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Evolutionary history of lamprey paired species *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch) as inferred from mitochondrial DNA variation

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Abstract

A remarkable trend in the evolution of lampreys is the occurrence in most genera of 'paired species', in which the parasitic anadromous lampreys are believed to have given rise to non-parasitic freshwater resident populations. The present work examines the phylogeography of the European paired species *Lampetra fluviatilis* and *Lampetra planeri*, in an attempt to elucidate species pair evolutionary history. We studied sequence variation in cytochrome *b* and ATPase 6, 8 mitochondrial genes in 63 individuals from 21 localities of the paired species throughout their distribution range. Results from the phylogenetic and nested clade analyses were largely consistent, suggesting the existence of three major evolutionary lineages: lineage I and possibly lineage II are widespread throughout Europe, while the most ancestral lineage III is apparently restricted to the Iberian Peninsula. The high genetic diversity observed in the Iberian Peninsula is probably the result of refugial persistence and subsequent accumulation of variation over several ice ages, whereas the low levels of genetic diversity observed in central and northern Europe should reflect a rapid postglacial colonization. Results suggest that *L. planeri* originated within at least two distinct evolutionary lineages, rejecting the single origin hypothesis. The observed lack of taxa monophyly within lineage I may be the result of ongoing gene flow if the two taxa are alternate life-history forms of a single species. However, structure within lineage I is also consistent with the hypothesis of divergence of taxa after postglacial dispersion (around 2000 generations ago) with incomplete lineage sorting. Further testing of the alternative hypotheses is warranted.

Keywords: anadromy, glacial refugia, parallel evolution, Petromyzontidae, phylogeography, species pair

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Introduction

Shifts from anadromous to freshwater life histories are known to have occurred repeatedly in some fish families, namely in Petromyzontidae (Potter 1980), Salmonidae (Taylor *et al*. 1996), Osmeridae (Taylor & Bentzen 1993), Gasterosteidae (Bell & Foster 1994), and Galaxiidae (Ovenden *et al*. 1993; Waters & Wallis 2001). Loss of migratory ability remains, however, a poorly understood aspect of the evolutionary biology of fishes. Because anadromous fish breed in fresh water, they have ample opportunities to colonize this environment, which is favoured when the cost of migration exceeds the value of marine food resources (Bell & Andrews 1997). Recent glaciation/deglaciation events during the past several millennia might have promoted evolution of nonmigratory species by blocking of migratory routes by ice or altering the ratio of freshwater productivity to the fitness cost of migration. The aquatic habitat uncovered by deglaciation was relatively inaccessible through fresh water but easily reached by anadromous fishes. Anadromous fishes had

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therefore access to largely unexploited resources, which might have favoured establishment of freshwater isolates (Bell & Andrews 1997; Lee & Bell 1999). It has been suggested that loss of anadromy might act as an initiator for radiation and speciation (Bell & Andrews 1997; Lee & Bell 1999; Taylor 1999; Waters & Wallis 2001), and that deterministic forces, that is natural and/or sexual selection, might play a significant role in driving the divergences (reviewed in Schluter 1996; Taylor 1999; but see Taylor & McPhail 2000).

A remarkable trend in the evolution of lampreys (Petromyzontidae) is the occurrence in most genera of 'paired species' (Zanandrea 1959), in which the larvae are morphologically similar but the adults adopt different life-history types, most commonly parasitic anadromous vs. nonparasitic freshwater resident. Nonparasitic species of lampreys share a large series of homoplasies, that can be grouped into three sets that reflect (i) precocious reproductive maturity, (ii) lack of growth after metamorphosis, and (iii) lack of feeding after metamorphosis (Vladykov & Kott 1979). It has been generally accepted that lamprey paired species are closely related, the nonparasitic freshwater species having evolved from a form similar to that of the extant parasitic anadromous lamprey (Zanandrea 1959; Hubbs & Potter 1971; Hardisty 1986a; Youson & Sower 2001). These inferences stem from close morphological similarities between paired species and the observation that geographical distribution of the nonparasitic resident species is typically contained within the range of the parasitic anadromous form (Hardisty 1986a). Nonparasitic forms may be allopatric to congeneric parasites (e.g. Holčík 1995; Renaud 1997), suggesting local extinction of the ancestor or range expansion of the nonparasitic form. In the view of the fact that more than one nonparasitic freshwater lamprey may appear to be related to a given parasitic anadromous species, Vladykov & Kott (1979) proposed the term 'satellite species' for such nonparasitic forms.

Some authors (e.g. Enequist 1937) questioned the merit of using life-history type as indication of separate species status and argued that parasitic anadromous and nonparasitic resident lampreys are alternate life forms of a single species that develop according to habitat characteristics encountered during the larval stage. Supporting this hypothesis, a lamprey population that may produce both parasitic and nonparasitic freshwater forms has been found (Beamish & Withler 1986; Beamish 1987). Genetic analysis based on allozyme (Beamish & Withler 1986; Engelhorn & Schreiber 1997; Schreiber & Engelhorn 1998; Yamazaki & Goto 1998) and mitochondrial DNA (mtDNA) markers (Tagliavini *et al*. 1994; Docker *et al*. 1999) revealed low or lack of genetic divergence between members of paired species. Some authors (Schreiber & Engelhorn 1998) interpreted these results to indicate that pair members might share the same gene pool and should be considered as a single species, while others (Docker *et al*. 1999; Salewski 2003) argued that they suggest very recent divergence of paired species from one another.

The present paper examined phylogeography of the European paired species *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch), in an attempt to elucidate species pair evolutionary history. These two species have similar geographical distributions occurring from northern Europe, especially in basins associated with the North and Baltic seas, to the western Mediterranean (Hardisty 1986b, c; Almaça & Collares-Pereira 1988; Almaça & Cortes 1991). The river lamprey, *L. fluviatilis*, is parasitic and exhibits a migratory life-history pattern, but landlocked populations have been reported (Hubbs & Potter 1971; Maitland 1980; Tuunainen *et al*. 1980; Maitland *et al*. 1994; Goodwin *et al*. 2006). The brook lamprey, *L. planeri*, is a purely freshwater form, generally assumed to have evolved from the migratory form and become nonparasitic (Hubbs & Potter 1971; Hardisty 1986a).

The origin and subsequent evolution of the nonparasitic resident form could follow any of three main scenarios. First, different resident populations may be derived from a single event of loss of the migratory ability, with vicariant divergence driven by drainage evolution. This model predicts that populations of *L. planeri* from different basins would form a monophyletic cluster distinct from all *L. fluviatilis* that would be contained in a second monophyletic cluster (Fig. 1a). Second, multiple occurrences of European brook lamprey may rather result from independent divergences from the river lamprey, with repeated loss of anadromy (Fig. 1b). This scenario leads to a phylogeographical pattern in which *L. planeri* is polyphyletic and *L. fluviatilis* is paraphyletic, resident and migratory populations from the same river being genetically closer to each other. If the multiple speciation events were very recent, the two recently formed species may have not yet achieved reciprocal monophyly via genetic drift and lineage sorting (Neigel & Avise 1986) and then it might not be possible to distinguish this second scenario from the third one where the river and brook lampreys are different ecotypes of a single species (Fig. 1c).

We examined genealogical relationships and geographical distribution of mtDNA haplotypes throughout the majority of *L. fluviatilis* and *L. planeri* geographical ranges in order to provide information on the mode and timing of divergence of pair members.

Materials and methods

Samples

Sixty-three specimens representing 21 localities throughout the geographical distribution of *Lampetra fluviatilis* and *Lampetra planeri* paired species were analysed (see Table 1;

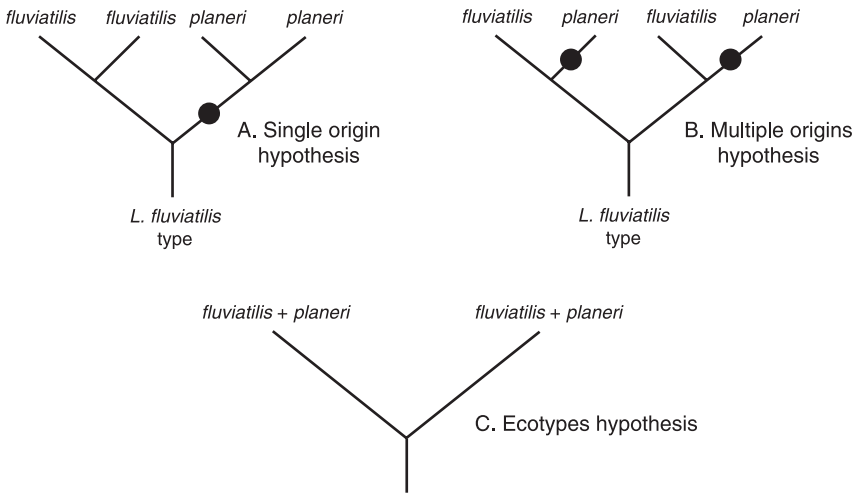

Fig. 1 Alternative hypotheses of relationships among populations of *Lampetra fluviatilis* and *Lampetra planeri*. Filled circles represent loss of anadromy and speciation events.

Table 1 Collection localities of specimens sequenced. Locality numbers refer to Fig. 2. The abbreviations for the life stage are: A, adult; M, macrophtalmia; and Am, ammocoete. *N* refers to sample size. The sequence for specimen with * was retrieved from EMBL database (Y186831)

Locality number	Basin	River	Country	*Lampetra* species	Stage	*N*
1	Ricklean	Ricklean	Sweden	*L. fluviatilis*	A	3
2	Vikedalseva	Vikedalseva	Norway	*L. planeri*	M	3
3	Gudenaa	Lilleaa	Denmark	*L. fluviatilis*	A	3
4	Peene	Tollense	Germany	*L. planeri*	Am	3
5	Vistula	Grzmiace	Poland	*L. planeri*	A	2
					Am	1
6	Elbe	Elbe	Germany	*L. fluviatilis*	A	3
7	Elbe	Blanice	Czech Rep.	*L. planeri*	A	1
					Am	1
8	Wadden Sea		Netherlands	*L. fluviatilis*	A	3
9	Danube	Poprad	Slovakia	*L. planeri*	A	1
10	Forth	Forth	Scotland	*L. fluviatilis*	A	3
11	Ure	Ouse	England	*L. fluviatilis*	A	3
				L. planeri	A	1
12	Loire	Allier	France	*L. planeri*	Am	1
13	Loire	Loire	France	*L. planeri*	Am	6
14	Rhone	Ain	France	*L. planeri*	Am	3
15	Garonne	Garonne estuary	France	*L. fluviatilis*	A	1*
16	Esmoriz		Portugal	*L. planeri*	A	3
17	Vouga	Águeda	Portugal	unidentified	Am	3
18	Mondego	Anços	Portugal	*L. planeri*	A	3
19	Tejo	Sorraia	Portugal	*L. planeri*	A	4
				L. fluviatilis	A	1
					M	1
20	Tejo	Tejo estuary	Portugal	*L. fluviatilis*	M	3
21	Sado	Sado	Portugal	unidentified	Am	3

Fig. 2). Adults from each species were distinguished based on differences in body size and mouth structure (Hardisty 1986b, c), and macrophtalmia were kindly identified by Juraj Holčík based on the shape of velar tentacles (Salewski *et al*. 1996). In localities where artificial barriers prevent the access of the anadromous form, ammocoetes were assigned to *L. planeri*. In rivers where ammocoetes were collected downstream barriers, no species assignment was made, and such ammocoetes are hereafter referred to as 'unidentified ammocoetes'.

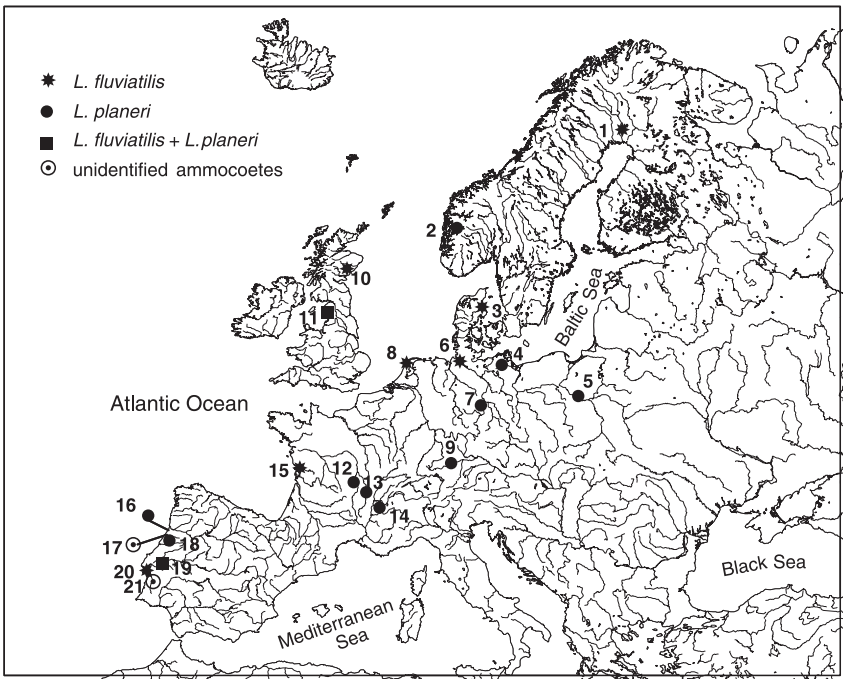

Fig. 2 Map of sampling localities. The numbers represent locality codes as presented in Table 1.

DNA extraction, amplification and sequencing

DNA was extracted from muscle tissue following the protocol described by Hillis *et al*. (1996). For the formalin-fixed macrophtalmia specimen of *L. fluviatilis* from the Sorraia River (Tejo basin) it was used the procedure described by Shedlock *et al*. (1996).

Polymerase chain reaction (PCR) amplification of 1173 bp of the cytochrome *b* (cyt *b*) gene used the primers LA (5′-GCGACTTGAAAAACCACCGTT-3′) and PRO (5′-TAGATACAGAGGTTTGAATCCC-3′). Internal primers were used: LB (5′-CTGCAGCTACTGCTTTCGTTGG-3′) and CB2H (5′-CCCTCAGAATGATATTTGCCCTCA-3′). For the amplification of the genes ATPase subunit 6 and 8 (828 bp), the primers used were ATPfor (5′-CCTTTTAA-GCTGAAGAAGATGGGTG-3′) and ATPrev (5′-TGGTAT-GCGTGAGCTTGGTGGG-3′). The primers were designed using the complete mtDNA sequence of *L. fluviatilis* available at the EMBL database (Y186831). Each 50-μL reaction contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer, 1 U of *Taq* DNA polymerase (Fermentas) and 1× of the buffer supplied. The reactions were run in a thermocycler Biometra Tgradient for 30 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min and extension at 72 °C for 2 min; the 30 cycles were preceded by an initial denaturation for 3 min at 94 °C and followed by a final 2-min 72 °C extension. For the formalin-fixed specimen, PCR amplification was preceded by DNA amplification using the GenomiPhi DNA Amplification Kit (Amersham Biosciences) following manufacturer's instructions.

The PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). PCR products were sequenced in both directions using an ABI PRISM 3700 DNA analyser at Macrogen (www.macrogen.com).

Data analyses

Alignment of the sequences was accomplished using the BIOEDIT software developed by Hall (1999). Sequences were translated into proteins using the vertebrate mitochondrial code available in the program MACCLADE (Maddison & Maddison 1992) to check for the presence of stop codons that may indicate sequencing errors or the presence of pseudogenes. The partition homogeneity test (Farris *et al*. 1994) performed in PAUP* (Swofford 1998) revealed that the two genes contained no significantly different phylogenetic signals ($P = 0.199$), and therefore subsequent analyses were performed with combined sequences. Haplotype diversity (h) and nucleotide diversity (π) were estimated using DNASP (Rozas *et al*. 2003). The GTR + gamma model (gamma shape parameter = 0.5013) was selected as the model of molecular evolution that best fitted our data by using the hierarchical likelihood-ratio test implemented in MODELTEST 3.0 (Posada & Crandall 1998).

Phylogenetic relationships among haplotypes were reconstructed using PAUP* (Swofford 1998) by the neighbour-joining algorithm (NJ) using the GTR + gamma distance matrix, by the maximum-parsimony criterion (MP), and by the maximum-likelihood criterion (ML) using the

parameter settings as estimated with MODELTEST (base frequencies: A = 0.29778, C = 0.24712, G = 0.12481, T = 0.33029; transition/transversion ratio = 2.20346). The MP and ML analyses were conducted using a heuristic search with tree-bisection–reconnection (TBR) branch swapping, and 10 random-addition replicates. The robustness of each branch was estimated using the bootstrap test (Felsenstein 1985). The sequences of *Petromyzon marinus* (L.) from the EMBL database (U11880) and *Eudontomyzon mariae* (Berg) from the Danube River obtained in the present study (AM051061) were used as outgroups. Most parsimonious trees were compared with those expected from alternate hypotheses as described by Templeton (1983) and implemented in PAUP*.

Partitioning of mtDNA variation was investigated by an analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992) using ARLEQUIN 2.000 (Schneider *et al*. 2000). This analysis produces estimates of variance components analogous to *F*-statistics (Wright 1951, 1965), reflecting the correlation of haplotypes at different levels of hierarchical subdivision. Sampling sites were first treated as individual 'populations' to test for overall genetic subdivision. Molecular variance was then partitioned into two hierarchical levels, nominal species and populations. Under the hypothesis of monophyletic origin of the resident form, most variation is expected to be found between the two forms (high Φ_{CT}), while under the hypotheses of multiple origins or the existence of a single species with ecotypes, most variation is expected to be due to differences between populations from different basins (high Φ_{SC} and Φ_{ST}). Ammocoetes from the Vouga and Sado basins were not included in this analysis because their specific taxonomic status is unknown. A third analysis was performed in which populations were grouped as suggested by the phylogenetic analysis. The significance of the observed variance components and Φ-statistics was tested against the respective null distribution generated by 1000 random permutations.

A frequency distribution of the pairwise number of mutational differences between haplotypes (mismatch analysis) for all the samples and for each clade identified in the phylogenetic analysis was constructed using ARLEQUIN 2.000 (Schneider *et al*. 2000). We compared observed frequencies of pairwise differences with those expected under various demographic models. Populations that have been historically stable are predicted to have multimodal mismatch distributions, whereas those that have undergone a recent expansion are predicted to be unimodal (Slatkin & Hudson 1991; Rogers & Harpending 1992). We used the raggedness index of Harpending (1994) to assess the significance of the fit of the distribution to that of an expanding population ($rg < 0.05$), using 1000 simulations. We also computed Tajima's (1989) *D* and Fu's (1997) F_S statistics. Significant negative values of Tajima's *D* and Fu's F_S are thought to be evidence of expanding populations.

A statistical parsimony (SP) cladogram among haplotypes was constructed using the TCS 1.13 software (Clement *et al*. 2000). A nested clade analysis was performed (NCA; Templeton 1998) to infer population history. The NCA nesting design was designed by hand on the SP cladogram following the rules given in Templeton (1998). The program GEODIS 2.2 (Posada *et al*. 2000) was used to calculate the various NCA distance measures (D_c, D_n and I-T) and their statistical significance. The statistical significance of the distance measures was calculated by comparison with a null distribution derived from 10 000 random permutations of clades against sampling locality. Fetzner & Crandall (2003) suggested that for freshwater, estuarine and costal species, standard geographical (great circle) distances may not adequately describe the actual distances involved between populations, and river and coastal distances should be used instead. Distances between populations were measured by following river courses and coastlines, using the software package ARCVIEW (version 3.2). Ambiguities present in the SP cladogram were solved using the three criteria suggested by Crandall & Templeton (1993): (i) frequency criterion, (ii) topological criterion, and (iii) geographical criterion. The inference key of Templeton (2004) was used to choose among the different events that may produce significant haplotype–geography associations.

Results

Mitochondrial DNA variation

Sequencing of 2001 bp across the cyt *b* and ATPase 6/8 genes from 63 lampreys revealed 37 haplotypes defined by 78 polymorphic sites, of which 37 were in cyt *b* and 41 in ATPase 6/8. Nucleotide composition showed anti-G bias (G = 12.48%). Overall haplotype diversity was 0.9462 and nucleotide diversity was 0.00389. Most haplotypes were found at single localities, with only five shared by two or more localities (Table 2). The most common haploype (H6) was found in 13 lampreys belonging to five populations. Haplotypes differed by 1–30 substitutions, with the largest differences found between the haplotypes from the Iberian Sado basin (H35-H37) and all others. Haplotypes H2, H4, H6, H7 and H9 were shared by both *Lampetra fluviatilis* and *Lampetra planeri*. Nucleotide sequences were deposited at the EMBL database with the accession numbers AJ937921–57.

Phylogenetic analysis

Phylogenetical analysis using NJ, MP and ML methods identified three distinct lineages (see Fig. 3), one widespread throughout all Europe including the Iberian Peninsula and two other exclusively present in Iberian basins. Lineage I grouped haplotypes found in both

Table 2 Distribution of haplotypes (rows) at each sampled locality (columns). Locality numbers refer to Table 1 and Fig. 2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total
H1															1							1
H2													3						1			4
H3																		1				1
H4	1	1																				2
H5	1																					1
H6	1	2		3		1	2													3		12
H7								1					1									2
H8								1														1
H9					2			1											1			4
H10														2								2
H11														1								1
H12												1										1
H13													2									2
H14					1																	1
H15			1																			1
H16			1																			1
H17			1																			1
H18										1												1
H19										1												1
H20										1												1
H21											1											1
H22											1											1
H23											1											1
H24											1											1
H25									1													1
H26																3						3
H27																	1					1
H28																	1					1
H29																	1					1
H30																		2				2
H31																			3			3
H32						1																1
H33						1																1
H34																			1			1
H35																					1	1
H36																					1	1
H37																					1	1

nominal species from basins throughout the entire geographical distribution, lineage II included haplotypes observed in adults of *L. planeri* from the Esmoriz river and in unidentified ammocoetes from the Vouga river (localities 16 and 17), and lineage III comprised haplotypes found in ammocoetes of unknown specific status from the Sado river (locality 21). The bootstrap values supporting the Iberian clades were high (88–100%), while those supporting lineage I were considerably lower (51–72%). Lineage I showed weak structure, with many polytomies and poorly supported subclades (~60%). Its subclade comprising the haplotypes found in the Rhone basin (H10 and H11) was an exception, being supported by high bootstrap values (80–81%).

Alternative hypotheses for the origin of the nonparasitic freshwater lamprey were examined by constraining trees to topologies predicted from hypothesized modes of origin, and contrasting these trees with the most parsimonious trees. In the first set of constrained trees, *L. fluviatilis* and *L. planeri* were constrained to form reciprocal monophyletic groups, as predicted by the hypothesis of single origin (Fig. 1a). In the second set of constrained trees, *L. fluviatilis* and *L. planeri* of lineage I were forced to form monophyletic groups. This would be consistent with the hypothesis of origin of *L. planeri* within multiple evolutionary lineages, having *L. fluviatilis* and *L. planeri* of lineage I achieved reproductive isolation (Fig. 1b). Statistical contrasts revealed that most parsimonious trees were significantly better ($P < 0.05$) than both sets of constrained trees, rejecting both hypotheses.

In order to investigate whether rejection of the null hypothesis of monophyly of each taxa within lineage I is

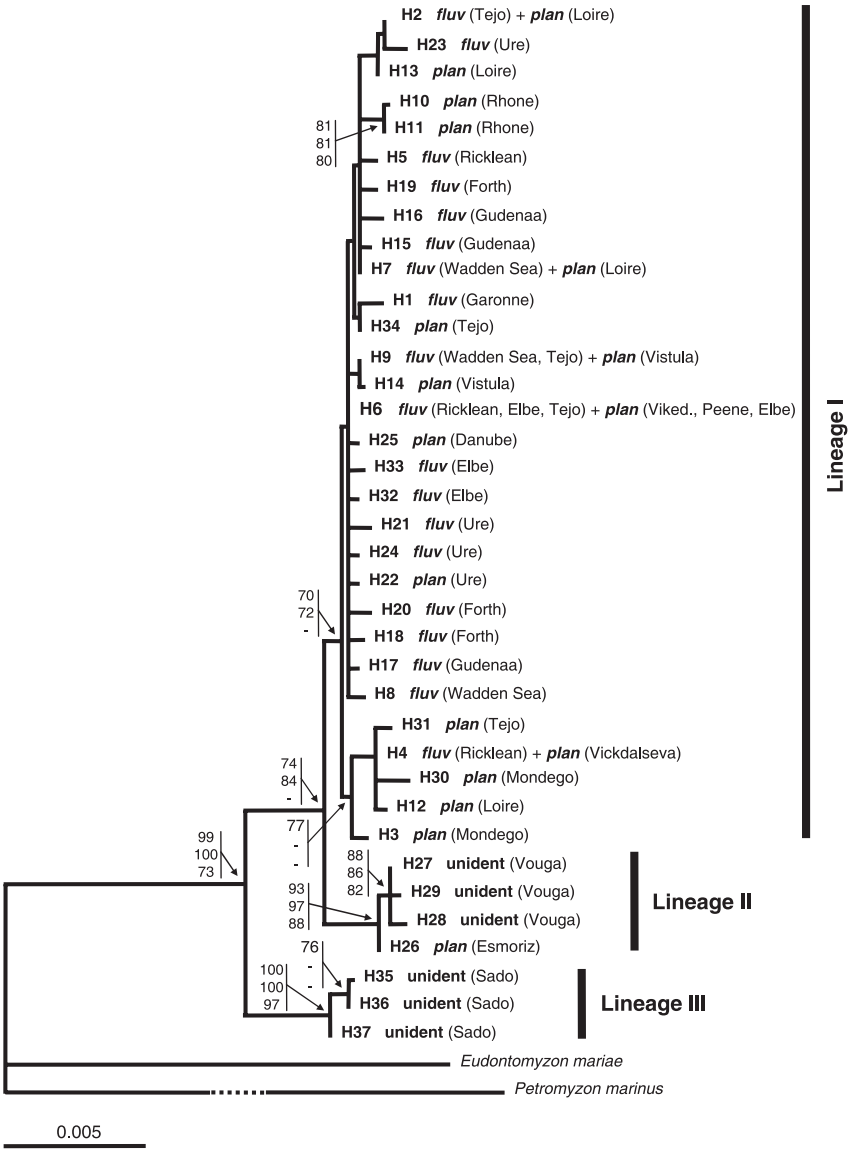

Fig. 3 Maximum-likelihood estimate of the phylogeny of 37 mitochondrial haplotypes of *Lampetra*. Species and rivers in which the haplotypes were detected are indicated. The abbreviations for the species are: *fluv*, *L. fluviatilis*; *plan*, *L. planeri*. Nodes supported by bootstrap values equal to or higher than 70% in either neighbour-joining, maximum-parsimony or maximum-likelihood analyses (top, middle, and bottom, respectively) are indicated.

the result of ongoing gene flow (hypothesis of different ecotypes of a single species, Fig. 1c) or may be the artefact of retained ancestral polymorphism, we used coalescent simulations to model the effect of time on lineage sorting. We used MESQUITE (Maddison & Maddison 2004) to simulate coalescent trees constrained within a population tree consistent with two independent taxa, with differing branch lengths expressed as number of generations. We simulated 1000 gene trees constrained within each population tree for effective population sizes, N_e, of 10 000 and 1000. We calculated Slatkin & Maddison's (1989) *s* statistic for each simulation, and graphed the distribution of *s* values for each population tree (Fig. 4). This statistic treats populations as categorical variables and measures the minimum number of sorting (or migration) events implied in the genealogies. Simulations show that, in the absence of gene flow and given an effective population size of 1000, complete lineage sorting ($s = 2$) within lineage I would require 200 000 generations, which assuming a generation length of 5–7 years (Hardisty & Potter 1971a) would correspond to about 1 million years. The *s* statistic calculated from our observed tree ($s = 12$) is consistent with the divergence of *L. fluviatilis* and *L. planeri* around 2000 generations (10 000–14 000 years) ago with no subsequent gene flow.

Hierarchical genetic diversity analysis

Analysis of molecular variance (Table 3) showed substantial subdivision among populations ($\Phi_{ST} = 0.705$), in keeping with the observation that many localities exhibited unique haplotypes. When molecular variance was partitioned into two hierarchical levels (nominal species and

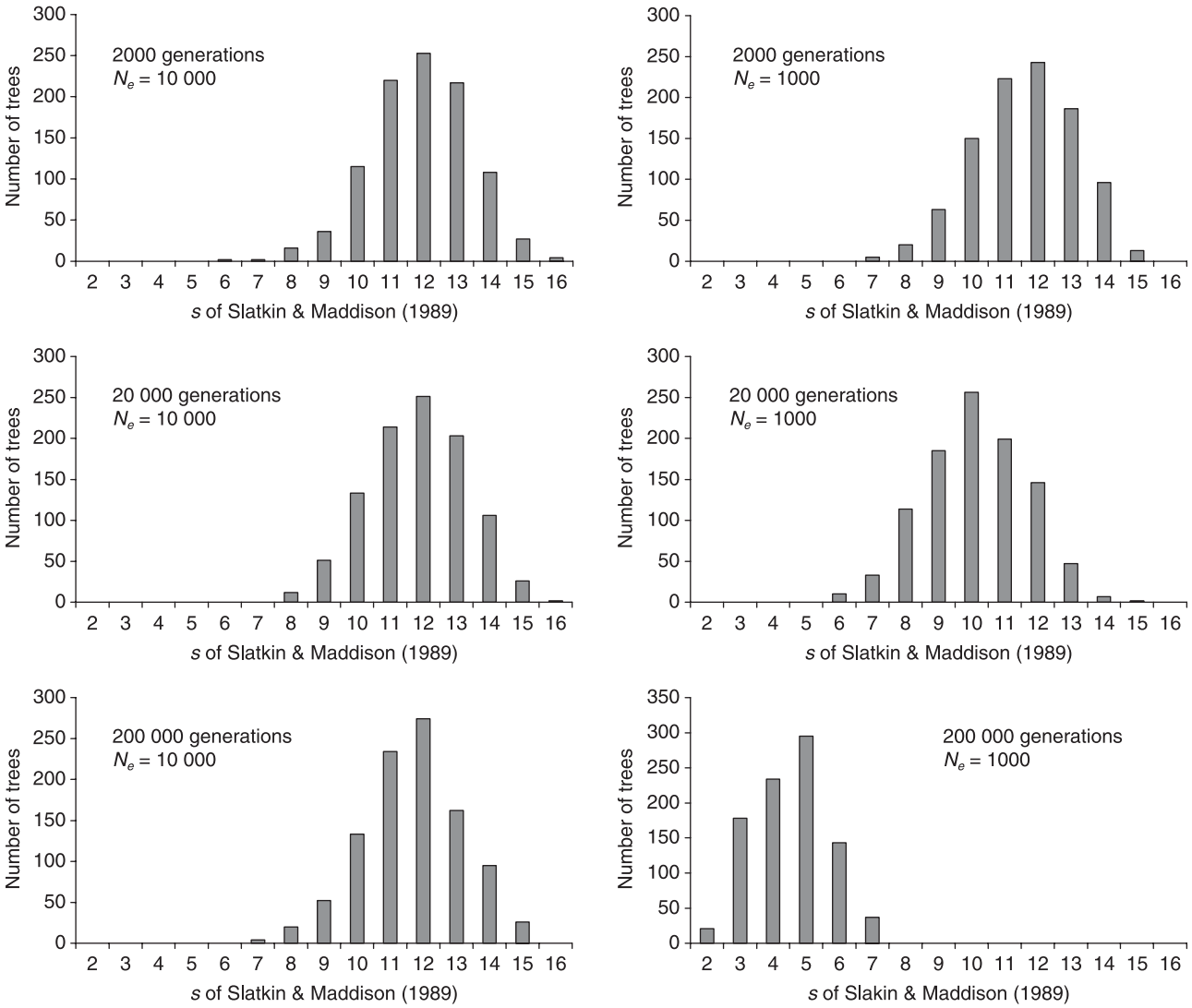

Fig. 4 Distributions of Slatkin & Maddison's (1989) gene flow statistic *s* for 1000 simulated gene trees within three population trees, considering two population sizes (N_e = 10 000 and 1000). Populations trees are consistent with two independent taxa within lineage I and differ in their branch lengths expressed as number of generations.

populations), variation between the two nominal species was low and not significant, while most variation (70.88%, $P < 0.001$) was due to differences among populations from the same nominal species. Accordingly, Φ_{CT} was very low, while Φ_{SC} and Φ_{ST} were high. Division of the populations into the three groups suggested by the phylogenetic analysis maximized among-group variance (i.e. the Φ_{CT} value), with over 78% ($P < 0.001$) of the overall variance explained by among-group variation; variation among populations within groups was low (9.04%, $P < 0.001$).

Mismatch distribution

Overall mismatch analysis fit the unimodal distribution as expected under a model of recent population expansion (Fig. 5). The raggedness statistic was significantly low ($rg = 0.0047$, $P < 0.01$) and both Fu's and Tajima's statistics produced significant negative values ($F_S = -18.599$, $D = -1.742$, $P < 0.01$). Observed distribution for lineage I separately also fits a unimodal distribution ($rg = 0.0078$, $F_S = -22.269$, $D = -2.091$, $P < 0.01$). A model of sudden expansion was rejected for lineage II ($rg = 0.1022$) and III ($rg = 0.2222$).

Nested clade analysis

In the SP cladogram performed by TCS, haplotypes from the Sado basin (H35-H37) were not connected to any other haplotypes because mutational steps exceeded the maximum number of mutational connections justified by the parsimony criterion. To connect these haplotypes to

Table 3 Analyses of molecular variance (AMOVA). In AMOVA 1, individuals collected in the same locality were grouped in the same population, independently of the taxonomic status; in AMOVA 2, two groups were formed, one with all *Lampetra fluviatilis* populations and another with *Lampetra planeri* populations; and in AMOVA 3, populations were grouped into the three lineages as suggested by the phylogenetic analysis (see Fig. 3)

Source of variation	Variance components	% variation	*P*	Φ statistics
AMOVA 1				
Among populations	2.97451	70.54		
Within populations	1.24229	29.46	< 0.001	Φ_{ST}: 0.70540
AMOVA 2				
Between species	0.03936	0.96	> 0.01	Φ_{CT}: 0.00959
Among populations within species	2.90837	70.88	< 0.001	Φ_{SC}: 0.71568
Within populations	1.15543	28.16	< 0.001	Φ_{ST}: 0.71840
AMOVA 3				
Among groups	7.62917	78.22	< 0.001	Φ_{CT}: 0.78220
Among populations within groups	0.88196	9.04	< 0.001	Φ_{SC}: 0.41519
Within populations	1.24229	12.74	< 0.001	Φ_{ST}: 0.87263

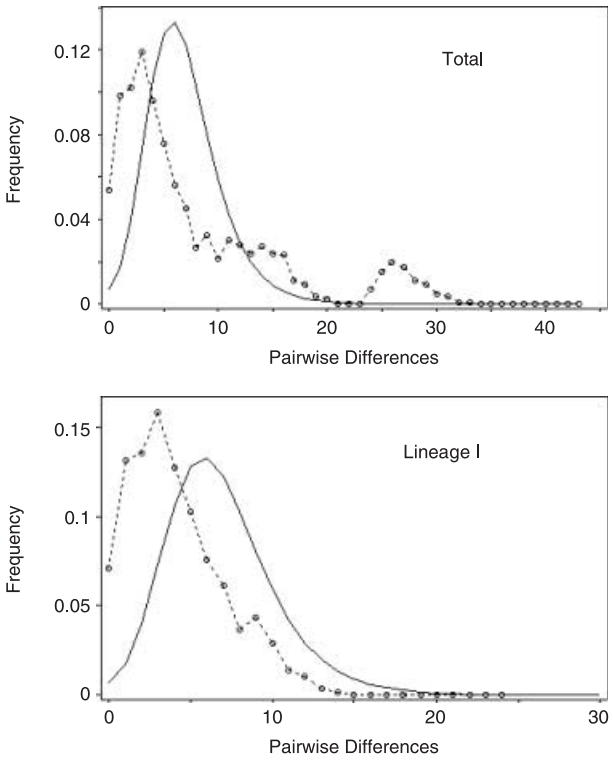

Fig. 5 Frequency distributions of pairwise number of mutational differences between *Lampetra* individuals observed in all mtDNA evolutionary lineages combined (top panel) and separately in lineage I (bottom panel). Circles represent the observed data, and the line is the model fitted to the data.

the cladogram, a minimum spanning network was constructed using ARLEQUIN. The parsimonious cladogram was not fully resolved, as five loops were encountered. Ambiguities were resolved by breaking the connections marked by a solid arrow in Fig. 6. In loop 1, the branch 1 A connecting H7 and the missing haplotype between H16 and H17 appeared the most unlikely according to the frequency, topology and geographical criteria, while the branch 1B was broken according to the geographical criterion. Connection between H6 and H2 (branch 1C) seemed more likely, because H2 is more frequent and co-occurs with H6 in the Tejo Basin. In loop 2, connection relating haplotype H15 and H7 (branch 2A) seemed the least likely according to the frequency criterion. In loop 3, the branch 3A connecting H10 with H6 was broken according to the geographical criterion. ARLEQUIN software connected the haplotype H37 from the Sado basin with both H6 (branch 4B) and H10 (branch 4A). According to the frequency and geographical criteria, the branch 4B was the most plausible. The most complex ambiguity was that represented by branches 5. Haplotype H4 is present in populations from Sweden and Norway (localities 1 and 2, respectively) that also have individuals with haplotype H6. Therefore, using the geographical and frequency criteria, the 5B connection was broken. The break of 5B was also supported by the fact that H3 co-occurs with H30.

The NCA (Fig. 7) detected three main clades, which correspond to the main lineages defined in the NJ, MP and ML trees. The 4-1 clade corresponds to lineage I; the 4-2 clade corresponds to lineage II; and the highly distant 4-3 clade corresponds to lineage III. The main discrepancy between the phylogenetic trees and SP cladogram was the position of the 3-5 clade, which in the former was placed within lineage I (clade 4-1) while in the latter was a subclade of clade 4-2 (lineage II).

The null hypothesis of no association between the position of haplotypes in the cladogram and geographical location was rejected ($P < 0.05$) for several clades (Table 4). The latest inference key of Templeton (2004) suggested

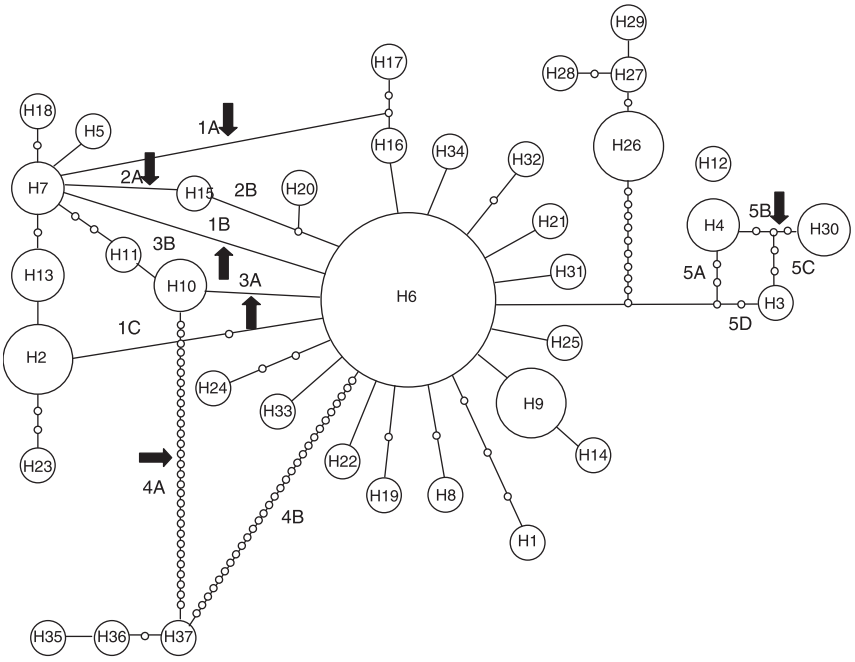

Fig. 6 Statistical-parsimony cladogram and loop solutions. Ambiguities in loops 1–5 were resolved by breaking the connections marked by a solid arrow. Haplotypes are identified as in Table 2. Haplotype frequencies are proportional to the area of the circle. Each line represents one mutational change. Empty circles indicate intermediate haplotypes not present in the sample.

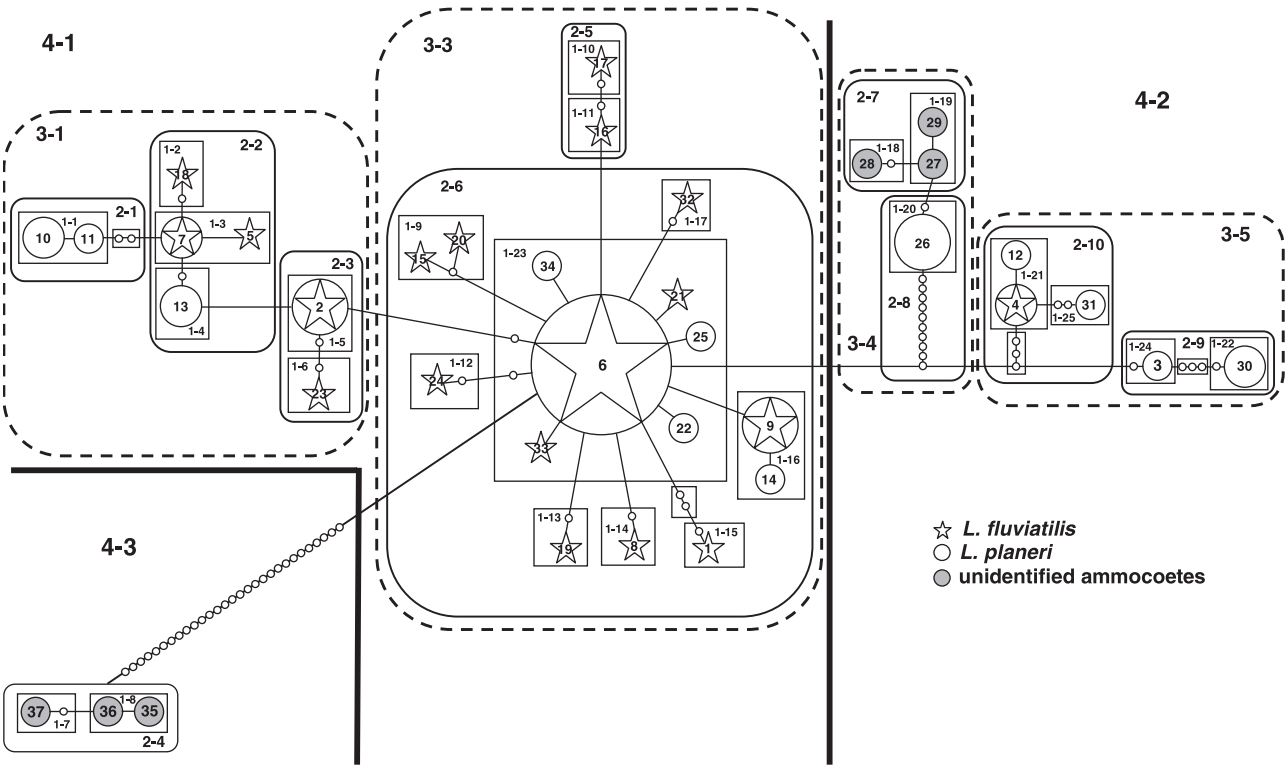

Fig. 7 Nesting design inferred from the cladogram estimation of the 37 haplotypes detected for *Lampetra*. Haplotypes belonging to the same clade level are boxed up to clade level 4-x. Clade designations are given with each box that contains observed haplotypes. Each line in the network represents one mutational change. Small empty circles represent the inferred, nonsampled interior haplotypes. The number inside each circle and stars identifies the haplotypes, as in Table 2.

Table 4 Chi-squared test of geographical association of clades and biological inference from nested clade. *P* is the probability of obtaining a χ^2 statistic larger than or equal to the observed statistic by randomly permuting the original contingency 999 times. For each clade with significant geographical associations as detected by permutation test, it is indicated the inference chain followed by a biological interpretation according to Templeton (2004)

Clade	Permutational χ^2 statistic	*P*	Inference chain	Inferred pattern
1-23	65.93	0.04	1-2-11-17-No	Inconclusive outcome
2-6	131.90	0.006	1-2-3-4-No	Restricted gene flow with isolation by distance
3-1	20.30	0.012	1-2-3-5-6-7	Restricted gene flow/Dispersal but with some long distance dispersal
4-1	22.20	0.033	1-2-3-5-15	Past fragmentation and/or long-distance colonization
4-2	12.00	0.006	1-2-3-5-15	Past fragmentation and/or long-distance colonization
Total cladogram	00.00	0.000	1-2-3-5-15	Past fragmentation and/or long-distance colonization

past fragmentation and/or long-distance colonization as the processes that might have produced the four-step clades. The same processes might have created the three-step clades inside both the 4-1 and 4-2 clades. Because of the lack of significant clade distances, no inference was possible for the three-step clades, except for clade 3-1 which has a geographical structure compatible with restricted gene flow or dispersal but with some long-distance dispersal. Restricted gene flow with isolation by distance might have produced the geographical pattern within the clade 2-6. At the one-step level, only the 1-23 clade showed significant values; however, results were inconclusive.

Discussion

Single origin vs. multiple origins vs. ecotypes?

Phylogeographical analysis and statistical contrasts of topologies predicted by the hypothesis of single origin of *Lampetra planeri* from *Lampetra fluviatilis* (Fig. 1a) suggest that the two taxa did not form reciprocal monophyletic groups, excluding this hypothesis. AMOVA analysis corroborates this result, as among-group variance (i.e. the Φ_{CT} value) was maximized when populations were grouped according to phylogroups rather than to specific status. Statistical contrasts of topologies consistent with the alternative hypothesis of multiple origins (Fig. 2a) rejected monophyly of the two taxa within evolutionary lineages. Lack of monophyly may be the result of ongoing gene flow (hypothesis of different ecotypes of a single species, Fig. 1c) but may also be the artefact of retained ancestral polymorphism. While the presence of reciprocal monophyly is a powerful evidence for the evolutionary independence of lineages, taxa may be evolving independently even in the absence of monophyly (Neigel & Avise 1986). Coalescent simulations examining the effect of time on lineage sorting showed that lineage sorting between taxa within lineage I would require at least 1 million years. Considering that central and northern European populations were founded after glaciers retreating around 10 000–12 000 years ago (discussed below), the time required for complete lineage sorting predates the populations' origin. Our observed tree is consistent with the divergence of the forms around 2000 generations (10 000–14 000 years) ago. Therefore, we failed to reject incomplete lineage sorting as an explanation for the observed pattern of nonmonophyly of each form within evolutionary lineages. The observed pattern of mtDNA geographical variation across the two taxa is therefore consistent with either the hypothesis of multiple independent origins of the nonparasitic resident form from the parasitic migratory one followed by reproductive isolation (Fig. 1b) or the hypothesis of alternate life-history forms of a single species (Fig. 1c).

Multiple independent divergences of freshwater populations out of diadromous populations through repeated independent evolution of the same reproductive isolating mechanism seems to be a common trend in diadromous fish (Schluter & Nagel 1995). Parallel speciation has been suggested for many examples of species pairs, namely for *Gasterosteus aculeatus* (reviewed in McPhail 1994; McKinnon & Rundle 2002), *Oncorhynchus nerka* (Foote *et al*. 1989; Taylor *et al*. 1996), *Oncorhynchus tshawytscha* (Healey 1991), *Salmo salar* (Thomaz *et al*. 1997), and *Salmo trutta* (Bernatchez *et al*. 1992). Parallel evolution of species pairs should implicate deterministic factors, that is natural and/or sexual selection, because it is unlikely that random factors could produce the same pattern several times (Endler 1986). *Lampetra fluviatilis* and *L. planeri* show marked differences in adult size, which appears to be a major determinant of assortative mating, as lampreys choose mates of similar sizes (Hardisty & Potter 1971b) and fertilization success decreases with increasing difference in body size (Hardisty & Potter 1971b; Malmqvist 1983; Beamish & Neville 1992).

Ecotypes are also known in many anadromous species, in which a small proportion of individual fish are non-anadromous (Gross 1987; Jonsson & Jonsson 1993). Because of this fact, McDowall (1997) considered that anadromy is

a trait with limited value in establishing phylogenetic relationships. It has been suggested that the body size attained at the age of maturation could be the main determinant for the larvae to choose which alternative life-history tactic maximizes its fitness. For example, in the Atlantic salmon the expression of alternative tactics is apparently dependent on a combination of additive genetic effects, parental life history and habitat quality that will ultimately shape juvenile growth rate (Garant *et al*. 2003). Hardisty (1986a) suggested that nonanadromous forms might be expected to arise from polymorphic lamprey species, such as *L. fluviatilis*, which is highly variable in what concerns age at metamorphosis, body size at the age of maturation and fecundity. Landlocked populations reported for Finland (Tuunainen *et al*. 1980), Lake Ladoga in the Baltic drainage (Hubbs & Potter 1971) and Loch Lomond in Scotland (Maitland 1980; Maitland *et al*. 1994) show lower body size and fecundity than those of the anadromous form. Also there is variability in the duration of the adult feeding phase. Most lampreys stay two or more years at sea before returning to fresh water to reproduce, but some lampreys known as praecox remain only one summer at sea. Lampreys with the first phenotype attain larger size and are expected to achieve higher fecundity than the praecox form. Hardisty (1986a) suggested that parasitic lampreys like *L. fluviatilis* are in a sensitive balance between the fitness advantage of being large, fecund and anadromous and that of being small, less fecund, strictly freshwater, but avoiding the fitness cost of migration. Aside from the energetic cost of migration, there is substantial 'cost of waiting' associated with running to sea. Longer generation time resulting from the time in the sea reduces the intrinsic rate of increase of the phenotype with longer generation time, and there is more probability to die before reproduction.

The hypothesis of multiple origins of nonparasitic resident lampreys from parasitic migratory ones (Fig. 1b) and the hypothesis of ecotypes of a single species (Fig. 1c) are not, however, mutually exclusive. Instead, and as suggested by Salewski (2003), the latter scenario might lead to future speciation due to assortative mate choice between the forms. If nonparasitic resident lampreys had multiple origins both in space and in time, it might be possible that speciation might still be in progress in some populations, where both forms have not reached the status of reproductive isolation. According to Huggins & Thompson (1970), hybridization can be a common phenomenon.

Phylogeography

European *Lampetra* is composed of three major evolutionary lineages. Phylogenetic analysis suggests that parasitic migratory and nonparasitic freshwater populations from central and northern European rivers belong to a single evolutionary lineage, the lineage I, which is also found in two Iberian rivers, the Mondego and Tejo rivers. The Iberian Peninsula presents two additional evolutionary lineages, the lineages II and III. NCA analysis suggests a different scenario, since the clustering of the clade 3-5 within the lineage II (clade 4-2) implies that this evolutionary lineage is found outside the Iberian Peninsula, in central and northern Europe.

During the late Pliocene and the Pleistocene, much of Europe was alternately covered by glaciers during cool periods and uncovered during the warmer interglacial periods when the glaciers retreated (Webb & Bartlein 1992). Lamprey populations from previously glaciated regions, such as those in central and northern Europe, show low levels of nucleotide diversity ($\pi = 0.00185$) and weak phylogeographical patterns, while populations from the Iberian Peninsula unaffected by habitat contractions show higher levels of nucleotide diversity ($\pi = 0.00661$) and stronger phylogeographical structure. The geographical distribution of genetic variation strongly suggests the Iberian Peninsula as a refugial area, providing suitable conditions for the survival of multiple ancestral lamprey populations over several ice ages. As the climate warmed and the ice retreated, anadromous populations had the opportunity to disperse northwards. Anadromous fishes had access to largely unexploited resources in the aquatic habitats uncovered by deglaciation, which according to Bell & Andrews (1997) might have favoured the establishment of freshwater isolates. McDowall (1997) suggested that in some species there is a latitudinal shift in the prevalence of diadromy. For example, temperature seems to be one of the main factors responsible for the limited occurrence of anadromy in *Salmo trutta* populations from the northern rivers of Iberian Peninsula (Weiss *et al*. 2000). Lampreys are largely affected by water temperature, exhibiting an antitropical distribution (Hardisty & Potter 1971b; Potter 1980). Therefore, one may hypothesize that during the interglacials while the *Lampetra* populations were expanding northwards, populations at lower latitudes would tend to abandon anadromy and eventually became restricted to freshwater. The fact that lineage III seems to be restricted to the Iberian Peninsula, suggests that by the time of the end of last glaciation (10 000–12 000 years ago) this lineage might have already lost the migratory form, precluding its ability to disperse northwards via sea. In contrast, lineage I and possibly lineage II presently include both the migratory and the resident forms, being widespread throughout central and northern Europe. Lineage I showed a star-like phylogeny with few high frequency ancestral haplotypes (H6) and numerous low frequency alleles separated from the ancestral ones by few mutational steps. This topology suggests a sudden demographic expansion, which was also supported by the mismatch analysis. NCA suggested that clades 4-1 and 4-2 (lineages I and II,

respectively) might have attained their current range by long distance colonization, which is concordant with the anadromous character of the *fluviatilis* form.

Hardisty & Potter (1971b) and Hardisty (1986a) suggested that last glaciation played an important role in the evolution of the European brook lamprey, due to blocking of migratory routes. For example, migration of *L. fluviatilis* from eastern Baltic would have ceased at the height of the last glaciation, favouring the emergence of resident populations. This scenario would imply the existence of additional refugia in central and northern Europe, where resident populations could have survived over the glacial period. Reproductive isolation among fragmented resident populations would result in allopatric divergence (Hewitt 2000). The present phylogenetic and NCA analyses failed, however, to identify such putative refugia, which is inconsistent with the hypothesis of Hardisty and Potter. The only exception may be the Rhone basin, a basin from southern France which drains to the Mediterranean Sea, as its haplotypes (H10 and H11) defined a well-supported clade (clade 2-1). A refugium located in southern France that allowed postglacial recolonization of western European rivers has been suggested for the perch *Perca fluviatilis* (Nesbø *et al*. 1999), and for the chub *Leuciscus cephalus* (Durand *et al*. 1999). However, the small sample sizes in neighbouring basins preclude testing the hypothesis that this area also acted as a source for foundation of lamprey populations in western basins.

The population of *L. planeri* from the Danube River, which drains to the Black Sea, belongs to lineage I. This result is consistent with the hypothesis of Holčík (1995) who suggested that this population has originated through postglacial interconnections among various river systems in central Europe.

Overall extent of lineages' divergence suggests that there has been a long-term barrier to the gene flow between populations. The most ancient separation would have involved the population from the Sado basin (lineage III) and all others. In the Iberian Peninsula, endorheic drainages developed along active geological faults during the Upper Miocene (Calvo *et al*. 1993). Lower Tejo and Sado probably formed a single basin until the Tortonian (Late Miocene), when the tectonic events related to the Betic orogens led to the formation of present-day rivers. In the Pliocene, when draining was already fluvial and exorheic, these two basins were probably independent because of the presence of the Arrábida Mountain (Azevêdo 1983). During the last Würm glaciation, which occurred 16 000–18 000 years ago, sea level along the Atlantic cost of Iberian Peninsula was estimated to be 130–140 m below the present level, resulting in the interconnection of many adjacent rivers (Dias *et al*. 1997). However, that did not happen with the Tejo and Sado rivers, which kept separated, as the bathymetry of the canyons associated with those rivers (the Cascais and Setúbal canyons, respectively) suggests (N. Pimentel, personal communication). NCA identified past fragmentation as the process that might have produced the main lineages' (four-step clades) divergence, corroborating this scenario.

Conclusion

The combined use of phylogeographical, nested-clade and coalescent analyses of mtDNA diversity proved to be very useful in improving knowledge on the evolutionary history of the European river and brook lampreys. Phylogeographical patterns across the two taxa suggested that *Lampetra planeri* originated within at least two distinct evolutionary lineages, the lineages I and II. The low number of specimens exhibiting mtDNA of lineage III together with the fact that they were all ammocoetes of unknown specific status did not allow telling what life-history types this lineage includes. Structure within lineage I is consistent with the hypothesis of divergence of both taxa around 2000 generations ago with no subsequent gene flow, approximately corresponding to last glacier retreat. The alternative hypothesis that the parasitic anadromous and nonparasitic freshwater lampreys are ecotypes of a single species could not, however, be excluded. The use of more variable genetic markers, like microsatellites, is necessary to test these two alternative hypotheses as recently derived species pairs are most times only distinguished by distinct frequencies of characters rather than by fixed differences (Taylor 1999).

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