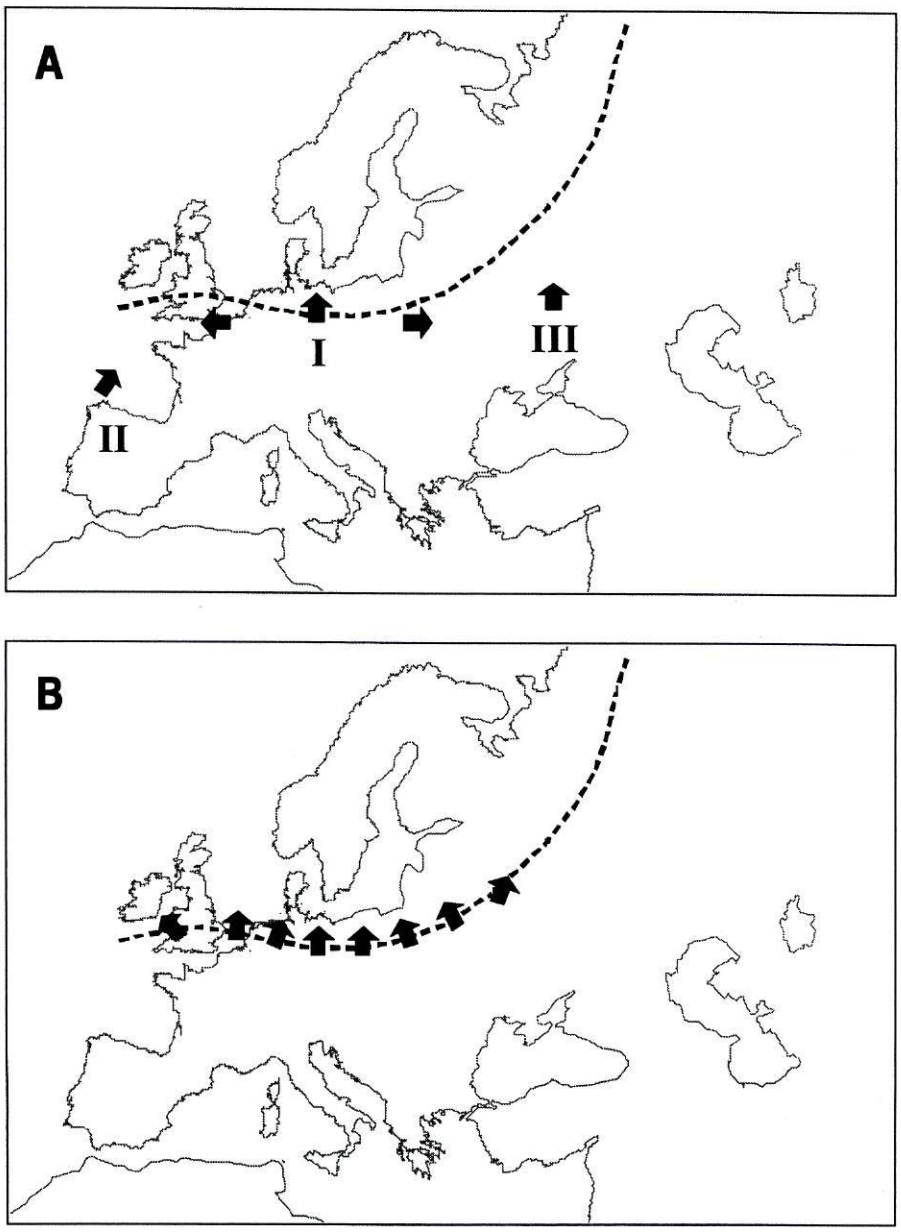

Figura 5.6. (*A*) Distribuição dos refúgios glaciares da truta intervenientes na recolonização do Norte da Europa (I, II, III) durante o último máximo glaciar (indicado pela linha a tracejado), segundo García-Marín *et al.* (1999a). As setas indicam as direcções de expansão durante a recolonização pós-glaciar. (*B*) Hipótese de dispersão pós-glaciar e recolonização do Norte da Europa, com base nas inferências propostas no Artigo III.

O padrão de distribuição geográfica e o grau de diferenciação haplotípica do DNA mitocondrial das populações Portuguesas, sugerem que estas terão divergido

das do Norte da Europa muito antes do último máximo glaciar há 18 mil anos (Artigo III). Este resultado não parece corroborar a hipótese de uma contribuição do Sudoeste Atlântico na colonização do Norte da Europa. Hynes *et al.* (1996), com base no estudo de 40 populações Atlânticas, encontraram dois haplótipos de DNA mitocondrial (RFLPs) privativos numa população do Norte da Península Ibérica. O alelo *TF*95*, típico do limite sul Atlântico (Artigo I, IV e VI) não foi observado em populações do Norte da Europa (Krieg & Guyomard 1985; Presa *et al.* 1994; Artigo VI). Por outro lado, de acordo com os resultados de Riffle *et al.* (1995), em sete populações localizadas a montante do Reno, o alelo *CK-A1*115* encontra-se fixado ou em elevadas frequências. García-Marín *et al.* (1999a) parecem não ter considerado estes dados, presumivelmente assumindo a influência de repovoamentos com *stocks* de piscicultura que parecem ocorrer nesta região. Contudo, apenas em dois destes sete casos existem registos de repovoamentos regulares e as populações não repovoadas exibem elevadas frequências do alelo *CK-A1*115* (0.77-1). O alelo **115* foi também observado em frequência elevada numa população da região do Mar Báltico, onde o alelo *LDH-C*90* e o haplótipo do DNA mitocondrial *At1* (característicos das regiões do Norte da Europa) se encontravam fixados (Bernatchez & Osinov 1995). Assim, parece existir uma ampla evidência de que o alelo *CK-A1*115* tenha existido num refúgio no Nordeste ou Centro da Europa durante o último máximo glaciar, diminuindo, assim, o poder informativo deste marcador para evidenciar a existência de fluxo génico da região do sudoeste Atlântico.

Existem várias evidências que são incompatíveis com uma dispersão inicial pós-glaciar da região do Ponto-Cáspio para o Norte Atlântico. Nenhuma população Atlântica exibiu a presença de haplótipos de DNA mitocondrial característicos do clado divergente do Danúbio (Bernatchez *et al.* 1992; Osinov & Bernatchez 1996; Bernatchez 2001). A análise de 24 *loci* aloenzimáticos em populações de regiões mais a este, incluindo os Mares Branco, Barents e Báltico, sugeriu que 16 dos 54 alelos observados, são exclusivos das populações do Ponto-Cáspio (Osinov & Bernatchez 1996). Desta forma, a existência de fluxo génico proveniente deste refúgio teria de ter ocorrido com a perda total de marcadores diagnósticos dessas populações.

A distribuição geográfica de *loci* aloenzimáticos e do DNA mitocondrial em regiões glaciadas do Norte da Europa parece ser mais bem interpretada por uma dispersão pós-glaciar a partir de um refúgio localizado a Norte da Península Ibérica e da região do Ponto-Cáspio (figura 5.6*B*). Esta inferência não apoia nem contradiz hipóteses prévias que sugerem a existência de múltiplas "ondas" de colonização (Ferguson & Fleming 1983; Hamilton *et al.* 1989; Hynes *et al.* 1996), apenas não permite a clarificação das populações originalmente envolvidas.

5.2. Importância da história das populações na definição de unidades de conservação

A análise da variação do DNA mitocondrial ao longo da distribuição nativa de *S. trutta* levou à descrição de cinco grandes linhagens evolutivas: Danúbio, Adriático, truta marmoreada, Mediterrâneo e Atlântico (Bernatchez *et al.* 1992). Estas foram posteriormente descritas como as principais "Unidades Evolutivas Significativas" (ESUs) em *S. trutta* (Bernatchez 1995, 2001) (figura 5.7). Contudo, o presente trabalho e outros evidenciam a existência, na região Atlântica, de uma considerável diferenciação das populações do sudoeste (em especial, as localizadas nos sistemas hidrográficos Ibéricos da vertente Atlântica), quer através da análise de polimorfismos proteicos (Hamilton *et al.* 1989; García-Marín & Pla 1996; Bouza *et al.* 1999; García-Marín *et al.* 1999a; Artigo I), quer de haplótipos de DNA mitocondrial (Machordom *et al.* 2000; Artigo III). Recentemente, Machordom *et al.* (2000) propuseram para a Península Ibérica cinco unidades de conservação, referidas como "Unidades Operacionais de Conservação" (*Operational Conservation Units* – OCUs) (figura 5.7). Curiosamente, a definição de ambas as unidades de conservação (ESUs e OCUs), baseou-se exclusivamente em dados provenientes da variação do DNA mitocondrial, apesar da existência de uma elevada quantidade de informação relativa a outros marcadores genéticos (p.ex. variação de proteínas) e a dados ecológicos. A noção de "ESUs" foi introduzida por Ryder (1986) para definir unidades intra-específicas de conservação, para populações com características adaptativas distintas, identificadas pela concordância de diferentes tipos de informação (p.ex. genética, morfológica e ecológica). O conceito foi aplicado pela primeira vez no salmão do Pacífico, tendo sido enfatizada a importância do isolamento reprodutivo (genético ou geográfico) entre populações (Waples 1991). Moritz (1994) propôs um critério objectivo para a definição de ESUs com base na identificação de populações com linhagens monofiléticas de DNA mitocondrial. No entanto, este critério vir-se-ia a revelar limitativo e controverso (Paetkau 1999; Crandall *et al.* 2000). Por outro lado, o conceito de OCUs pretendia conjugar a interacção de componentes biológicos e sócio-económicos (Dodsen *et al.* 1998). Contudo, em *S. trutta*, grandes unidades de conservação, ainda que definidas exclusivamente com base em clados distintos de DNA mitocondrial, parecem ser demasiado heterogéneas para reflectir uma "unidade" a conservar.

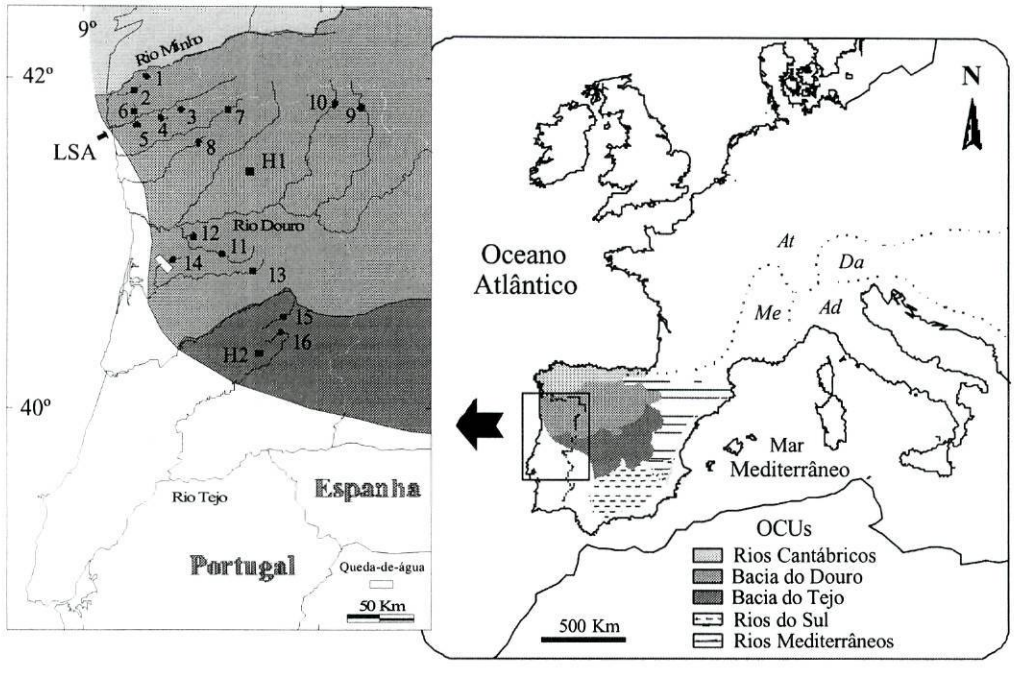

Figura 5.7. Localização geográfica das populações analisadas com base na variação do DNA mitocondrial: Rio Minho (1 e 2); Rio Lima (3 a 6); Rio Cávado (7); Rio Ave (8); Rio Douro (9 a 12); Rio Vouga (13 e 14); Rio Mondego (15); Rio Tejo (16); pisciculturas (H1 e H2). A sigla LSA representa o limite sul de ocorrência da forma migradora (Rio Lima; SNPRCN 1991). As "OCUs" apresentadas para a Península Ibérica, são as sugeridas por Machordom *et al.* (2000), com algumas adaptações para clarificar que a espécie não está presente em algumas regiões do Sul da Península. Assumiu-se que pelo menos as populações de 3 a 14 pertencem a uma mesma "OCU". As linhas a tracejado identificam os grandes sistemas hidrográficos que correspondem a quatro das cinco linhagens evolutivas do DNA mitocondrial: Danúbio (*Da*), Adriático (*Ad*), Mediterrâneo (*Me*) e Atlântico (*At*) (Bernatchez *et al.* 1992) (posteriormente descritas como ESUs; Bernatchez 1995, 2001). A linhagem da truta marmoreada (Adriático) não se encontra representada.

A variação do DNA mitocondrial em 16 populações de truta em Portugal permitiu criticar alguns aspectos relacionados com a definição de unidades de conservação nesta espécie altamente fragmentada. A detecção de uma profunda estruturação em mosaico, resultante de processos de fragmentação antiga e fluxo génico restrito, sugerem a existência de uma história evolutiva complexa (Artigo V). A considerável heterogeneidade genética, mesmo entre populações de uma única bacia hidrográfica, constitui um desafio para a definição de unidades de conservação, em particular se essas pretenderem reflectir uma análise hierárquica em termos evolutivos. De facto, 12 das 16 populações analisadas (figura 5.7), encontram-se num único OCU proposto por Machordom *et al.* (2000), mas foi detectada uma elevada heterogeneidade genética, mesmo entre populações da

mesma bacia hidrográfica. O estudo recente de 62 populações do rio Douro (Espanha) com base na variação de 34 *loci* aloenzimáticos, evidenciou igualmente uma elevada estruturação (G_{ST} = 46%; Bouza *et al.* 2001). Apesar de os sistemas hidrográficos poderem constituir uma unidade lógica para a gestão da qualidade da água, não são, necessariamente, um bom indicador sobre as relações genéticas entre as populações (Currens *et al.* 1990; McGlashan and Hughes 2000).

A existência de uma elevada estruturação populacional em *S. trutta*, reflectindo uma história complexa, sugere que a conservação desta espécie politípica requer a revisão de alguns conceitos relativos à definição de unidades de conservação. Segundo Laikre *et al.* (1999), as unidades básicas para a conservação de *S. trutta* deverão ser as populações locais. Os resultados obtidos corroboram essa ideia, sugerindo que em pelo menos algumas regiões geográficas, populações ou metapopulações sejam as únicas unidades de conservação que poderão ser adequadamente consideradas para a gestão e preservação da extensa heterogeneidade genética ao nível intra-específico. Esta perspectiva populacional de conservação apresenta-se em conformidade com a existência de elevados níveis de subestruturação em *S. trutta* (p.ex. Ryman 1983; Ferguson 1989; Guyomard 1989; Laikre *et al.* 1999), bem como em outras espécies de salmonídeos (p.ex. Allendorf & Leary 1988). Existem poucas evidências para considerar que as grandes linhagens evolutivas do DNA mitocondrial em *S. trutta* coincidam com uma real divergência em termos de caracteres com valor adaptativo. Eventualmente grandes compósitos populacionais, tais como populações migradoras no Norte Atlântico, poderão possuir caracteres de valor adaptativo com um ancestral comum. Contudo, nestas regiões, a diversidade genética foi frequentemente identificada a uma escala mais local (p.ex. populações simpátricas reprodutivamente isoladas; Allendorf *et al.* 1976; Ryman *et al.* 1979; Ferguson & Mason 1981; Ferguson & Taggart 1991).

Deste modo, a prioridade de conservação deverá exercer-se, em certos casos, numa perspectiva hierarquicamente invertida, com as populações e metapopulações mais distintas a requererem o maior esforço de protecção. Esta perspectiva está também em maior equilíbrio com a realidade política da Europa, uma vez que a consideração de grandes unidades de conservação, englobando diferentes províncias ou países, representa uma estratégia de difícil concretização prática. Outros organismos que apresentem uma elevada fragmentação populacional, poderão igualmente revelar padrões de estruturação em mosaico em que a elevada diversidade genética não será necessariamente preservada com estratégias delineadas com base na criação de grandes unidades de conservação.

5.3. Filogeografia da truta na região Euroasiática

5.3.1. Variação geográfica dos polimorfismos do gene da *transferrina*

O estudo da variação electroforética do *locus* da *transferrina* (*TF*) revelou a existência de um padrão geográfico da distribuição dos electromorfos (p.ex. Guyomard 1989; Giuffra *et al.* 1996; Largidèr & Scholl 1996). O alelo *TF*100* encontra-se geralmente fixado nas populações do Atlântico e *stocks* de piscicultura originados a partir destes. Por outro lado, o alelo *TF*102* está geralmente fixado na truta do Mediterrâneo, o *TF*75* na truta marmoreada (*S. trutta marmoratus*) e o *TF*78* no *carpione* Italiano (*S. trutta carpio*). Observaram-se ainda outros electromorfos, geralmente com frequências moderadas a altas, tais como *TF*95* nas populações do Sudoeste Atlântico de Portugal e Espanha (Artigo I, IV e VI), *TF*101* nas populações Mediterrâneas de Espanha (Artigo VI) e *TF*80* nas populações Mediterrâneas de Espanha, França (ArtigoVI) e Córsega (Krieg & Guyomard 1985; Presa *et al.* 1994; Berrebi 1995). *TF*80* e *TF*102* foram também identificados no salmão do Atlântico (*S. salar*) (Giuffra *et al.* 1996).

Apesar de se registar uma elevada estruturação geográfica no *locus TF*, a análise dos seus variantes proteicos, em termos filogeográficos, apresenta limitações, uma vez que a relação de ancestralidade entre alelos permanece desconhecida. A investigação das possíveis mutações ao nível do gene *TF* e o consequente conhecimento da filogenia dos alelos assumiram um papel fundamental para avaliar a utilidade de genes nucleares na reconstrução de histórias evolutivas ao nível intra-específico (Artigo VI). Contudo, diversos obstáculos técnicos e biológicos dificultam a determinação de genealogias nucleares (Avise 2000). Dentro desses, documentar a incidência de fenómenos de recombinação intra-génica constitui uma das maiores dificuldades. Recentemente, algumas metodologias objectivas foram propostas para superar analiticamente esse problema, possibilitando detectar fenómenos de recombinação estatisticamente significativos (Crandall & Templeton 1999; Templeton *et al.* 2000a) e identificar a estrutura cladística de um gene que evoluiu na ausência de recombinação (Templeton *et al.* 2000b).

A detecção de eventos de recombinação/conversão génica, segundo o algoritmo "CT" proposto por Crandall & Templeton (1999), inicia-se com a estimativa de uma árvore de haplótipos de máxima parcimónia estatística (*statistical parsimony* – SP; Templeton *et al.* 1992; Crandall 1994; Crandall & Templeton 1996), partindo de uma hipótese nula de ausência de recombinação ou

conversão génica. Essa árvore pode não reflectir adequadamente a história evolutiva do gene se a hipótese nula for rejeitada. A hipótese é testada através da associação entre a posição de aparentes homoplasias na árvore e a ordem da localização física na sequência de DNA. Ao traçar percursos ao longo da árvore, essa associação permite a identificação de potenciais sequências recombinantes, através do teste da relação entre homoplasias num local e a sua ocorrência noutro local da árvore. O algoritmo permite ainda a identificação de intervalos prováveis de recombinação e sequências parentais candidatas (para mais detalhes ver Crandall & Templeton 1999; Templeton *et al.* 2000a).

O método proposto por Templeton *et al.* (2000b) para a estimativa da estrutura evolutiva de um gene na presença de fenómenos de recombinação ou conversão génica tem uma aplicação mais vasta relativamente ao método de subdivisão de um gene em porções mais pequenas que apresentem reduzidas ou nenhumas evidências de recombinação (p.ex. Templeton & Sing 1993). O método inicia-se com a remoção de todos os eventos de recombinação/conversão génica estimados como estatisticamente significativos para a região de DNA analisada. Isto significa que as posições homoplásicas que foram usadas para definir recombinantes segundo a algoritmo CT e todos os haplótipos e ramos da árvore que são produtos de recombinação sejam removidos. A restante porção da árvore reflecte idealmente a diversidade haplotípica não afectada por recombinação na coalescência desta região de DNA. Contudo, poderá ser identificada uma estrutura cladística adicional em linhagens haplotípicas que tenham evoluído a partir de um haplótipo recombinante (para mais detalhes ver Templeton *et al.* 2000b).

A aplicação das metodologias propostas por Crandall & Templeton (1999) e Templeton *et al.* (2000a, 2000b) a sequências relativas de uma porção do gene *TF* (3,7 kb) representando os principais variantes electroforéticos de *S. trutta*, permitiu a elaboração da genealogia do gene (Artigo VI). A utilização do algoritmo CT permitiu a detecção estatisticamente significativa de dois eventos de recombinação e cinco de conversão génica. Apesar da identificação destes fenómenos na porção de DNA analisada, foi ainda possível identificar uma considerável estrutura cladística. A árvore resultante da remoção de todos os eventos de recombinação/conversão génica estimados reteve os ramos que definem os três grupos principais (G-1, G-2 e G-3). A diferenciação destes grupos resulta, em parte, de alterações genómicas raras (RGCs; Rokas & Holland 2000), como sejam as deleções que definem G-1 e G-2, e a inserção de um codão em G-3, sugerindo uma estrutura evolutiva bastante antiga. Foi ainda detectada uma substancial estrutura cladística, que terá emergido posteriormente aos eventos de recombinação/conversão génica. Curiosamente, alguns dos eventos de

recombinação e conversão génica parecem fornecer evidências para a existência passada de contactos secundários entre distintos subgrupos.

5.3.2. Hipóteses para a origem e radiação do complexo *S. trutta*

O conhecimento das relações genealógicas entre os diversos electromorfos do *locus TF*, para além da sua distribuição geográfica e frequências populacionais, permitiu a formulação de hipóteses sobre a origem e evolução do complexo *S. trutta* na Eurásia. As populações mais ancestrais, são caracterizadas pelo subgrupo TF-BCA e localizam-se nos sistemas hidrográficos dos Mares Negro, Cáspio e Aral. Esta situação parece ser também corroborado por registos fósseis. O mais antigo fóssil de truta foi encontrado no Cáucaso e parece datar do Pleistoceno inferior, há cerca de 2 milhões de anos (discutido em Osinov & Bernatchez 1996). A radiação da espécie poderá ter começado durante este período ou mesmo antes.

A posição filogenética e considerável variação do subgrupo TF-BCA, bem como a sua relação com o electromorfo *TF*102*, sugere a dispersão inicial da espécie do Oeste Asiático para a Europa com uma ampla distribuição pela região Mediterrânea (figura 5.8*A*). A expansão geográfica poderá ter incluído dispersão através de conexões hidrográficas nas regiões a montante do Danúbio, tal como foi sugerido para a presença de alguns haplótipos de DNA mitocondrial *Da* em populações de drenagem Adriática (Bernatchez *et al.* 1992; Apostolidis *et al.* 1997), ou pelo mar, a partir do Mar Negro para o Mediterrâneo. Esta última possibilidade de dispersão foi sugerida pela análise de aloenzimas em populações da Grécia (Karakousis & Triantaphyllidis 1990; Apostolidis *et al.* 1996a) e Turquia (Togan *et al.* 1995). A divergência populacional terá depois surgido pela separação das duas grandes drenagens hidrográficas.

O subgrupo pós-recombinante TF75 (grupo G-2), correspondendo ao electromorfo *TF*75*, foi detectado na truta marmoreada (*S. trutta marmoratus*), fenotipicamente e ecologicamente distinta, ocorrendo actualmente na Itália, Eslovénia, Croácia e Albânia (drenagem Adriática; Giuffra *et al.* 1996; Berrebi *et al.* 2000a). Com base em dados proteicos, Giuffra *et al.* (1996) propuseram duas hipóteses alternativas para a colonização da bacia hidrográfica do Pó, dependentes da ordem relativa de chegada das duas formas naturais que aí ocorrem (truta marmoreada e do Mediterrâneo). Evidências adicionais sugerem que a truta marmoreada foi a última a chegar à região Adriática (Berrebi *et al.* 2000a), situação que é corroborada pelas inferências resultantes da análise genealógica do gene *TF*. A elevada divergência deste subgrupo e o electromorfo distinto exibido indica que a truta marmoreada descende de uma linhagem evolutiva bastante

antiga. A existência de alguns haplótipos de DNA mitocondrial característicos da truta marmoreada em três populações da Grécia (drenagem dos Mares Jónico-Adriático), bem como a conversão génica com o subgrupo TF-BCA, sugerem que a origem do subgrupo TF75 poderá ter sido a sul da sua ocorrência actual (figura 5.8*B*).

O subgrupo TF95 (grupo G-2), que corresponde ao electromorfo *TF*95*, tem uma distribuição bastante distinta do subgrupo TF75. O electromorfo *TF*95* foi observado em populações "relíquias" localizadas nas regiões a montante de alguns sistemas hidrográficos, nomeadamente no Ródano (Mediterrâneo) e Pó (Adriático), onde exibe baixas frequências (Largiadèr & Scholl 1995; Largiadèr *et al.* 1996), e em alguns afluentes no Sudoeste Atlântico, com frequências moderadas a altas (Artigo I, IV e VI). No Artigo I, é sugerido que se existisse uma ancestralidade comum entre o electromorfo *TF*95* de diferentes regiões geográficas, isso seria uma indicação de dispersão ancestral da região Mediterrânea para a Atlântica. Os resultados apresentados no Artigo VI indicam que este electromorfo possui uma considerável antiguidade, nomeadamente em relação ao *TF*100* presente na maioria das populações Atlânticas. A relação filogenética de *TF*95* com *TF*75*, associada aos seus actuais padrões de distribuição, sugere que a presença do electromorfo *TF*95* na região do Sudoeste Atlântico resultou de uma dispersão de longa distância a partir do Adriático (figure 5.8*B*). Uma vez que as regiões do Adriático e Mediterrâneo se encontravam já colonizadas, a dispersão deste electromorfo nessas regiões não terá sido tão bem sucedida. A vertente Atlântica da Península Ibérica, com zonas não colonizadas, terá permitido a continuação da expansão da espécie para oeste.

O grupo G-3 é definido pela inserção de um codão no exão 2, que constitui uma característica derivada da truta relativamente a outras espécies de salmonídeos (Kvingedal *et al.* 1993; Lee *et al.* 1995, 1998; Tange *et al.* 1997; Ford *et al.* 1999; Ford 2000). A mutação representa o testemunho de uma dispersão mais recente ao longo de uma grande parte da distribuição actual da espécie (figure 5.8*C*). Dentro do grupo G-3, TF78.80 foi observado em populações dos Mares Negro ao Mediterrâneo, enquanto TF100 se encontra presente na maioria das populações Atlânticas. A divergência entre os subgrupos reflecte a segunda grande dispersão a partir de sistemas hidrográficos do Mediterrâneo para os da vertente Atlântica. As vias de dispersão parecem não ter sido usadas muito frequentemente, mas antes em alturas precisas quando o clima e a geografia assim o permitiram.

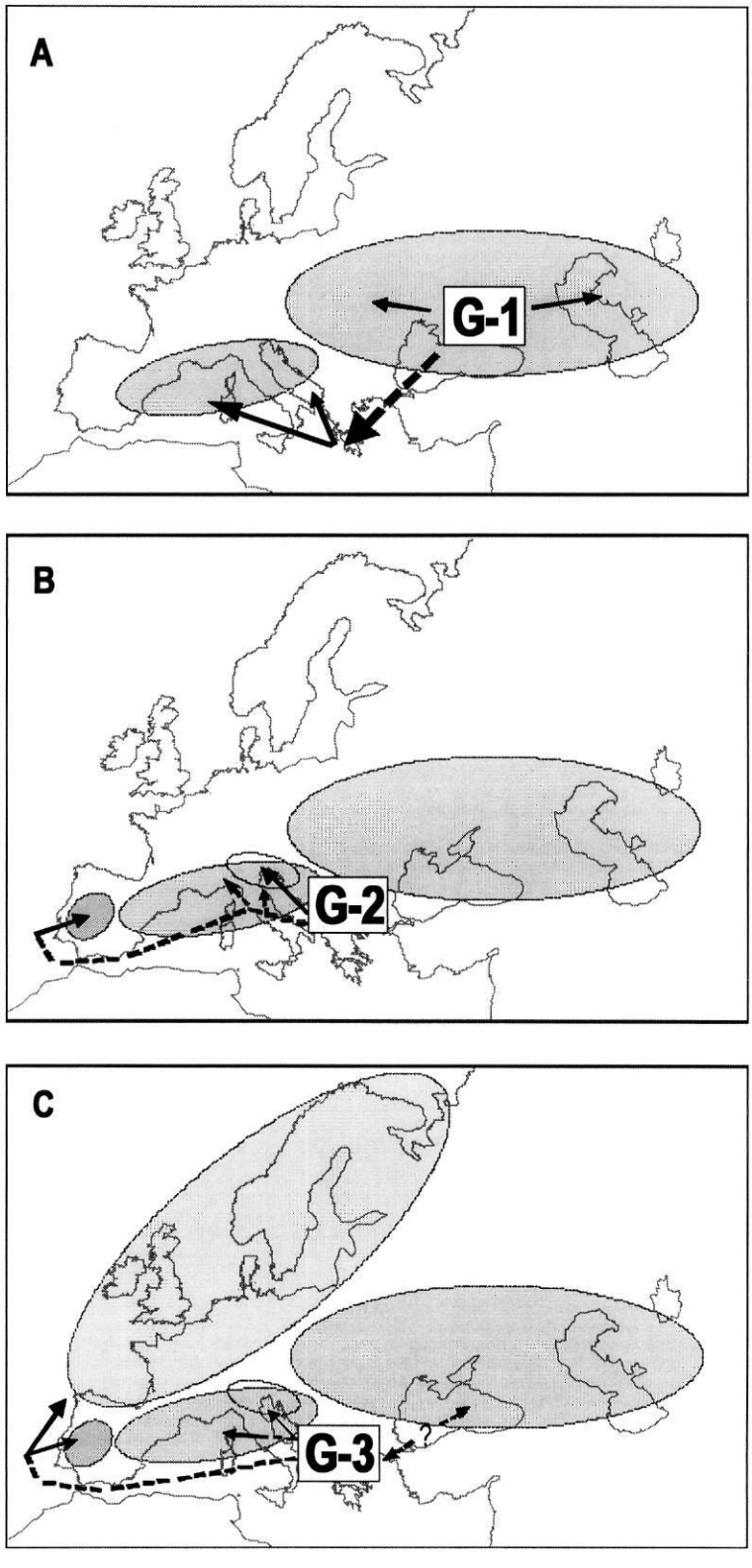

Figura 5.8. (*A*, *B* e *C*) Cenários sobre a origem e dispersão do complexo *S. trutta* inferidos com base na genealogia do gene da *transferrina*.

O electromorfo *TF*78* encontra-se fixado para o *carpione* Italiano (*S. trutta carpio*), uma forma endémica do lago Garda (drenagem do rio Pó) considerada como tendo uma origem pós-glaciar resultante da hibridação entre a truta marmoreada e a truta do Mediterrâneo (Giuffra *et al.* 1994, 1996). Os dados da genealogia do gene *TF* (Artigo VI) parecem apoiar a origem por hibridação desta forma, uma vez que foi detectado um indivíduo com uma sequência relacionada com o subgrupo da truta marmoreada. A semelhança dos haplótipos de dois indivíduos do lago Garda (drenagem no Mar Adriático) e um indivíduo do longínquo rio Kodori (drenagem no Mar Negro) é de alguma forma surpreendente, uma vez que sugere fluxo génico recente entre estas duas áreas. Por outro lado, foi observada alguma divergência entre haplótipos correspondendo ao electromorfo *TF*80*, actualmente observado em algumas populações do Mediterrâneo (Espanha, França e Córsega; Presa *et al.* 1994; Berrebi 1995; Artigo VI). As distribuições de *TF*78* e **80* sugerem um reduzido sucesso na dispersão destes electromorfos para as populações previamente estabelecidas nas vertentes do Adriático e Mediterrâneo. Por outro lado, a ampla distribuição do subgrupo TF100 (correspondendo ao electromorfo *TF*100*) ao longo da vertente Atlântica sugere uma rápida expansão geográfica. A região Atlântica foi a mais directamente influenciada pelas glaciações do Pleistoceno em termos de perda de habitat (Hewitt 2000). A extinção de populações terá sido elevada nesta região, com excepção de refúgios glaciares, nomeadamente, no sudoeste Atlântico (p.ex. Sanz *et al.* 2000; Bouza *et al.* 2001; Artigo III, IV, V). Isto explicaria parte da complexa história evolutiva detectada nas populações do limite sul da distribuição Atlântica e a persistência de algumas "relíquias" populacionais exibindo o subgrupo TF95 e alguma heterogeneidade no subgrupo TF100.

5.3.3. Filogeografia nuclear *versus* mitocondrial

O padrão filogeográfico do gene *TF* é em parte sobreponível com o obtido com base nas linhagens de DNA mitocondrial. As trutas da região dos Mares Negro, Cáspio e Aral (englobando a linhagem mitocondrial *Da*) parecem caracterizar-se principalmente pela presença do subgrupo TF-BCA. A truta marmoreada (linhagem mitocondrial *Ma*) é caracterizada por um único subgrupo (TF75). Contudo, *S. trutta* de outras regiões geográficas exibem haplótipos do gene *TF* de diferentes subgrupos. Foi este o caso das populações das drenagens Mediterrânea e Adriática (linhagens mitocondriais *Me* e *Ad*, respectivamente) que possuem os subgrupos TF102 e TF78.80. Igualmente, as populações da vertente Atlântica (linhagem mitocondrial *At*) exibem os subgrupos TF95 e TF100.

Considerando as relações filogenéticas do gene *TF*, os haplótipos da região dos Mares Negro, Cáspio e Aral são aqueles que exibem maior semelhança com o salmão do Atlântico. Alguma desta homologia resulta de alterações genómicas raras que estão pouco sujeitas a evolução convergente, contribuindo para um baixo nível de homoplasia (Rokas & Holland 2000). Ao nível do DNA mitocondrial, foi sugerida a divergência ancestral da linhagem *At* de todas as outras quando comparada com o salmão do Atlântico (Giuffra *et al.* 1994; discutida em detalhe por Bernatchez 2001). Com base na análise de sequências de 1,25 Kb (porções da região de controlo, citocromo *b* e subunidade VI da ATPase), a distinção da linhagem *At*, é determinada por quatro posições nucleotídicas partilhadas com *S. salar*. Curiosamente, assumindo a divergência do clado *At* a partir da linhagem *Me*, ou de um ancestral comum, duas soluções estatisticamente parcimoniosas foram identificadas na ligação das duas linhagens com base num fragmento da região de controlo de 464 pb (Artigo V). Este resultado sugere que, devido à elevada taxa de mutação do DNA mitocondrial, exista um maior número de homoplasias quando as comparações são efectuadas ao nível inter-específico. De acordo com os resultados do Artigo VI, o subgrupo TF100, característico da maioria das populações Atlânticas, é o mais divergente do salmão do Atlântico.

Em termos gerais, o cenário filogeográfico inferido com base na genealogia do gene *TF* parece anteceder o obtido a partir do DNA mitocondrial. Enquanto as linhagens do DNA mitocondrial tiveram tempo suficiente para se tornarem reciprocamente monofiléticas, o mesmo não se verificou para as do gene *TF*. Por um lado, o DNA mitocondrial evolui mais rapidamente e por outro, sendo haploide, o seu efectivo (N_e) é quatro vezes inferior ao de um gene nuclear autossómico (Birky *et al.* 1989). Efectivos populacionais reduzidos, influenciados pela deriva génica, permitem, em média, uma maior fixação e/ou extinção de linhagens evolutivas (Hoelzer 1997). O estudo da genealogia do gene *TF*, com uma coalescência mais antiga relativamente ao DNA mitocondrial, permitiu identificar diferentes cenários filogeográficos e elaborar uma sequência de acontecimentos para a expansão de *S. trutta* ao longo da sua área de distribuição, fornecendo novos dados para o conhecimento da sua história evolutiva.

6. Conclusões

Esta tese representa um contributo para o conhecimento da história evolutiva de *S. trutta*. De acordo com os objectivos previamente definidos expõem-se de forma resumida os principais resultados e conclusões deste trabalho:

6.1. História evolutiva de *S. trutta* no limite sul da distribuição Atlântica

A análise de variação genética com base em polimorfismos proteicos veio corroborar a existência de três grupos geográficos bem diferenciados, que representam as populações do noroeste Atlântico, do sudoeste Atlântico (onde se incluem as populações de Portugal) e do Mediterrâneo.

As populações de truta da vertente Atlântica da Península Ibérica evidenciam a existência de uma elevada heterogeneidade, em particular, a sul do LSA (limite sul de anadromia). As frequências de *CK-A1*100*, *GPI-B2*135* e *MPI-2*105* apresentam um decréscimo clinal para sul, o que sugere a existência de fluxo génico contínuo associado à presença da forma migradora. Por outro lado, a detecção em elevadas frequências dos alelos *PEPLT*70* e *TF*95* mais a sul, estando estes ausentes ou em reduzidas frequências no norte da Península Ibérica, indica a persistência de "relíquias" populacionais que permaneceram isoladas durante longos períodos de tempo.

A variação genética de quatro microssatélites em 11 populações de truta do Sudoeste Atlântico veio corroborar os padrões de estruturação obtidos através de polimorfismos proteicos. Contudo, os microssatélites por exibirem um grau mais elevado de polimorfismo, indicam com maior sensibilidade a perda de variabilidade genética. A distribuição alélica evidencia um padrão norte-sul caracterizado pela redução do número de alelos, variância e heterozigotia, que se intensifica nas populações localizadas a sul do LSA. Essa tendência, foi também observada ao nível do *locus* proteico muito polimórfico, *PX*, descrito em *S. trutta*.

Os padrões de distribuição e divergência do DNA mitocondrial em 16 populações de truta em Portugal revelaram a existência de uma elevada estruturação genética (ϕ_{ST} = 0,76) sugerindo uma complexa e antiga história de isolamento geográfico (pelo menos 200 mil anos). A aplicação da "análise

cladística hierárquica" revelou que o padrão geográfico de variação do DNA mitocondrial parece ter sido determinado principalmente por processos de fragmentação antiga e fluxo génico restrito recorrente.

A interpretação dos padrões de estruturação genética nas populações do Sudoeste Atlântico indica um cenário histórico caracterizado por condições de maior estabilidade demográfica a norte da bacia hidrográfica do rio Lima (LSA) e condições ecológicas limitantes a sul desse rio, responsáveis por flutuações profundas e recorrentes dos efectivos populacionais. Os sucessivos estrangulamentos populacionais nesta região ocorridos ao longo dos ciclos de oscilação climática do Pleistoceno terão determinado um padrão de fragmentação populacional e redução da diversidade genética. A história evolutiva destas populações foi ainda influenciada por um fluxo génico mais ou menos constante a partir de populações localizadas no norte da Península Ibérica.

A análise dos padrões de diversidade genética forneceu reduzidas evidências para suportar uma influência substancial desta região na recolonização do Norte da Europa depois do último máximo glaciar. A distribuição geográfica da variação genética, tanto de *loci* proteicos como do DNA mitocondrial, nas populações do Norte da Europa indica que a dispersão pós-glaciar se deu provavelmente a partir de um refúgio localizado a norte da Península Ibérica.

A elevada estruturação populacional em *S. trutta* sugere que a conservação desta espécie politípica requer a revisão de alguns conceitos relativos à definição de unidades de conservação. Os resultados obtidos demonstram que, em pelo menos algumas regiões geográficas, populações ou metapopulações sejam as únicas unidades de conservação que podem ser adequadamente consideradas para a gestão e preservação da extensa heterogeneidade genética ao nível intra-específico.

6.2. História evolutiva do complexo *S. trutta* na região Euroasiática

O estudo de uma porção do gene *TF*, com cerca de 3,7 kb, correspondendo aos principais variantes electroforéticos, permitiu a elaboração da genealogia do gene. A árvore obtida, assumindo a hipótese nula de ausência de recombinação ou conversão génica, pôs em evidência três grupos haplotípicos (G-1; G-2 e G-3). A diferenciação destes grupos resulta, em parte, de alterações genómicas raras que indicam uma estrutura evolutiva bastante antiga.

Apesar da existência de fenómenos de recombinação e conversão génica na porção de DNA analisada, foi possível identificar uma considerável estrutura cladística, nomeadamente a que define os três grupos principais (G-1, G-2 e G-3).

Foi ainda detectada uma estrutura adicional, que terá surgido posteriormente aos eventos de recombinação e conversão génica. Curiosamente, alguns desses eventos parecem indicar a existência de contactos secundários entre distintos subgrupos.

O conhecimento das relações genealógicas entre os diversos electromorfos do *locus TF*, para além da sua distribuição geográfica e frequências populacionais, permitiu a formulação de hipóteses sobre a origem e evolução do complexo *S. trutta* na Eurásia. Os resultados obtidos demonstram que as populações mais ancestrais estão presentes nas drenagens dos Mares Negro, Cáspio e Aral. Dentro do grupo G-1, a posição filogenética e a considerável variação do subgrupo TF-BCA, bem como a sua relação com subgrupo TF102, existente nas populações do Mediterrâneo, sugerem a dispersão inicial da espécie do Oeste Asiático para a Europa.

No grupo G-2, o subgrupo TF75, detectado na truta marmoreada (*S. trutta marmoratus*), sugere que esta, embora descendendo de uma linhagem evolutiva bastante antiga, terá chegado à região Adriática depois da truta do Mediterrâneo. A relação filogenética do subgrupo TF95 (grupo G-2), associada aos actuais padrões de distribuição do electromorfo *TF*95*, sugere uma dispersão ancestral a partir da região Adriática-Mediterrânea para a região do Sudoeste Atlântico.

Dentro do grupo G-3, a divergência entre os subgrupos TF78.80 e TF100 reflecte a segunda grande dispersão a partir de sistemas hidrográficos do Mediterrâneo para os do Atlântico. Dentro do subgrupo TF78.80, os dados da genealogia do gene suportam a hipótese de origem do *carpione* Italiano (*S. trutta carpio*; fixada para o electromorfo *TF*78*) por hibridação entre a truta marmoreada e a truta do Mediterrâneo. Por outro lado, foi detectada alguma variação haplotípica dentro do electromorfo *TF*80*, actualmente observado em algumas populações do Mediterrâneo. A ampla distribuição do subgrupo TF100 ao longo da vertente Atlântica evidencia uma rápida expansão geográfica em consequência das oscilações climáticas do Pleistoceno. A existência de refúgios glaciares no Sudoeste Atlântico permitiu a persistência de algumas "relíquias" populacionais que exibem o subgrupo TF95 e alguma estruturação no subgrupo TF100.

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