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## Phylogeographical analysis reveals multiple conservation units in brook lampreys *Lampetra planeri* of Portuguese streams

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The populations of brook lamprey *Lampetra planeri* of Portuguese Rivers were analysed phylogeographically using a fragment of 644 bp of the mitochondrial control region of 158 individuals from six populations. Samples representing *L. planeri* and migratory lampreys *Lampetra fluviatilis* of rivers draining to the North Sea and the Baltic Sea were also included to assess the relationships of Portuguese samples. The data support a clear differentiation of all the populations studied. Several populations, which are isolated among themselves and also from the migratory lampreys, proved to be entirely composed of private haplotypes, a finding that supports some time of independent evolutionary history for these populations. This, combined with the geographic confinement to small water bodies, justifies the recognition of at least four conservation units in the Portuguese rivers Sado, São Pedro, Nabão and Inha.

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Key words: allopatric speciation; conservation units; *Lampetra*; phylogeography; Portugal.

### INTRODUCTION

Lampreys (Petromyzonidae) are remarkable not only for their assortment of very primitive characters but also for their diverse life histories and patterns of speciation (Hardisty, 2006). All lampreys spawn in fresh water and have a larval phase that typically lasts for several years. The eyeless ammocoetes, which are filter feeders, live buried in the sediment. After metamorphosis, their development may take one of two courses: (1) individuals may migrate downstream spending one or more years in the sea or lakes, where they adopt a parasitic or predatory way of life. Upon the

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approach of sexual maturation, they migrate back into fresh water where they spawn and die; (2) alternatively, recently metamorphosed lampreys may remain in fresh water without feeding, reaching sexual maturity at a much smaller size, spawning and dying after a short period (Kottelat & Freyhof, 2007).

While the majority of extant lamprey genera include one or more migratory species, in most of them non-migratory forms are also present (Lang *et al.*, 2009). This observation indicates that the shift between the two life-history patterns occurred independently several times in lamprey evolution. Non-migratory forms are viewed as derived from the migratory ones, leading to the concept of paired or satellite species (Zanandrea, 1959; Vladykov & Kott, 1979).

There is still a considerable debate about the taxonomic status of at least some of the non-migratory species. Exchange of genes between the two forms has not been ruled out in areas where they occur sympatrically (Schreiber & Engelhorn, 1998). On the other hand, it has been suggested that different environmental conditions could trigger different life histories, a situation that would not by itself exclude the role of genetic factors in the determination of different ontogenetic pathways (Kucheryavii *et al.*, 2007). Additionally, some authors considered that the same species could be polymorphic for feeding type and life history (Manion & Purvis, 1971; Beamish, 1987).

In Western Europe, two species of the genus *Lampetra* have been traditionally recognized: the lampera or river lamprey *Lampetra fluviatilis* (L.) and the brook lamprey *Lampetra planeri* (Bloch).

*Lampetra fluviatilis* is composed of predatory migratory fish, mostly distributed in north-west Europe, from western France and the British Isles to Scandinavia, spawning in rivers draining to the Atlantic Sea, North Sea and Baltic Sea. In south-west Europe, it is absent from Spain, where the last record of its presence dates back to 1974 in the Tagus drainage (Doadrio, 2001). In Portugal, there are apparently few records in recent years, although its previous presence in the Tagus is well documented (Doadrio, 2001).

*Lampetra planeri*, a non-parasitic and non-migratory fish, occupies a similar range in the fresh waters of north-west Europe and is absent from Spain, except for a stream close to the French border in the Pyrenees (Doadrio, 2001). In Portugal, many populations ascribed to this species are found in numerous rivers and streams (Cabral *et al.*, 2005). Some populations are present in Italy (Kottelat & Freyhof, 2007).

Both species have been assigned various levels of protection in western European countries (Kelly & King, 2001). In Portugal, fish of the genus *Lampetra* were considered critically endangered by Cabral *et al.* (2005).

Blank *et al.* (2008) compared a substantial number of mitochondrial genes and found that none of them could serve to detect consistent differences between *L. fluviatilis* and *L. planeri* in the area where both co-occur, a finding that points either to a persistent gene flow between forms or to a differentiation process which is so recent that lineage sorting in mtDNA has not yet taken place.

Espanhol *et al.* (2007), using mtDNA (ATPase 6, 8 and cytochrome *b* genes), demonstrated that *L. planeri* is polyphyletic, and thus, according to the phylogenetic species concept, does not correspond to a valid species. They showed that *L. planeri* possesses haplotypes that are shared with *L. fluviatilis* and others that are derived by numerous independent pathways from *L. fluviatilis*. Although these authors used only a few samples per river, they also showed that several Portuguese haplotypes

of non-migratory lampreys are quite distinctive and may represent ancient branches in the radiation of *Lampetra*.

The study of Espanhol *et al.* (2007) included only a small number of samples per drainage, which precludes any phylogeographic analysis. A population approach, however, is urgently needed in terms of conservation, because many Portuguese populations are confined to small or very small streams and are isolated from the sea. Therefore, they are reproductively isolated from other populations and, according to the results of Espanhol *et al.* (2007), may represent evolutionary units.

In this article, a fragment of the control region of the mtDNA was used to compare several Portuguese *L. planeri* populations, their genetic diversity, their level of differentiation and their relationships with north European populations.

### MATERIALS AND METHODS

#### SAMPLING

DNA sequences were obtained from 267 individuals for the control region (GenBank accession numbers from EU595965 to EU596199 and from GQ340523 to GQ340554; for collection site locations, see Fig. 1). Ammocoetes and adults were collected by electrofishing. While the ammocoetes of migratory and non-migratory *Lampetra* are not easily distinguishable, the

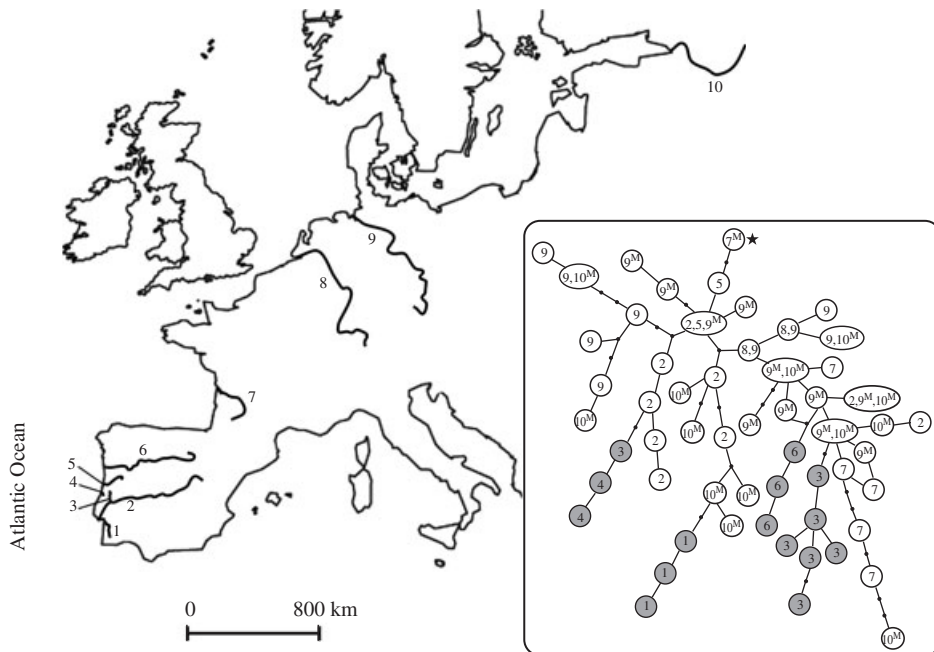


FIG. 1. Map with collection site locations and haplotype network. Haplotype number identifications refer to rivers. 1, Sado; 2, Tagus; 3, Nabão; 4, São Pedro; 5, Mondego; 6, Inha; 7, Garonne; 8, Elbe; 9, Rhine; 10, Neva. Haplotypes of migratory *Lampetra fluviatilis* are identified with superscript M. \* corresponds to the GenBank accession number Y18683. Private haplotypes present in the Portuguese populations suggested as conservation units in this article (see Discussion section) are marked with grey.

mature adults are readily classified according to size and morphology. The diagnostic criteria given by Kottelat and Freyhof (2007) were used. Ammocoetes from populations of drainages where there are no records of predatory lamprey were assigned to the non-migratory form. These include the Rivers Sado, Nabão, São Pedro, Mondego and Inha. In all these rivers, all the adults captured were small non-migratory lampreys. Because of the historical presence of the migratory form in the Tagus River, the absence of migratory fish was not sure and refrained from classifying them as migratory or non-migratory.

## MOLECULAR PROCEDURES

Total genomic DNA was extracted from fin clips or pieces of muscle preserved in ethanol by an SDS–proteinase-k-based protocol (Sambrook *et al.*, 1989 with modifications). A total of 644 base pairs (bp) of the control region (non-coding region I according to Blank *et al.*, 2008) were amplified using the primers LampFor 5'-ACACCCAGAAACAGCAACAAA-3' and LampRev 5'-GCTGGTTTACAAGACCAGTGC-3' (Almada *et al.*, 2008). PCR conditions followed Almada *et al.* (2008). Sequencing reactions were performed in StabVida on a 3700 ABI DNA sequencer (Applied Biosystems; www.appliedbiosystems.com) using the Big Dye terminator DNA sequencing kit (<http://www.stabvida.com>).

## DATA ANALYSIS

Sequences were aligned with Clustal X (Thompson *et al.*, 1997). In the control region, an indel of 39 bp was widespread, which is homologous to part of the repetitive motif described by White and Martin (2009) in *Lampetra (Okkelbergia) aepyptera* (Abbott). The insertion had a conserved sequence and did not vary in the number of repeats, and most populations were polymorphic for the indel. Thus, it was coded as a transversion to make the data amenable to subsequent analysis.

Phylogenetic analysis was performed with PAUP 4.0b10 (Swofford, 2003) using maximum parsimony (MP). Relationships among haplotypes were analysed with a parsimony network estimated by the software TCS version 1.18 (Clement *et al.*, 2000). Because the river Inha was comparatively overrepresented (49 samples) with only three haplotypes, the number of samples included in the haplotype network was reduced to 32. Care was taken to ensure that the reduction kept the relative frequency of haplotypes constant and that all of them were included.

Arlequin software package version 3.01 (Excoffier & Schneider, 2005) was used to estimate genetic diversity indices, to assess population differentiation and to perform a Mantel test between geographic and  $F_{ST}$  values and an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992). Mean numbers of inter-population pair-wise differences were corrected by subtracting the average within-population differences for the populations of each pair. To further test the relationships among populations, the corrected mean number of pair-wise differences was used to perform a multidimensional scaling using the software SPSS 16.0 (SPSS Inc., 2008).

## RESULTS

A total of 267 sequences were obtained. The number of variable sites was 31 (one of which corresponds to an indel of 39 bp, coded as a transversion, see Materials and Methods for further details), of which 20 were parsimony informative. Fifty-eight haplotypes were found. The average percentage of divergence among haplotypes was 1.97% (corresponding to 11 differences), while the most divergent haplotypes differed by 2.16% (corresponding to 13 differences).

In Table I, the number of samples, number of haplotypes, percentage of private haplotypes, genetic diversity indices and mean and maximum number of differences

TABLE I. Number of *Lampetra* spp. samples, number of haplotypes, percentage of private haplotypes, genetic diversity indices, maximum and mean numbers of pair-wise differences per population

N	Number of haplotypes	Percentage of private haplotypes	Gene diversity	Nucleotide diversity	Maximum number of differences between haplotypes	Mean number of pair-wise differences within population
Rhine*	8	62.5	0.8409 ± 0.0328	0.007450 ± 0.004185	10	4.492424 ± 2.269317
Elbe*	2	0	0.4583 ± 0.0954	0.000764 ± 0.000793	1	0.458333 ± 0.425019
Garonne*	5	100	0.8571 ± 0.1083	0.006596 ± 0.004202	8	3.964286 ± 2.216707
Inha*	3	100	0.3452 ± 0.0764	0.000597 ± 0.000654	2	0.358844 ± 0.354095
Mondego*	2	50	0.1250 ± 0.1064	0.000208 ± 0.000376	1	0.125000 ± 0.202014
São Pedro*	2	100	0.3913 ± 0.0912	0.000652 ± 0.000705	1	0.3913 ± 0.379463
Tagus†	11	63.6	0.8824 ± 0.0643	0.006036 ± 0.003589	7	3.627451 ± 1.927931
Nabão*	7	100	0.6608 ± 0.1143	0.002890 ± 0.001965	11	1.736842 ± 1.056511
Sado*	3	100	0.6552 ± 0.0418	0.001442 ± 0.001160	2	0.866935 ± 0.626696
Neva‡	13	38.5	0.9384 ± 0.0255	0.006629 ± 0.003827	10	4.003623 ± 2.073226
Rhine‡	28	53.8	0.8862 ± 0.0416	0.004513 ± 0.002748	9	2.73059 ± 1.493020

\**Lampetra planeri* non-migratory population.

†Uncertain life-history population.

‡*Lampetra fluviatilis* migratory populations.

between haplotypes for each population are shown. Inspection of Table I shows that the highest levels of genetic diversity are found in populations of the Rivers Neva, Rhine, Garonne and Tagus, while the Portuguese Rivers Mondego, Inha, Nabão, São Pedro and Sado plus the Elbe population show the lowest genetic diversity levels. The percentage of private haplotypes reaches 100% in Rivers Sado, Nabão, Inha, São Pedro and Garonne. Thus, the Rivers Sado, São Pedro, Inha and Nabão in Portugal stand out for both the low levels of genetic diversity and the presence of private haplotypes. The Mantel test failed to detect a correlation between geographic and genetic distances among populations (correlation coefficient =  $-0.302$ ;  $P > 0.05$ ), providing evidence against an isolation by distance model of population differentiation. Indeed, the most distinct populations are found in a restricted geographical area in Portugal.

An AMOVA including all populations detected significant inter-population variation ( $V_a = 2.01047$ ;  $P < 0.001$ ;  $V_b = 0.96110$ ).

In Table II, the net average differences among all pairs of populations and the corresponding  $F_{ST}$  values are shown. Both values were significant for all pairs of populations, also allowing for a Bonferroni correction. It is interesting to note that, even within the same drainage, significant differences could be detected among populations, although with low-to-moderate  $F_{ST}$  values. These are the cases of the migratory and non-migratory fish from the Rhine and those of the Tagus and its tributary Nabão. The highest  $F_{ST}$  values were those found in comparisons involving the Sado population, and this was also true for net average differences. A very high level of population differentiation was also found in comparisons involving the São Pedro River.

In Fig. 2, the relationships among populations are summarized using a multidimensional scaling based on the corrected average pair-wise differences (stress = 0.1240; RSQ = 0.9513). Inspection of Fig. 2 reveals two salient features: there is a grouping near the centre of the graph, which includes migratory and non-migratory fishes from the Rhine, and samples from the Neva, Garonne, Tagus and Mondego rivers. Several Portuguese populations of small rivers occupy very peripheral positions and are also quite separated from each other.

Maximum parsimony analysis produced 50 000 equally parsimonious trees, which failed to recover the distinction between migratory and non-migratory lampreys, confirming the results of Espanhol *et al.* (2007). The MP trees were basically unresolved, except for a few haplotype groups with weak supports.

In Fig. 1, a parsimony haplotype network is presented. Inspection of this figure supports the following conclusions: (1) Lack of monophyly of the non-migratory. Indeed, *L. planeri* include haplotypes that were independently derived from haplotypes found in migratory *L. fluviatilis* at least nine times. Moreover, at least 3 haplotypes were shared between migratory and non-migratory forms. (2) The populations of the Rivers Sado, São Pedro, Inha, Nabão and Garonne, in which only private haplotypes were found, contained sequences that were derived from other haplotypes of the same river, strongly suggesting a considerable history of local independent evolution. Nabão and Garonne populations are polyphyletic, while the remaining three are monophyletic. (3) In contrast to these clades confined to specific rivers, several haplotypes found in migratory *L. fluviatilis* have a wide geographic distribution, with some found in the Tagus in Portugal and in the Neva near the Russian Baltic Sea. (4) In the Tagus, where migratory *L. fluviatilis* were recorded

TABLE II. Population pair-wise  $F_{ST}$  (distance method; pair-wise differences) (above diagonal) and corrected average pair-wise differences (below the diagonal) for sample populations of *Lampetra* spp.

	Rhine*	Elbe*	Garonne*	Inha*	Mondego*	São Pedro*	Tagus†	Nabão*	Sado*	Neva‡	Rhine‡
Rhine*	4.49242	0.29818	0.32733	0.598	0.30385	0.65838	0.24835	0.5123	0.68412	0.23138	0.27667
Elbe*	1.42803	0.45833	0.62208	0.93389	0.89394	0.94561	0.56165	0.78268	0.91306	0.49514	0.46655
Garonne*	2.16558	2.47619	3.96429	0.84337	0.6625	0.83559	0.38379	0.54133	0.81941	0.20909	0.17001
Inha*	2.97263	5.40136	4.21088	0.35884	0.9082	0.88304	0.5167	0.89089	0.94037	0.63347	0.71425
Mondego*	1.42424	2.45833	2.51786	3.00425	0.125	0.95299	0.403	0.77923	0.90899	0.4069	0.48106
São Pedro*	5.37632	7.26268	6.0722	2.78819	5.80435	0.3913	0.62164	0.88646	0.94112	0.69338	0.75153
Tagus†	1.39629	2.75572	2.3083	1.23928	1.35294	2.87951	3.62745	0.57483	0.76801	0.27395	0.33164
Nabão*	3.70833	4.16886	2.70207	5.97579	3.55263	7.65961	3.58686	1.73684	0.86873	0.50532	0.51412
Sado*	5.86767	7.71237	6.37345	8.80038	6.25403	10.62088	6.07225	7.84285	0.86694	0.66474	0.77302
Neva‡	1.29364	2.59194	1.05771	2.62149	1.73777	4.9692	1.45298	3.08591	4.35118	4.00362	0.08351
Rhine‡	1.41468	1.70933	0.55456	3.01235	1.70412	5.0107	1.51762	2.47703	5.90235	0.29829	2.73016

\*All values were significant ( $P < 0.05$ ) *Lampetra fluviatilis* non-migratory population.

†Uncertain life-history population.

‡*Lampetra fluviatilis* migratory populations.



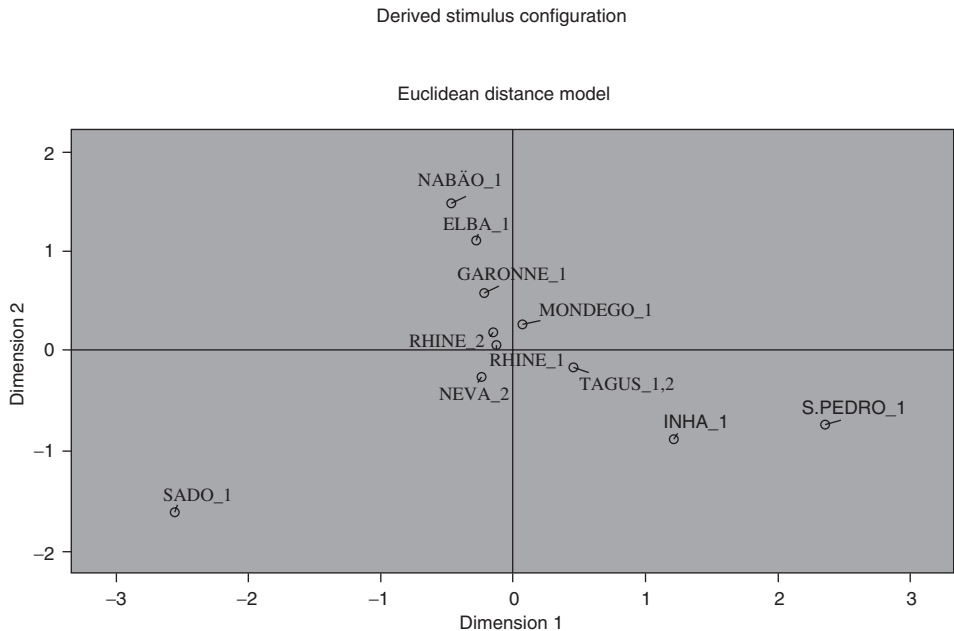


FIG. 2. Multidimensional scaling based on corrected average pair-wise differences between pairs of *Lampetra* spp. populations. 1, *L. planeri* non-migratory population; 2, *Lampetra fluviatilis* migratory populations. 1,2, uncertain life-history population (population in which most specimens are non-migratory but the presence of some migratory individuals cannot be ruled out).

with some consistency until relatively recent dates, the haplotypes now present are shared or derived by very few steps from the ones found in migratory fish.

## DISCUSSION

A striking result of the present study is the very high level of genetic diversity found in *L. fluviatilis* migrating to the North Sea and Baltic Sea. This finding stands in sharp contrast with the extremely low genetic diversity and lack of population differentiation in *Petromyzon marinus* L. samples collected in western Europe, from the Rhine to the Tagus, by Almada *et al.* (2008). Indeed, using the same DNA fragment, these authors found only 18 haplotypes in 273 specimens, in contrast with the 58 haplotypes in 267 specimens found in this study. This difference could be the result of the contrasting behaviour of *P. marinus* and the migratory *L. fluviatilis*. Indeed, *P. marinus* apparently lacks homing (Bergstedt & Seelye, 1995), and *P. marinus* travel extensively in the sea, with a high probability of migrating to a river different from the one where they were born. This means that a large area such as the west coast of Europe may harbour a single gene pool, subjected to a common process of lineage sorting, likely to be especially severe during population bottlenecks. The behaviour of *Lampetra* may be conducive to a more rapid population differentiation and retention of a higher level of genetic diversity. It is known that *L. fluviatilis* tends to perform more restricted migratory movements than *P. marinus*, with individuals



staying within or near the mouth of estuaries (Maitland, 2003). This migratory pattern may restrict but not suppress gene flow between rivers, favouring some level of population differentiation and retention of an overall higher level of genetic diversity, as haplotypes lost in a river by drift will likely persist in other drainages.

In glacial conditions, it is likely that migratory *L. fluviatilis* must have moved south, which would have increased their abundance in Portuguese rivers. In interglacials, like the present one, the bulk of the migratory populations must have moved northward again. In this situation, the Portuguese populations would have become increasingly isolated from the migratory fish from North Europe. The reduction of population size in small streams, combined with a lack of gene flow among rivers, would have favoured a rapid lineage sorting and loss of genetic diversity.

The low percentage of divergence among haplotypes of European *Lampetra* spp., when compared, for instance with the ones found in *L. aepyptera* (Martin & White, 2008), another non-parasitic lamprey, and the finding that some *Lampetra* populations are polyphyletic are consistent with the hypothesis that brook lamprey populations in different Portuguese rivers may have originated from the migratory stock in relatively recent times during the Pleistocene. The polyphyletic populations could represent cases where lineage sorting did not take place yet, a condition that is typical of the initial stages of population isolation (Avice, 2000). An exception to this scenario of small localized populations getting isolated from a large migratory stock may be represented by the population of the River São Pedro, which contained haplotypes derived from the ones found in Nabão. The two rivers have their sources <5 km apart, which means that minor geological changes may have allowed previous contacts between the two headwaters. This would identify dispersal among rivers as a less important, but plausible, process in *L. planeri* phylogeography, which agrees with the findings of Martin and White who found substantial phylogeographical structure in *L. aepyptera*.

The low overall level of divergence observed, and the use of a single marker, makes a discussion on the status of local populations as potential species obviously premature. Regardless of future conclusions on this point, this article describes mechanisms that, given sufficient time, will promote allopatric speciation *sensu* Futuyma (2005).

For the populations of the Rivers Sado, Inha and São Pedro, there is no evidence that migratory *Lampetra* were present in historical times, which means that it may be almost sure that gene flow does not exist among populations. On the other hand, even the mitochondrial data alone clearly demonstrate the distinctiveness and uniqueness of the Portuguese *L. planeri* populations, suggesting that the populations of the Rivers Sado, Nabão, São Pedro and Inha qualify as distinct conservation units following the Convention of Biological Diversity (CBD). They are composed of unique haplotypes, isolated from other populations, and are confined to small streams (in the Sado River, *L. planeri* only occur in a few tributaries), and are thus highly vulnerable to extinction if proper management action is not taken.

The need to recognize conservation units for these Portuguese *L. planeri* populations is made more urgent for two reasons. The first is that the total area of territory where *Lampetra* species are present is very small (Cabral *et al.*, 2005). This means that population sizes are likely small. Secondly, these non-migratory lampreys occupy southern locations in the overall range of *Lampetra* spp. in western Europe and may

have acquired adaptive traits favourable in the high temperature conditions where they live.

One could argue that these populations are the less valuable, from a conservation point of view, because of their very low level of genetic diversity, which contrasts with the high levels found in large rivers such as the Neva, Rhine and Tagus. The low genetic diversity, however, negates neither the distinctiveness of populations nor the possibility of independent evolutionary histories. Indeed, it may simply reflect a prolonged condition of low population size, with accelerated lineage sorting, due to the small habitat areas where these populations occur.

The population from the River Garonne also contained only private haplotypes. Because of small sample size, however, it is premature to draw conclusions on the status of this population or species. It is likely that a more comprehensive phylogeographical analysis of *Lampetra* spp. in western Europe may uncover additional populations with high levels of distinctiveness, restricted distributions and low gene flow with other populations.

One positive contribution of the present work is the demonstration that much more phylogeographic work must be undertaken to properly understand and conserve the genetic biodiversity of *Lampetra* spp. in Europe.

In conclusion, these data provide clear evidence supporting the genetic distinctiveness and consequent need for conservation of the *Lampetra* spp. populations of small Portuguese streams. It also demonstrates the need for detailed phylogeographical analysis of *Lampetra* spp. across its western European range.

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