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Contrasts in the phylogeography of two migratory lampreys in western Europe

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Abstract. The ways that organisms respond to climatic oscillations depend on a wide range of factors, including life-history, behaviour, thermal preferences and physiology, and ecology. To investigate these processes, we compared the phylogeographic patterns found in *Lampetra fluviatilis* and *Petromyzon marinus* in Europe. We reanalysed all molecular data available for the mitochondrial non-coding region subunit I for both species. For *L. fluviatilis*, we also analyzed new and existing data for ATPase subunits 6 and 8, which cover a wider geographic range. In *L. fluviatilis*, both gene diversity and nucleotide diversity are at least three times higher than in *P. marinus* in Europe. *L. fluviatilis* shows population differentiation in Europe and displays a deeper haplotype network, with no predominance of an ancestral haplotype, which contrasts with a star-like pattern for *P. marinus*. Bayesian skyline plots for the two species fit exponential models and, with estimates of the times to the most recent common ancestor in each species, indicate that *P. marinus* has much younger populations in Europe, supporting the hypothesis of its relatively recent migration from North America. The differences in phylogeographic structures of these two species are discussed considering the likely effects of differences in their thermal preferences, migration abilities, and times available for diversification.

Keywords. *Lampetra fluviatilis*, *Petromyzon marinus*, phylogeography, co-distributed species, Europe

Introduction

The way organisms respond to climatic oscillations like the glacial cycles of the Pleistocene is dependent on a wide range of factors, like life-history, behaviour, thermal preferences and physiology, ecological optima previously evolved and specific habitat requirements. This means that even closely related species may respond very differently to historical climatic conditions and also to modern ecological discontinuities that sometimes represent a dispersal barrier for a number of species, while being highly permeable to migration by others (Domingues et al. 2006, Patarnello et al. 2007, Domingues et al. 2008, Neethling et al. 2008, Ayre et al. 2009, Larmuseau et al. 2009, Pelc et al. 2009; but see Dawson 2012). Almada et al. (2012) argued that for north temperate fish, small differences in thermal tolerance may affect drastically the responses to cold periods, affecting both the areas available for refugia and speed of colonization of the deglaciated areas.

Migratory lampreys in Western Europe provide an opportunity to compare phylogeographic and historical demographic patterns in two related animals with similar European distributions. Two migratory lamprey species occur in West Europe: the sea lamprey *Petromyzon marinus* L., 1758 and the river lamprey *Lampetra fluviatilis* (L., 1758). The sea lamprey occurs on both sides of Atlantic (from the Labrador coast to Florida in North America, and from Norway to the Mediterranean in Europe) while *L. fluviatilis*, the river lamprey, is restricted to the eastern margin of the Atlantic (from Norway to the Mediterranean) (Kelly and King 2001). Both being anadromous, they have to cross inshore waters twice in their life cycle and find streams with appropriate substratum, where they spawn and die. The larvae stay in freshwater filter-feeding in the sediment, and migrate to the sea after four to nine years (Renaud 2011).

Genner et al. (2012) performed a phylogeographic study of Atlantic *P. marinus* using mitochondrial non-coding region I (*NCR-I*). They

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found that the European population has a very low genetic diversity, little population structure, and is dominated by two haplotypes. The North American population is more diverse, and estimation of population size and age point to a larger effective population size and an older origin (Genner et al. 2012). In view of this, these authors raised the possibility of a very severe bottleneck in the European sea lamprey and/or a recent origin from an older American lineage.

There are no similar phylogeographic data describing *L. fluviatilis*. However some fragmentary information is available that suggests the genetic diversity in *L. fluviatilis* is higher than in European *P. marinus* (Pereira et al. 2010). This information came from studies that focused on the relationship between *L. fluviatilis* and *L. planeri. L. planeri* is a collective designation for the non-feeding and non-migratory adult populations similar in the larval phase to *L. fluviatilis*, but living permanently in freshwater, spawning and dying soon after metamorphosis at a much smaller size. These studies have shown that these populations have evolved several times independently at different times and places from a *L. fluviatilis* ancestor, making the species *L. planeri* polyphyletic (Espanhol et al. 2007, Blank et al. 2008, Pereira et al. 2010).

The underlying hypothesis of this work is that the distinct distribution of the genetic diversity in *P. marinus* and *L. fluviatilis* in Europe may be the consequence of differences in behaviour and thermal preferences, together with an older history of evolution in Europe in *L. fluviatilis*.

Material and Methods

We studied the phylogeography of *L. fluviatilis* and reanalysed the data available from European *P. marinus* to compare the phylogeographic patterns and the historical demography between these two species. We have used all the sequences of a fragment of the *NCR-I* available in Genbank that enable population comparisons in both species. For *Lampetra fluviatilis*, 52 DNA sequences of the *NCR-I* from two locations (Rhine river, North Sea, and Neva river, Baltic) were

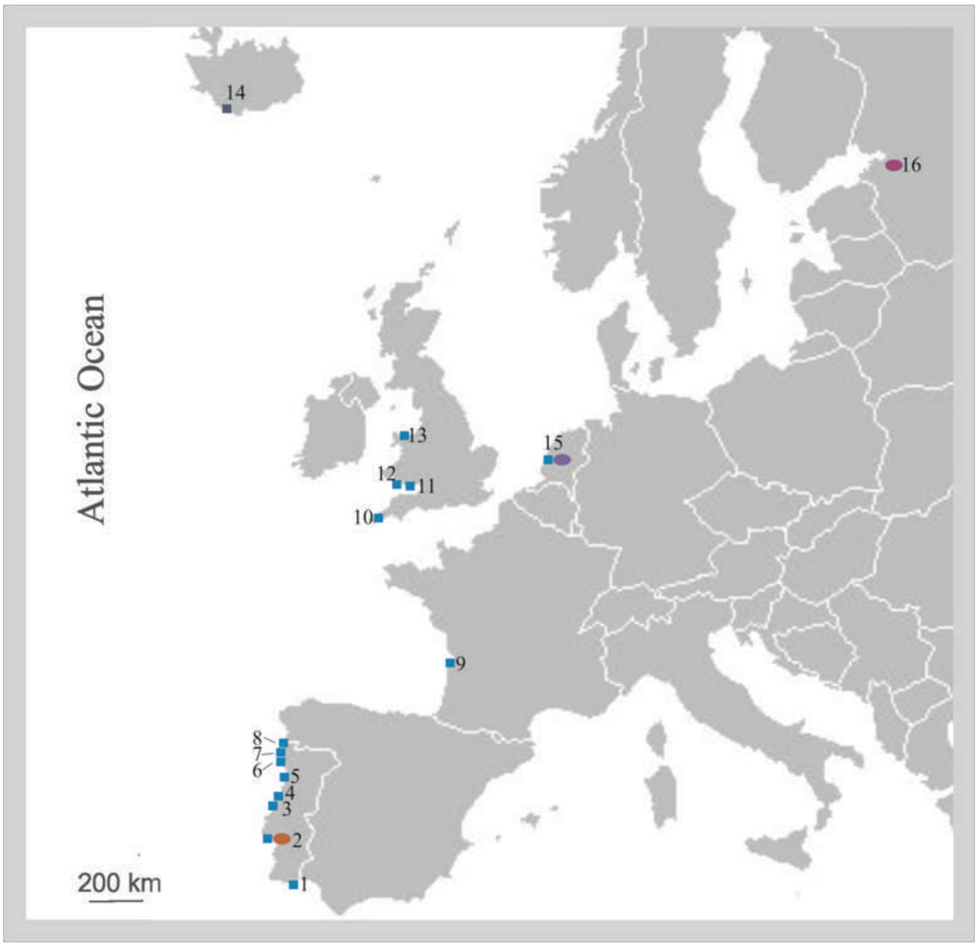

Figure 1. Map with collection sites for *P. marinus* (blue squares) and *L. fluviatilis* (ellipses).

Species	Genetic marker	Sampling location	Sample size	GenBank accession number	Reference
Petromyzon marinus	*NCR-I*	Iceland	4	HM245344-HM245347	Pereira et al. 2012
		Dee	52		
		Humber	17		
		Usk	10	JX484145-JX484295	Genner et al. 2012
		Wye	30		
		Tamar	13		
		Rhine	10		
		Garonne	26		
		Minho	31		
		Lima	37		
		Cavado	26		
		Douro	37	EF5655470-EF564742	Almada et al. 2008
		Vouga	28		
		Mondego	60		
		Tejo	17		
		Guadiana	1		
Lampetra fluviatilis	*NCR-I*	Neva	24	EU596126-EU596149	Pereira et al. 2010
		Rhine	28	EU596118-EU596144, GQ340546	Pereira et al. 2010
	ATP6 and *ATP8*	Neva	14	JX910230-JX910238 EU596200- EU596204	This study
		Rhine	18	JX910226-JX910229, JX910239, EU596219-EU596230	This study
		Tagus	18	FN641848, FN641846, AJ937926	Espanhol et al. 2007 Mateus et al. 2012

Table 1. Summary of the information on the mitochondrial non-coding region I (*NCR-I*) and ATPase subunit 6 (*ATP6*) and ATPase subunit 8 (*ATP8*) sequences.

downloaded from GenBank (Table 1, Fig. 1). For *Petromyzon marinus*, 399 sequences of the same marker from sixteen locations in Europe were downloaded (Table 1, Fig. 1).

There is a large distributional gap in *L. fluviatilis* from western France to the river Tagus in central Portugal. As there are a large number of the mitochondrial ATPase subunit 6 (*ATP6*) and ATPase subunit 8 (*ATP8*) sequences available in GenBank for the Portuguese population, we sequenced samples from the river Rhine (which drains to the North Sea) and river Neva (which drains to the Baltic). This procedure allowed a comparison of the Portuguese (18 published sequences), North Sea (18 new sequences) and Baltic (14 new sequences) river lampreys (Table 1, Fig. 1; for frequency of each haplotype see Espanhol et al. 2007, Mateus et al. 2012). Total genomic DNA was extracted with a REDExtract-N-Amp^{TM} tissue PCR kit (Sigma-Aldrich, Saint-Louis, USA) from 14 samples from the Baltic (Neva river) and 18 from the North Sea (Rhine river; Table 1, Fig. 1). Mitochondrial *ATP6* and *ATP8* were amplified, using the primers and protocol described in Espanhol et al. (2007). Sequencing reactions were performed in StabVida (Lisbon) on a 3700 ABI DNA sequencer (AppliedBiosystems) using the BigDye terminator DNA sequencing kit.

For both datasets (*NCR-I* for both species and *ATP6* and *ATP8* for *L. fluviatilis*), DNA sequences were aligned using ClustalX v.1.81 with default parameter values (Thompson et al. 1997) and truncated to obtain fragments of the same size. Relationships among haplotypes were analyzed with parsimony networks estimated by TCS v.1.18 (Clement et al. 2000). ARLEQUIN v.3.01 (Excoffier and Schneider 2005) was used to calculate genetic diversity indices, to perform analysis of molecular variance (AMOVA) and assess population differentiation in European populations of both species. ARLEQUIN also was used to estimate the number of migrants between populations in *L. fluviatilis*.

There is no fossil-calibrated molecular clock for lamprey mtDNA. Genner et al. (2012), following previous calibration for cyclostomes based on a large number of genes (Kuraku et al. 2006), found a mutation rate of 0.34% per site per million years for the *NCR-I*, which we adopt here. Past population demography was inferred in European populations of both species with the non-coding region datasets using linear Bayesian skyline plots, as implemented in BEAST v.1.6 (Drummond et al. 2005, Drummond and Rambaut 2007), using the HKY substitution model. The posterior probability distribution was generated using results from five independent runs of 100 million MCMC steps for *L. fluviatilis* and 50 million steps for *P. marinus* (burn-in of 10%), obtaining combined effective samples sizes of parameter estimates of over 200. The median and 95% confidence interval for the estimate of the age of the most recent common ancestor (t_{MRCA}) were depicted using Tracer v.1.4 (Rambaut and Drummond 2007). The evolution in time of the median effective population size of each species was analysed using regression analysis with IBM SPSS Statistics v.20. The model with the best fit to the data was retained.

Results

Genetic diversity indices for *NCR-I* for *Lampetra fluviatilis* and *Petromyzon marinus* and for *ATP6* and *ATP8* for *L. fluviatilis* are presented in Table 2. Diversity is at least three times higher in *L. fluviatilis NCR-I*, even considering the differences in sampling effort. In *P. marinus*, only 2.6% of the genetic variation in *NCR-I* is attributed to among population variation (AMOVA: p = 0.022 ± 0.005), and after Bonferroni corrections none of the pairwise comparisons between populations is significant. In *L. fluviatilis*, 9.2% of the genetic variation observed in *NCR-I* is attributed to among population variation (AMOVA: p = 0.00196 ± 0.00136),

Species	Genetic marker	N	Population	Number of haplotypes	Allelic richness	Gene diversity	Nucleotide diversity	Number of polymorphic sites	Mean number of pairwise differences
P. marinus	*NCR-I*	399	Pooled data	14	3.51%	0.3040 ± 0.0294	0.000667 ± 0.000698	11	0.383962 ± 0.363404
L. fluviatilis	*NCR-I*	52	Pooled data	21	40.4%	0.9133 ± 0.0224	0.005290 ±.003082	15	3.200603 ± 1.680498
		24	Neva river (Baltic Sea)	13	54.2%	0.9384 ± 0.0255	0.006201 ± 0.003614	11	3.739130 ± 1.954770
		28	Rhine river (North Sea)	13	46.4%	0.8862 ± 0.0416	0.004054 ± 0.002517	11	2.452381 ± 1.367719
	ATP6 & *ATP8*	14	Neva river (Baltic Sea)	6	42.9%	0.7692 ± 0.0895	0.002436 ± 0.001667	8	1.846154 ± 1.125593
		36	Rhine river (North Sea) and Portugal	5	13.9%	0.2127 ± 0.0904	0.000438 ± 0.000499	5	0.331746 ± 0.339995

Table 2. Genetic diversity indices for *P. marinus* using *NCR-I* sequences, and for *L. fluviatilis* using *NCR-I* and ATPase subunit 6 (*ATP6*) and ATPase subunit 8 (*ATP8*) sequences.

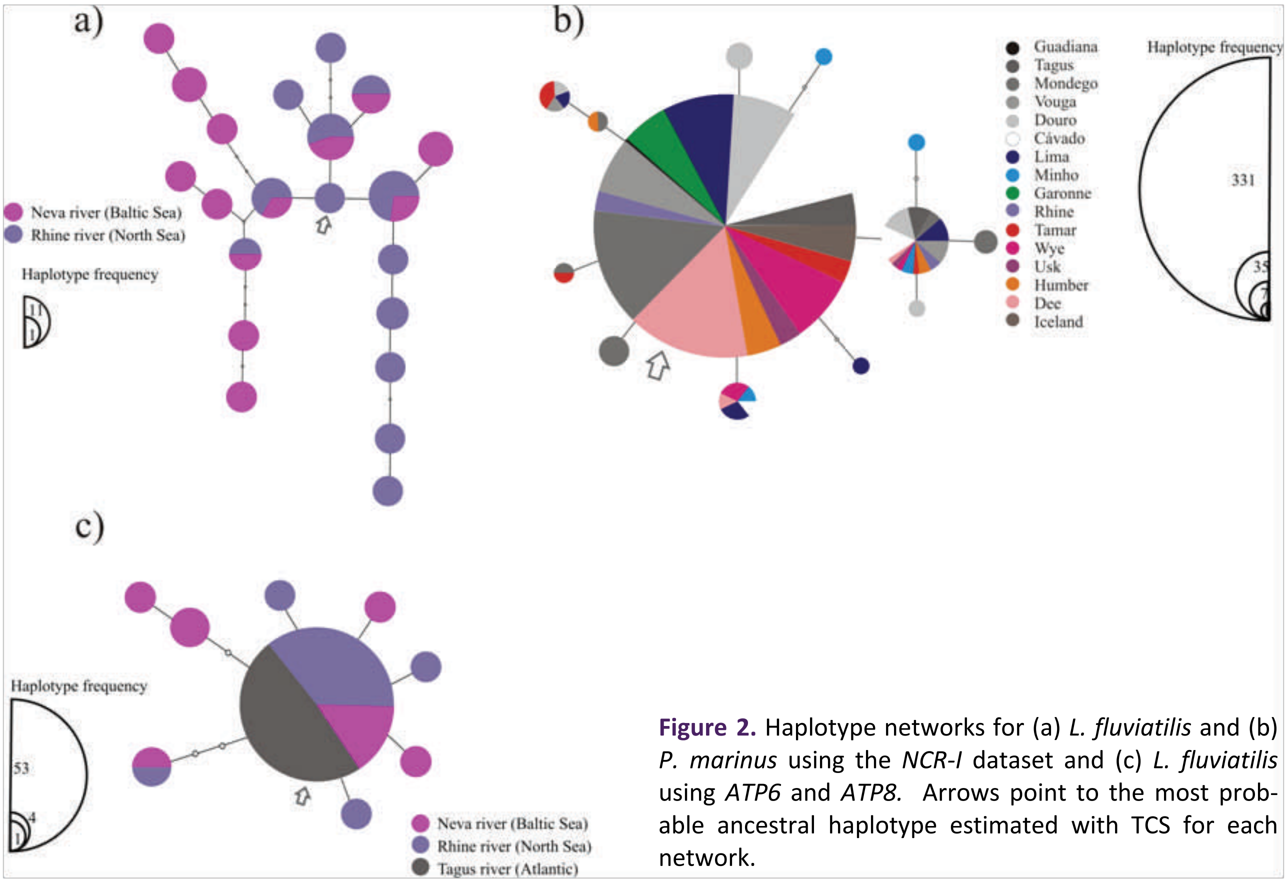

Figure 2. Haplotype networks for (a) *L. fluviatilis* and (b) *P. marinus* using the *NCR-I* dataset and (c) *L. fluviatilis* using *ATP6* and *ATP8.* Arrows point to the most probable ancestral haplotype estimated with TCS for each network.

and the two populations studied are genetically distinct ($F_{ST} = 0.092$, $p < 0.001$). Analysis of three populations of *L. fluviatilis* using *ATP6* and *ATP8*, showed no significant differences between the Tagus river (Portugal) and Rhine river (North Sea) populations ($F_{ST} = 0.009$, $p = 0.333$). AMOVA using two samples, Rhine+Tagus versus Neva (Baltic Sea), showed 24.5% of variation between groups ($p < 0.001$). However, the estimated number of migrants using *ATP6* and *ATP8* and *NCR-I* datasets is 1.54 and 4.9, respectively, which indicates that the populations are not isolated from each other. Estimated divergence between the Neva river (Baltic) and Rhine river (North Sea) in *L. fluviatilis* using the corrected number of pairwise differences with *NCR-I* and the molecular clock points to time of divergence around 74,000 years ago.

Haplotype networks for *NCR-I* show a deep genealogy, with haplotypes separated by a high number of mutational steps and without a predominant ancestral haplotype, for *L. fluviatilis*, (Fig. 2a) but a star-like pattern dominated by a central haplotype in *P. marinus* (Fig. 2b). The haplotype network for *ATP6* and *ATP8* of *L. fluviatilis* is also star-like and shallow (Fig. 2c). It is worth noting that all specimens from the Tagus, which are at the southern limit of the species range, display the ancestral haplotype.

The t_{MRCA} estimated with BEAST for *L. fluviatilis* is around 1 million years (median: 1.0022 Ma, 95% confidence intervals: 0.3733 Ma, 1.8279 Ma), and for *P. marinus* is median 375,200 years ago (95% confidence intervals: 0.018257 Ma, 0.8869 Ma). Bayesian skyline plots show that *L. fluviatilis* effective population sizes fit an exponential regression ($N_{ef} = 3.043e^{-0.001t}$, where N_{ef} is the effective population size in million females, and t is time in thousands of years; adjusted $R^2 = 0.656$; regression coefficient t-test d.f. = 50, t = -9.907, $p < 0.001$; constant t-test, d.f. = 50, t = 45.498, $p < 0.001$; Fig. 3). Other curve fitting attempts with linear and logistic models yielded worse fits (adjusted $R^2 \leq 0.584$). In *P. marinus*, the effective population size also fits an exponential regression ($N_{ef} = 0.758e^{-0.002t}$; adjusted $R^2 = 0.577$; regression coefficient t-test, d.f. = 397, t = -23.319,

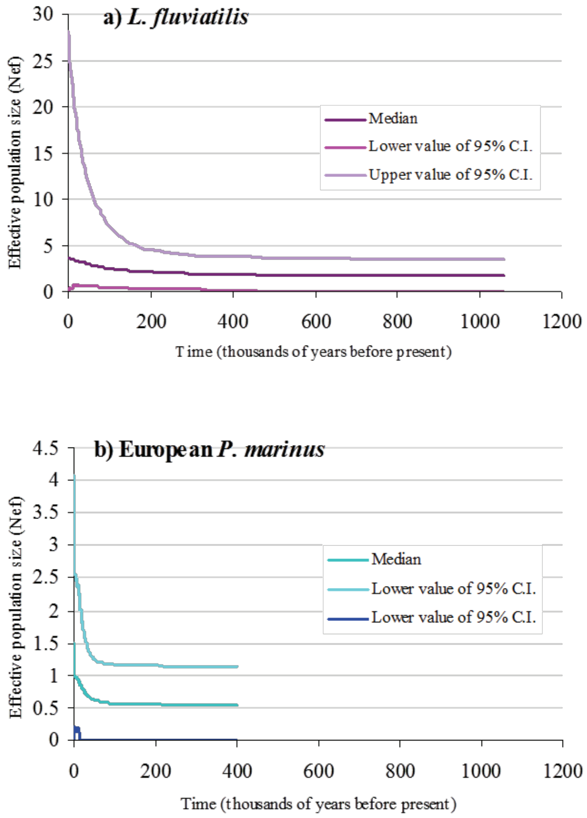

Figure 3. Past population demography estimated for (a) *L.. fluviatilis* (n = 52) and (b) *P. marinus* (n = 399) using *NCR-I*. Note different scales for effective population size. Confidence intervals (C.I.) for the median are presented.

$p < 0.001$; constant t-test, d.f. = 397, t = 444.708, $p < 0.001$; Fig. 3); linear and logistic models yielded worse fits (adjusted $R^2 \leq 0.555$).

Discussion

Phylogeographic patterns in *Lampetra fluviatilis* differ sharply from those of *Petromyzon marinus* in Europe. *L. fluviatilis* diversity is higher, geographically structured, and older than that of *P. marinus* in Europe. The effective population size of *L. fluviatilis* is greater, and population expansion began earlier for *L. fluviatilis*, than corresponding demographic metrics for *P. marinus* in Europe. These results are consistent with the hypothesis of an American origin of the sea lamprey, with an invasion of Europe in the last three hundred thousand years (Genner et al. 2012).

In many fish species of temperate waters, an acceleration of growth has been documented for the present interglacial and has been interpreted as the result of an expansion driven by deglaciations and ocean warming (Larmuseau et al. 2009, Francisco et al. 2011). In contrast, there is no indication of an acceleration of population growth in the last ten thousand years in either species, thus failing to provide evidence for a recent expansion related with warming of the North Atlantic. The observations with the two lampreys suggest that exponential growth which was ongoing during glacial periods became especially obvious as time approached the present, as a simple result of continued exponential growth. Reanalysis of previous publications on other species may be used to test the generality of this hypothesis.

Both lamprey species endured at least the last glaciation in Europe. Why then the differences in their genetic diversity? Although *L. fluviatilis* survives at temperatures similar to those tolerated by *P. marinus*, its reproduction and metamorphosis require substantially lower temperatures: between 8.5 ºC and 12 ºC in *L. fluviatilis* versus 15 ºC in *P. marinus* (Maitland 2003). These thermal requirements should have limited the areas where it would be possible for these species to breed and metamorphose, *P. marinus* being more restricted to southern areas than *L. fluviatilis*. The Iberian Peninsula was proposed to have been an important refugium for *P. marinus* during the last glaciation (Almada et al. 2008, Genner et al. 2012). *L. fluviatilis* also may have been abundant in the Iberian Peninsula during glaciations and even in the Mediterranean, where several non-migratory *Lampetra* populations persist. The fact that genetically distinct non-migratory *Lampetra* populations are found in many Portuguese rivers, and the finding that two haplotypes in the *ATP6* and *ATP8* and three in the *NCR-I* of *L. fluviatilis* were recovered from the strictly freshwater populations (Pereira et al. 2010, Mateus et al. 2011), suggest this species was once more abundant and widespread in Iberia.

The present restriction of *L. fluviatilis* to the Tagus River and its extremely low diversity in Iberia contrasts with the abundance and higher genetic diversity of *P. marinus* in Portugal. It is likely that *L. fluviatilis* in the Iberian Peninsula is at the extreme south of its range, being a relict population. A slight increase in temperatures both in sea and rivers may bring it to rapid local extinction. In the northern part of its range, the lower thermal requirements to breed and metamorphose of *L. fluviatilis* may have allowed a wider use of unglaciated areas than in *P. marinus*, what is in agreement with the old divergence between populations that are now in the North Sea and the Baltic. The divergence calculated between the Baltic and North Sea populations in *L. fluviatilis* is estimated to have occurred 74,000 years ago. Since the North Sea was partially covered by an ice sheet, and partially emerged, due to the lowering of the sea-level (Andersen and Borns Jr. 1997), this area would be unavailable for lampreys at that time. We suggest that for *L. fluviatilis* it seems plausible that survival in distinct refugia, with reduced migration among them, may have generated the patterns observed. Presently, *L. fluviatilis* is much more abundant in the southern Baltic than *P. marinus*, and the differences seem to have been consistent for hundreds of years (Thiel et al. 2009). The fact that a number of glacial lakes seem to have acted as glacial refugia from which the Baltic was recolonized by *Cottus gobio* (Kontoula and Vainola 2001), suggests that similar processes could have operated in *L. fluviatilis*, which can migrate to large freshwater bodies, surviving in absence of routes to the sea.

As a point of contrast to *L. fluviatilis* and *P. marinus*, some anadromous fishes—of which salmonids are the most studied and best known examples—use olfactory cues from the water of their natal river when adults migrate to the spawning grounds (Dittman and Quinn 1996). This behaviour tends to promote a strong differentiation between salmonids of different streams and rivers. In lampreys, although in some species larval pheromones (petromyzonol sulphate and allocholic acid) are used by migrating adults as cues to find suitable spawning habitats (Fine et al. 2004), there is no evidence of site fidelity and homing to natal waters. This could explain why anadromous lampreys, in contrast to anadromous salmonids, in general tend to show low interpopulation differentiation with haplotypes scattered in many and often distant rivers (Almada et al. 2008, Goodman et al. 2008). However, differential homing behaviour is unlikely to explain the differences between *L. fluviatilis* and *P. marinus* because both species lack homing ability. They do, however, differ in other traits like adult size, migration behaviour, and life cycle duration. Adults of *P. marinus* reach a larger size and perform long off-shore movements. Their feeding mode, which may involve being attached to large fishes or sea mammals, may promote long range migrations (Maitland 2003, Hocik 2004). In contrast, *L. fluviatilis* adults are smaller and stay in areas near the estuaries (Maitland 2003), having a higher probability of breeding in their natal river. This difference in behaviour may promote the retention of an overall higher genetic diversity in *L. fluviatilis* than in *P. marinus*. The genetic differentiation in populations present in *L. fluviatilis* may reduce the effect of the genetic drift in this species when compared with *P. marinus*, because haplotypes lost in a river by drift will likely persist in other drainages (Avise 2000). Shorter life cycle, characteristic of *L. fluviatilis* (Kelly and King 2001), may also have consequences in what concerns genetic diversity, as for the same period of time, a higher number of generations have elapsed in this species, which may have promoted a higher level of diversification.

In summary, we propose that differences in thermal requirements, in size, and in behaviour affect dispersal and can account for the different diversity patterns found in *P. marinus* and *L. fluviatilis*. We are aware however, that while *L. fluviatilis* persisted in Europe for many glacial cycles (for at least 1 million years), *P. marinus* may have migrated from North America (probably not before 300,000 years ago) having a much shorter evolutionary history in Europe, that would cause by itself a smaller scope for diversification. The evidence presented in this paper is compatible with our initial hypothesis, but is far from demonstrating the links we postulate. More work will be needed to assess the specific contributions of the different candidate factors discussed in this study.

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