Genetic and morphological diversity of the genus *Lampetra* (Petromyzontidae) in Europe

Catarina Sofia Pereira Mateus

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Biologia

ORIENTADORES: *Professor Doutor Pedro Raposo de Almeida*
Doutora Maria Judite Alves

ÉVORA, DEZEMBRO DE 2013

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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Aos meus pais

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List of publications

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Other relevant publications

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Abstract

This study aims at contributing to the knowledge on genetic and morphological diversity of European *Lampetra*. Mitochondrial DNA was used to infer the phylogeography of this genus, and further define conservation units in the Iberian Peninsula. Morphological data was then combined to describe three new species endemic to Portugal. These results support evidence of the high diversity of the Iberian Peninsula, a region that acted as glacial refugium during the Pleistocene glaciations. The analysis of microsatellite loci allowed to understand the postglacial colonization processes and to assess contemporary gene flow between *Lampetra* species in Europe. The use of genome-wide sequencing significantly contributed for a better knowledge about the taxonomic validity of lamprey paired species, identifying strong divergence between species. The information gathered with this study greatly contributes to the knowledge on lampreys, a group of particular interest in evolutionary studies that constitutes a great model system to study speciation processes.

Resumo

Diversidade genética e morfológica do género *Lampetra* (Petromyzontidae) na Europa

Este estudo pretende contribuir para o conhecimento da diversidade genética e morfológica do género *Lampetra* na Europa. A filogeografia deste género, e posterior definição de unidades de conservação na Península Ibérica, foi inferida através de ADN mitocondrial. A junção de dados morfológicos permitiu a descrição de três espécies novas, endémicas de Portugal. Estes resultados suportam as evidências acerca da elevada diversidade da Península Ibérica, uma região que atuou como refúgio glaciar durante o Plistocénico. A análise de microssatélites permitiu estudar os processos de colonização pós-glaciais e detetar fluxo genético recente entre espécies de *Lampetra* na Europa. O uso de genómica contribuiu significativamente para um melhor conhecimento da validade taxonómica de espécies pares, identificando forte divergência entre espécies. A informação recolhida no âmbito desta tese contribuiu significativamente para o conhecimento das lampreias, um grupo de particular interesse em estudos evolutivos que constitui um excelente modelo para estudar os processos de especiação.

Chapter 1

General Introduction

Chapter 1 | General Introduction

Lampreys are agnathans because they lack jaws, an absence regarded as a primitive character state. Due to their phylogenetic position, lampreys are key species to study the evolutionary sequence of events in the history of the vertebrates. Despite their importance in evolutionary studies, lamprey phylogenies and relationships remained poorly studied for several decades. This dissertation aims to be a contribution to lamprey taxonomy, morphology and phylogeography, especially in what concerns populations of the genus *Lampetra* inhabiting an important glacial refugium, the Iberian Peninsula.

The origin and evolution of lampreys

The fossil record indicates that, during the Cambrian period, there was a great elaboration in the diversity of animal body plans. This included the emergence of a lineage with several characteristics shared with modern-day vertebrates, such as a cartilaginous skeleton that encases the central nervous system (cranium and vertebral column) and provides a support structure for the branchial arches and median fins. Subsequent diversification of this lineage gave rise to the jawless (agnathans) and jawed vertebrates (gnathostomes) (Smith *et al.* 2013). The jawless vertebrates are represented by only two extant orders, the lampreys (order Petromyzontiformes) and the hagfishes (order Myxiniformes), and several fossil groups known collectively as ostracoderms (bony-skinned) (Nelson 2006). Lampreys and hagfishes belong to the phylum Chordata and subphylum Craniata and together are informally referred to as cyclostomes ("round mouth"). Agnathans are distinguished from the gnathostomes (craniates with jaws) by the absence of both jaws and pelvic fins; gills covered with endoderm and directed internally; and gills opening to surface through pores rather than through slits (Nelson 2006). The phylogenetic relationships between the three groups of Craniates (lampreys, hagfishes and gnathostomes) have been an issue of intense debate, and most opinions diverge towards two possible scenarios (Figure 1). According to the cyclostome hypothesis, the ancestral jawless craniate would have given rise to two sister groups, the gnathostomes and the cyclostomes, and thus

lampreys and hagfishes form a monophyletic clade (Cyclostomata). This classical hypothesis, first supported by morphological characters, usually the feeding apparatus and characters therein (e.g. Yalden 1985), is currently mainly supported by molecular data (e.g., Stock & Whitt 1992; Mallatt & Sullivan 1998; Kuraku *et al.* 1999; Delarbre *et al.* 2002; Kuraku & Kuratani 2006; Mallatt & Winchell 2007). The second hypothesis is the vertebrate theory, which became fashionable in the 1970s. According to this hypothesis, lampreys and gnathostomes are more closely related and form the clade Vertebrata, whereas hagfishes are a sister group to the vertebrata, making cyclostomes paraphyletic. This hypothesis is based mainly on morphological and paleontological data (e.g., Løvtrup 1977; Janvier 1978; Hardisty 1979; Janvier & Blieck 1979).

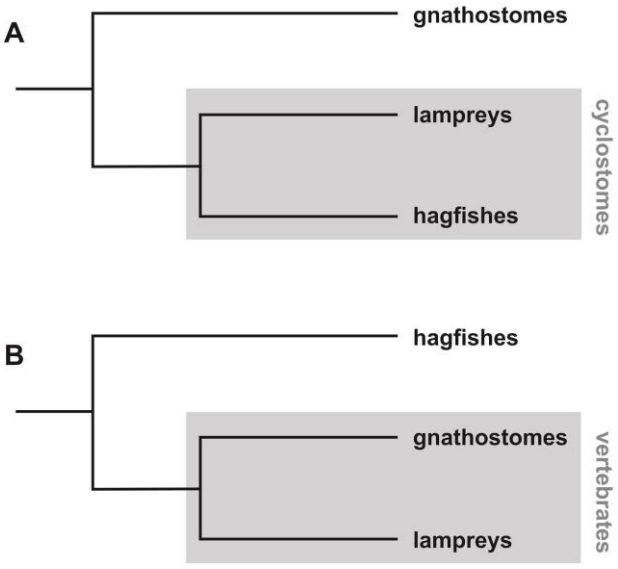

Figure 1 - Two possible hypotheses for Craniate phylogeny. A) Cyclostome hypothesis, hagfishes and lampreys form a monophyletic group, the Cyclostomata; B) Vertebrate hypothesis, gnathostomes and lampreys form a monophyletic group, the Vertebrata. The cyclostome hypothesis currently receives more support than the vertebrate hypothesis. Adapted from Osório & Rétaux (2008).

The recently reconstructed phylogenetic trees based on molecular data, in particular the study of Heimberg *et al.* (2010) employing microRNAs and a reanalysis of morphological characters, present overwhelming support for the cyclostome monophyly. Also, new insights into hagfish morphology from a developmental point of view have demonstrated that they have lost, and not primitively lacked, many of the characteristics used previously to diagnose a lamprey-gnathostome clade. For instance, these animals present vertebra-like elements that are homologous to gnathostome vertebrae, implying a secondary reduction of vertebrae in most of the

trunk. Hence, hagfish can no longer be excluded from the Vertebrata simply due to the absence of a cartilaginous axial skeleton (Ota *et al.* 2011).

The monophyly of cyclostomes has resulted in a dispute over when cyclostomes diverged from gnathostomes, and when hagfishes and lampreys split from each other in the cyclostome lineage. Until the late twentieth century, cyclostomes were assumed to be "degenerate" descendants of armoured jawless vertebrates (ostracoderms) that lived from the Ordovician period to the Devonian period, 490 million to 358 million years ago (Mya). Hagfishes and lampreys were thought to share a common ancestor that derived from certain ostracoderms, and their fossil record was based on two species found in North American Carboniferous deposits (300 and 330 Myr old). The evolutionary divergence of the two cyclostome groups from each other was regarded as relatively recent, possibly occurring during the Mesozoic period 251–65 Mya (reviewed in Janvier 2006). In the year 2006 two new lamprey fossils were found, *Mesomyzon mengae* from the Cretaceous (125 Myr) of China (Chang *et al.* 2006) and *Priscomyzon riniensis* from the Devonian period (360 Myr) of South Africa (Gess *et al*. 2006), both looking very similar to modern lampreys. These findings proved that lampreys and hagfishes had already diverged by late Devonian times, earlier than previously thought. Also, cyclostomes would have diverged from other craniates (gnatosthomata) before the ostracoderms, implying that ostracoderms although jawless are more closely related to jawed vertebrates than to cyclostomes (reviewed in Janvier 2008). Using nuclear-encoded protein sequence data along with complete genome sequences, and a Bayesian local molecular clock method, Blair & Hedges (2005) estimated molecular divergence times for the major lineages of deuterostomes (hemichordates, echinoderms, cephalochordates, urochordates, and vertebrates). Within vertebrates, the divergence between gnathostomes and cyclostomes was estimated as 605–742 Mya, and the divergence between lampreys and hagfishes as 461–596 Mya. Different divergence times have been proposed by several authors, however. For instance, Kuraku & Kuratani (2006), using nucleotide and amino acid sequences, estimated the lamprey-hagfish split to date back to 470–390 Mya in the Ordovician–Silurian–Devonian Periods, 30–110 million years after the cyclostome

lineage split from the future gnathostome lineage. The cyclostome-gnathostome spilt is generally assumed to have occurred ~500 million years ago (e.g., Smith *et al.* 2013).

Considering the monophyly of cyclostomes, another question raised is whether this group diverged before or after the genome duplications thought to have occurred in the lineage leading to vertebrates. Ohno (1970) was the first to propose that whole genome duplication (WGD) occurred in the lineage leading to vertebrates. As opposed to smaller scale events such as tandem duplications, WGD generates enormous amounts of genetic raw material which is susceptible to acquire novel functions, leading to the generation of new gene networks used for biological innovations (Ohno 1970). This phenomenon is believed to be one of the major evolutionary events that shaped the genomes of vertebrates, enabling the evolution of phenotypic complexity, diversity and innovation, and the origin of novel gene functions (Holland *et al.* 1994; Holland 1999; Meyer & Schartl 1999).

The actual existence of WGD and the existence of one or two rounds (1R and 2R hypothesis) and their timings have been subjects of intense debate, with some studies supporting 2R (e.g., Escriva *et al.* 2002; Kuraku *et al.* 2009), others indicating only a single round of WGD (e.g., McLysaght *et al.* 2002), and still others proposing multiple independent events of tandem duplication and translocation as an alternative to the whole-genome duplication scenario (e.g., Hughes *et al.* 2001; Friedman & Hughes 2003). During the 1990s a large number of genes were cloned from amphioxus (cephalochordate), ascidia (urochordate) and basal vertebrates, and comparison of gene numbers cloned, together with molecular phylogenetic analyses, revealed a remarkably consistent scenario: presence of single *versus* multiple protein-coding gene copies (one-to-four rule) in invertebrates *versus* vertebrates, supporting the 2R hypothesis (summarized in Holland 1999). The timing for the first ploidy event has been more consensual, being generally assumed that it post-dated the divergence of the vertebrate, cephalochordate and urochordate lineages. Panopoulou *et al.* (2003), through a large catalogue of genes from amphioxus, provided evidence for large-scale gene duplication immediately after the separation of cephalochordates and vertebrates at 650 Mya. Posterior evidence that the urochordates, rather than the cephalochordates, are the invertebrates most closely related to vertebrates (e.g.,

Delsuc *et al.* 2006) updated the 2R hypothesis, and the first ploidy event was assumed to have occurred after the split between urochordates and vertebrates. Jawless vertebrates, represented by hagfish and lampreys, occupy an intermediate phylogenetic position between urochordates and jawed vertebrates, and there has been controversy whether the second round of WGD occurred before (e.g., Kuraku *et al.* 2009) or after (e.g., Escriva *et al.* 2002) the cyclostome-gnathostome split. Recently, Smith *et al.* (2013) reported the whole-genome sequence of the sea lamprey, *Petromyzon marinus*, providing new insights into vertebrate evolution. Their analyses provided evidence for two whole-genome duplication events occurring before the divergence of the ancestral lamprey and jawed vertebrate (gnathostome) lineages. An additional (third) entire genome duplication, commonly called fish-specific genome duplication (FSGD or 3R), took place during the evolution of teleost fish, leading at least initially to up to eight copies of the ancestral deuterostome genome (Meyer & Van de Peer 2005; Ravi & Venkatesh 2008; Sato & Nishida 2010). This event, which occurred ~350 Mya before the diversification of teleosts (Christoffels *et al.* 2004) is thought to explain the remarkable diversity in teleost morphology, behaviour, and adaptations and their evolutionary success.

The sea lamprey and a number of hagfish species are known to undergo programmed genome rearrangement (PGR) events during early embryogenesis (Kubota *et al.* 1993, 1997; Smith *et al.* 2009). Genomic rearrangements are known in different organisms (e.g., nematodes, copepods, scriarid flies) and result in the selective removal of repetitive sequences, entire chromosomes, or single-copy genes. This phenomenon mediates the deletion of ~20% of germ line DNA from somatic tissues of the sea lamprey, with a substantial fraction of the somatically deleted DNA corresponding to single-copy and protein-coding DNA (Smith *et al.* 2009, 2012). The fact that PGR seems to occur in both cyclostome lineages raises the possibility that this mechanism is conserved within this group, and it remains to be established whether PGR is an ancestral feature of all vertebrates or a derived feature that originated in cyclostomes (Sémon *et al.* 2012).

Lamprey morphology, ecology and life cycles

Lampreys are aquatic animals, eel-like shaped and with smooth, scaleless skins. Paired fins and jaws are absent and the mouth is circular, with a tongue-like structure and bearing horny teeth. There is a single median nostril, located on the top of the head.

There are a total of 43-47 extant lamprey species (see next section, "Lampreys of the world" and Table 1), including anadromous parasitic, freshwater parasitic and freshwater non-parasitic *taxa* (Table 1). All species breed in fresh water; some species migrate to the sea as juveniles (anadromous) but well over half of the species spend their entire life cycle in fresh waters. Some species are predacious as adults, using their sucking mouth to attach to the body of hosts (mainly teleost fishes), using the tongue to open a wound through which they can suck the blood and tissue fragments from their prey. Predacious species are commonly anadromous, with a freshwater larval stage feeding by filtration, and a saltwater post-metamorphic stage (Figure 2), but species confined to fresh waters (resident) can also be parasitic, with a feeding phase generally restricted to large river basins or lakes (Hardisty & Potter 1971a). Resident species are, however, generally non-parasitic, not feeding as adults; they reproduce and dye shortly after metamorphosis (post-metamorphic phase is limited to a short period of 3 to 9 months) (Figure 2), attaining smaller sizes and exhibiting lower fecundity when compared to the closely related parasitic species.

The common stage of the life cycle, the larval stage, is the longest period, and is spent entirely in fresh water. The duration of the larval phase results from the time needed to attain a critical size and the necessary energetic reserves to initiate metamorphosis (Youson 1980), and varies greatly between geographic regions with different climatic regimes (Beamish & Potter 1975; Beamish 1980a; Morkert *et al.* 1998). This freshwater period lasts for 2-8 years (Hardisty & Huggins 1970; Beamish & Potter 1975; Morkert *et al.* 1998; Quintella *et al.* 2003), depending on the location and the environmental conditions.

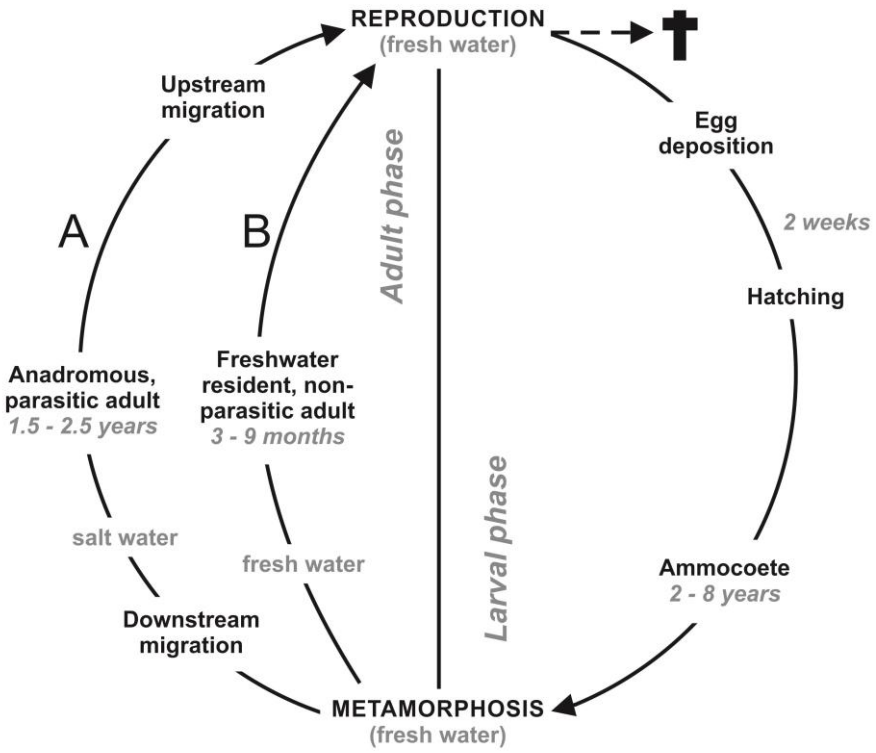

Figure 2 - Life cycle of parasitic, anadromous species (A) and freshwater resident, non-parasitic species (B). The larval stage is the longest period of the life cycle. Overall life span is similar in both cycles, in brook lampreys the adult life has been reduced to the same extent as the larval life has been extended.

Lamprey larvae are so different from the adults that they were formerly seen as a separate species, *Ammocoetes branchialis* (Norman 1949), and for this reason usually called ammocoetes. The word “ammocoete” derives from the Greek, meaning “sleeping in sand”. The ammocoetes have a worm-like body shape. They are toothless and the eyes lying below the skin surface are barely visible externally and motor responses to light are largely mediated through the photosensitivity of the tail region. On the dorsal surface of the head is the pineal spot, below which is the pineal organ, a structure concerned with the diurnal rhythm of body colour change. The fins are not well developed, consisting of a low, continuous dorsal fold, beginning in the trunk region and extending around the tail as a caudal fin. The blunt anterior end of the animal is formed by an oral hood containing oral cirri that prevent large particles from entering the pharyngeal chamber (Hardisty & Potter 1971b). After an initial pro-larval stage, during which they absorb the yolk, the larvae become filter feeders. The pro-ammocoetes emerge from the sand and are carried downstream to sites where the

current slackens (Hardisty 1986). Larval distribution is strongly dependent upon sediment characteristics, especially particle size composition. For instance, Almeida & Quintella (2002) found that smaller individuals (total length between 20 and 60mm) of sea lamprey were commonly found on silty, sandy bottoms, whereas ammocoetes with a total length between 60 and 140mm prefer a more heterogeneous substrate, with equal contributions of gravel and silt (gravel-silt-sand), and larger ammocoetes between 140 and 200mm prefer coarse-grained sediments, substratum composed of sand or gravelly-sand. Other variables such as the organic content of the substratum, presence of macrophytes, abundance of periphyton, shading and water temperature are also considered important in determining the distribution of the ammocoetes (Potter *et al.* 1986). For periods of several years, the ammocoetes live burrowed in fine sediment deposits of rivers and streams, straining of from the water the organic particles and microorganisms, diatoms in particular, on which they feed (Hardisty & Potter 1971b; Moore & Mallatt 1980). This protracted freshwater larval stage is, probably, one of the main reasons why lampreys are regarded as a highly successful group. Due to this life cycle strategy, not only the ammocoetes are relatively protected but also, during this period, the limitations on growth are those imposed by the mechanics of microphagous feeding rather than the availability of nutrients (Hardisty & Potter 1971b). Larvae then go through metamorphosis, which involves important remodelling of the cephalic region and of the digestive apparatus. This includes the development of the oral disc and a protrusible tongue-like piston, the appearance of teeth, extension of the preorbital region, modifications in the structure of the gill openings, eruption of the eyes, enlargement of the fins and changes in pigmentation (Hardisty & Potter 1971a; Youson 1980). In the majority of Northern Hemisphere species, the main external changes associated with metamorphosis are initiated from mid-July to September (Hardisty & Potter 1971a). There is a tendency for transforming animals to change their habitat in favour of coarse sand or gravel often lying in mid-stream, probably reflecting an alteration in oxygen requirements resulting from the anatomical changes in the branchial region taking place at this time (Hardisty 1961). This is in accordance to the results mentioned above attained by Almeida & Quintella (2002), who found that smaller individuals were commonly found on silty/sandy bottoms, whereas larger ammocoetes (pre-metamorphosis) prefer coarse-grained

sediments. The term “transformer” is normally applied to those animals in which the more obvious external changes are still taking place, while the term “macrophthalmia” or juvenile is used to describe the phase immediately after the completion of metamorphosis when animals are fully transformed. During this final phase, lampreys bear a general resemblance to the adult form and the term macrophthalmia refers to the relatively large size of the eye, which is characteristic of the parasitic species (Hardisty & Potter 1971a).

After metamorphosis, the life cycle undergoes in one of two main directions: a parasitic feeding phase (either in salt or fresh water) or a non-parasitic, freshwater phase (Figure 2). There are 18 parasitic species, nine of which are anadromous (Table 1). Anadromous species initiate, after metamorphosis, a downstream migration to salt water. The majority of studies on the migrations of anadromous species have been directed to the sea lamprey. For North American populations of this species, it was estimated that the period of downstream migration extends from the autumn (late October) through the middle of April, being greatest during late March and early April with a lesser peak of activity in November (Applegate & Brynildson 1952). This bimodal distribution, with one peak in spring and another in the autumn, however, is not found in all populations of this species. An European population (River Ulla, Spain) showed a unimodal distribution with a progressive increase in the number of individuals migrating, and peak in March (Silva *et al.* 2012). Distinct environmental conditions are probably related with the divergent distributions in the number of juveniles moving downstream; climatic conditions in North America (i.e., onset of the winter freeze-up and the break-up of the ice in the following spring caused by rising temperatures and inevitably leading to high water levels) are such as to encourage this separation of autumn and spring migrations (Hardisty 2006), while in Western Europe, the milder weather with higher water temperatures may explain the more continuous and gradual downstream migration (Silva *et al.* 2012).

Anadromous species spend the adult phase of their life cycle in salt water, where they feed parasitically on a wide variety of bony fish (Hardisty & Potter 1971a; Beamish 1980b; Farmer 1980; Halliday 1991; Schwartz 2006), having also been reported feeding on sharks (Halliday 1991; Jensen & Schwartz 1994; Gallant *et al.* 2006) and cetaceans

(Pike 1951; Nichols & Hamilton 2004; Nichols & Tscherter 2011; Samarra *et al.* 2012). *Caspiomyzon wagneri*, however, presents adult feeding habits as those of a scavenger (Renaud 1997). Information on the parasitic phase of anadromous lampreys is very limited and in general restricted either to isolated references of attacks (Hardisty & Potter 1971a), or to descriptions of the short initial parasitic period which occasionally takes place in rivers and estuaries (Potter & Beamish 1977; Silva *et al.* 2012, 2013a). The extent of the marine phase of anadromous species is also still poorly known; Beamish (1980b) proposed a period of 23 to 28 months for the sea lamprey, and recently Silva *et al.* (2013b) suggested a shorter period of 18 to 20 months between completion of metamorphosis and reproduction. Adults return to rivers for reproduction, where they become sexually mature, spawn and die. The passage from seawater to freshwater habitats implies the shift from a saline osmoregulation process to a freshwater adaptation, and lampreys tend to assemble off the river mouths or in estuaries, used as an acclimation chamber to undergo this process. After this period of acclimation, the adults begin their upstream movement in rivers and streams to spawning sites (Hardisty 1986). The timing and extent of the spawning migration varies throughout the geographical range of the species, the earlier migrations tending to occur in streams at the lower latitudes, and is triggered by temperature and water levels (Hardisty & Potter 1971a; Beamish 1980a; Hardisty 1986). For the sea lamprey inhabiting the east coast of North America, spawning migrations occur between March and September (Beamish 1980a), whereas for instance in Portugal, it is reported from December to June (Almeida *et al.* 2002; Oliveira *et al.* 2004). From the beginning of the upstream migration, feeding ceases and there is a great atrophy of the intestine; during this period lampreys depend on tissue reserves, mainly lipids, to provide the energy for migrating and spawning (Hardisty 1986). The great development of the gonads is accompanied by very marked reductions in length and weight (Hardisty & Potter 1971a; Hardisty 1986). Lampreys are negatively phototaxic, moving upstream mainly during dusk and darkness, and avoiding the light in the daytime, when they seek out resting places under rocks or river banks (Hardisty & Potter 1971a; Almeida *et al.* 2002; Andrade *et al.* 2007). Once spawning has begun, lampreys lose daytime light avoidance responses, and even tolerate bright sunlight (Hardisty 1986).

Lampreys are apparently an exception to the rule of homing in anadromous fishes (Waldman *et al.* 2008). It is known that migrating adult lampreys detect and are attracted by pheromones released by larvae (e.g., Li *et al.* 1995; Vrieze & Sorensen 2001), suggesting that, instead of returning to natal streams, lampreys use chemical signalling to locate spawning habitat that is suitable for larvae. This tactic, referred to as the "suitable river" strategy by Waldman *et al.* (2008), may have coevolved with parasitism, as individuals carried far from their natal streams by hosts are allowed to locate suitable spawning and rearing habitats. A number of studies suggest a lack of geographic population structure, supporting this hypothesis; for instance, several authors found no significant genetic differences among anadromous populations of sea lamprey along the North American Atlantic coast (Bryan *et al.* 2005; Waldman *et al.* 2008) or along the European Atlantic coast (Almada *et al.* 2008). Rodríguez-Muñoz *et al.* (2004), however, found significant differences between European and North American sea lampreys, suggesting that they are reproductively isolated. Goodman *et al.* (2008) failed to find significant differences among populations of another anadromous species, the Pacific lamprey *Entosphenus tridentatus*.

Five of the nine anadromous species include landlocked forms (Table 1); adults live and feed parasitically in lakes or large rivers (Applegate 1950; Tuunainen *et al.* 1980). The faculty of adult lampreys to feed and grow in fresh waters demonstrates the ability of anadromous populations to respond to, for instance, extreme adverse conditions to migration or limited trophic resources. This evolutionary step has involved a decline in the ability to osmoregulate in high salinities and a reduction in body size and fecundity (Potter & Beamish 1977).

Nine of the 18 parasitic species are freshwater resident (Table 1) and have a life cycle similar to the landlocked forms of anadromous species. The restriction of freshwater parasitic species to the larger river systems presumably reflects the requirement of a sufficiently large host population (Hubbs & Potter 1971).

Table 1 - Extant species of lamprey and their adult mode of life. Based on Renaud (1997, 2011), Yamazaki *et al.* (2006), Lang *et al.* (2009) and Mateus *et al.* (2013a). Global distribution of the genera is presented in Figure 3.

Species	Adult mode of life
Family Petromyzontidae	
Genus *Caspiomyzon* Berg 1906	
C. wagneri (Kessler, 1870)	Anadromous, scavenger
Genus *Entosphenus* Gill 1862	
E. folletti Vladykov & Kott, 1976	Freshwater resident, non-parasitic
E. hubbsi Vladykov & Kott, 1976	Freshwater resident, non-parasitic
E. lethophagus (Hubbs, 1971)	Freshwater resident, non-parasitic
E. macrostomus (Beamish, 1982)	Freshwater resident, parasitic
E. minimus (Bond & Kan, 1973)	Freshwater resident, parasitic
E. similis Vladykov & Kott, 1979	Freshwater resident, parasitic
E. tridentatus Gairdner in Richardson, 1836	Anadromous, parasitic; also freshwater resident form
Genus *Eudontomyzon* Regan 1911	
E. danfordi Regan, 1911	Freshwater resident, parasitic
E. graecus Renaud & Economidis 2010	Freshwater resident, non-parasitic
E. hellenicus Vladykov, Renaud, Kott & Economidis, 1982	Freshwater resident, non-parasitic
E. mariae (Berg, 1931)	Freshwater resident, non-parasitic
E. morii (Berg, 1931)	Freshwater resident, parasitic
E. stankokaramani Karaman, 1974[1]	Freshwater resident, non-parasitic
E. vladykovi Oliva & Zanandrea 1959[2]	Freshwater resident, non-parasitic
Genus *Ichthyomyzon* Girard 1858	
I. bdellium (Jordan, 1885)	Freshwater resident, parasitic
I. castaneus Girard, 1858	Freshwater resident, parasitic
I. fossor Reighard & Cummins, 1916	Freshwater resident, non-parasitic
I. gagei Hubbs & Trautman, 1937	Freshwater resident, non-parasitic
I. greeleyi Hubbs & Trautman, 1937	Freshwater resident, non-parasitic
I. unicuspis Hubbs & Trautman, 1937	Freshwater resident, parasitic
Genus *Lampetra* Bonnaterre 1788	
L. aepytera (Abbott, 1860)	Freshwater resident, non-parasitic
L. ayresi (Günther, 1870)	Anadromous, parasitic; also freshwater resident form
L. fluviatilis (Linnaeus, 1758)	Anadromous, parasitic; also freshwater resident form
L. lanceolata Kux & Steiner, 1972	Freshwater resident, non-parasitic
L. pacifica Vladykov, 1973	Freshwater resident, non-parasitic
L. planeri (Bloch, 1784)	Freshwater resident, non-parasitic
L. richardsoni Vladykov & Follett, 1965	Freshwater resident, non-parasitic
L. lusitanica Mateus, Alves, Quintella & Almeida 2013	Freshwater resident, non-parasitic
L. auremensis Mateus, Alves, Quintella & Almeida 2013	Freshwater resident, non-parasitic
L. alavariensis Mateus, Alves, Quintella & Almeida 2013	Freshwater resident, non-parasitic
Genus *Lethenteron* Creaser & Hubbs 1922	
L. alaskense Vladykov & Kott, 1978	Freshwater resident, non-parasitic
L. appendix (DeKay, 1842)	Freshwater resident, non-parasitic
L. camtschaticum (Tilesius 1811)[3]	Anadromous, parasitic; also freshwater resident form
L. kessleri (Anikin, 1905)	Freshwater resident, non-parasitic
L. ninae Naseka, Tuniyev & Renaud 2009	Freshwater resident, non-parasitic
L. reissneri (Dybowski, 1869)	Freshwater resident, non-parasitic
L. sp. N Yamazaki & Goto 1996[4]	Freshwater resident, non-parasitic
L. sp. S Yamazaki & Goto 1996[4]	Freshwater resident, non-parasitic
L. zanandreai (Vladykov, 1955)	Freshwater resident, non-parasitic

Table 1 - continued

Species	Adult mode of life
Genus *Petromyzon* Linnaeus 1758	
P. marinus (Linnaeus, 1758)	Anadromous, parasitic; also freshwater resident form
Genus *Tetrapleurodon* Creaser & Hubbs 1922	
T. geminis Alvarez, 1964	Freshwater resident, non-parasitic
T. spadiceus (Bean, 1887)	Freshwater resident, parasitic
Family Geotriidae	
Genus *Geotria* Gray, 1851	
G. australis Gray, 1851	Anadromous, parasitic
Family Mordaciidae	
Genus *Mordacia* Gray, 1851	
M. lapicida (Gray, 1851)	Anadromous, parasitic
M. mordax (Richardson, 1846)	Anadromous, parasitic
M. praecox Potter, 1968	Freshwater resident, non-parasitic

[1]Previously synonymized with *Eudontomyzon mariae* (Berg 1931), but redescribed as a valid species (Holčík & Šorić 2004).

[2]Often synonymized with *Eudontomyzon mariae* (Berg 1931), some authors consider it a valid species (Kottelat & Freyhof 2007; Lang *et al.* 2009).

[3]Also known as *Lethenteron japonicum*.

[4]Missing formal description; considered *L. reissneri* by Renaud (2011).

Most of the extant lamprey species are non-parasitic, not feeding in the adult stage (Table 1). The adult life of these so-called brook lampreys has been reduced to the same extent as their larval life has been extended, implying a shifting in the timing of metamorphosis relative to the timing of sexual maturation without changing the overall life span. Sexual maturation begins immediately after metamorphosis (Hardisty 2006; Docker 2009).

Typical spawning grounds are generally found in upper river regions, and spawning adults require specific ecological conditions that are distinct from those required by ammocoetes. Two important factors involved in the location of the spawning grounds are the presence of substrate suitable for the excavation of nests and development of the embryos, and relatively stable current flow. Adult lampreys are attracted to those rivers containing larvae through the pheromones released by the ammocoetes (Vrieze *et al.* 2010, 2011), and females are subsequently attracted to the spawning grounds by sexual pheromones released by mature males (Li *et al.* 2002), the first to arrive and begin nesting activities (Hardisty 1986).

Lampreys of the world

Lampreys have an antitropical distribution and are represented by three distinct monophyletic groups, currently recognized as distinct families. Two of these, Geotriidae and Mordaciidae, are endemic to the southern hemisphere, and the third, Petromyzontidae, is restricted to the northern hemisphere (Hubbs & Potter 1971; Gill *et al.* 2003) (Figure 3).

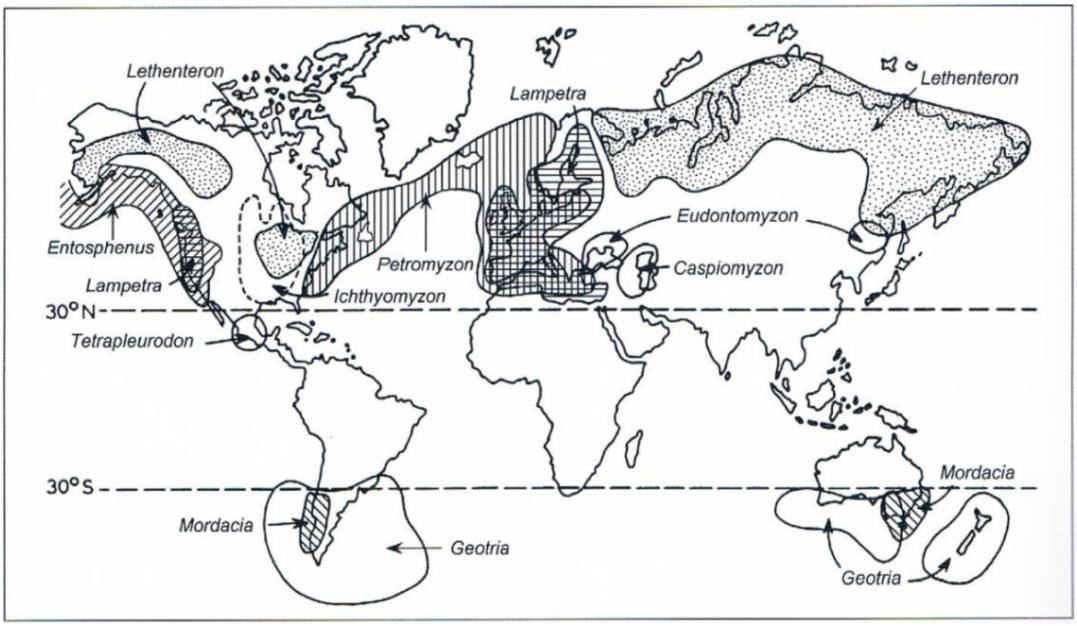

Figure 3 – Global distribution of extant lamprey genera. Species belonging to each genus are presented in Table 1. Figure from Hardisty (2006).

The phylogeny and taxonomy of lampreys have been updated making use of the increasing available amount of molecular data (Docker *et al.* 1999; Yamazaki *et al.* 2006; Lang *et al.* 2009) and a number of species of the family Petromyzontidae have been recently validated or described using both morphological and genetic data (e.g., Reid *et al*. 2011; Mateus *et al*. 2013a). Molecular data is especially valuable for taxonomic studies in a group, such as the lampreys, that possesses so few morphological characters and such a conserved morphology (Lang *et al.* 2009). However, the number of extant lamprey species is not consensual due to the lack of a formal description of some putative species that are currently not accepted as valid by some taxonomists. This is the case of two nonparasitic species in Japan, *Lethenteron* sp. N and *Lethenteron* sp. S, previously treated together as *Lethenteron reissneri*,

which are morphologically identical (Yamazaki & Goto 1997) but genetically distinct (Yamazaki & Goto 1996, 1998; Yamazaki *et al.* 2003, 2006). Also, some species considered valid by some authors are considered in synonymy by others, like *Eudontomyzon vladykovi* and *Eudontomyzon stankokaramani*, considered synonyms of *Eudontomyzon mariae* in the last revision of Renaud (2011), but recognized as valid species by other authors (Holčík & Šorić 2004; Kottelat & Freyhof 2007; Lang *et al.* 2009). Accordingly, and considering our study describing three new species from Portugal (Mateus *et al*. 2013a; chapter 3), there are 43-47 extant species of lampreys, all presented in Table 1.

Northern lampreys differ both in the number of species and in generic diversity from that of the Southern Hemisphere (Hubbs & Potter 1971); the family Petromyzontidae contains 39-43 species and eight genera (Lang *et al.* 2009; Renaud 2011; Mateus *et al.* 2013a), whereas southern lampreys (Geotriidae and Mordaciidae) are composed solely of four species and two genera. Geotriidae is represented by a single species, *Geotria australis*, and Mordaciidae by three species, *Mordacia mordax*, *Mordacia praecox* and *Mordacia lapicida* (Hubbs & Potter 1971) (Table 1).

Lampreys of the Iberian Peninsula

The Iberian Peninsula is inhabited by six species of lampreys: sea lamprey (*P. marinus*), European river lamprey (*Lampetra fluviatilis*), European brook lamprey (*Lampetra planeri*), Costa de Prata lamprey (*Lampetra alavariensis*), Nabão lamprey (*Lampetra auremensis*) and Sado lamprey (*Lampetra lusitanica*), the last three have been described during the development of the present study (chapter 3). The first two (*P. marinus* and *L. fluviatilis*) are parasitic and anadromous whereas the remaining are brook lampreys, i.e., non-parasitic and freshwater resident (see "Lamprey morphology, ecology and life cycles", above, for a description of life cycles). All species are of conservation concern in this region; the rare *L. fluviatilis* and *L. planeri* are classified as *Critically Endangered*, with *L. fluviatilis* being *Regionally Extinct* in Spain (Doadrio 2001; Cabral *et al.* 2005). The three new cryptic brook lampreys, with very restricted distributions, are proposed to be classified as *Critically Endangered* (Mateus *et al.*

2013a; chapter 3). The sea lamprey is the only with economic value both in Portugal and Spain, and is classified as *Vulnerable* (Doadrio 2001; Cabral *et al.* 2005). In Portugal it occurs in all the major river basins, and in Spain it occurs in most rivers flowing into the Cantabrian Sea and the Atlantic Ocean, as well as in some flowing into the Mediterranean (Mateus *et al.* 2012; chapter 3). The river lamprey *L. fluviatilis*, although migratory, has a very restricted distribution, currently occurring solely in Tagus river basin, in the Portuguese territory until the first insurmountable obstacles, with an estimated 273 km of available habitat (Mateus *et al.* 2012). The brook lampreys are confined to fresh waters: *L. planeri* occurs from the Tagus basin in the south to the Douro basin in the north, and in two rivers in northern Spain; *L. alavariensis* occurs in the Esmoriz and Vouga rivers basins, in northwestern Portugal; *L. auremensis* is restricted to the river Nabão in Portugal, a tributary from the Tagus basin; and *L. lusitanica* occurs in the Sado river basin, southwestern Portugal (Mateus *et al.* 2012; Mateus *et al.* 2013a).

The Iberian Peninsula was one of the most important Pleistocene glacial refugia in Europe, and a number of studies have been supporting the existence of several minor refugia within Iberia (Gómez & Lunt 2006). Espanhol *et al.* (2007), Pereira *et al.* (2010) and Mateus *et al.* (2011; chapter 3) identified unique evolutionary lineages of *Lampetra* in this region, and high genetic diversity, probably the result of refugial persistence and subsequent accumulation of variation over several ice ages. This is in contrast to the low levels of genetic diversity observed in central and northern Europe, that probably reflect a rapid postglacial colonization (Espanhol *et al.* 2007).

Lamprey paired and satellite species

Lamprey “paired species” consist of a couple of closely related lampreys with distinct life histories as adults: one is parasitic and anadromous, and the other is a non-parasitic, freshwater resident form, derived from a form similar to that of an extant parasitic one (Hubbs 1925, 1940; Zanandrea 1959). Some parasitic ancestors have given rise to two or more different non-parasitic derivatives, and these are called “satellite species” (Vladykov & Kott 1979).

"Paired" or "satellite" species occur in seven of the ten lamprey genera. It is assumed that non-parasitism has evolved independently in different taxa, in different locations, and multiple times within recognized species (reviewed in Docker 2009). Not all non-parasitic species can be obviously paired with parasitic forms; the former have an extreme southerly distribution that seems to reflect their status as "relicts" of groups with a previously more widespread distribution (Hardisty & Potter 1971c; Hubbs & Potter 1971). Relict species include *Lethenteron zanandreai*, *Lampetra aepyptera*, and *Entosphenus hubbsi*, that presumably represent more ancient non-parasitic derivates occurring at or near the extreme southern limits of the distribution of Northern Hemisphere lampreys (Hubbs & Potter 1971). Also, some parasitic species do not have a non-parasitic counterpart; this is the case of the three monotypic genera *Petromyzon*, *Caspiomyzon*, and *Geotria* (Potter 1980; Docker 2009).

It has been suggested that the evolution of non-parasitism has involved a change in the timing of metamorphosis relative to the timing of sexual maturation, without changing the overall life duration. This implies that both parasitic and non-parasitic members of a species pair spawn and die at a similar age; the adult life of the brook lamprey has been reduced to the same extent as its larval life has been extended (Hardisty 2006). Due to their smaller adult size, fecundity has been substantially reduced in the non-parasitic species, but this has been compensated by the reduced mortality in non-parasitic and non-migratory lampreys (Hardisty & Potter 1971c; Hardisty 1986).

The taxonomic validity of members of species pairs has long been questioned. The fact that they co-occur on breeding grounds and often spawn in common nests (Huggins & Thompson 1970; Lasne *et al.* 2010), produce viable offspring when crossed artificially (e.g., Enequist 1937), the larvae of the two forms are morphologically indistinguishable (Potter 1980), they often largely overlap in geographical distribution (Hubbs 1925; Hubbs & Potter 1971), and the increasing molecular studies failing to detect significant genetic differences between paired species have been suggesting that differences in adult size alone may not represent a barrier to gene flow, and consequently, some authors argue that members of paired species are morphs of a single species. For example, for the paired European river and brook lampreys,

Schreiber & Engelhorn (1998), comparing allelic frequencies at 24 allozyme loci, failed to find significant genetic differences between this species pair. Mitochondrial DNA variation was also analyzed in this pair, but no diagnostic differences were found (Espanhol *et al.* 2007; Blank *et al.* 2008; Mateus *et al.* 2011; chapter 3). This is common to other paired lamprey species; for example, Hubert *et al.* (2008) and Docker *et al*. (2012) using mtDNA and microsatellite markers found no significant differences between the paired silver (*Ichthyomyzon unicuspis*) and northern brook (*Ichthyomyzon fossor*) lampreys.

The sharing of mtDNA haplotypes by paired species and the associated lack of monophyly is compatible with two alternative scenarios: it may reflect ongoing gene flow between members of species pairs, implying that these are not valid species but instead morphs of a single species that share the same gene pool, or alternatively, it may be a sign of recent speciation, where the two recently formed species may have not yet achieved reciprocal monophyly via genetic drift and lineage sorting (Espanhol *et al.* 2007; Blank *et al.* 2008). This long-standing ambiguity in the evolution of lamprey pairs might be resolved by high-resolution genetic data. We examined one species pair in detail by means of restriction site-associated DNA sequencing (RADseq) and found, for the first time, significant differences between the paired European brook and river lampreys (Mateus *et al.* 2013b; chapter 4). These results clearly suggest that sympatric populations of *L. fluviatilis* and *L. planeri* are not experiencing gene flow and each constitute a valid species.

Aims and structure of the thesis

Many are the studies identifying new endemic species and a great diversity in populations from the Iberian glacial refugium, especially since the advent of molecular tools. Lampreys, however, were still poorly studied in this region, with the exception of the biology and ecology of the migratory *P. marinus*. A first molecular approach by Espanhol *et al.* (2007) revealed high levels of diversity in *Lampetra* populations from the Iberian Peninsula, compared with northern populations, and showed that Iberian populations required further comprehensive studies, not only from the evolutionary

and conservationist point of view, but also as model systems for the understanding of the paired species conundrum. Using up to date molecular markers and morphological studies, with this thesis I intend to understand the evolutionary history of *Lampetra* in this peninsular region, through the analysis of phylogeographic patterns and population structure across their distributional range in Europe, and give new insights into the long standing question about lamprey paired species taxonomy. Ultimately, it is my objective that this thesis may contribute to the conservation of the unique Iberian *Lampetra* species and populations.

To achieve these goals, the present study was focused in the following specific objectives:

1. To access the distributional range and conservation status of *Lampetra* in the Iberian Peninsula;
2. To infer the phylogeography and define conservation and management units of the genus *Lampetra* in the Iberian Peninsula, using mitochondrial DNA;
3. To analyse the morphological variation in Portuguese populations of the resident form;
4. To infer the population structure, patterns of colonization and gene flow among European species and populations of *Lampetra* using microsatellite markers;
5. To examine whether the sympatric paired *L. fluviatilis* and *L. planeri* are two valid species or instead represent products of phenotypic plasticity within a single species, using restriction site-associated DNA sequencing (RADseq).

This thesis is organized in five chapters. **Chapter 1** comprises the present general introduction, highlighting the main aspects regarding lamprey evolution, life cycles, extant species, and some of the major issues in debate on lamprey research. **Chapter 2** is entitled “Distribution, threats and conservation of lampreys in the Iberian Peninsula” and comprises two papers published in international journals. **Paper I**, entitled “Presence of the genus *Lampetra* in Asturias (Northern Spain)”, is published in *Cybium*, and redefines the distributional range of the genus *Lampetra* in Spain, representing a significant extension of the occurrence of this genus in the Iberian Peninsula. **Paper II** is entitled “Lampreys of the Iberian Peninsula: distribution, population status and

conservation", is published in the international journal *Endangered Species Research*, and constitutes a review paper about the historical and present distribution, main threats and conservation status of the species of lampreys known to occurr in the Iberian Peninsula at the time. **Chapter 3** is entitled "Genetic and morphological variation of *Lampetra*" and comprises three papers, two of which published in international journals and the other in preparation. **Paper III** is entitled "MtDNA markers reveal the existence of allopatric evolutionary lineages in the threatened lampreys *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch) in the Iberian glacial refugium", is published in *Conservation Genetics*, and analyses the phylogeography of *Lampetra* in the Iberian Peninsula using mitochondrial DNA, identifying a number of conservation units. **Paper IV** is published in *Contributions to Zoology*, entitled "Three new cryptic species of the lamprey genus *Lampetra* Bonnaterre, 1788 (Petromyzontiformes: Petromyzontidae) from the Iberian Peninsula", and combines previous results from mtDNA and new data from morphology to describe three new cryptic species endemic to Portugal, corresponding to the conservation units attained in the previous chapter. **Paper V** constitutes a paper in preparation for submission in the international journal *Molecular Ecology*, entitled "European lamprey species: new insights on postglacial colonization processes and gene flow using microsatellite loci", that analyses the population structure, gene flow and colonization processes among *Lampetra* species and populations from Europe, using microsatellite loci. **Chapter 4** is entitled "Lamprey species pairs: real species or morphs of a single species?" and comprises one paper: **Paper VI**, published in the international journal *Current Biology*, is entitled "Strong genome-wide divergence between sympatric European river and brook lampreys", and describes the genetic population structure of sympatric *L. fluviatilis* and *L. planeri* using restriction site-associated DNA sequencing (RADseq), showing strong genetic differentiation between the two forms, corroborating their classification as distinct taxonomic units. In this paper, we also assign fixed and diagnostic single nucleotide polymorphisms (SNPs) between the two species to specific genes in the sea lamprey genome. In **chapter 5**, "General discussion and conclusions" the results attained throughout the thesis are discussed and combined, some priority conservation measurements are suggested, and future research objectives are outlined.

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Chapter 2

Distribution, threats and conservation of lampreys in the Iberian Peninsula

Mateus CS, Quintella BR, Rodríguez-Muñoz R, Almeida PR (2011) Presence of the genus *Lampetra* in Asturias (northern Spain). *Cybium*, **35**, 162−164.

Mateus CS, Rodríguez-Muñoz R, Quintella BR, Alves MJ, Almeida PR (2012) Lampreys of the Iberian Peninsula: distribution, population status and conservation. *Endangered Species Research*, **16**, 183–198.

Paper I | Presence of the genus *Lampetra* in Asturias (Northern Spain)

Catarina S. Mateus[1, 2, 3,*], Bernardo R. Quintella[2, 4], Rolando Rodríguez-Muñoz[5] & Pedro R. Almeida[1, 2]

[1]Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, Largo dos Colegiais 2, 7000 Évora, Portugal. [csmateus@fc.ul.pt] [pmra@uevora.pt]

[2]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal. [bsquintella@fc.ul.pt]

[3]Centro de Biologia Ambiental, Museu Nacional de História Natural, Universidade de Lisboa, Rua da Escola Politécnica 58, 1250-102 Lisboa, Portugal.

[4]Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

[5]School of Biosciences, Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Tremough, Penryn TR10 9EZ, UK.[R.Rodriguez-Munoz@exeter.ac.uk]

[*]E-mail: csmateus@fc.ul.pt

Abstract

In July 2009, larvae of *Lampetra* sp. were caught in the river Deva, northern Spain. This is the first record of this genus in the Cantabrian coast and represents a significant extension of its range in the Iberian Peninsula. The analyses of two mitochondrial genes and some morphologic and meristic characters confirmed the identification of the genus.

***Keywords*:** Petromyzontidae, *Lampetra* sp., river lamprey, brook lamprey, Iberian Peninsula, Cantabrian coast, new record.

Introduction

Three species of lampreys are known in the Iberian Peninsula: the European river lamprey (*Lampetra fluviatilis* L., 1758), the European brook lamprey (*Lampetra planeri* Bloch, 1784) and the sea lamprey (*Petromyzon marinus* L., 1758) (Doadrio 2001; Cabral *et al*. 2005). *L. fluviatilis* and *L. planeri* are considered 'paired species', i.e., the larvae are morphologically similar but the adults adopt different life histories (Zanandrea 1959).

Present known distribution of *L. fluviatilis* in the Iberian Peninsula is restricted to the Portuguese part of the Tagus River basin. In the Spanish part, it is presumed to vanish after the construction of the Cedillo dam (Cáceres) in River Tagus in 1976 (Doadrio 2001). The confirmed range of *L. planeri* is wider. Although in Spain it is reported exclusively in Olabidea River (Navarra) (Doadrio 2001) its presence has been confirmed in several river basins in Portugal (Espanhol *et al*. 2007). Both species are threatened in the Iberian Peninsula (Doadrio 2001; Cabral *et al*. 2005).

In this paper, we describe the results of a sampling survey carried out in Asturias (Northern Spain).

Material and methods

Sampling of lamprey larvae was carried out in July 2009 by electric fishing on the four main watersheds of Asturias (rivers Eo, Nalón, Sella and Deva). A total of 50 larvae were captured in the Deva River basin, in Northern Spain (43º19'53'' N; 04º34'37'' W) (Fig. 1). The captured specimens were anaesthetised and measured for total length (Lt ± 1 mm) and total weight (Wt ± 0.1 g). A piece of tissue was removed from the dorsal fin and the larvae were released near the capturing sites. Tissue samples were deposited in the National Museum of Natural History, Portugal (voucher numbers MB85-8697 to 8721 and MB85-8743 to 8767). One specimen (MB85-8703) was photographed and measured for morphometric and meristic traits. Morphological characteristics of the collected specimens such as the distribution and intensity of pigmentation in the branchial region and caudal fin were registered.

Total genomic DNA was extracted from the tissue of eight larvae. The mitochondrial genes ATPase (subunits 6 and 8) and cytochrome *b* were sequenced (2002 bp). Nucleotide sequences were grouped in haplotypes and analysed for phylogenetic relationships with other *Lampetra* sequences by the method of neighbour-joining. A sequence from *Petromyzon marinus* (L.) from the EMBL database (U11880) was used as outgroup.

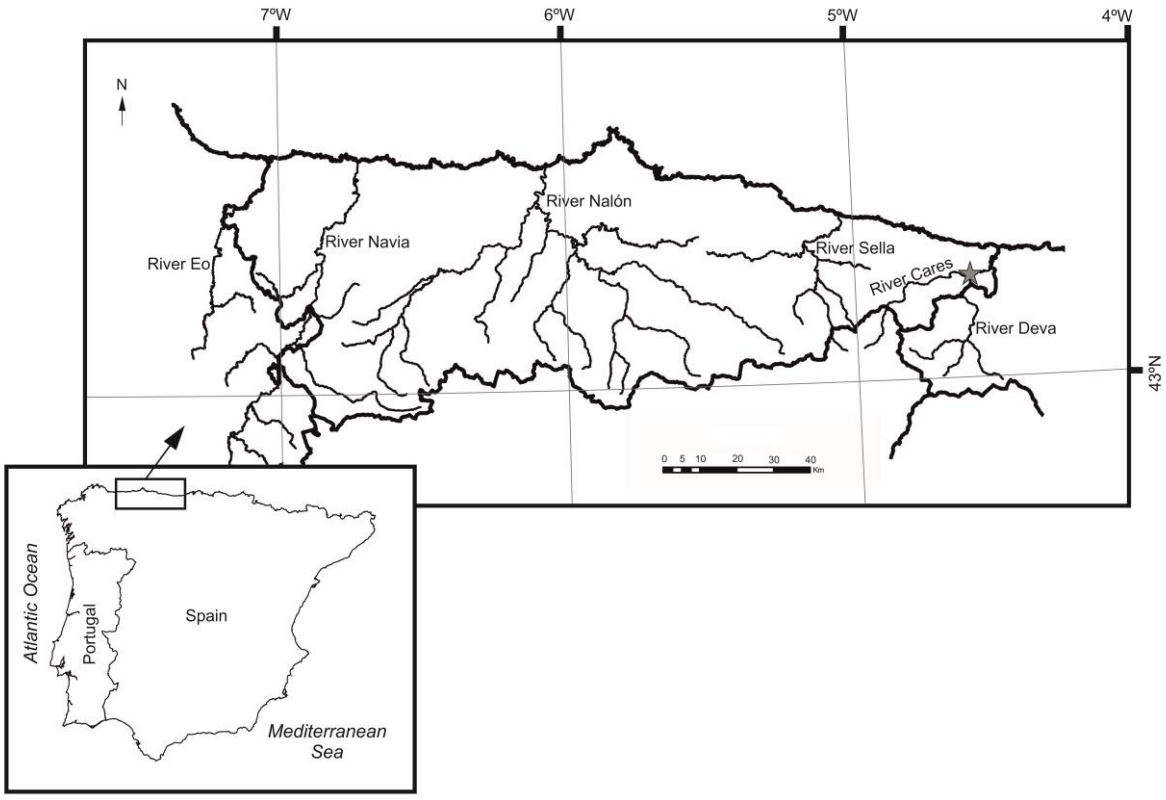

Figure 1 - Map of the Iberian Peninsula showing the Spanish region of Asturias and the capture locality of the *Lampetra* specimens (★).

Results

The total length (Lt) and weight (Wt) (mean ± SD) of the captured individuals ranged from 54 mm to 160 mm (108.88 ± 27.20) and 0.34 g to 7.49 g (2.55 ± 1.78), respectively.

Morphological characteristics, body measurements and meristic counts are congruent with the reported data in the literature for the genus *Lampetra* (Fig. 2; Tab.

1). Also, the neighbour-joining phylogenetic tree placed the five Spanish haplotypes (H73-H77) together with other *Lampetra* nucleotide sequences (Fig. 3).

Nucleotide sequences were deposited at the EMBL database (accession numbers FN641859-63).

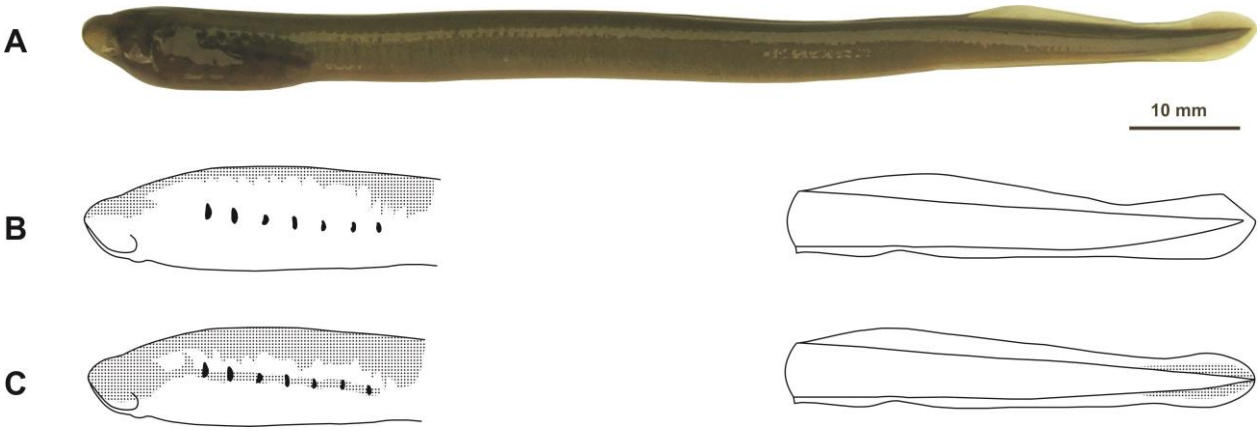

Figure 2 - Ammocoete of *Lampetra* sp. captured in River Deva, Spain (MB85-8703, 113 mm Lt) A: Lateral view; B and C: Diagrams of the head and tail of *Lampetra* sp. and *P. marinus* ammocoetes, respectively, illustrating important pigmentation recognition features (diagrams adapted from Potter & Osborne 1975).

Table 1 - Biometric characteristics of a *Lampetra* sp. ammocoete collected in River Deva, Spain (MB85-8703).

	MB85-8703
Morphometrics (mm)	
Total lenght	113.44
Head lenght	21.46
Trunk lenght	60.96
Tail lenght	30.64
Branchial lenght	13.31
Body depht	6.43
Meristics	
Number of trunk myomeres	61
Total weight (g)	2.35

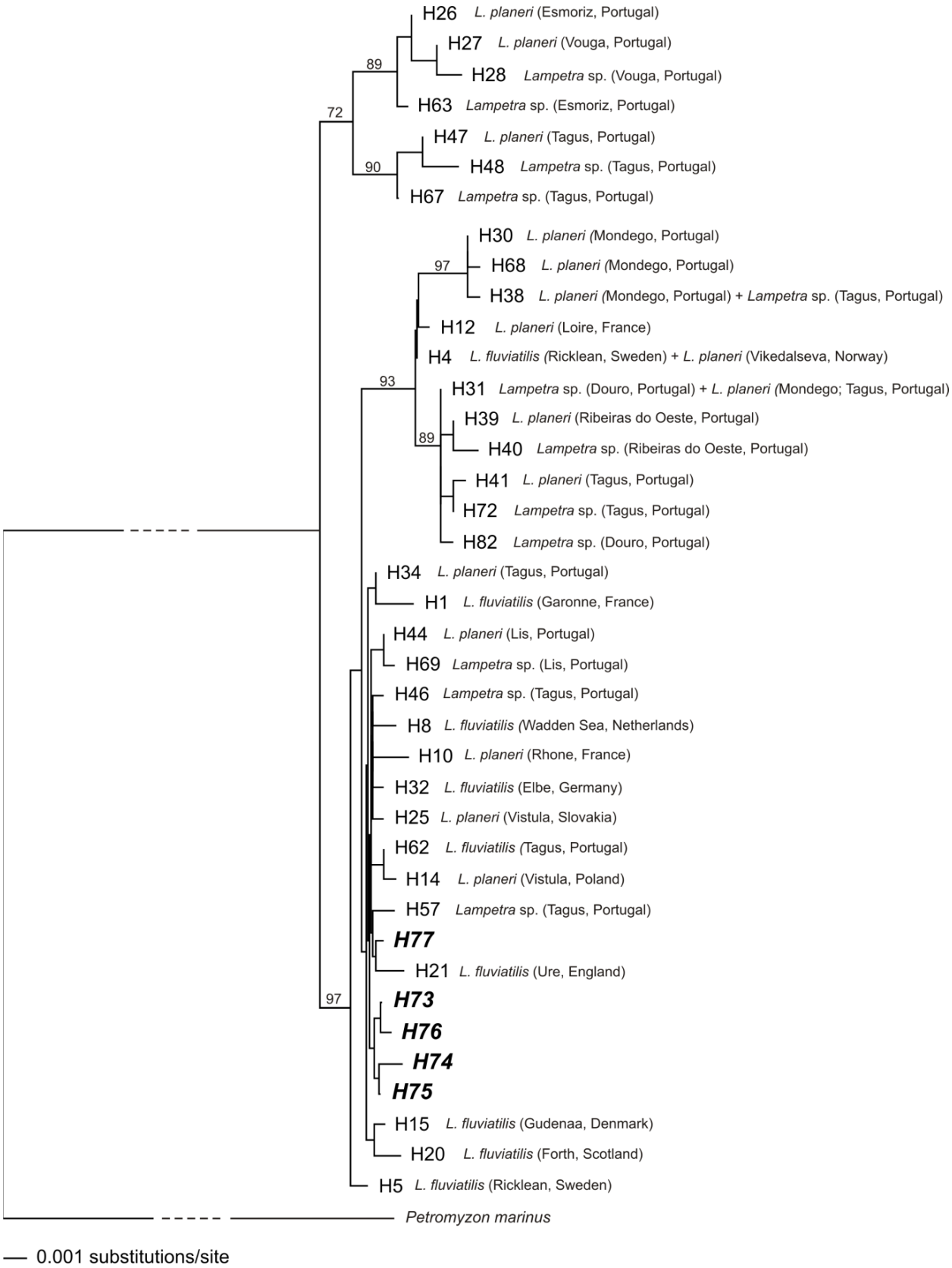

Figure 3 - Neighbour-joining phylogenetic tree of 39 mitochondrial haplotypes of *Lampetra*. For each haplotype, species, river basin and country are indicated. Haplotypes 73 to 77 (in bold) refer to the five haplotypes from River Deva. Numbers are the bootstrap support values.

Discussion

This record represents the first confirmed incidence of the genus *Lampetra* in the Cantabrian coast and the second in Spain.

The nucleotide sequences from our specimens are embedded in a widely distributed *Lampetra* clade, evidencing the correct identification of the genus (Fig. 3). The pigmentation patterns of the individuals caught in River Deva are consistent with the morphologic characteristics that discriminate *Lampetra* sp. and *P. marinus* larvae (see Potter & Osborne 1975), (Fig. 2). Sixty-one trunk myomeres were counted in the specimen from River Deva (Tab. 1), well within the range described for *L. fluviatilis* and *L. planeri* (56-69; Holčík 1986), and below that described for *P. marinus* (69-75; Holčík 1986).

These records represent a significant extension of the distributional range of the genus *Lampetra* in the Iberian Peninsula.

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Paper II | Lampreys of the Iberian Peninsula: distribution, population status and conservation

Catarina S. Mateus[1,2,3,*], Rolando Rodríguez-Muñoz[4,5], Bernardo R. Quintella[1,6], M. Judite Alves[3] & Pedro R. Almeida[1,2]

[1]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal

[2]Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, Largo dos Colegiais, 7000 Évora, Portugal

[3]Museu Nacional de História Natural e da Ciência & Centro de Biologia Ambiental, Universidade de Lisboa, 1250-102 Lisbon, Portugal

[4]Centre for Ecology & Conservation, School of Biosciences, University of Exeter, Cornwall Campus, Penryn, TR10 9EZ, UK

[5]Área de Zoología, Facultad de Biología, Universidad de Oviedo, Calle Catedrático Rodrígo Uría s/n, 33006 Oviedo, Spain

[6]Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal

Abstract

The 3 lamprey species, sea lamprey *Petromyzon marinus* L., European river lamprey *Lampetra fluviatilis* L. and European brook lamprey *L. planeri* Bloch, that inhabit the Iberian Peninsula are of conservation concern. They are considered either Vulnerable, Critically Endangered, and even Extinct in different regions of this area mainly due to habitat loss and population fragmentation. Although several other factors contribute to the decline of lamprey populations in Iberian rivers, obstacles to migration (dams and weirs) are probably the most widespread and significant, causing an estimated 80% loss of accessible habitat in most river basins. We analysed historical records from all main Iberian rivers before the construction of impassable dams became widespread, and found that lampreys were consistently present in the upper reaches. The unblocking of the lower stretches of major river basins and the restoration of former spawning sites and larval habitats should be considered as priority measures for the conservation of these species. Identification of Special Areas of Conservation to be included in the Natura 2000 European network can also be very relevant for lamprey conservation.

***Keywords*:** *Petromyzon marinus*, *Lampetra fluviatilis*, *Lampetra planeri*, historical distribution, habitat loss, conservation options.

Introduction

Living lampreys are represented by 3 families with antitropical distributions. Two families are endemic to the southern hemisphere and the third to the northern hemisphere (Hubbs & Potter 1971, Gill *et al*. 2003). Northern lampreys belong to the family Petromyzontidae, which contains 38 of the 42 current species (Lang *et al*. 2009). Three of those species occur in the Iberian Peninsula (i.e. sea lamprey *Petromyzon marinus* L., 1758, European river lamprey *Lampetra fluviatilis* L., 1758 and European brook lamprey *L. planeri* Bloch, 1784). The first 2 are anadromous and parasitic, and the other is freshwater resident and non-parasitic. The European river and brook lampreys are considered "paired species", i.e. they are closely related and morphologically very similar but have different modes of adult life (Zanandrea 1959).

The Iberian Peninsula was one of the most important refugia in the European subcontinent during the Pleistocene glaciations, acting intermittently as a refugium and a source for postglacial expansion. Many species display a strong population substructure within Iberia and are actually composed of isolated populations in distinct Iberian subrefugia as a consequence of extended periods of isolation throughout the ice ages (Gómez & Lunt 2006). The recognition of refugia and subrefugia has implications for conservation genetics, highlighting the areas where conservation efforts should be concentrated. Overall, the southern regions are of particular interest because they support most of the current genetic variation of taxa not adapted to very cold environments. Thus, overall long-term conservation may benefit from the preservation of genetic diversity in these areas (Taberlet *et al*. 1998).

All lampreys of the Iberian Peninsula are of conservation concern. The sea lamprey is the only one that has economic value both in Portugal and Spain and is subjected to intensive exploitation. However, it is considered the least threatened of the 3 lampreys. In Portugal, *Lampetra fluviatilis* and *L. planeri* are currently included in the Critically Endangered category of the Portuguese Red List of Threatened Vertebrates, and *Petromyzon marinus* is classified as Vulnerable (Cabral *et al*. 2005). In Spain, *L. fluviatilis* is considered Regionally Extinct, *L. planeri* is Critically Endangered, and *P. marinus* is Vulnerable (Doadrio 2001). Globally and in Europe, the 3 species are

considered of Least Concern according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Freyhof & Kottelat 2008a,b,c), and the European Red List of Freshwater Fishes (Freyhof & Brooks 2011), respectively. Yet *P. marinus* is considered threatened in the European countries holding the main populations (i.e. France, Spain and Portugal). Table 1 lists the current IUCN categories for each species in the countries of their natural range for which this information exists.

In this paper, we analyse the distribution, conservation status and population trends of Iberian lampreys. In particular, we compare historical and recent records of lampreys in Iberian rivers, estimate habitat loss, discuss the factors that contributed most to their decline and recommend conservation measures that may contribute to their recovery.

Systematics

Lampreys (Superclass Petromyzontomorphi) together with the exclusively marine hagfishes (Superclass Myxinomorphi) represent the most primitive extant vertebrates (Renaud 1997). Living lampreys are represented by 3 distinct monophyletic groups, currently recognised as distinct families. Two of these are endemic to the southern hemisphere (Geotriidae and Mordaciidae), and the third is restricted to the northern hemisphere (Petromyzontidae; Hubbs & Potter 1971, Gill *et al*. 2003).

The term “paired species” is applied to pairs of closely related and morphologically similar lampreys of which one is non-parasitic (brook lamprey) and the other a parasitic species (Zanandrea 1959). Vladykov & Kott (1979) introduced the more general term “satellite species” to apply to those cases in which more than 1 brook lamprey species has apparently derived from a single parasitic species.

Table 1 - *Petromyzon marinus* and *Lampetra* spp. 2001 International Union for Conservation of Nature (IUCN) Red List categories for countries where information exists across their natural range. In Italy, *P. marinus* and *L. fluviatilis* are often classified as Regionally Extinct, but these species still reproduce at least in the River Magra (Bianco & Delmastro 2011). In Slovenia, *P. marinus* is present in the Adriatic river basin (Povž 2002). In Lithuania, *L. fluviatilis* and *L. planeri* are common, not being included in the Red data book (T. Virbickas & R. Repecka pers. comm.). RE: Regionally Extinct; CR: Critically Endangered; EN: Endangered; VU: Vulnerable; NT: Near Threatened; LC: Least Concern; DD: Data Deficient; NE: Not Evaluated. Other categories are R: Rare; n/t: not threatened; LR: Lower Risk; NA: not applicable; X: species occurrence not confirmed; –: no data available/not included in the Red data book.

Country	*P. marinus*		*L. fluviatilis*		*L. planeri*	
	IUCN	**Source**	**IUCN**	**Source**	**IUCN**	**Source**
Russia	EN	Russian Academy of Sciences (2001)	–	–	–	–
Finland	NA[a]	Rassi *et al*. (2010)	NT	Rassi *et al*. (2010), Urho & Lehtonen (2008)	LC	Kaukoranta *et al*. (2000)
Norway	LC[b]	Kålås *et al*. (2010)	LC[b]	Kålås *et al*. (2010)	LC[b]	Kålås *et al*. (2010)
Sweden	NT	Gärdenfors (2010)	LC	Gärdenfors (2010)	LC	Gärdenfors (2010)
Estonia	NE[c]	Lilleleht *et al*. (2008)	LC	Lilleleht *et al*. (2008)	DD	Lilleleht *et al*. (2008)
Ireland	VU	Maitland (2004)	LR	Maitland (2004)	LR	Maitland (2004)
Great Britain	VU	Maitland (2000)	VU	Maitland (2000)	VU	Maitland (2000)
Denmark	VU	Carl *et al*. (2004)	DD[d]	Carl *et al*. 2004	LC	Carl *et al*. (2004)
Lithuania	EN[e]	Rašomavičius (2007)	–	–	–	–
Poland	EN	Głowaciński *et al*. (2002)	VU	Głowaciński *et al*. (2002)	VU	Witkowski *et al*. (2003)
Belgium - Flanders	RE	Kestemont (2010)	R	Kestemont (2010)	VU	Kestemont (2010)
Belgium - Wallonia	RE[f]	Philippart (2007), Kestemont (2010)	RE[f]	Philippart (2007); Kestemont (2010)	VU	Philippart (2007); Kestemont (2010)
Germany	n/t	Freyhof (2002)	n/t	Freyhof (2002)	n/t	Freyhof (2002)
Czech Republic	RE	Lusk *et al*. (2004)	RE	Lusk *et al*. (2004)	EN	Witkowski *et al*. (2003), Lusk *et al*. (2004)
Ukraine	X	X	–	–	LC	Witkowski *et al*. (2003)
Slovakia	–	–	X	X	CR	Witkowski *et al*. (2003)
Switzerland	–	–	RE	Kirchhofer *et al*. (2007)	EN	Kirchhofer *et al*. (2007)
France	NT	IUCN France *et al*. (2010)	VU	IUCN France *et al*. (2010)	LC	IUCN France *et al*. (2010)
Slovenia	EN[g]	Povž (2011)	X	X	–	–
Croatia	DD	Mrakovčić *et al*. (2007)	X	X	NT	Mrakovčič *et al*. (2007)
Italy	–	–	–	–	NT	Bianco *et al*. (2011)
Spain	VU[h]	Doadrio (2001)	RE	Doadrio (2001)	CR[i]	Doadrio (2001)
Portugal	VU	Cabral *et al*. (2005)	CR	Cabral *et al*. (2005)	CR	Cabral *et al*. (2005)

[a]Recorded, but only occasionally and/or not reproducing; [b]Little information available on the distribution and status in Norway. It is assumed that <1% of the total European stock occurs in Norway (E. Thorstad pers. comm.); [c]Rare in Estonian waters. No reliable data available about the reproduction of sea lamprey in Estonia (Saat *et al*. 2002); [d]Species is rare and may be threatened, but data are missing from several of the suspected habitats; therefore categorised as DD; [e]Population abundance is very low, has been officially recorded in Lithuania a few times (T. Virbickas & R. Repecka pers. comm.); [f]Likely to return (Philippart 2007); [g]In Slovenia it is very rare and is restricted to the Pirano Bay and inflowing rivers in the North Adriatic Sea (Povž 2011); [h]Endangered according to decree no. 139/2011 (BOE 2011), but only for populations from the Rivers Guadiana, Guadalquivir and Ebro and those from the southern basins; [i]Vulnerable according to decree no. 139/2011 (BOE 2011).

There has been much controversy about the taxonomic status of paired lamprey species. Some earlier authors suggested that parasitic and non-parasitic forms are not fully differentiated species (e.g. Eneqvist 1937, McPhail & Lindsey 1970), and more recently, genetic studies using distinct molecular markers (especially mitochondrial DNA) failed to find genetic differences between lamprey species pairs (e.g. Docker *et al*. 1999, Espanhol *et al*. 2007, Blank *et al*. 2008, Hubert *et al*. 2008).

Are the ecological differences between species pairs associated with distinct gene pools, or do environmental factors trigger a divergent adult phase? There is likely not a single answer for all lamprey species pairs (Docker 2009). Schreiber & Engelhorn (1998) studied allozyme markers of the paired species *Lampetra fluviatilis* and *L. planeri* and suggested that there was gene flow between these species where they occurred in sympatry and that the 2 are therefore not distinct species. Also, Espanhol *et al*. (2007) and more recently Mateus *et al*. (2011a) analysed the phylogeography of Iberian and European populations of the species pair *L. fluviatilis* and *L. planeri* using the cytochrome *b* and ATPase (subunits 6 and 8) genes, and in both studies, the clades recovered were not species specific. Analysis of more variable genetic markers, like microsatellite loci, is needed to help understand whether this species pair is composed of 2 recently diverged species or of 2 forms of the same species. Microsatellite loci are highly polymorphic and have high mutation rates, making them especially useful for the study of fine-scale population structure as they are capable of detecting differences among closely related populations or recently diverged species (O'Connell & Wright 1997).

Mateus *et al*. (2011a) identified highly divergent allopatric lineages in the Iberian Peninsula and suggested the existence of a complex of incipient or cryptic species in this region. These authors identified a number of Iberian populations that merit separate management and have high priority for conservation as Evolutionary Significant Units (ESUs) or Management Units (MUs). Four ESUs were defined for *Lampetra planeri*, 3 exclusive to the Iberian Peninsula (Sado basin, River Nabão and Esmoriz/Vouga basins) and another that is distributed across Europe. For *L. fluviatilis*, a unit including not only the threatened Iberian population but also populations from across Europe was suggested (Mateus *et al*. 2011a). Both Espanhol *et al*. (2007) and

Mateus *et al*. (2011a) found that genetic diversity is considerably higher in Iberian populations compared to European populations, which reflects the persistence and independent evolution in refugia and subrefugia during the ice ages.

For the sea lamprey, analysis of the mitochondrial control region has been used to compare populations from the Iberian Peninsula with populations from North America (Rodríguez-Muñoz *et al*. 2004). Iberian samples showed an almost identical frequency of the observed haplotypes, but none of these haplotypes was found among North American populations, suggesting that sea lamprey populations living on each side of the Atlantic have a long history of reproductive isolation. The authors suggested that the low number of haplotypes observed in sea lamprey from Spanish rivers is evidence for bottlenecking and suggest that analysis of nuclear DNA microsatellite variation is required (Rodríguez-Muñoz *et al*. 2004). Bryan *et al*. (2005) used microsatellite loci to investigate the spatial structure, invasion dynamics and origins of sea lamprey populations in the Great Lakes and anadromous populations in North America. The authors included an anadromous population from the River Mondego (central Portugal) in the analysis and concluded that this population had the lowest average number of alleles per locus of any sea lamprey population examined, showing evidence of a genetic bottleneck probably due to a large reduction in population size, and reflecting the vulnerable state of sea lamprey populations in Europe (Bryan *et al*. 2005). Population bottlenecks reduce genetic variation and, consequently, the population's capacity to face environmental changes. These results support the assumption that European and North American sea lamprey populations are reproductively isolated and should be managed independently.

Distribution

Present distribution

The genera *Petromyzon* (monospecific) and *Lampetra* are represented both in Europe and North America, and within the genus *Lampetra*, 2 species, the European river and brook lampreys, are endemic to Europe (Hardisty 1986a).

Petromyzon marinus is distributed on both sides of the North Atlantic. In North America, it occurs on the east coast from Labrador (Canada) in the north (53°N) to Florida (USA) in the south (30°N). In Europe, it can be found from the Barents Sea (Kola Peninsula, 70°N) in the north to the Iberian Peninsula (38°N) in the southwest and Adriatic Sea (40°N) in the southeast (Hardisty 1986b). It has also been documented in the Aegean Sea (Economidis *et al*. 1999) and the Levantine Sea (eastern Mediterranean; Cevik *et al*. 2010). Occasionally, it occurs off Iceland, Greenland and in the North and Baltic Seas (Hardisty 1986b). It has occasionally been found at lower latitudes in northern Africa (Boutellier 1918, Dollfus 1955). Several landlocked populations inhabit the North American Great Lakes, but none has been reported for Europe (Kottelat & Freyhof 2007). Fig. 1A shows the present distribution of *P. marinus* on the Iberian Peninsula. In Spain, it occurs in most rivers flowing into the Cantabrian Sea and the Atlantic Ocean, as well as some of the Mediterranean (Fig. 1A). Along the Cantabric coast, it is present in nearly all river basins located west of the River Deva. These basins include the Mera, Ouro, Masma and Eo in Galicia (Cobo *et al*. 2010), and the Navia, Nalón, Sella and Deva in Asturias (Rodríguez-Muñoz 1992). It occurs at the lower reaches of the River Bidasoa (Navarra), in the Bay of Biscay and at the eastern end of the Cantabrian Sea (Doadrio 2001). Along the Atlantic coast, it can be found in the basins of the Rivers Mandeo, Anllóns, Tambre, Ulla, Umia and Lérez in Galicia (Cobo *et al*. 2010), and in the Guadiana, Guadalquivir estuary, Guadalete and Barbate in Andalusia. In the Mediterranean, it is found in the Guadiaro and Ebro (Doadrio 2001; Fig. 1A). In Portugal, it occurs in all major river basins (Minho, Lima, Cávado, Douro, Vouga, Mondego, Tagus and Guadiana, Fig. 1A), being more abundant in the central and northern regions of the country (Almeida *et al*. 2008).

Lampetra fluviatilis is restricted to European watersheds, where its range extends from southern Norway (around Bergen), along the Baltic and North Sea coasts, the Atlantic waters of the British Isles, France and the Iberian Peninsula (River Tagus), to the western Mediterranean (along French and western Italian coasts; Hardisty 1986c). It has also been reported for Turkey (Erguven 1989). In contrast to the rare sea lamprey, the river lamprey is generally a common and widely distributed member of the ichthyofauna of the Baltic Sea (Thiel *et al*. 2009). There are occasional records in

Adriatic and Ionian seas. Landlocked populations are known from Lakes Ladoga and Onega and the Volga basin (Russia), Loch Lomond (Scotland), some lakes in Finland and possibly Lough Neagh (Northern Ireland; Kottelat & Freyhof 2007). On the Iberian Peninsula (Fig. 1B), the river lamprey lives as a single isolated population in the Portuguese part of the Tagus river basin (Almaça & Collares-Pereira 1988), which is extremely reduced (Cabral *et al*. 2005). Its distribution is limited by the Belver dam in the Tagus (150 km from the river mouth), Castelo de Bode dam in the River Zêzere (12 km from the confluence with the Tagus), Montargil dam in the River Sôr (91 km from the confluence with the Tagus) and Gameiro weir in the River Raia (20 km from the confluence with the Sôr; Table 2, Fig. 2).

The distributional range of *Lampetra planeri* coincides for the most part with that of *L. fluviatilis*, although the former penetrates farther inland in central and northern Europe (Hardisty 1986d). *L. planeri* occurs in rivers draining into the North Sea north to Scotland and around Stavanger (Norway), in the Baltic Sea basin and in the Atlantic Ocean basin as far south as Portugal, in the Mediterranean basin in France and in western Italy. It occurs in the upper and middle parts of the Volga basin and in the Danube basin. On the Iberian Peninsula, the brook lamprey is more widely distributed than its parasitic counterpart (Fig. 1C). It is widespread in the west Iberian basins, with confirmed presence in the Douro, Esmoriz, Vouga, Mondego, Lis, Ribeiras do Oeste, Tagus and Sado river basins (Espanhol *et al*. 2007, Mateus *et al*. 2011a). In Spain, a population inhabits the River Olabidea (Navarra) close to the Pyrenees, a tributary of the River Adour in France, which flows into the Cantabrian Sea at the Bay of Biscay (Doadrio 2001). The genus *Lampetra* was recently reported during a sampling survey in the River Deva in the central Cantabrian Sea, northern Spain (Mateus *et al*. 2011b); this population was later assigned to *L. planeri* (Perea *et al*. 2011).

On the Iberian Peninsula, the 3 species live in sympatry in a single basin, the River Tagus, and within this basin, the co-occurrence of all 3 species has only been confirmed in the River Sorraia, the main tributary of the Tagus basin (C.S. Mateus *et al*. pers. obs.). The sea and brook lampreys co-occur in other rivers, such as the Vouga and Mondego in Portugal and the Deva in Spain.

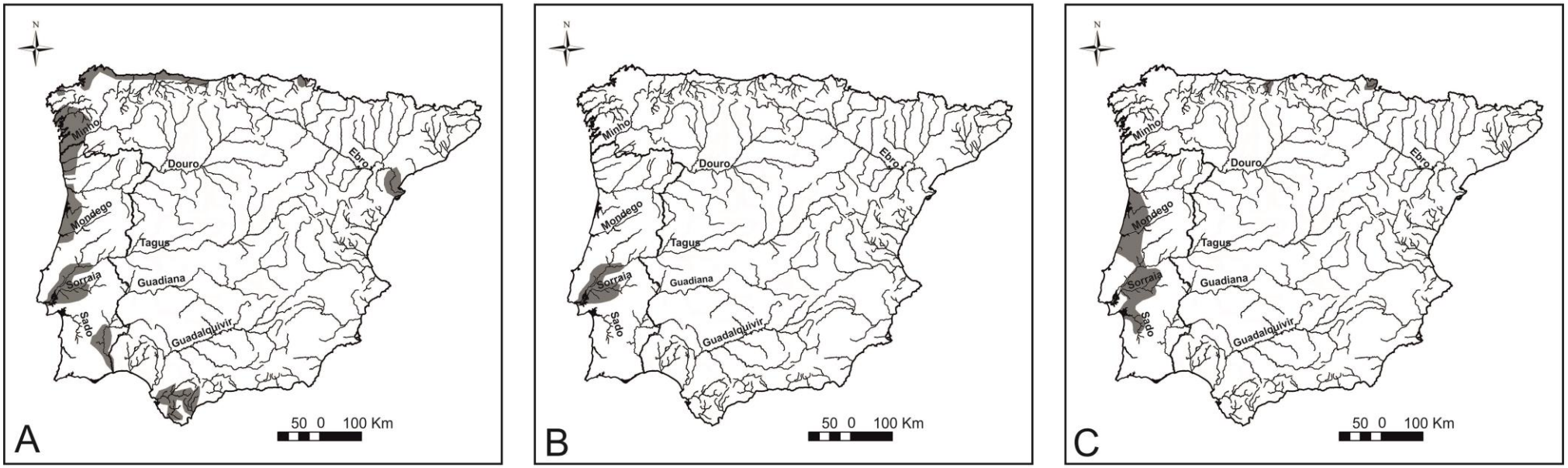

Figure 1 - *Petromyzon marinus* and *Lampetra* spp. Geographic distribution (gray shading) of (A) *P. marinus*, (B) *L. fluviatilis* and (C) *L. planeri* in the Iberian Peninsula.

Historical distribution

Historical data on lamprey distribution in the Iberian Peninsula are very scarce. First records were published in the late 19th and early 20th centuries (Steindachner 1866, Gibert 1912), but some data on lamprey distribution are also available from references on geography and history, with no details on species identity (e.g. Miñano 1827a, Escolar 1865). Because lampreys are rarely confused with other fish, these references are probably quite reliable in terms of how widespread lampreys were in the recent past. Before the building of insurmountable dams, lampreys were present at the headwaters and tributaries of all major Iberian basins (Miñano 1827a,b, Escolar 1865, Baldaque da Silva 1891, Granado-Lorencio 1991, 1996, Abel 1998, Fernández Pasquier 1999, Torrente 1999, Doadrio 2001, Pérez-Bote 2002, Elvira 2004, Pérez-Bote *et al*. 2005, Frutos 2011; Fig. 2). After the building of most of the dams during the second half of the 20th century (Santo 2005, Cea Azañedo & Sánchez Cabezas 2007), upstream migration became blocked at the lower stretches of all major rivers, interrupting the movement of lampreys along most of the main stem and principal tributaries, with an estimated 80% loss of accessible habitat (Table 2, Fig. 2). In the Guadalquivir river basin, some of the most important migratory species (i.e. sea lamprey, sturgeon *Acipenser sturio* L. and shad *Alosa alosa* L.) seem to have disappeared completely (Granado-Lorencio 1991).

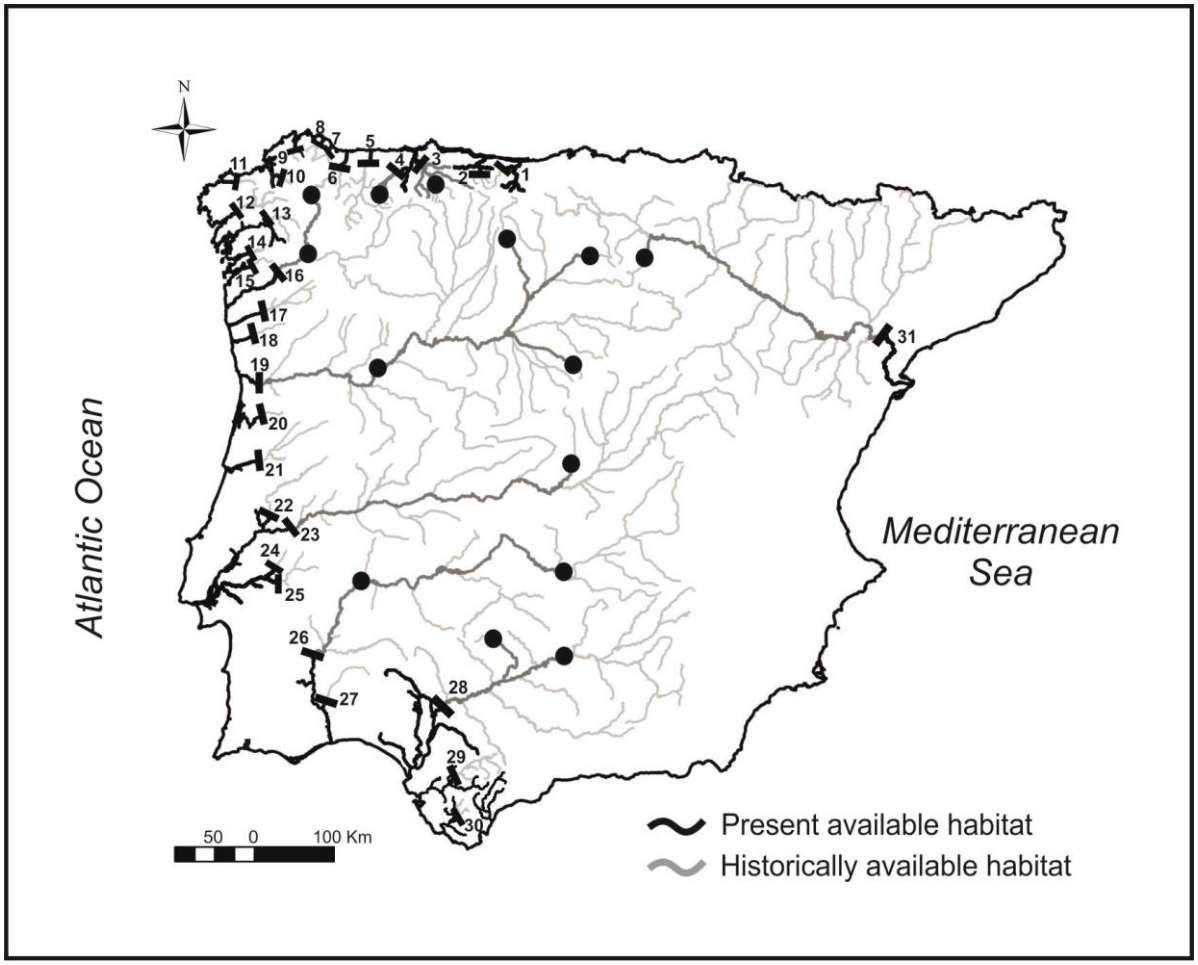

Figure 2 - Iberian Peninsula, showing the first insurmountable obstacles (black bars, numbered as in Table 2) to the migration of *Petromyzon marinus* and *Lampetra fluviatilis*, present available habitat (in black) and historically available habitat (dark grey) in the main river basins, and location of historical lamprey records (black dots). Historically available habitat was considered as the river stretch between the first insurmountable obstacle and the historical record located more upstream in the main course. When records were located in tributaries, the upper limit was considered their confluence with the main course. Only rivers with lamprey records are included. Data sources are given in the text ('Distribution' section).

Table 2 - First obstacles to the migration of *Petromyzon marinus* and *Lampetra fluviatilis* in the main Iberian rivers and some main tributaries where they occur, with reference to their construction year, distance from the river mouth and, for the main basins with historical records, habitat loss quantification in the main stream. When the obstacle is located in a tributary, the main stream is given in parentheses. Numbers correspond to obstacles shown in Fig. 2. Present available habitat and habitat loss were both measured along the main stream, with no information on tributaries. Data sources: Assis (1990), Granado-Lorencio (1991, 1996), Rodríguez-Muñoz (1992), Almeida *et al*. (2000, 2002), Santos *et al*. (2002), Quintella (2006), Andrade *et al*. (2007), Cobo *et al*. (2010), unpubl. data. NA: not applicable; –: lack of historical data.

Country	River	Obstacle	No.	Construction year	Present available habitat (km)	Habitat loss (km)
Spain						
Asturias	Cares (Deva)	Niserias weir	1	Unknown	24	–
	Sella	Caño weir	2	Unknown	35	–
	Nalón	Valduno dam	3	2000	29	–
	Narcea (Nalón)	Calabazos dam	4	1966	33[a]	–
	Navia	Arbón dam	5	1967	15	–
Galicia	Eo	Pé de Vinã weir	6	1993	32	–
	Masma	Celeiro weir	7	Unknown	7	–
	Ouro	Piscifactoría do Ouro weir	8	Unknown	9	–
	Mera	Natural obstacle	9	N/A	11	–
	Mandeo	Maquias de Chelo weir	10	Unknown	12	–
	Anllóns	C.H. Anllóns dam	11	Unknown	13	–
	Tambre	Barrié de La Maza dam	12	1958	16	–
	Ulla	weir	13	Unknown	60	–
	Umia	Segade waterfall	14	N/A	26	–
	Lérez	Bora weir[b]	15	Unknown	7	–
	Minho[c]	Frieira dam	16	1970	80	174 (69%)
Andalusia	Chanza (Guadiana)	Chanza dam	27	1989	0.5[d]	–
	Guadalquivir	Alcalá del Río dam	28	1930	104	290 (74%)
	Guadalete	Arcos dam	29	1965	84	–
	Barbate	Barbate dam	30	1992	50	–
Tarragona	Ebro	Flix dam	31	1948	116	564 (83%)
Portugal	Lima	Touvedo dam	17	1993	48	–
	Cávado	Penide dam	18	1951	27	–
	Douro[c]	Crestuma-Lever dam	19	1985	20	496 (96%)
	Vouga	Grela dam	20	1993	53	–
	Mondego	Açude-Ponte dam	21	1981	35	–
	Zêzere (Tagus)	Castelo de Bode dam	22	1951	12[e]	–
	Tagus[c]	Belver dam	23	1952	150	483 (76%)
	Sôr (Tagus)	Montargil dam	24	1958	91[e]	–
	Raia (Tagus)	Gameiro weir	25	1960	20[f]	–
	Guadiana[c]	Pedrogão dam[g]	26	2005	132	516 (80%)

[a]From the confluence with the Nalón; [b]This weir does not block lamprey access completely, as larvae have been caught upstream from it. Upper limit for lamprey migration is unknown; [c]River flows both in Portugal and Spain; [d]From the confluence with the Guadiana; [e]From the confluence with the Tagus; [f]From the confluence with the Sôr; [g]In years of normal meteorological conditions, the first obstacle is a waterfall called 'Pulo do Lobo', located 85 km from the river mouth.

Habitat Requirements

Lampreys distribute via 2 contrasting behaviours: upstream spawning migration of adults and downstream drift of ammocoetes. Through these opposing movements, adult lampreys find suitable spawning conditions, and ammocoetes locate the silted bottoms of the middle and lower courses, where conditions are favourable for their feeding and burrowing activities (Hardisty & Potter 1971).

Ammocoetes

Studies of the influence of environmental variables on the distribution and abundance of larval lampreys have primarily focused on small-scale analysis of general preferences and requirements (e.g. Malmqvist 1980, Almeida & Quintella 2002, Sugiyama & Goto 2002). Recently, the need to develop studies that allow the evaluation and prediction of lamprey occurrence/abundance at multiple spatial scales has been emphasised. For example, Almeida *et al*. (2011a) found that the presence of *Lampetra* ammocoetes in Portuguese basins is strongly associated with abiotic macro-scale predictors related to lithology, altitude, water availability and temperature. This large-scale approach was particularly important, as the information gathered could be used as a tool to prioritise rivers for conservation of these species.

Ammocoetes select soft substrates where the current is slow but relatively constant, in mainstream areas protected from major environmental fluctuations and with a plentiful food supply in the form of microalgae and particulate organic matter. Such conditions are commonly found in eddies, backwaters, at bends or behind obstacles, where organic material tends to accumulate. These areas, which are often partially shaded by trees, are favourable for the growth of diatoms, the preferred food item (Hardisty & Potter 1971, Hardisty 1979). Ammocoete colonisation is most dependent on stream gradients which dictate overall current velocity, the type of substrate particles that are deposited and the accumulation of organic debris (Hardisty & Potter 1971). The influence of the sediment particle size and current velocity on ammocoetes distribution was recognised early on by many authors (e.g. Hardisty 1944, Baxter 1957, Malmqvist 1980, Morman *et al*. 1980, Potter *et al*. 1986, Almeida &

Quintella 2002). Other variables such as the organic content (e.g. Hardisty 1944, Potter *et al*. 1986, Waterstraat & Krappe 1998), presence of macrophytes (e.g. Potter *et al*. 1986, Waterstraat & Krappe 1998), shading (e.g. Hardisty 1944, Potter *et al*. 1986, Waterstraat & Krappe 1998) and water temperature (e.g. Morman *et al*. 1980) were also considered important in determining the distribution of the ammocoetes.

Studies on the distribution and habitat selection of larval lampreys in the Iberian Peninsula have mainly focused on sea lamprey (Rodríguez-Muñoz 2000, Almeida & Quintella 2002, Quintella *et al*. 2003, Cobo *et al*. 2010). Most of the unobstructed lengths of Iberian rivers run through low-slope landscapes, which makes larval habitats potentially more abundant than those required for nest building. On the Cantabrian coast, sea lamprey larvae are usually restricted to the middle and lower reaches of the main rivers, where suitable substrate of sand and clay are more common (Rodríguez-Muñoz 1992). In the River Sella (Cantabrian coast, northern Spain), larvae under 70 mm are more abundant in the middle than in the upper and lower river reaches, whereas no pattern was observed for larger larvae (Rodríguez-Muñoz 2000). In the River Mondego (central Portugal), sea lamprey ammocoete distribution is strongly dependent upon sediment particle size. Smaller individuals (20 to 60 mm) are commonly found on silty, sand bottoms. Ammocoetes of 60 to 140 mm prefer a more heterogeneous substrate, with equal contributions of gravel and silt. Larger ammocoetes (140 to 200 mm) prefer coarse-grained sediments of sand or gravelly-sand (Almeida & Quintella 2002). In this river, possibly due to a severe reduction in the habitat available for adult sea lampreys, ammocoete abundance is higher in areas where spawning activities are observed (Almeida & Quintella 2002).

In Europe, spawning in sea lampreys starts at 15°C, whereas in river and brook lampreys it starts at 10 to 11°C (Hardisty & Potter 1971). In the River Sella, sea lampreys spawn at water temperatures between 13 and 16°C, and eggs hatch and survive at temperatures between 15 and 23°C. Temperatures below this range limit survival (Rodríguez-Muñoz *et al*. 2001). Larvae from age 0+ collected from the same river and maintained in the laboratory at different constant temperatures within the range of 5 to 30°C increased their body mass when reared between 15 and 27.5°C (Rodríguez-Muñoz 2000).

Adults

Typical spawning habitats for adults are generally found in upper river regions, in contrast with ammocoete habitats that are usually found more downstream (Waterstraat & Krappe 1998). Ammocoetes release pheromones (bile acids) into the water, which play an important role in attracting anadromous adult lampreys towards those rivers containing larvae. Adult females are subsequently attracted to the spawning grounds by sexual pheromones released by mature males, the first to arrive and begin nesting activities (Hardisty 1986a).

The 2 most important factors involved in the location of the spawning grounds are the presence of substrate suitable for the excavation of redds and relatively stable current flow. Both vary with the body size of the species. Larger lampreys, such as the sea lamprey, utilise sites where the gravels vary in diameter from 1.5 to 11.0 cm, whereas smaller brook lampreys choose particle sizes smaller than 0.5 cm (Hardisty 1986a). Sea lampreys frequently spawn immediately downstream from weirs or other obstructions to upstream migration in strong currents (1 to 2 m s^{-1}), whereas brook lampreys prefer sites with current velocity between 0.2 and 0.3 m s^{-1}. Other variables such as stream order, stream size, water depth, shading and water temperature are also important in spawning site selection (Hardisty 1986a).

The spawning behaviour during the riverine adult phase of the parasitic *Lampetra fluviatilis* from the Iberian Peninsula is still poorly understood, mostly because of the impediments derived from the conservation status and small size of the single existent population. Results of a study on the spawning migration of this species using radio transmitters (ATS-Model 1415; dimensions: 0.5 g in air, 6 mm in diameter and 12 mm in length) in the River Almansor, a small tributary of the Tagus river basin (Almeida *et al*. 2011b), revealed that diurnal resting sites are generally found in covered locations near the banks, in sites with moderate water velocity (0.22 m s^{-1} on average), about 35 cm in depth and mainly sandy bottom. Upstream spawning migration was exclusively nocturnal, characterised by a discontinuous movement alternating between periods of migration and periods of stationary rest. Surprisingly, an apparently transposable weir made of loose stones was actually insurmountable for

the upstream-migrating adults. In fact, the relatively small size of spawning adults from this population (ca. 25 to 30 cm total length and 30 to 40 g weight) suggests that their swimming capability is probably much lower than that of *Petromyzon marinus*. The migrating adults exhibited, on average, a swimming velocity of 0.40 km h^{-1} (1566 body lengths h^{-1}). This study was conducted for 3 yr, and during that period, only 12 adult river lampreys were caught, which is indicative of the rareness of this population (Almeida *et al*. 2011b).

Legislation and protection

The 3 lamprey species are listed in Annex III (protected fauna species) of the Bern Convention (Convention on the Conservation of European Wildlife and Natural Habitats) and in Annex II of the European Union Habitats Directive (92/43/EEC), which lists animal and plant species of interest to the European Community whose conservation requires the designation of Special Areas of Conservation (SACs) by the member states. *Petromyzon marinus* is listed in the OSPAR convention list (Convention for the Protection of the Marine Environment of the North-East Atlantic) of threatened and/or declining species, and their European populations are protected by Annex B-II of the European Habitats Directive and Annex III of the Bern Convention. The river lamprey is also listed in Annex V of the Habitats Directive, which lists animal and plant species of community interest, for which capture and exploitation may be controlled by management measures.

According to the Habitats directive, a SAC is a site of Community importance designated by the Member States through a statutory, administrative and/or contractual act where the necessary conservation measures are applied for the maintenance or restoration, at a favourable conservation status, of the natural habitats and/or the populations of the species for which the site is designated. These areas are then associated in a European ecological network called Natura 2000. This network, composed of sites hosting the natural habitat types listed in Annex I and habitats of the species listed in Annex II of the Habitats Directive, shall enable the natural habitat types and the species' habitats concerned to be maintained or, where

appropriate, restored at a favour able conservation status in their natural range. The SACs for lampreys should be characterised by good water quality, clean coarse substrate at spawning grounds and the presence of fine sand/silt sediment down-stream of spawning areas which may constitute ammocoete beds. Access from the sea to spawning areas must also be ensured for anadromous lampreys. Following this directive, several countries have already defined sites important for lamprey species to form part of the Natura 2000 network. For example, in 2004, Germany proposed a number of SACs in German Baltic waters to the EU Commission. These SACs cover parts of the estuarine Szczecin Lagoon and adjacent waters, covering the main migration route of river lampreys to their most numerous spawning sites (Thiel *et al*. 2009). Also, a list of SACs for sea, brook and river lampreys has been proposed for Ireland (Kelly & King 2001). The designated sites give particular emphasis to channels in which the 3 species are known to co-occur. Similarly, the primary reason for the selection of the River Teith (Scotland) to be proposed as a SAC was that, unlike many other British rivers, it supports populations of all 3 lamprey species (Maitland & Lyle 2003). When species co-occur, since many of the habitat requirements of the 3 species are the same, particularly during the larval phase, the creation of SACs should benefit all 3 species (Maitland 2004). In France, 84 Natura 2000 sites were designated due to, among other reasons, their importance to *Petromyzon marinus*. For *Lampetra fluviatilis* and *L. planeri*, 49 and 215 Natura 2000 sites, respectively, were defined in France (Muséum National d'Histoire Naturelle 2003–2012).

For the genus *Lampetra* in Portugal, following an extensive sampling campaign where the presence and abundance of larvae were related to the characteristics of the habitat, a total of 31 river stretches from 8 river basins with the potential to be designated as SACs were identified (Almeida *et al*. 2011a). Ten locations have been selected to be proposed as SACs only in the Tagus basin, of which 8 presumably support populations of the 3 species, as no obstacle to the migration of the 2 anadromous species is known to occur. However, site designation by the proper authorities will not be sufficient to ensure the conservation and protection of this genus, classified with the most threatened conservation status (Critically Endangered), as management actions will be required to ensure their conservation.

In the Iberian Peninsula, besides the classification in the threatened category of the Red Lists of both Portugal and Spain (Doadrio 2001, Cabral *et al*. 2005), the 3 species are protected by several laws. In Portugal, all 3 species are included in the following laws: decree no. 140/99 (DR 1999), Appendix B-II (and B-V for the river lamprey), transposition to the Portuguese legislation of the Habitats Directive (92/43/CEE), 21 May; decree no. 316/89 (DR 1989), transposition to the Portuguese legislation of the Bern Convention (Appendix III); law no. 7/2008, which governs fishing activities in inland waters (DR 2008), and respective publication of regulations. Additionally, the sea lamprey, as an important economic resource in Portugal, is also protected by the following: decree no. 43/87 (DR 1987) and decree no. 7/2000, which governs fishing activities in non-oceanic inland waters (DR 2000), and complementary legislation for each river basin. In general, the fishing period for the sea lamprey is established between the beginning of January and the end of April. Captures are limited to lampreys over 350 mm in body length and to a maximum of 30 ind. d^{-1} for each fisherman. In river basins where the species is less abundant, the quota is lower (e.g. 6 specimens in the River Guadiana and 10 specimens in the Rivers Vouga and Cávado for the year 2011).

In Spain, all 3 species are listed for protection under decree no. 1997/95 (BOE 1995), Appendix B-II (and B-V for the river lamprey), transposition to the Spanish legislation of the Habitats Directive (92/43/CEE). The sea lamprey is included in decree no. 1095/89 (BOE 1989a), which determines the species subject to fisheries and hunting in Spain and the regulations that assure their protection, and decree no. 1118/89 (BOE 1989b), which determines commercial species subject to fisheries and hunting and the related rules. Laws no. 8/98 (DOE 1998) and 9/2006 (DOE 2006) protect threatened species of the Autonomic Region of Extremadura and their habitats. Recently, decree no. 139/2011 (BOE 2011) classified *Lampetra planeri* as Vulnerable in Spain. *Petromyzon marinus* has been classified as Endangered, but this applies only to populations from the Rivers Guadiana, Guadalquivir, Ebro and those from the so-called southern basins. However, Doadrio (2001) classified *L. planeri* as Critically Endangered due to the existence of a single small population in Spain (the

only confirmed population at the time), which is declining due to the reduction of available spawning habitat.

Sea lamprey fisheries still persist in Galicia, where captures are allowed in the Rivers Tea and Ulla (DOG 2011). In the autonomic regions, this species is classified as follows: Vulnerable in the List of Threatened Species of Galicia, law no. 9/2001 (BOE 2001) and decree no. 88/2007 (DOG 2007); Endangered in the List of Threatened Species of Extremadura (Fallola et al. 2010a); Protected species of the autochthonous wild fauna of Cataluña, law no. 12/2006 (DOGC 2006); and Vulnerable in the Catalogue of Threatened Species of Vertebrates of Asturias, decree no. 32/90 (BOPA 1990).

The brook lamprey is considered Of Special Interest in Navarra according to the Catalogue of Threatened Species of Navarra, decree no. 563/95 (BON 1995), and the river lamprey is considered Regionally Extinct in Extremadura (Fallola *et al*. 2010b).

Factors contributing to population declines

European populations of sea lamprey have declined dramatically over the last 25 yr (Lelek 1987), and several authors have pointed out a reduction in sea lamprey abundance in Iberian rivers (e.g. Guimarães 1988, Almaça 1990, Assis 1990, Granado-Lorencio 1991, Almeida & Quintella 2002). This decline is also severe in the other 2 species occurring on the Iberian Peninsula, the river and brook lampreys, and several factors contribute to this reduction.

River impoundments

Habitat fragmentation and reduction by construction of large dams, weirs and other man-made barriers are among the main threats to lamprey populations both in Portugal (Cabral *et al*. 2005) and Spain (Doadrio 2001). The 2 anadromous species are severely affected by this activity, which has fewer effects on the brook lamprey due to its non-migratory ecology. Table 2 shows the percentage of habitat lost with the construction of dams in the lower stretches of all the major basins, and on average

80% of the habitat that was historically used by lampreys in each river basin is now unavailable.

Dams and weirs block the longitudinal continuity of a river, limiting the access of adults to suitable spawning grounds. This reduces the available habitat for adults to spawn and for the growth and development of ammocoetes (Table 2, Fig. 2). Spawning grounds are usually located in upstream reaches, where temperature and oxygen conditions are suitable for spawning, egg incubation and early larval development. In a study on sea lamprey in the River Mondego (central Portugal), Quintella *et al*. (2003) observed that the abundance of ammocoetes was higher in areas around sea lamprey nests, due to the severe reduction in the area available for both spawning and larval growth. In this river, the sea lamprey is confined to the lower 35 km, and adults concentrate to spawn in the uppermost 5 km downstream from the Açude-Ponte dam.

On the Iberian Peninsula, most of the dams and weirs were built in the second half of the last century (Santo 2005, Cea Azañedo & Sánchez Cabezas 2007). During this period, about 20 dams yr^{-1} were constructed in Spain, and fish migration was blocked at most of the major Spanish rivers (Cea Azañedo & Sánchez Cabezas 2007). Portuguese rivers are impounded by 166 dams and more than 3000 small weirs (Quintella 2006). The number of weirs and dams with fish passes is extremely low in Portugal, and only a small percentage of the fish passes installed are still functional (Santo 2005).

Pollution

First signs of river pollution caused by human activities have been timed around 5000 yr ago (Davis *et al*. 2000), but it is not until the dramatic increase in mining, industrial and urban development that water contamination became the key and widespread problem we experience at present (Prenda Marín *et al*. 2006, Gros *et al*. 2007, Lorenzo *et al*. 2007). The beginning of the industrial era during the 19th century set the start of the decline of Spanish river ecosystems (Pérez Cebada 2008).

Lampreys are known to be sensitive to pollution and, although few data are available, entire populations probably disappeared from rivers that became polluted.

This is most likely the case of the River Ave in northern Portugal (Quintella 2006), where sea lampreys were once considered common by Baldaque da Silva (1891) and have now vanished. Industrial pollution is probably also responsible for the extremely low density of sea lamprey larvae populations in the lower reaches of the River Cávado (Almeida *et al*. 2008). Anadromous species are especially affected by pollution barriers during their spawning migration, but in the larval phase, both resident and anadromous species are affected.

Dredging and habitat destruction

Besides the loss of spawning and larval habitats caused by dams and weirs, several other anthropogenic actions may modify the physiographic features of rivers and streams. Sand extraction may drastically modify riverbeds and cause the destruction of larval habitats; it is therefore considered to be among the main threats to lamprey larval stages (Quintella *et al*. 2007). Dredging also causes the removal of areas of riffles and associated spawning gravels, which will disturb the spawning activity of the lampreys. Channel and bank regulation can also cause the destruction of suitable spawning and larval habitats through removal of areas of riffles and dredging of suitable silt beds, respectively, and it can eliminate populations from entire river stretches.

Commercial exploitation

On the Iberian Peninsula, overfishing from commercial harvesting is a serious threat for *Petromyzon marinus*, particularly in the central and northern regions (Renaud 1997, Doadrio 2001, Cabral *et al*. 2005). The high economic value of the sea lamprey in Portugal and some Spanish regions makes them a preferred target for both poaching and legal fisheries, creating a major threat to the sustainability and conservation of this species. The fishing gears traditionally used by professional fishermen to harvest adult sea lampreys in Portugal are drift trammel nets and large fyke nets (Quintella 2006).

The gastronomic importance of sea lampreys is reflected by their high commercial value, which can easily reach €50 per animal during the peak of the season (Quintella 2006). Sea lampreys are sold directly to restaurants or intermediaries without being taxed. For that reason, the official records of sea lamprey captures are far from being realistic. In the River Mondego (central Portugal), a study by Duarte *et al*. (2003) to assess the catch rate of a large fyke net used to harvest sea lampreys in the estuary is indicative of the number of animals that are captured annually. During the 2002 spawning season, between 6 January and 13 April, 555 lampreys were captured by a single fyke net with a catch rate of 7.4 ind. tide^{-1} (12 h). The same authors gathered additional unverified information about the catch rates of 6 local fishermen who used the same fishing gear. Between January and April 2002, in a total of 6 nets, 2846 lampreys were captured. These numbers are reflective of the threat that this activity, if not properly regulated, may pose to the survival of the exploited sea lamprey populations. The impact of poachers is also not negligible in Portuguese rivers. In a study by Andrade *et al*. (2007) that was aimed at investigating the spawning migration of sea lampreys in the Vouga basin (central Portugal) via radio telemetry, 76% of the tagged lampreys were recaptured by poachers, who delivered the transmitters to the researchers involved in the study in exchange for a €50 reward.

Climate change and water availability

Most Iberian rivers are within the temperature and oxygen concentration ranges required to sustain lamprey populations. However, a shift in these ranges due to global warming, especially in the southern basins, may cause the local extinction of lamprey populations. For *Lampetra planeri*, Hardisty (1961) found that even when spawning activity is well under way, a sudden but slight drop in temperature has often resulted in the almost complete disappearance of lamprey adults from the nests.

In a recent study, Lassalle *et al*. (2008) projected that, under a climate change scenario, by the end of the 21st century *Petromyzon marinus* will show a decrease in the basins bordering the east coast of the Adriatic Sea, in most of the Italian basins and in the Iberian Peninsula. The authors calculated that this species can disappear from

the largest basins in the south of the Iberian Peninsula, remaining present only in the northern Minho basin. As the predictive model for this species included both temperature and precipitation as explanatory variables, a change in climate is projected to severely negatively affect the distribution of this species in its southern limit (Lassalle *et al*. 2008).

Populations in the southern distribution of these species are inherently at risk of extinction because in addition to anthropogenic pressures, these basins are situated in a region at risk of being significantly affected by climate change. In the southern basins, where water availability is often critical during the summer period, activities such as water abstraction accentuate the pollution impact by diminishing the dilution capacity of the streams. This may be particularly alarming in the Sado basin, which represents the southern distribution of *Lampetra*. The population inhabiting this basin has been classified as an ESU by Mateus *et al*. (2011a), constituting an important source of genetic variability, and should be prioritised in terms of conservation.

Final remarks and recommendations

On the Iberian Peninsula, like in many countries, the 3 species are classified as threatened (i.e. Critically Endangered, Endangered or Vulnerable. See Table 1). Based on genetic analyses that suggest differentiation between European and North American sea lamprey populations (Rodríguez-Muñoz *et al*. 2004), we recommend that European and North American sea lampreys be considered as different populations that should be managed independently. In view of that, and considering its conservation status in the countries holding the main populations (i.e. France, Spain and Portugal), we propose that the European population of *Petromyzon marinus* be revised to a threat category in the IUCN Red List. Also, we recommend that the conservation units identified by Mateus *et al*. (2011a) for Iberian populations of *Lampetra fluviatilis* and *L. planeri*, following mitochondrial DNA analysis, be considered in future IUCN Red List revisions.

The recently confirmed presence of the genus *Lampetra* in a river basin in Asturias (Mateus *et al*. 2011b) is indicative that this genus may occur in other rivers

from this Spanish region and possibly in neighbouring regions like Galicia and Cantabria. Further data on the distribution of *Lampetra* in Spain is needed, especially in these regions, where its presence is expected.

During the larval phase, the 3 species occupy similar (often the same) habitats. Thus, factors that affect 1 species are likely to affect the other 2. Similarly, conservation requirements to enhance and restore populations are likely to be very similar for all 3 species (Maitland & Lyle 2003). *Petromyzon marinus* and *Lampetra fluviatilis*, however, require a pathway from their adult feeding grounds in the marine environment to their spawning grounds, whereas *L. planeri* is a purely freshwater species, requiring access only between larval and spawning habitats. One of the main problems in the adult phase of migratory species is related to river impoundments, since the habitat that was historically used by these species is now unavailable. Based on historical records of lampreys in the upper reaches of the main river basins, we quantified range contraction caused by the construction of insurmountable obstacles in the lower reaches of most rivers to be no less than 80% of the original area. In the River Douro, the largest basin on the Iberian Peninsula, the loss was 96%, since the first dam is located just 20 km from the river mouth (Table 2, Fig. 2). Management should focus on unblocking the lower stretches of all major river basins, so that adult and juvenile migration can be resumed. Unblocking can be accomplished by either the removal of barriers and weirs or the construction of functional fish passages in rivers where spawning and larval habitats are situated. Delimiting viable areas suitable to be used by ammocoetes, in conjunction with the restriction of economic activities such as sand extraction, can be effective conservation measures for the protection of larval habitats (Quintella *et al*. 2007).

From data obtained through a predictive distribution model of the genus *Lampetra* in Portugal, Almeida *et al*. (2011a) identified a number of river stretches with the potential to become SACs. These cover the main migratory routes of river lampreys to their most numerous spawning sites and the most suitable larvae habitats for both river and brook lampreys. The identification of protected areas constitutes an important measure for the conservation of these species in Iberian rivers, and is particularly important for the protection of the single Iberian population of European

river lampreys. Efforts should be made to restore lost spawning sites and the connectivity between them, as well as the nursery habitats. Such efforts would benefit all 3 species. The identification of conservation units following molecular studies (e.g. Mateus *et al*. 2011a) is also of great importance to support plans focused on the maintenance of gene flow and the preservation of gene diversity.

In Portugal and some regions of Spain, the sea lamprey is a species with high economic value. In Portugal, it supports commercial fisheries in most of the major river systems. Despite the actual legislation controlling fisheries, this activity may lead to an over-exploitation of this resource. Promoting the sustainable management of commercial exploitation can minimise the negative impacts of fisheries. It is important to gather reliable records of the professional captures in each river basin where this species occurs, and professional fishing regulations should be reviewed according to scientific background information.

Lamprey populations from the southern basins are particularly vulnerable to climate changes, and additional efforts should be taken when implementing management plans. In these basins, actions causing hydraulic stress and pollution should be the first to be minimised. Knowledge on the effects of pollution is very scarce, and research needs to be done to identify important pollution problems and their geographical location, so that actions to reduce or eliminate contamination sources can be implemented.

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Chapter 3

Genetic and morphological variation of *Lampetra*

Mateus CS, Almeida PR, Quintella BR, Alves MJ (2011) MtDNA markers reveal the existence of allopatric evolutionary lineages in the threatened lampreys *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch) in the Iberian glacial refugium. *Conservation Genetics*, **12**, 1061–1074.

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Paper III | MtDNA markers reveal the existence of allopatric evolutionary lineages in the threatened lampreys *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch) in the Iberian glacial refugium

Catarina S. Mateus[1,2,3,4,*], Pedro R. Almeida[1,2], Bernardo R. Quintella[1,5] & M. Judite Alves[3,4]

[1]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal

[2]Departamento de Biologia, Escola de Ciência e Tecnologia, Universidade de Évora, Largo dos Colegiais, 7000 Évora, Portugal

[3]Museu Nacional de História Natural, Universidade de Lisboa, Rua da Escola Politécnica 58, 1250-102 Lisbon, Portugal

[4]Centro de Biologia Ambiental, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal

[5]Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal

*E-mail: csmateus@fc.ul.pt

Abstract

The Iberian Peninsula has been identified as an important glacial refugium during the Pliocene and Pleistocene epochs for the genus *Lampetra*, providing intermittent refuge and postglacial opportunities for colonization and expansion. We used mitochondrial DNA markers to investigate the processes that have shaped present-day genetic constitution of the genus *Lampetra* within the Iberian Peninsula. We surveyed 1,173 bp of the cytochrome *b* gene and 829 bp of the genes ATPase subunits 6 and 8 in 233 individuals of *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch) from 21 localities along their distribution range in the Iberian Peninsula. We identified four highly divergent allopatric evolutionary lineages that evolved by fragmentation during the Pliocene and Pleistocene likely driven by environmental factors, namely regional geomorphic events. The high level of genetic divergence between the four lineages suggests that sufficient time has apparently passed by to originate a complex of incipient or cryptic resident species and allows the definition of four evolutionary significant units (ESUs) for *L. planeri* and one ESU for *L. fluviatilis*. These findings have important consequences for the interpretation of refugia biological diversity and have major implications on the conservation of these threatened species.

Keywords**:** Iberian Peninsula, allopatric speciation, species pairs, cryptic species, critically endangered, conservation units.

Introduction

The population genetic structure of organisms is expected to reflect historical patterns of dispersal and isolation (Avise 2000; Wilson *et al*. 2004). Comparing the genetic diversity levels in marine, freshwater and anadromous fishes, there is evidence that freshwater fish tend to show higher population differentiation than marine fish, with the data from anadromous species indicating that this group occupies an intermediate position (Gyllensten 1985; Ward *et al*. 1994). These differences can be endorsed primarily to differences in average levels of gene flow, with gene flow in marine species thought to be less restricted than in freshwater species. On one hand, physical barriers to adult movement will be less pronounced in the marine environment than in freshwater habitats and on the other hand it is possible that historical founder effects and drift, brought about by the effects of Pleistocene glaciations, have impinged to a greater degree of population differentiation on freshwater species than on marine species (Gyllensten 1985; Ward *et al*. 1994).

The lampreys constitute a good model to investigate how intrinsic factors (life-history type and dispersal capability) interact with historical extrinsic factors to shape genetic structuring, as many genera present anadromous species and closely related freshwater residents. It is generally assumed that the freshwater lampreys have evolved from the migratory parasitic form and become non-parasitic (reviewed in Docker 2009).

The Iberian Peninsula seems to have played a major role as a glacial refugium for the European river lamprey *Lampetra fluviatilis* (L.) and the European brook lamprey *L. planeri* (Bloch), as Iberian populations reveal higher genetic diversity when compared to those of central and northern Europe (Espanhol *et al*. 2007). *L. fluviatilis* and *L. planeri* are ‘paired species’, i.e. the larvae are morphologically similar but the adults adopt different life history types (Zanandrea 1959). The parasitic and anadromous *L. fluviatilis* is most easily distinguished in its sexually mature stage from the non-parasitic *L. planeri* by its generally greater body size (Hardisty 1986a). Phylogeographical analysis revealed that the two taxa are not reciprocally monophyletic, suggesting that loss of the migratory ability may have occurred multiple

times (Espanhol *et al*. 2007). Many Iberian populations of *L. planeri* are apparently composed of private mitochondrial DNA (mtDNA) haplotypes, suggesting some time of independent evolutionary history for these populations (Espanhol *et al*. 2007; Pereira *et al*. 2010). As suggested for other brook lamprey species, these isolated populations may represent a complex of incipient or cryptic resident species, despite their highly conserved body form (cf. Docker 2009). This finding is in agreement with the realization that many species display a strong population substructure within glacial refugia (reviewed in Goméz and Lunt 2006) and indicates that phylogeography of *Lampetra* within the Iberian glacial refugium warrants further investigation.

Here, sequence variation in two mtDNA genes is used to evaluate the relationship between *L. planeri* and *L. fluviatilis* within Iberian river basins and the existence of divergent allopatric evolutionary lineages. We also investigate the processes that have shaped genetic structure in the genus *Lampetra*. Our results from population genetic, phylogenetic and phylogeographical analyses indicate a complex and dynamic evolutionary history of expansion and fragmentation in multiple, independent lineages, with important conservation implications.

Materials and methods

Sampling, extraction, amplification and DNA sequencing

In total, we collected 233 individuals of *Lampetra*, comprising 66 adults of *L. planeri*, 16 adults of *L. fluviatilis* and 151 ammocoetes of unknown specific status in 21 sites throughout the distributional range of the species in the Iberian Peninsula, covering all the major basins (Fig. 1; Table 1). Tissue samples were deposited in the zoological collections 'Museu Bocage' (MB85) of Museu Nacional de História Natural, Portugal (Table 1).

We extracted the total genomic DNA from muscle tissue preserved in alcohol pro-analyses by the conventional SDS-proteinase K/phenol–chloroform protocol. We quantified DNA samples using NanoDrop ND-1000 Spectrophotometer and established standard working stocks of 40 ng μl^{-1} in sterile water for all individuals.

We amplified the mitochondrial genes ATPase subunits 6 and 8 (ATPase 6/8) and cytochrome *b* (cyt *b*) by polymerase chain reaction (PCR) in a thermocycler Biometra Tgradient. A total of 1,173 bp of the cyt *b* gene were amplified using the primers LampLA and LampPRO and the internal primers LampLB and LampCB2-H (Espanhol *et al*. 2007). The primers used for the amplification of the 829 bp of the genes ATPase subunits 6 and 8 were ATPfor and ATPrev (Espanhol *et al*. 2007). PCR reactions were performed in a final volume of 25 µl, with 1 µl of total genomic DNA, 1.5 mM $MgCl^2$, 0.2 mM dNTP mix, 0.5 µM of each primer, 1 U of Taq DNA polymerase (Fermentas) and 1x of the reaction buffer supplied. PCR conditions were as follows: an initial denaturation step of 94ºC for 3 min followed by 30 cycles consisting of denaturation at 94ºC for 1 min, primer annealing at 60ºC for 1 min, extension at 72ºC for 2 min, and a final extension step of 72ºC for 2 min. The resulting PCR products were purified using the ExoSAP kit (Fermentas) and sequenced using an ABI PRISM 3730 DNA Analyser at Macrogen (www.macrogen.com).

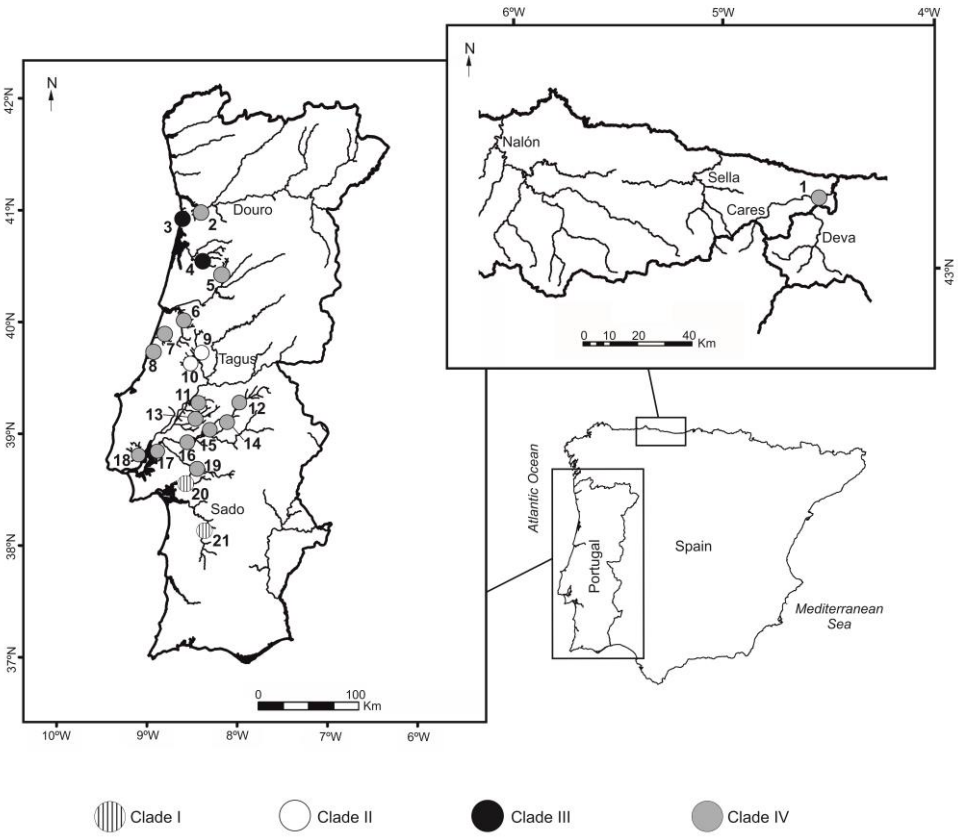

Figure 1 - Geographical distribution of the 21 sampling sites (dots) assayed in this study and of the four clades suggested by the phylogenetic analysis. Sites are numbered as in Table 1. A detailed distribution of all haplotypes is described in Table 2.

Table 1 - Collection data for the individuals analyzed in this study.

Locality	Drainage	River/Tributary	*N*	Species	Voucher no.
1	Deva*	Deva	8	unknown	(MB85) 8698-8700; 8716-8720
2	Douro	Inha	10	unknown	(MB85) 5673, 5674; 9630-9637
3	Esmoriz	Esmoriz	15	8 unknown; 7 *L. plan*	(MB85) 5497; 5502-5505; 5507-5510; 5544-5546; 9424-9426
4	Vouga	Águeda	10	6 unknown; 4 *L. plan*	(MB85) 5649-5652; 5655; 5657; 9457-9460
5	Mondego	Criz	9	*L. plan*	(MB85) 5619-5622; 5624-5628
6	Mondego	Anços	10	*L. plan*	(MB85) 5606-5610; 5612-5614; 5617; 5618
7	Lis	Lis	14	9 unknown; 5 *L. plan*	(MB85) 5639-5645; 5647; 5648; 9359-9363
8	Ribeiras do Oeste	Ribeira de S. Pedro	13	8 unknown; 5 *L. plan*	(MB85) 5629; 5631-5636; 5638; 9392-9396
9	Tagus	Nabão	9	unknown	(MB85) 5596-5603; 5605
10	Tagus	Ribeira do Olival	13	8 unknown; 5 *L. plan*	(MB85) 5587-5592; 5594; 5595; 9461-9465
11	Tagus	Ulme	32	unknown	(MB85) 5466-5486; 5576-5586
12	Tagus	Longomel	9	*L. plan*	(MB85) 5566-5571; 5573-5575
13	Tagus	Muge	7	unknown	(MB85) 5556-5559; 5562; 5564; 5565
14	Tagus	Sôr	6	unknown	(MB85) 5972-5977
15	Tagus	Erra	15	8 unknown; 2 *L. fluv*; 5 *L.plan*	(MB85) 5487-5495; 5498; 5499; 5547; 9515-9517
16	Tagus	Sorraia	1	*L. plan*	(MB85) 5501
17	Tagus	Ponta de Erva	13	*L. fluv*	(MB85) 5669-5671; 6175-6184
18	Tagus	Tagus	1	*L. fluv*	(MB85) 5971
19	Tagus	Canha	11	10 unknown; 1 *L. plan*	(MB85) 5496; 5548-5552; 5554; 5555; 5968-5970
20	Sado	Marateca	19	14 unknown; 5 *L. plan*	(MB85) 5452-5465; 9530-9534
21	Sado	Sado	8	unknown	(MB85) 5659-5665; 5667

Sampled localities are presented from north to south and locality numbers correspond to locations as in Fig. 1. Sample sizes (*N*) and specific status are also presented. Voucher numbers correspond to zoological collections 'Museu Bocage' (MB85) of Museu Nacional de História Natural, Portugal.

L. plan, *L. planeri*; *L. fluv*, *L. fluviatilis*

*Spanish river basin

Data analysis

We aligned and edited the DNA sequences manually, using Sequencher V4.8 (Gene Codes Corporation, Ann Arbor, USA). Sequences from *Petromyzon marinus* (L.) from the EMBL database (U11880) and *Eudontomyzon mariae* (Berg) from the EMBL database (AM051061) were used as outgroups.

For either gene, we performed a χ^2 test of homogeneity to test the assumption of base-compositional homogeneity. Prior to combining the two genes into single analyses, we implemented the incongruence length difference test (ILD) to access the significance of incongruence between the two data sets. Both analyses were implemented in PAUP* version 4.0b10 (Swofford 2002). All further analyses were performed in the concatenated alignment of both genes.

Levels of gene diversity were described as haplotype (h) and nucleotide (π) diversities. The definition of the haplotypes and the estimation of the levels of gene diversity were attained with the software package DnaSP version 4.50 (Rozas *et al*. 2003).

Haplotypes were connected on a network obtained using the 95% parsimony criterion implemented in the program TCS version 1.21 (Clement *et al*. 2000). We performed the phylogenetic analysis by three methods, maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML), all implemented by using PAUP*. We used the Modeltest version 3.8 software (Posada & Crandall 1998) associated with PAUP* to select the most appropriate evolutionary model of nucleotide substitution and its parameters, according to the Bayesian information criterion (BIC), following Posada & Buckley (2004). We calculated the maximum-likelihood (ML) and neighbour-joining (NJ) phylogenetic trees according to the selected model, HKY + G substitution model (gamma distribution shape parameter = 0.2102; base frequencies: A = 0.3029, C = 0.2424, G = 0.1180, T = 0.3366; transition/transversion ratio = 5.0898). For the three methods, the optimal trees were found by a heuristic search with tree-bisection-reconnection (TBR) as the branch-swapping algorithm. Initial trees were obtained via stepwise addition with 100 replicates of random addition sequence and gaps were treated as missing data. Bootstrap proportions (Felsenstein 1985) were obtained to

access node robustness, using PAUP*. In MP and NJ analyses, 1,000 heuristic pseudoreplicates were generated, each consisting of 100 heuristic TBR searches of random addition sequence. In ML analysis, 500 heuristic pseudoreplicates were generated with TBR searches of as-is addition sequence.

We performed Bayesian analyses using MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003) to produce a population of trees and parameter values used then to estimate a 50% majority-rule consensus tree. We estimated the probabilities of the phylogenetic trees by a Metropolis-coupled, Markov chain Monte Carlo sampling algorithm (MCMCMC). For each analysis, a total of 2×10^6 samples were taken (2 separate concurrent runs), with successive samples separated by 100 generations. Model selection was carried out separately for each mtDNA data set with MrModeltest version 2.2 (Nylander 2004), allowing different values for each parameter for each partition.

We also performed an analysis of molecular variance, AMOVA (Excoffier *et al*. 1992). This analysis accomplishes three components of genetic variation: among groups (F_{CT}), among populations within each group (F_{SC}), and within populations (F_{ST}). Molecular variance was first partitioned into two hierarchical levels, where individuals were assembled into two different groups, reflecting their specific taxonomic status (AMOVA I). Consequently, the 151 ammocoetes of unknown specific status were not included in this analysis. A second analysis was performed in which individuals from the same locality were treated as individual populations to test for overall genetic subdivision (AMOVA II), regardless their specific taxonomic status. Finally, molecular variance was partitioned into two hierarchical levels, where localities were assembled into different groups reflecting the results from the phylogenetic analyses (AMOVA III), regardless their specific taxonomic status. The significance of the observed variances for each hierarchical comparison was tested by 10,000 permutations.

We quantified genetic differentiation among populations by computing pairwise F_{ST} estimates calculated using conventional *F*-statistics based on mtDNA haplotype frequencies and the pairwise difference distance method. Significance of pairwise population comparisons was assessed by 1,000 permutations. All analyses were conducted using Arlequin version 3.11 (Excoffier *et al*. 2005).

We performed Fu's *Fs* (Fu 1997) and Tajima's *D* (Tajima 1989) statistics using DnaSP version 4.50. Fu's *Fs* tends to be negative under an excess of recent mutations and a significant negative value will be taken as evidence of population growth and/or selection. A positive value of *Fs* is evidence for a deficiency of alleles, as would be expected from a recent population bottleneck or from overdominant selection. Tajima's *D* statistic is influenced by changes over time in the size of populations, population structure and the action of natural selection. The test uses the nucleotide diversity and the number of segregating sites observed in a sample of DNA sequences to make two estimates of the amount of variation. In a constant-size neutral equilibrium population, the expectation of Tajima's *D* is nearly zero because the expectations of both estimates are the same. When some kind of balancing selection is acting, Tajima's *D* tends to be positive. On the other hand, purifying selection can generate negative values of Tajima's *D*.

Results

The incongruence length difference test showed that the two genes were not incongruent (ILD $P = 0.45$). For both genes, the null hypothesis of homogeneity in base composition across sequences was not rejected by the χ^2 test (ATPase: $\chi^2 = 4.06$, *d.f.* = 165, $P = 1.00$; cyt *b*: $\chi^2 = 1.31$, *d.f.* = 165, $P = 1.00$). All further results refer to concatenated alignments of both genes.

The 233 samples were grouped in 56 composite haplotypes. Haplotype codes follow Espanhol *et al*. (2007). No haplotype was observed exclusively in *L. fluviatilis*, while nine were detected only in *L. planeri* (Table 2). Considering the geographical distribution of the haplotypes, 47 were found at single localities, nine (H6, H31, H37, H38, H47, H60, H62, H65, H66) were observed in two or more localities and none was detected at all localities (Table 2). Haplotype H31 was the most frequently observed haplotype (N = 44) and was found in seven localities of the Tagus, Mondego and Douro river basins. The otherwise most frequent haplotype H6 (N = 31) was found in six localities of the Tagus, Lis and Mondego river basins. The 56 haplotypes were distinguishable by 82 polymorphic sites (*S*), 42 in cyt *b* and 40 in ATPase 6/8 (see

supplementary information, Table S1). Overall haplotype diversity (*h*) was 0.929 and nucleotide diversity (π) was 0.00633. Tajima's *D* was not significantly different from zero (D =-0.30872; *P*>0.10) but Fu's *Fs* produced a significant negative value (*Fs* = -50.260).

The tree topologies obtained from analysis of the mtDNA data by the three methods MP, NJ and ML were highly concordant and revealed four clades (I–IV), which were well supported by bootstrap and Bayesian credibility values. Clade IV presents further well supported subdivision (subclades IV-A to IV-C) (Fig. 2). As previously observed in Espanhol *et al*. (2007), clades recovered are not species specific: clades I, II and III are composed of adults of *L. planeri* and ammocoetes of unknown specific status, while clade IV includes ammocoetes and both migratory and resident adults. Clades do not apparently overlap geographically: clade I includes the samples from Sado basin, represented by 14 haplotypes; clade II includes the individuals from River Nabão and its tributary Ribeira do Olival; clade III includes the populations from Esmoriz and Águeda rivers; and clade IV shows a wider distribution from Tagus river basin to the northern Spanish River Deva. Subclade IV-A groups individuals from the Tagus, Ribeiras do Oeste, Mondego, and Douro river basins; subclade IV-B includes individuals from the Mondego and Tagus river basins; and subclade IV-C groups individuals from the Tagus, Lis, Mondego, and Deva river basins (Fig. 2; Table 2). The parsimony network of haplotypes (Fig. 3) revealed that clade IV has a double star-like structure, with two dominant haplotypes H31 and H6 in the centre of subclades IV-A and IV-C, respectively.

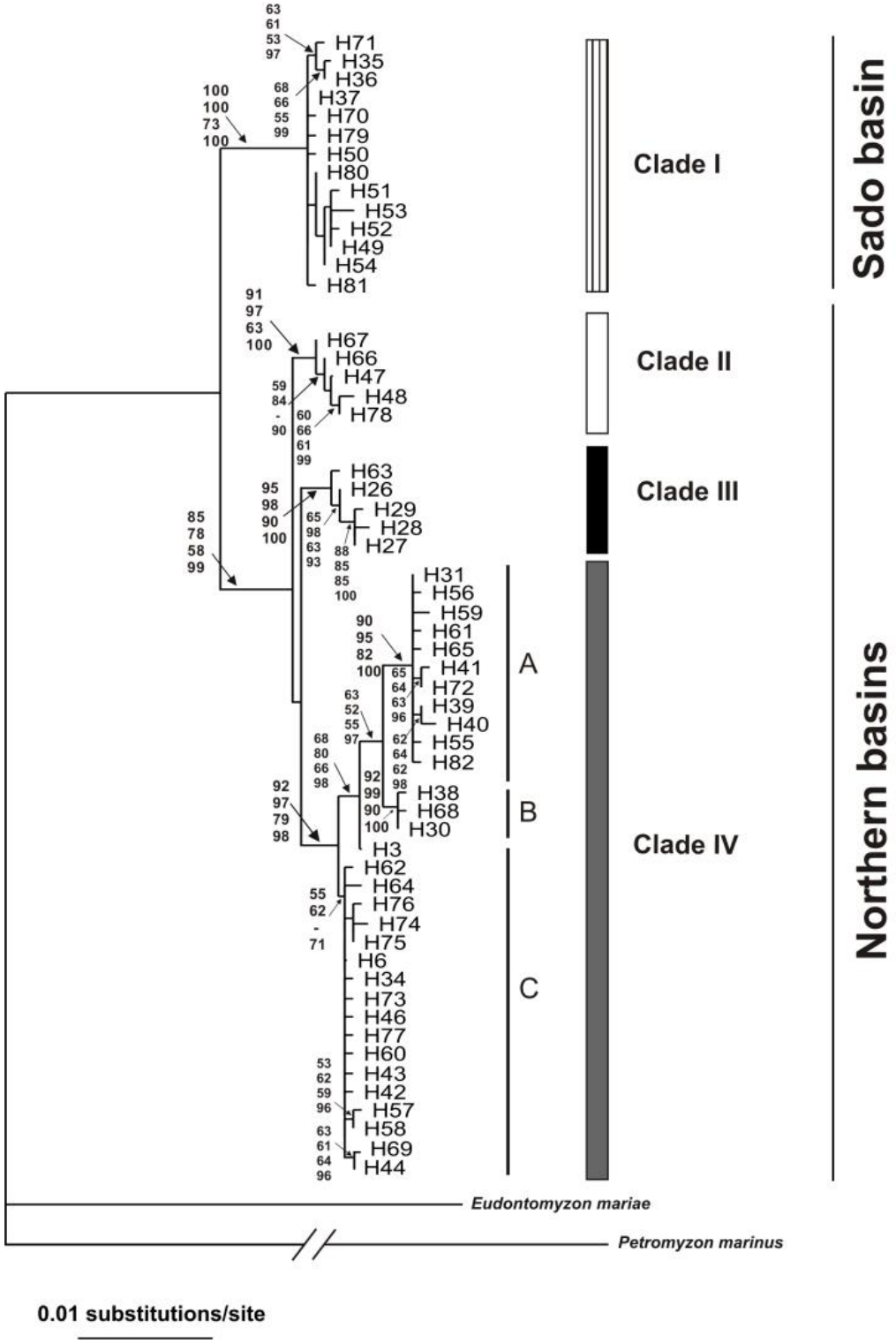

Figure 2 - Maximum-likelihood phylogenetic tree of 2002 bp of the ATPase 6/8 and cyt *b* mtDNA genes. Numbers in terminal nodes refer to the haplotype numbers as in Table 2. Main geographical division is indicated as two lineages, Sado basin and Northern basins and inside each division the respective clades are presented: clades I to IV. Clade IV is divided in three subclades: A, B and C. Numbers are the bootstrap support values equal to or higher than 50% obtained from maximum parsimony, neighbour-joining, and maximum likelihood and the Bayesian credibility value, respectively.

Table 2 - Distribution of haplotypes across samples, according to their specific taxonomic status and across the 21 sampled localities, regardless the specific taxonomic status of the samples.

Haplotype	Species			Locality																			NS_H
	L. fluv	*L. plan*	Am	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
H3		1							1																1
H6	7	13	11					8		10								1	1	6		5			31
H26		7	7			14																			14
H27		4	4				8																		8
H28			1				1																		1
H29			1				1																		1
H30		2							2																2
H31		9	35		9				1					11	4	7	4	8							44
H34		1	1																			2			2
H35			1																					1	1
H36			2																					2	2
H37			3																				1	2	3
H38		3	1						3					1											4
H39		5	7								12														12
H40			1								1														1
H41		2													2										2
H42		1						1																	1
H43			1							1															1
H44		1	1							2															2
H46			1																			1			1
H47		4	13									7	10												17
H48			1									1													1
H49			6																				6		6
H50			1																				1		1
H51			1																				1		1
H52			1																				1		1
H53			2																				2		2
H54			2																				2		2
H55			15											15											15
H56			1											1											1

Table 2 - continued

Haplotype	Species			Locality																				NS_H
	L. fluv	*L. plan*	Am	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
H57			2											2											2
H58			2											2											2
H59		1	1															2							2
H60	5		2															2		4		1			7
H61			1															1							1
H62	4		2															1		3	1	1			6
H63			1			1																			1
H64			1																			1			1
H65		3	1												3		1								4
H66			2									1	1												2
H67			1										1												1
H68		3							3																3
H69			1							1															1
H70			1																					1	1
H71			2																					2	2
H72			1														1								1
H73			1	1																					1
H74			2	2																					2
H75			3	3																					3
H76			1	1																					1
H77			1	1																					1
H78		1											1												1
H79		2																					2		2
H80		2																					2		2
H81		1																					1		1
H82			1		1																				1
NS	16	66	151	8	10	15	10	9	10	14	13	9	13	32	9	7	6	15	1	13	1	11	19	8	233
NH	3	20	47	5	2	2	3	2	5	4	2	3	4	6	3	1	3	6	1	3	1	6	10	5	
NH_P	0	9	34	5	1	2	3	1	3	3	2	1	2	4	1	0	1	2	0	0	0	3	9	4	

For each species/locality, number of samples (NS), number of haplotypes (NH) and number of private haplotypes (NH_P) are presented. Number of samples in each haplotype is also indicated (NS_H). *L. fluv*, *L. fluviatilis*; *L. plan*, *L. planeri*; Am, ammocoete

In AMOVA I, where populations were grouped according to their specific status, variance between species was virtually null, while most of the variance was distributed among localities within species (79.05%, *P*<0.001; Table 3). This result supports the non-existence of specific clades for *L. planeri* and *L. fluviatilis* as revealed in the phylogenetic analysis and the assumption that the two *taxa* do not form reciprocal monophyletic groups. In AMOVA II, where individuals from the same locality, regardless their specific taxonomic status, were grouped in the same population, statistically significant amounts of the molecular variance (76.52%, *P*<0.001) was attributed to differences among localities. When molecular variance was partitioned into two hierarchical levels reflecting the results of the phylogenetic analysis (AMOVA III), most of the variance (71.41%, *P*<0.001) was distributed among groups, while variance among localities within each group accounted for 12.92% (*P*<0.001) and variance within localities for 15.67% (*P*<0.001) (Table 3).

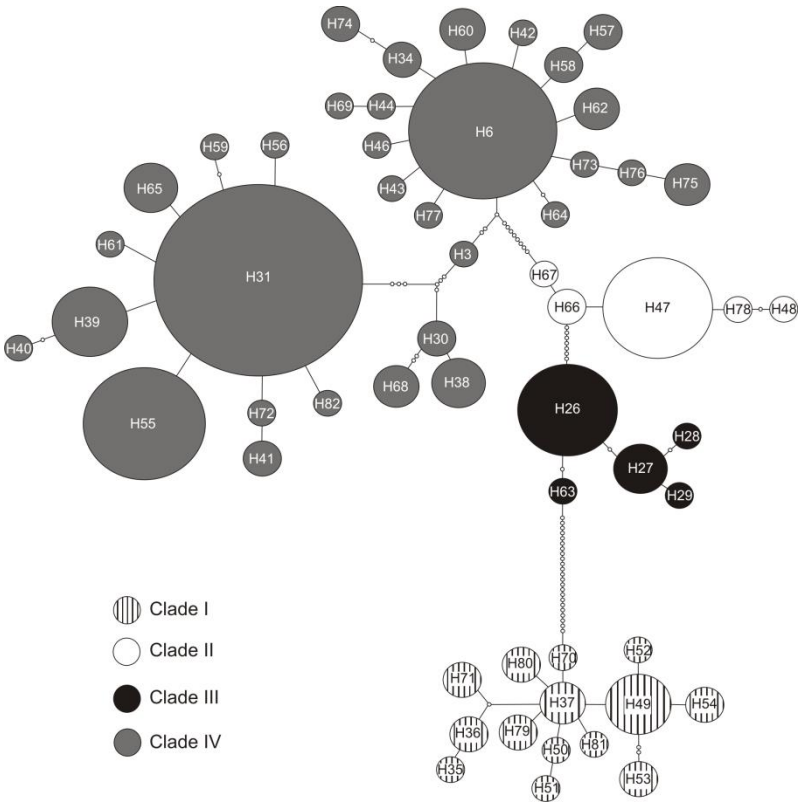

Figure 3 - Haplotype network inferred by the criterion of parsimony with TCS 1.21 representing the 56 haplotypes obtained in this study. Haplotype numbers are identified as in Table 2. The cladogram was estimated under the 95% statistical limits of parsimony. Circle size represents haplotype frequency. Each line in the network represents a single mutational change and empty circles indicate hypothetical, missing haplotypes.

Pairwise comparisons among geographical samples revealed that for 72% of the pairwise F_{ST} values there are significant differences (P<0.05) in allele frequencies (see supplementary information, Table S2). These results are congruent with the results of the AMOVA analysis.

Haplotype diversity in the four clades ranged from 0.407 in clade II (River Nabão and Ribeira do Olival) to 0.932 in clade I (Sado basin; Table 4). Nucleotide diversity was lower, ranging from 0.00035 in clade II to 0.00254 in clade IV (Table 4).

Nucleotide sequences are available at the EMBL database under the accession numbers AJ937923, AJ937926, AJ937946–AJ937951, AJ937954–AJ937957, FN641825–FN641863 and FR669668–FR669672.

Table 3 - Analysis of molecular variance (AMOVA).

Source of variation	*d.f.*	Sum of squares	Variance components	Percentage of variation	*P*	Fixation Indices
AMOVA I						
Among species	1	77.660	-0.40648	-3.79	>0.05	F_{CT}: -0.03795
Among populations within species	13	578.443	8.46731	79.05	<0.001	F_{SC}: 0.76163
Within populations	67	177.555	2.65007	24.74	<0.001	F_{ST}: 0.75258
Total	81	833.659	10.71090			
AMOVA II						
Among populations	20	1961.794	8.73964	76.52		
Within populations	212	568.450	2.68137	23.48	<0.001	F_{ST}: 0.76522
Total	232	2530.245	11.42101			
AMOVA III						
Among groups	3	1523.157	12.22215	71.41	<0.001	F_{CT}: 0.71410
Among populations within groups	17	438.637	2.21197	12.92	<0.001	F_{SC}: 0.45204
Within populations	212	568.450	2.68137	15.67	<0.001	F_{ST}: 0.84334
Total	232	2530.245	17.11548			

In AMOVA I individuals of *Lampetra fluviatilis* and *L. planeri* were assembled into two different groups, in AMOVA II individuals from the same locality were grouped in the same population, independently of the taxonomic status, and in AMOVA III populations were grouped into the four clades suggested by the phylogenetic analyses, independently of the taxonomic status.

Table 4 - Summary of genetic variability, Tajima's *D* and Fu's *Fs* neutrality tests in the four clades based on cyt *b* and ATPase 6/8 mtDNA genes.

Clade	*N*	*NH*	*h*	*π*	*S*	*k*	Tajima's *D*	Fu's *Fs*
I	27	14	0.932	0.00124	14	2.934	-1.35071 NS	-14.785***
II	22	5	0.407	0.00035	5	2.400	0.00000 NS	-2.680 NS
III	25	5	0.603	0.00071	7	3.200	-0.33192 NS	-2.116 NS
IV	159	32	0.869	0.00254	37	6.569	-1.10353 NS	-35.376***

N, sample size; *NH,* number of haplotypes; *h*, haplotype diversity; *π*, nucleotide diversity; *S*, polymorphic sites; *k*, average number of pairwise nucleotide differences

***Significant at the 0.1% level; NS, not significant (P>0.05)

Discussion

Genetic diversity and population structuring

This study revealed a significant level of phylogenetic structuring in the mitochondrial cyt *b* and ATPase genes of the genus *Lampetra* in the Iberian Peninsula. Clades recovered are not species specific but instead follow a geographic pattern, confirming the results of Espanhol *et al*. (2007) who concluded that *L. planeri* and *L. fluviatilis* do not form reciprocal monophyletic groups. The present results suggest the existence of four highly divergent clades with apparent allopatric distribution. Clades I–III apparently only include adults of the resident form and they have restricted distribution, each being confined to a few localities of the same or close small river basins. Clade IV shows a wider distribution, extending from throughout Tagus river basin to Deva river basin, in northern Spain, and it includes both adults of the migratory and resident forms. Clades II and IV both occur in the Tagus river basin, but so far they have been found in different rivers; nevertheless, they may be sympatric at some localities that remained undetected in the present sampling. The AMOVA analysis showed concordant results, revealing substantial levels of genetic fragmentation both between localities and between groups of localities, independently of the taxonomic specific status (Table 3).

The present results suggest the past occurrence of repeated landlocking of diadromous forms in freshwater isolates, leading to the lost of the migratory ability. The new founded resident populations would have become isolated from other

resident and migratory populations (discussed below), with very little or no subsequent gene exchange, allowing for the accumulation over time of new local mutations. Reduced gene flow combined with small population sizes in small streams promoted allopatric divergence, leading to monophyly in most populations. The most ancient long-term barriers to the gene flow involved the populations from Sado basin and the northern basins. Before the establishment of the exorheic network in the Plio-Pleistocene, most river systems drained to a large number of inland lakes (Calvo *et al*. 1993). Since the uplifting of the Arrábida Chain in the Late Miocene (Antunes *et al*. 1995; Choffat 1908) and probably the posterior establishment of the Cascais and Setúbal canyons (Alves *et al*. 2000; Coppier & Mougenot 1982) Tagus and Sado basins remained independent basins. The differentiation of a Sado phylogroup has been also observed in other freshwater fishes, namely in *Chondrostoma lusitanicum* (Mesquita *et al*. 2001) and *Squalius pyrenaicus* (Sousa-Santos *et al*. 2007).

Clade IV is widely distributed in the Tagus basin, with the exception of populations from River Nabão, which are apparently monophyletic for the clade II (discussed above). The Nabão river is the only tributary of the right bank of Tagus basin where *L. planeri* is known to occur. The strong divergence of these populations is probably related with events from the Late Miocene that extended through the Pliocene. Different tectonic movements (subsidence and uplift) of both banks produced distinct systems with own characteristics. The subsidence of the right bank in the beginning of the Superior Miocene promoted the existence of lower areas, protected from sea level changes, with retention of small lakes. In this system, drainage was predominantly endorheic, retaining the water with no outflow to other bodies of water. In the left bank, however, the fluvial system was opened, with exorheic drainage, disabling the existence of lakes (Barbosa 1995). Populations from the right bank may have remained isolated in the endorheic lakes through this period and would have time to diverge from the other populations. Tectonic movements between banks remained through the Pliocene, with inversion of the tectonic subsidence to the left bank, as marked by the accumulation of Pliocene sediments (Barbosa 1995). The dissimilarity of ecological conditions between the tributaries of

both banks may have promoted the isolation and differentiation of populations when the Tagus river basin gained its present configuration.

The differentiation of the populations from the Vouga river basin and its close neighbour the Esmoriz river is somehow surprising, considering the paleogeological evidences (Rodrigues & Dias 1989) and previous phylogeographic studies with other freshwater fishes (Aboim *et al*. 2009; Sousa-Santos *et al*. 2007), which suggest recent connections between these basins and the Douro and the Mondego drainages. In fact, in the Pleistocene, connections between the Mondego and Douro and the Mondego and Vouga drainages were still possible (Rodrigues & Dias 1989), allowing the dispersal of freshwater fishes between these basins (Sousa-Santos *et al*. 2007). This high differentiation, together with the high structuring within the Tagus river basin (discussed above), suggests limited dispersal capabilities in continuous freshwater systems (further discussed below).

The star-like structure and the geographical distribution of haplotypes within clade IV are consistent with a scenario of dispersal and demographic expansion. The Fu's *Fs* significant negative value for clade IV indicates that this expansion was recent (Table 4). This phylogenetic lineage is apparently the only one that still includes the migratory form, *L. fluviatilis*, and postglacial sea dispersal by the anadromous form, followed by demographic expansion and establishment of resident populations, has been postulated to explain the widespread distribution of clade IV in central and northern Europe (Espanhol *et al*. 2007). Nevertheless, structuring within clade IV suggests that movements of the migratory form were probably restricted during the glacial times, favouring population differentiation. The most ancestral haplotype of subclade IV-A (H31) is most common in the left tributaries of the Tagus basin, suggesting that this subclade has differentiated most probably in this basin, having attained its current distribution through long distance colonization. In fact, this subclade is present in the Douro basin, in the Mondego basin and also in the Ribeira de S. Pedro. Subclade IV-B seems to have differentiated in the left tributaries of the Mondego basin, and its restricted distribution suggests reduced dispersal. In fact, only one haplotype from this clade has been observed outside the Mondego basin, in the River Ulme (Tagus basin). Finally, the most ancestral haplotype of subclade IV-C (H6) is

most common in the right tributary of the Mondego basin but also in some left tributaries of the Tagus basin (Erra, Sorraia and Canha), making it difficult to infer the geographic origin of this subclade. This subclade is also present in the neighbour basin Lis, and also in the distant Spanish Deva river basin, suggesting long distance colonization. The observation that subclade IV-C is apparently absent from the right tributaries of the Tagus basin reinforces this hypothesis, as it suggests that gene flow between populations from the Mondego and Tagus basins was established not by inland connections through river captions, as it has been suggested for other freshwater fishes (Sousa-Santos *et al*. 2007), but instead by sea.

The high levels of genetic diversity and population structuring attained in this study for the Iberian Peninsula can be explained by the persistence of multiple glacial refugia. Our findings are in agreement with a number of other phylogeographical studies (e.g., Alexandrino *et al*. 2002; Gante *et al*. 2009; Martínez-Solano *et al*. 2006; Paulo *et al*. 2001; Ribera & Vogler 2004), which provided evidence of considerable genetic divergence within the peninsula and suggested that a strong fragmented distribution may be considered a set of interglacial refugia. Haplotype diversity was higher in clades I and IV, which also showed the highest values of nucleotide diversity (Table 4). The high levels of genetic diversity observed in the Sado clade are somehow surprising considering its present restricted distribution, and suggest that environmental conditions in this basin may have allowed the stability of population through time, despite the climatic crisis of Upper Pliocene which was responsible for the disorganization of the Sado drainage network (Pimentel 1997), and have apparently caused declines in other freshwater fishes (Sousa-Santos *et al*. 2007).

On the dispersal ability of *Lampetra fluviatilis* and *L. planeri*

The Tagus basin is the only river basin where *L. fluviatilis* has been recorded in the Iberian Peninsula (Almaça & Collares-Pereira 1988; Doadrio 2001), and the sampled individuals all belong to subclade IV-C. This subclade is present in the Tagus, in the Lis, in the Mondego and in the Deva river basins, but is apparently absent from all other basins, suggesting that the migratory form stopped at some point visiting these basins

to spawn. Even between the former river basins, gene flow should have become restricted as they all show unique derived haplotypes, in particular River Deva, where all five haplotypes are exclusive of that population (Table 2). Also, the majority of the population pairwise F_{ST} between these localities revealed significant differences (see supplementary information, Table S2). Two hypotheses may account for this scenario: the development of inappropriate local conditions to the migratory form; and/or the reduction of the dispersal ability of *L. fluviatilis*. The absence of the other migratory lamprey, *Petromyzon marinus* in some of these basins (Cabral *et al*. 2005) provide some support to the first hypothesis. As for the second hypothesis, there are evidences that lampreys are largely affected by temperature (Hardisty & Potter 1971; Potter 1980), which may have caused populations at lower latitudes to abandon anadromy while temperature raised during the interglacials (Espanhol *et al*. 2007). In particular, the persistence of *L. fluviatilis* in the Tagus basin may have been possible due to the size of its estuary (c. 300 km^2), allowing individuals to remain in the estuary during the adult stage feeding on estuarine species, a known behaviour for this species (Hardisty 1986b).

The presence of private haplotypes in most *L. planeri* populations (Table 2) and the fact that the majority of the population pairwise F_{ST} revealed significant differences (see supplementary information, Table S2) suggest that the resident form presents very low dispersal ability within river basins. This pattern is particularly evident for the two populations sampled in the Sado basin (localities 20 and 21), which share only one haplotype among the 14 identified and present a pairwise F_{ST} value significant at the 0.1% level; or for the population from Ulme river in the Tagus basin (locality 11), whose the most common haplotype (H55) is apparently absent elsewhere in the basin. In fact, the spawning migrations of brook lampreys are known to be limited, since the spawning grounds are usually located only a short distance upstream from the silt beds inhabited by the ammocoetes and transforming stages (Hardisty 1986b). Furthermore, transformed brook lampreys are unable to feed, and the limited energy supply stored in their tissues might prevent efficient long-distance journeys during the few months of life as an adult (Schreiber & Engelhorn 1998).

Existence of cryptic species and implications for conservation

Genetics is an important focus of conservation biology as measuring genetic variation and interpreting these data in a phylogeographic and population genetics context enables us to understand the evolutionary context of species and the development of improved management strategies (Hurt & Hedrick 2004). The assessment of biodiversity within and among populations is central to identifying and prioritizing areas for monitoring, management and protection and the main goal of management should be to maintain levels of gene flow and maximum gene diversity, as inferred from molecular data (Crandall *et al*. 2000; Moritz & Faith 1998). Particular emphasis should be placed on those populations with highly diverged haplotypes and unique environmental traits (Hurt & Hedrick 2004). In 1986, Oliver Ryder referred to the evolutionarily significant unit (ESU) as a population unit that merits separate management and has a high priority for conservation. Moritz (1994) suggests the distinction between two types of conservation units, the ESUs which are concerned with historical population structure, mtDNA phylogeny and long-term conservation needs, and the management units (MUs) which address current population structure, allele frequencies and short-term management issues.

Conservation units have already been proposed for *L. planeri* (Pereira *et al*. 2010), but the low overall level of divergence and the low phylogenetic resolution observed in that study, due to the use of a single marker, suggest that a more indepth evaluation is needed. The present study revealed high levels of mtDNA divergence and clear phylogeographical patterns of genetic structuring. The high genetic diversity attained for the Iberian glacial refugia is even more obvious when compared with the distribution of the haplotypes of samples from across Europe (see Espanhol *et al*. 2007 and supplementary information, Figure S1).

L. planeri and *L. fluviatilis* remain widely distributed across Europe, and, in terms of the current conservation status, they are globally considered Least Concern according to the IUCN Red List of Threatened Species due to a markedly recover following earlier pollution problem in central and western Europe (Freyhof & Kottelat 2008a; Freyhof & Kottelat 2008b). Nevertheless, both species are considered

threatened in the Iberian Peninsula. In Portugal they are currently included in the Critically Endangered category of the red list of endangered species (Cabral *et al*. 2005). According to the red list of continental fish in Spain *L. fluviatilis* is considered Regionally Extinct and *L. planeri* Critically Endangered (Doadrio 2001).

The highly divergent clades recovered for *L. planeri* within the Iberian Peninsula are evidence for a long history of local independent evolution, suggesting that they should be considered significant for conservation. These Iberian populations have higher levels of divergence than populations from across Europe, which haplotypes are embedded in Clade IV, the widest distributed clade (see supplementary information, Figure S1). Accordingly, we suggest the definition of four evolutionarily significant units (ESUs) for *L. planeri* in the Iberian Peninsula, namely populations from clades I, II, III and IV. Clades I, II and III are exclusive to the Iberian Peninsula (Sado basin, River Nabão and Esmoriz/Vouga basins, respectively) and clade IV is distributed across Europe (see supplementary information, Figure S1). As suggested by Docker (2009), isolated populations of brook lampreys that are genetically very distinct may represent cryptic species. In fact, the number of brook lamprey species in the genus *Lampetra* may be underestimated as differentiated populations are often considered the same species due to their relatively conserved body form (Boguski 2009; Martin 2006). As most species-level characters in lamprey taxonomy are from the adult stage, morphological analysis of adult specimens representing each ESU identified in the present study is under way, and may provide further clarification on this issue.

The identification of cryptic species has important implications for conservation and natural resource protection and management (Bickford *et al*. 2007; Cook *et al*. 2008). These species require special consideration in conservation planning because the prevalence of cryptic complexes in already endangered nominal species presents a dual problem: species already considered endangered or threatened might be composed of multiple species that are even more rare than previously supposed; and the different species might require different conservation strategies (Bickford *et al*. 2007). To consider the genetically unique brook lamprey populations as individual species would maximize their need for protection, as each putative cryptic species

raise more serious conservation concerns, considering its extremely reduced distribution.

The proposed ESUs also include populations with some degree of divergence, as they are almost entirely composed of private haplotypes (Table 2) and the majority of the population pairwise F_{ST} revealed significant differences (see supplementary information, Table S2). These populations should be managed separately for the conservation of biodiversity, constituting independent management units (MUs). These are the case of populations from Deva, Esmoriz, Águeda, Anços,Ribeira de S. Pedro, Nabão, Ribeira do Olival, Marateca and Sado.

The fact that the sympatric populations of *L. planeri* and *L. fluviatilis* are both included in the same clade (IV) raises the question whether the two species should be considered together or separately for conservation purposes. Several authors have questioned the validity of the classification of these species pair as two separate *taxa*, pointing out the possibility of two ecotypes instead (e.g., Eneqvist 1937; Schreiber & Engelhorn 1998) but the uncertainty about this issue (cf. Espanhol *et al*. 2007) makes the inclusion of individuals of both species in the same conservation units somehow premature. Although paired species presumably have very similar habitat requirements and similar or identical vulnerabilities in the larval stage, these differ considerably following metamorphosis. In particular, migratory and parasitic adults will be impacted by barriers to migration and by depletion of their prey base, factors that presumably would have little or no effect on nonparasitic brook lampreys. Consequently, until the taxonomic issue is fully understood it is important to conserve phenotypic diversity, protecting both parasitic and brook lampreys (Docker 2009). In view of that, we suggest that populations of the migratory *L. fluviatilis* should constitute a separate ESU. In this unit we include not only the threatened Iberian population but also populations from across Europe.

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Supplementary information

Table S1 - Variable base positions in the 2002 bp segment of the ATPase 6/8 and cyt *b* mtDNA genes defining 56 haplotypes (*H*). Dots represent matches with nucleotides present in haplotype 3

H	*Nucleotide position*																																																																																		
	30	39	51	59	96	97	132	135	196	237	243	318	357	373	390	432	489	492	502	565	576	586	612	630	657	714	723	748	768	819	825	846	858	879	885	915	927	972	1024	1082	1083	1122	1175	1211	1279	1289	1302	1310	1363	1440	1481	1494	1503	1510	1511	1512	1521	1530	1539	1559	1572	1584	1605	1608	1611	1620	1633	1644	1647	1679	1721	1848	1881	1893	1908	1941	1949	1956	1968	1977	1987	1992
H3	A	A	T	G	G	G	T	G	G	C	G	C	T	G	T	C	T	T	T	T	C	A	C	T	A	T	A	G	G	G	A	T	A	T	C	T	C	C	C	T	G	T	A	C	C	G	C	A	T	A	C	C	A	A	C	T	C	A	A	C	C	C	C	A	T	T	A	G	A	C	C	T	G	A	C	A	T	A	C	T	A	T
H6	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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H76	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
H77	.	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H78	.	.	.	.	A	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	G	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	G	.	.	.	.
H79	G	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C	.	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
H80	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C	.	.	.	.	T	G	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
H81	.	.	A	A	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C	.	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
H82	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

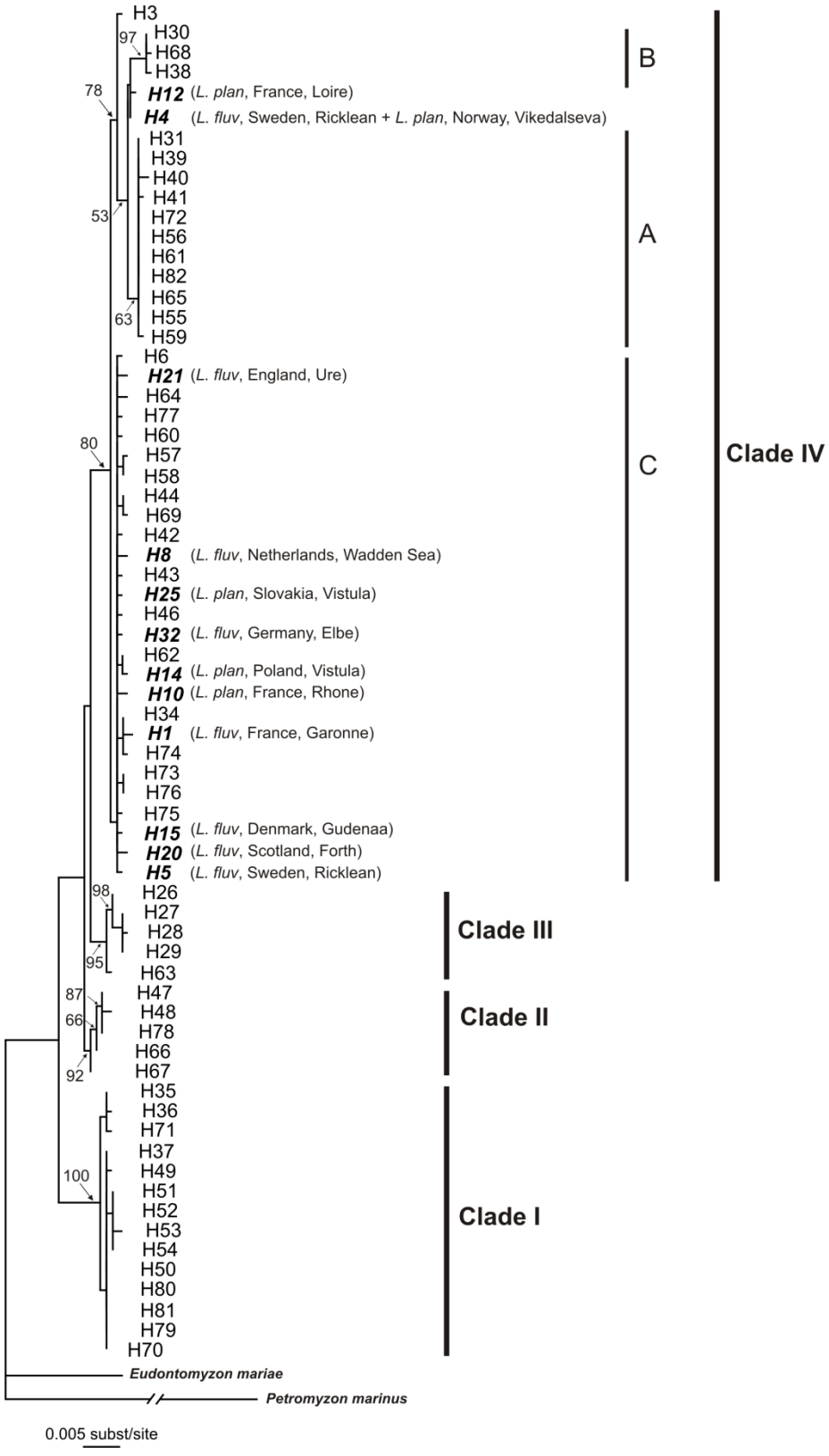
Table S2 - Pairwise *F*ST values and significance among the 21 sampled localities, calculated using conventional *F*-statistics based on mtDNA haplotype frequencies and the pairwise difference distance method

Locality	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1		***	***	***	***	***	*	***	***	***	***	***	***	***	***	NS	***	NS	NS	***	***
2	0.8841		***	***	***	***	***	***	***	***	NS	*	NS	NS	NS	NS	***	NS	***	***	***
3	0.9136	0.9430		***	***	***	***	***	***	***	***	***	***	***	***	NS	***	NS	***	***	***
4	0.9675	0.9901	0.4631		***	***	***	***	***	***	***	***	***	***	***	NS	***	NS	***	***	***
5	0.1454	0.7368	0.8646	0.9171		**	NS	***	***	***	***	***	***	***	***	NS	*	NS	NS	***	***
6	0.6150	0.6080	0.6755	0.7140	0.5294		***	***	***	***	***	*	***	*	***	NS	***	NS	**	***	***
7	0.1592	0.5918	0.7863	0.8284	-0.0521	0.4652		***	***	***	***	**	***	***	***	NS	NS	NS	NS	***	***
8	0.7446	0.4411	0.9037	0.9399	0.6612	0.5658	0.5769		***	***	***	NS	***	***	***	NS	***	NS	***	***	***
9	0.8725	0.9205	0.6607	0.7748	0.8154	0.6020	0.7331	0.8737		NS	***	***	***	***	***	NS	***	NS	***	***	***
10	0.7798	0.8432	0.5898	0.6721	0.7276	0.5600	0.6625	0.8107	-0.0529		***	***	***	***	***	NS	***	NS	***	***	***
11	0.6077	0.0703	0.8922	0.9171	0.5533	0.6243	0.5236	0.3422	0.8738	0.8282		*	NS	NS	NS	NS	***	NS	***	***	***
12	0.5595	0.1523	0.8199	0.8573	0.4663	0.3418	0.4090	0.1815	0.7607	0.6979	0.1770		NS	NS	**	NS	***	NS	**	***	***
13	0.8746	-0.0396	0.9372	0.9911	0.7030	0.5598	0.5532	0.4004	0.9079	0.8230	0.0408	0.1002		NS	NS	NS	***	NS	***	***	***
14	0.8251	0.0556	0.9275	0.9818	0.6608	0.5282	0.5263	0.3180	0.8921	0.8072	0.0429	0.0368	0.0278		NS	NS	***	NS	***	***	***
15	0.5550	0.1279	0.8976	0.9334	0.4601	0.5785	0.4161	0.3685	0.8692	0.8048	0.0707	0.1837	0.0894	0.0795		NS	***	NS	***	***	***
16	-0.2088	0.9718	0.8945	0.9830	-1.0000	0.3194	-0.7051	0.6429	0.8119	0.6773	0.4518	0.2051	1.0000	0.8667	0.3042		NS	NS	NS	NS	NS
17	0.3847	0.9259	0.9322	0.9793	0.0700	0.6823	0.1463	0.7925	0.9092	0.8272	0.6316	0.6363	0.9263	0.8888	0.5943	-0.5714		NS	*	***	***
18	0.2517	0.9753	0.8978	0.9835	-0.4375	0.3485	-0.4276	0.6732	0.8176	0.6881	0.5155	0.2619	1.0000	0.8824	0.3965	1.0000	0.2143		NS	NS	NS
19	0.1806	0.5753	0.7541	0.7989	-0.0217	0.4018	-0.0639	0.5541	0.6879	0.6164	0.5235	0.3641	0.5289	0.4975	0.4122	-0.6378	0.1932	-0.4602		***	***
20	0.8643	0.9080	0.9185	0.9404	0.8406	0.8120	0.7994	0.8822	0.8904	0.8475	0.8661	0.8274	0.8994	0.8890	0.8646	0.8362	0.8924	0.8421	0.7888		***
21	0.7939	0.8526	0.8023	0.8274	0.7565	0.6380	0.7173	0.8238	0.7155	0.6791	0.8533	0.7176	0.8259	0.8046	0.8228	0.6363	0.8490	0.6453	0.6768	0.5230	

Below diagonal, pairwise *F*ST values; above diagonal, significance of the pairwise *F*ST values

*Significant at the 5% level; **significant at the 1% level; ***significant at the 0.1% level; NS, not significant

Figure S1 - Neighbour-joining phylogenetic tree of 68 mitochondrial haplotypes of *Lampetra* (56 from this study and 12 (highlighted) from European populations of *L.planeri* and *L.fluviatilis* included in Espanhol *et al*. (2007)). Numbers in terminal nodes refer to the haplotype numbers. For the haplotypes from European populations, species, country and river basin are indicated. The abbreviations for the species are: *L. fluv*, *L. fluviatilis*; *L. plan*, *L. planeri*. Clades I to IV and subclades IV-A to IV-C refer to clades obtained in the ML phylogenetic tree of 56 haplotypes (Fig. 2) from this study. The evolutionary model of nucleotide substitution and its parameters was calculated according to the Bayesian Information Criterion (BIC) (HKY + *I* + *G*; proportion of invariable sites = 0.4841; gamma distribution shape parameter = 0.7302; base frequencies: A = 0.3049, C = 0.2405, G = 0.1218, T = 0.3328; transition/transversion ratio = 5.2256). Numbers are the bootstrap support values equal to or higher than 50% obtained from neighbour-joining



Paper IV | Three new cryptic species of the lamprey genus *Lampetra* Bonnaterre, 1788 (Petromyzontiformes: Petromyzontidae) from the Iberian Peninsula

Catarina S. Mateus[1, 2, 3,*], M. Judite Alves[3], Bernardo R. Quintella[1, 4] & Pedro R. Almeida[1, 2]

[1]Centro de Oceanografia, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[2]Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, Largo dos Colegiais 2, 7004-516 Évora, Portugal

[3]Museu Nacional de História Natural e da Ciência and Centro de Biologia Ambiental, Universidade de Lisboa, Rua da Escola Politécnica 56/58, 1250-102 Lisboa, Portugal

[4]Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

*E-mail: csmateus@fc.ul.pt

Abstract

The Iberian Peninsula is a repository for biodiversity, presenting high levels of endemism in both plants and animals. In this peninsular region, brook lampreys confined to small, isolated river basins evolved in allopatry giving rise to evolutionary lineages, as revealed by mitochondrial DNA markers. For a better understanding of the taxonomic status and relationships of Iberian populations of the genus *Lampetra*, we combined previous data from genetics and morphological analysis (assessed here), and describe three new species of the lamprey genus *Lampetra* Bonnaterre, 1788 in Portugal. In this region *L. planeri* actually represent a complex of cryptic species, each having smaller geographic ranges than *L. planeri*, and consequently, greater vulnerability to extinction. The description of *Lampetra alavariensis* sp. nov. is based on 36 specimens collected on Ribeira de Mangas, a tributary of river Esmoriz, in Northern Portugal. *Lampetra auremensis* sp. nov. is described on the basis of 31 specimens collected on Ribeira do Olival, a small tributary of river Nabão (Tagus basin). Finally, *Lampetra lusitanica* sp. nov. is described based on 38 specimens from Ribeira da Marateca, Sado river basin, the southernmost distribution of the genus *Lampetra*. The recognition of these new species will contribute to the conservation of these already imperilled taxa and will help prevent the extinction of three important evolutionary lineages.

Keywords: critically endangered, cryptic species complex, non-parasitic, *Lampetra alavariensis* sp. nov., *Lampetra auremensis* sp. nov., *Lampetra lusitanica* sp. nov.

Introduction

The genus *Lampetra* is a Holarctic genus presently composed of two parasitic (anadromous) and five non-parasitic (freshwater resident) species distributed across Eurasia and North America in both Atlantic and Pacific watersheds (Holčík 1986a).

Europe is inhabited by the European river lamprey, *Lampetra fluviatilis* (L., 1758) and the European brook lamprey, *Lampetra planeri* (Bloch, 1784), which are 'paired species', i.e. the larvae are morphologically similar but the adults adopt different life history types: the brook lamprey is non-parasitic while the river lamprey is parasitic (Zanandrea 1959; Hardisty & Potter 1971). The distribution ranges of both species are similar, currently occurring from northern Europe, along the Baltic and North Sea coasts, to the western Mediterranean (Kottelat and Freyhof 2007). They are both present in the Iberian Peninsula. *Lampetra fluviatilis* is presumed to be extinct in Spain (Doadrio 2001) and in Portugal is restricted to the Tagus river basin (Mateus *et al*. 2012). *Lampetra planeri* shows a wider distribution in the Iberian Peninsula: in Spain it is reported exclusively in the river Olabidea (Alvarez & Doadrio 1986) and more recently in the river Deva, in Asturias (Mateus *et al*. 2011a; Perea *et al*. 2011), but its presence has been confirmed in several river basins in Portugal (Espanhol *et al*. 2007; Mateus *et al*. 2011b).

Brook lampreys presumably derive from a parasitic ancestor. In some cases, the origin of non-parasitism may occur at different times or in different locations, resulting in morphological and genetic differences among the non-parasitic derivatives (Docker 2009). Recently, following mitochondrial DNA (mtDNA) analyses using the cytochrome *b* (cyt *b*) and ATPase (subunits 6 and 8) (ATPase 6/8) genes, we recognized the existence of highly divergent allopatric evolutionary lineages of *L. planeri* from the Iberian Peninsula, and suggested the existence of a complex of incipient or cryptic species (Mateus *et al*. 2011b). We identified four clades (I-IV) that do not overlap geographically (Fig. 1): clade I includes the populations from Sado basin; clade II includes the individuals from river Nabão and its tributaries, in the Tagus river basin; clade III includes the populations from Esmoriz and Vouga basins; and clade IV shows a wide distribution, from Tagus river basin to the northern Spanish river Deva and

presents further subdivision (subclades IV-A to IV-C). The uniqueness of Iberian populations from clades I, II and III is even more evident when they are placed in a phylogenetic context including *L. planeri* populations from throughout the European range, showing greater levels of genetic divergence, and falling outside the *L. planeri* clade (clade IV) (Mateus *et al*. 2011b). Accordingly, we suggested the definition of four evolutionarily significant units (ESUs) for *L. planeri*, as defined by clades I, II, III and IV. Morphological differentiation between these ESUs remains, however, to be investigated.

Suitable data for taxonomic descriptions has been a subject of controversy within the taxonomists' community, especially between the use of molecular markers and morphological differences (e.g. Packer *et al*. 2009; Hołyński 2010; Ebach 2011; Mitchell 2011). Consensus opinion suggests that species delimitation should rely on several sorts of data and not solely on a particular gene fragment or on morphological characters that can vary with life history stage or gender (e.g. Will *et al*. 2005; Perkins & Austin 2009; Page & Hughes 2011). Genetic data are increasingly being included in taxonomic decisions, and even if not directly included in species descriptions, authors have used genetic data to verify morphology-based decisions before publishing solely morphological descriptions and diagnoses (Cook *et al*. 2010). If species descriptions included both morphological and DNA-based data, a more universal taxonomy would result. When faced with a group, such as the lampreys, that possesses so few of the morphological characters traditionally used in taxonomy, molecular data represent an incredibly valuable source of information (Lang *et al*. 2009). DNA-sequence data have the advantage that it can be used to identify all life history stages, which is sometimes impossible through morphology alone (Page & Hughes 2011), and it is not influenced by subjective assessments, being reproducible at any time and by any person (Tautz *et al*. 2003). In fact, most of the morphological characters used in lamprey taxonomy are limited to adult specimens (Hubbs & Potter 1971), and some are based on shape and pigmentation of different parts of the body (Renaud 2011), making them subjective and potentially erroneous. Furthermore, extreme environmental conditions might impose stabilizing selection on morphology, reducing or eliminating morphological change that can accompany speciation (Bickford *et al*. 2007).

Until now, the recognition of new species of lampreys has been generally based exclusively on morphology (e.g. Vladykov & Kott 1979; Vladykov *et al*. 1982; Holčík & Šorić 2004; Renaud & Economidis 2010) but some authors have used molecular data to resolve phylogenetic relationships among lampreys (e.g. Lang *et al*. 2009; Boguski *et al*. 2012) and to suggest the existence of new morphologically cryptic species (e.g. Yamazaki & Goto 1996, 1998; Boguski *et al*. 2012).

In this context, we analysed the morphology of immature adults of brook lampreys from previously recognized genetically-distinct populations and used both genetic and morphological evidence to describe three new species. Morphological characters of the three new species show statistically significant differences, but also some degree of overlap, so we consider the new species to be cryptic. The description of these three cryptic lamprey species follows the evolutionary species concept of Wiley (1978): "a species is a lineage of ancestral descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate".

The identification and description of cryptic species can contribute to defining patterns of biodiversity that may be important for conservation, and have important implications for natural resource protection and management (Bickford *et al*. 2007; Cook *et al*. 2008). *Lampetra planeri* is currently included in the Critically Endangered category of the Portuguese Red List of Threatened Vertebrates (Cabral *et al*. 2005) and listed as Critically Endangered in the Spanish Red List of Continental Fish (Doadrio 2001). The present study suggests that *L. planeri* has a much more restricted distribution and revealed new cryptic species with an even more limited distribution, making them highly vulnerable to extinction. Consequently, this study is extremely important for conservation of these imperilled taxa.

Material and methods

Sampling and material

Adult brook lampreys from six sampling sites representing the previously recognized allopatric lineages (Mateus *et al*. 2011b) were captured by electric fishing during the months of November and January in four consecutive years, 2009 to 2012 (Fig. 1).

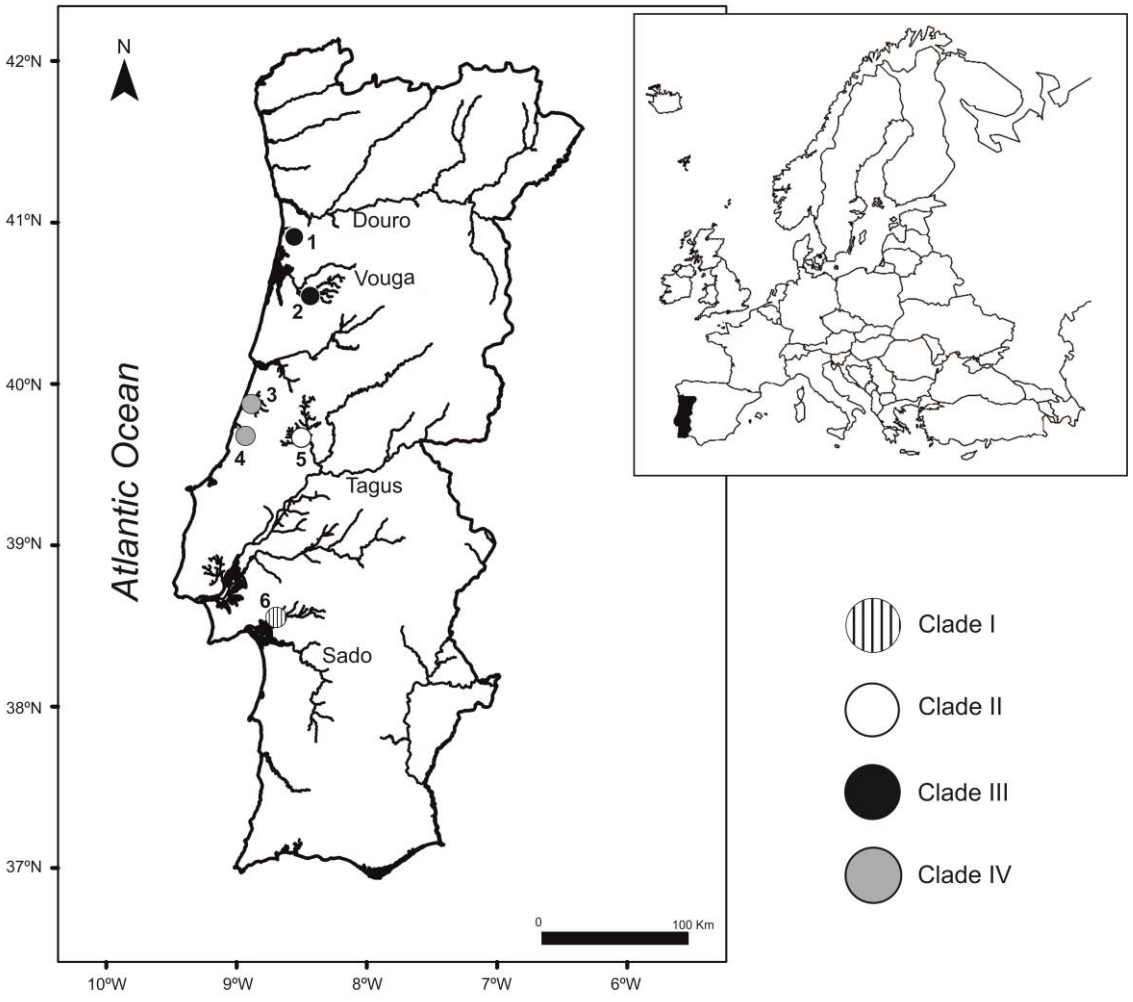


Figure 1 - Collection sites of brook lampreys in Portugal (circles). Circles are filled according to the clades recognized in Mateus *et al*. (2011b). Site locations: 1, river Esmoriz; 2, river Vouga; 3, river Lis; 4, Ribeiras do Oeste; 5, river Nabão; 6, river Sado.

Placement of the individuals into the different clades was inferred from their collection sites. Rivers Esmoriz and Vouga represent clade III, river Lis subclade IV-C, Ribeiras do Oeste subclade IV-A, river Nabão clade II and river Sado clade I (Fig. 1). In total, 163 immature adults were used in the morphological analyses (n=36 Esmoriz, n=27 Lis, n=31 Ribeiras do Oeste, n=31 Nabão and n=38 Sado). The Vouga population was not included in the morphological analysis due to the reduced number of samples. Maturation stage was determined according to criteria given for *L. planeri* by Bird & Potter (1979).

Specimens analysed in this study were not compared with museum material because the preserved specimens analysed had, in general, their original body shape deformed. Because lampreys lack a rigid endoskeleton, shrinkage due to initial fixation in formalin followed by preservation in ethanol can be significant, and has been estimated at 1-3% of the total length (Renaud 2011).

From each population sampled, some individuals were deposited in the zoological collections 'Museu Bocage' of the Museu Nacional de História Natural e da Ciência (MNHNC) (Lisbon, Portugal) as reference material:

Lampetra alavariensis sp. nov.: MB-002866, 1 ex., female, holotype, Ribeira de Mangas, Carvalheira de Maceda, Ovar (40º55'27.30" N; 8º37'19.20" W), Esmoriz drainage, Portugal. 127.6 mm Tl. Coll. C.S. Mateus and C.M. Alexandre. 09.XII.2009; MB05-002867, 2 ex., paratypes, type locality. Coll. C.S. Mateus and C.M. Alexandre. 09.XII.2009; MB05-002868, 4 ex., non-type, river Águeda, Falgoselhe, Águeda (40º34'06.27" N; 8º21'19.58" W), Vouga drainage, Portugal. Coll. C.S. Mateus and C.M. Alexandre. 10. XII.2009.

Lampetra auremensis sp. nov.: MB05-002869, 1 ex., female, holotype, Ribeira do Olival, Caxarias, Ourém (39º42'15.60'' N; 8º32'06.84'' W), Tagus drainage, Portugal. 121.0 mm Tl. Coll. C.S. Mateus and C.M. Alexandre. 05.I.2012; MB05-002870, 3 ex., paratypes, type locality. Coll. C.S. Mateus and C.M. Alexandre. 17.XII.2009.

Lampetra lusitanica sp. nov.: MB05-002871, 1 ex., female, holotype, Ribeira da Marateca, Landeira, Vendas Novas (38º35'39.46'' N; 8º38'43.86'' W), Sado drainage,

Portugal, 132.8 mm Tl. Coll. C.S. Mateus and C.M. Alexandre. 05.I.2012; MB05-002872, 22 ex., paratypes, type locality. Coll. C.S. Mateus and C.M. Alexandre. 28.XI.2009.

Lampetra planeri: MB05-002873, 3 ex., Ribeira de Monte Redondo, Monte Redondo, Leiria (39º55'38.18'' N; 8º50'55.85'' W), Lis drainage, Portugal. Coll. C.S. Mateus and C.M. Alexandre. 11.XII.2009; MB05- 002874, 3 ex., Ribeira de São Pedro, Marinha Grande, Leiria (39º46'14.63'' N; 09º00'34.26'' W), Ribeiras do Oeste, Portugal. Coll. C.S. Mateus and C.M. Alexandre. 11.XII.2009.

Tissue samples (fin clips or a piece of muscle, in the case of preserved specimens) and photographs of all individuals were deposited in the tissue and DNA collection and digital collection, respectively, of the MNHNC (Lisbon, Portugal).

The holotype and two paratypes of each new species were sequenced for both cyt *b* and ATPase 6/8 following the protocol in Mateus *et al*. (2011b). All sequences exhibit haplotypes attained in that study, except for the holotype of *L. auremensis*, which has a single substitution (cyt *b*-285: T > C) in relation to the other five haplotypes already identified for the species. This sequence is available in the EMBL-Bank accession number HF546517. Both the holotype and the paratypes of *L. alavariensis* exhibit haplotype 26 (EMBL-Bank accession number AJ937946), the paratypes of *L. auremensis* present haplotype 47 (EMBL-Bank accession number FN641833), the holotype and one paratype of *L. lusitanica* show haplotype 50 (EMBL-Bank accession number FN641836) while the other paratype shows haplotype 37 (EMBL-Bank accession number AJ937957).

Morphological analyses

The morphological characters were selected according to Holčík (1986b). The morphometric character H (body depth) was measured below the base of first dorsal fin, and not in the position presented in Holčík (1986b), to avoid measurement errors. We also added a character not present in Holčík (1986b), HW (head width). A total of 19 morphometric characters were recorded. Meristic characters included the number of trunk myomeres and dentition (Fig. 2, Table 1).

Because *L. planeri* is a threatened species in Portugal, morphological data were collected without euthanizing the specimens. The lampreys were taken to the laboratory, anaesthetised by immersion in 2-phenoxyethanol (0.3 ml L^{-1}) and after all specimens were analysed they were released at the capture sites (except for the type material, as described above). For this reason, characters that would imply the death of the specimens (e.g. velar tentacles) were not analysed.

Specimens were photographed for morphometric measurements (Sony Handycam HDR-XR200VE, Sony Corp., Japan) and the image analysis software package SigmaScan Pro V5.0 (SPSS Inc., Chicago) was later used to make measurements on digitized images. Trunk myomeres were counted between the posterior edge of the last branchial opening and the anterior edge of the cloacal slit, using a stereomicroscope (Wild M3C, Heerbrugg, Switzerland). The number, type (unicuspid, bicuspid or tricuspid) and arrangement of teeth were recorded using a stereomicroscope (Leica MZ9.5, Leica Microsystems, Germany) that allowed photo capture for further analysis (Leica DFC320, Leica Microsystems, Germany). Terminology of the disc teeth follows that proposed by Vladykov & Follett (1967). All counts and measurements were made on the left side of the body following the procedure summarized by Holčík (1986b).

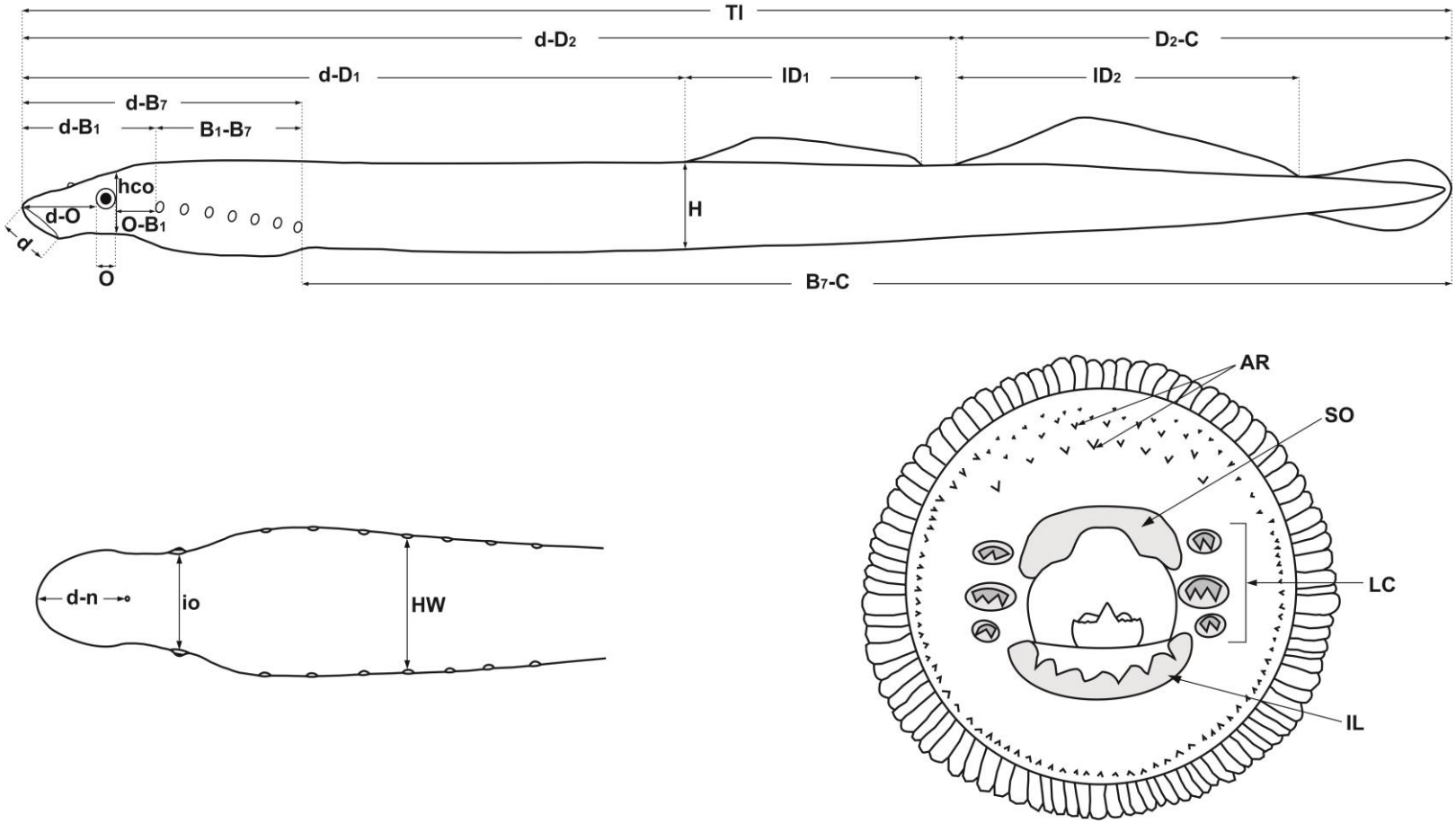


Figure 2 - Scheme of the morphometric measurements and meristic counts used to examine morphological variation of adult brook lampreys. Variables: Tl, total length; d, disc length; d-O, preocular length; O, eye diameter; O-B_1, postocular length; d-n, prenostril length; hco, head depth; io, interocular distance; HW, head width; d-B_1, prebranchial length; B_1-B_7, branchial length; d-B_7, head length; d-D_1, predorsal distance; d-D_2, distance between disc and base of second dorsal fin; D_2-C, dorsal part of caudal fin length; lD_1, first dorsal fin length; lD_2, second dorsal fin length; H, body depth; B_7-C, postbranchial length; AR, anterial rows; SO, supraoral lamina; LC, lateral circumorals or endolaterals; IL, infraoral lamina.

Table 1 - Morphometrics and trunk myomeres in *Lampetra*. Data are the mean ± standard deviation and range for the morphometrics, and mode and range for the trunk myomeres. See Fig. 2 for character acronyms. *Lampetra* species and populations are presented from North to South.

Characters	***L. alavariensis* (n=36)**	***L. planeri* (Lis) (n=27)**	***L. planeri* (Ribeiras do Oeste) (n=31)**	***L. auremensis* (n=31)**	***L. lusitanica* (n=38)**
Morphometric	mean ± SD	mean ± SD	Mean ± SD	mean ± SD	mean ± SD
	[range]	[range]	[range]	[range]	[range]
Tl (mm)	131.1±10.6	116.1±7.5	101.7±6.2	114.3±7.0	124.7±7.7
	[109.1-152.3]	[103.7-127.6]	[89.3-114.8]	[101.4-129.3]	[109.7-140.0]
d (% Tl)	4.2±0.3	3.7±0.4	3.9±0.3	4.1±0.2	3.7±0.3
	[3.8-5.1]	[2.9-4.7]	[3.2-4.3]	[3.6-4.6]	[3.0-4.2]
d-O (% Tl)	5.4±0.3	5.0±0.5	5.1±0.3	5.2±0.3	4.7±0.4
	[4.7-6.0]	[4.2-6.3]	[4.4-5.7]	[4.6-5.7]	[3.8-5.7]
O (% Tl)	1.4±0.1	1.3±0.1	1.5±0.1	1.5±0.1	1.5±0.1
	[1.3-1.6]	[1.2-1.5]	[1.3-1.7]	[1.4-1.7]	[1.3-1.9]
O-B_1 (% Tl)	3.0±0.1	3.2±0.1	3.2±0.1	3.1±0.1	2.9±0.1
	[2.7-3.2]	[2.9-3.4]	[2.9-3.6]	[2.9-3.3]	[2.6-3.2]
hco (% Tl)	4.5±0.1	4.6±0.2	4.4±0.2	4.5±0.2	4.3±0.3
	[4.2-4.9]	[4.3-5.3]	[3.8-4.7]	[4.1-4.8]	[3.6-5.2]
d-B_1 (% Tl)	9.7±0.4	9.6±0.6	9.8±0.5	9.8±0.4	9.0±0.5
	[10.5-9.0]	[8.5-11.1]	[9.0-10.6]	[9.1-10.6]	[7.8-10.4]
B_1-B_7 (% Tl)	10.2±0.3	10.4±0.4	10.3±0.3	10.2±0.3	10.2±0.3
	[9.7-10.8]	[9.8-11.6]	[9.8-11.0]	[9.4-10.7]	[9.3-11.1]
d-B_7 (% Tl)	19.9±0.5	19.9±0.9	20.1±0.6	20.0±0.5	19.2±0.7
	[18.9-21.3]	[18.5-22.7]	[18.8-21.5]	[21.0-19.1]	[17.5-21.4]
d-n (% Tl)	3.7±0.3	3.3±0.4	3.5±0.3	3.6±0.3	3.2±0.3
	[3.0-4.3]	[2.4-4.3]	[2.7-4.1]	[3.2-4.3]	[2.6-4.2]
io (% Tl)	4.0±0.2	3.9±0.2	3.9±0.2	4.0±0.2	3.9±0.2
	[3.7-4.4]	[3.6-4.5]	[3.5-4.5]	[3.7-4.3]	[3.5-4.4]
HW (% Tl)	4.2±0.3	4.1±0.3	4.0±0.2	4.1±0.3	4.3±0.3
	[3.6-4.9]	[3.6-4.8]	[3.6-4.5]	[3.5-4.6]	[3.5-4.8]
B_7-C (% Tl)	80.1±0.5	80.1±0.9	79.9±0.6	80.0±0.5	80.8±0.6
	[78.7-81.1]	[77.3-81.5]	[78.5-81.2]	[79.1-80.9]	[78.9-82.5]
lD_1 (% Tl)	15.0±1.0	14.1±1.0	15.1±0.9	15.8±0.8	15.3±0.8
	[12.1-16.7]	[12.5-16.2]	[11.7-16.3]	[14.3-17.4]	[13.5-16.8]
lD_2 (% Tl)	23.3±1.0	22.6±0.9	23.0±1.1	23.1±1.1	24.0±1.1
	[21.1-25.1]	[20.8-24.2]	[20.7-25.0]	[20.6-25.3]	[22.0-26.1]
D_2-C (% Tl)	34.1±0.9	32.5±0.8	33.7±0.9	33.3±0.8	34.6±0.8
	[32.4-36.1]	[29.8-33.9]	[32.2-35.6]	[32.2-36.0]	[33.1-36.9]
d-D_2 (% Tl)	65.9±0.9	67.5±0.8	66.3±0.9	66.7±0.8	65.4±0.9
	[63.9-67.6]	[66.1-70.2]	[64.4-67.8]	[64.0-67.8]	[63.1-67.3]
d-D_1 (% Tl)	47.9±1.1	49.1±1.1	48.5±1.0	48.8±1.1	47.6±1.0
	[45.8-50.1]	[46.6-50.9]	[46.9-50.8]	[46.5-51.1]	[45.8-49.6]
H (% Tl)	6.2±0.3	6.1±0.2	5.6±0.3	6.0±0.2	6.0±0.2
	[5.6-6.8]	[5.8-6.7]	[5.2-6.3]	[5.7-6.5]	[5.5-6.5]
Meristic	mode	mode	mode	mode	mode
	[range]	[range]	[range]	[range]	[range]
myTr (counts)	61	61	57	60	60
	[58-63]	[57-65]	[55-58]	[58-62]	[57-62]

Data analysis

For morphometric analysis, each individual was considered as one multivariate observation, and all morphological characters were transformed to logarithms to approximate multivariate normality. All 18 morphometric characters showed a linear relationship with total length (P<0.001) and were, therefore, standardised to the overall mean total length by applying a modified formula of Claytor & MacCrimmon (1987):

$$ACij = \ln(OCij+1) - [ß \times (\ln(Tlj+1) - \ln(Tl+1))]$$

where ACij is the adjusted character measurement i of the j specimen; OCij is the unadjusted character measurement i of the j specimen; ß is the common within- group regression coefficient of that character against total length after the logarithmic transformation of both variables; Tlj is the total length of the j specimen; and Tl is the mean total length of all specimens. Analysis of covariance (ANCOVA) was employed to estimate the common within-group regression slopes (ß) (Claytor & MacCrimmon 1987).

Kruskal–Wallis was used to compare the number of trunk myomeres between groups. No significant relationship (P>0.05) was found between the number of trunk myomeres and total length.

A Multiple Discriminant Analysis (MDA) was employed to identify the morphometric variables that most contribute to group segregation (see Almeida *et al*. 2008). In the performed stepwise method independent variables are entered into the discriminant function one at a time on the basis of their discriminating power. The selection rule in this procedure is to maximize the Mahalanobis distance (D^2) between groups (Hair *et al*. 1998). The discriminatory power of the classification matrix relative to chance was measured with Press's Q statistic. Also, a potency index was used to assess the relative importance of each independent variable in discriminating between groups across all significant discriminant functions (Hair *et al*. 1998). Discriminant Z scores and group centroids from discriminant functions 1 and 2 were plotted for representation of the relationships between groups. All these analyses were conducted using SPSS Statistics V19.0 software (SPSS Inc., Chicago).

Results

The total length (Tl) and weight (Tw) (mean ± SD) of the immature adults ranged from 89.3 mm to 152.3 mm (118.3 ± 12.9 mm) and from 0.8 g to 5.66 g (2.37 ± 0.85 g), respectively (n=163).

The stepwise MDA performed on morphometric data revealed that of 18 initial variables (Table 1), 10 were included in the analysis. Four statistically significant discriminant functions (P<0.001) were computed (Table 2).

Table 2 - Results of Wilk's lambda (Λ) tests to verify the hypothesis that the means (centroids) of all functions are equal in the five groups when their morphometric characters were compared by stepwise Multiple Discriminant Analysis.

Test of Function(s)	Λ	χ^2	*d.f.*
1-4	0.058	440.544*	40
2-4	0.209	241.748*	27
3-4	0.445	125.265*	16
4	0.717	51.459*	7

*significant at the 0.1% level.

The first discriminant function was mainly correlated with O (eye diameter; negative correlation) and O-B_1 (postocular length; positive correlation), the second function was negatively correlated with d (disc length) and d-O (preocular length), the third function positively correlated with H (body depth) and io (interocular distance), and the fourth function positively correlated with D_2-C (dorsal part of caudal fin length) and negatively correlated with lD_1 (first dorsal fin length) (Table 3). The first two discriminant functions accounted for 55.1% and 23.7% of total variance, respectively (Table 4). The scatter plot obtained from the discriminant analysis of the morphometric data revealed differentiation between populations along both discriminant functions 1 and 2 (Fig. 3). Discriminant function 1 separates Lis and Sado from the group formed by Ribeiras do Oeste / Nabão / Esmoriz, although Sado overlaps slightly with Nabão, and discriminant function 2 separates Esmoriz from the rest of the watersheds, although

there is some overlap with Nabão. The pairwise *F*-test for the equality of groups revealed that all groups were significantly different ($P<0.001$) and 76% of the individuals were correctly classified (Table 5). Press's Q test revealed that the classification accuracy is significantly better than chance (Press's Q = 320.321, $df = 1$, $P<0.001$).

Kruskal-Wallis test for the number of trunk myomeres showed that there are significant differences between populations ($\chi^2 = 85.352$; $df = 4$; $P<0.001$). Myomere counts ranged from 55 to 65, the higher counts occurring in Lis and the lower counts occurring in Ribeiras do Oeste (Table 1).

Table 3 - Summary of discriminant loadings and potency index for adjusted morphometric characters.

Characters	Discriminant loadings				Potency index
	function 1	function 2	function 3	function 4	
d	-0.117	-0.740*	-0.236	0.033	0.14
d-O	0.074	-0.696*	-0.343	-0.062	0.13
O	-0.468*	-0.134	0.108	-0.260	0.13
D_2-C	-0.412	-0.009	-0.005	0.695*	0.13
io	-0.124	-0.592*	0.384	0.000	0.11
O-B_1	0.423*	0.078	-0.217	-0.182	0.11
d-B_1	0.092	-0.584*	-0.319	-0.163	0.10
H	0.076	-0.512*	0.447	-0.222	0.10
hco	0.266	-0.452*	0.170	-0.149	0.09
lD_1	-0.292	0.021	-0.260	-0.416*	0.07

*Largest absolute correlation between each variable and any discriminant function.

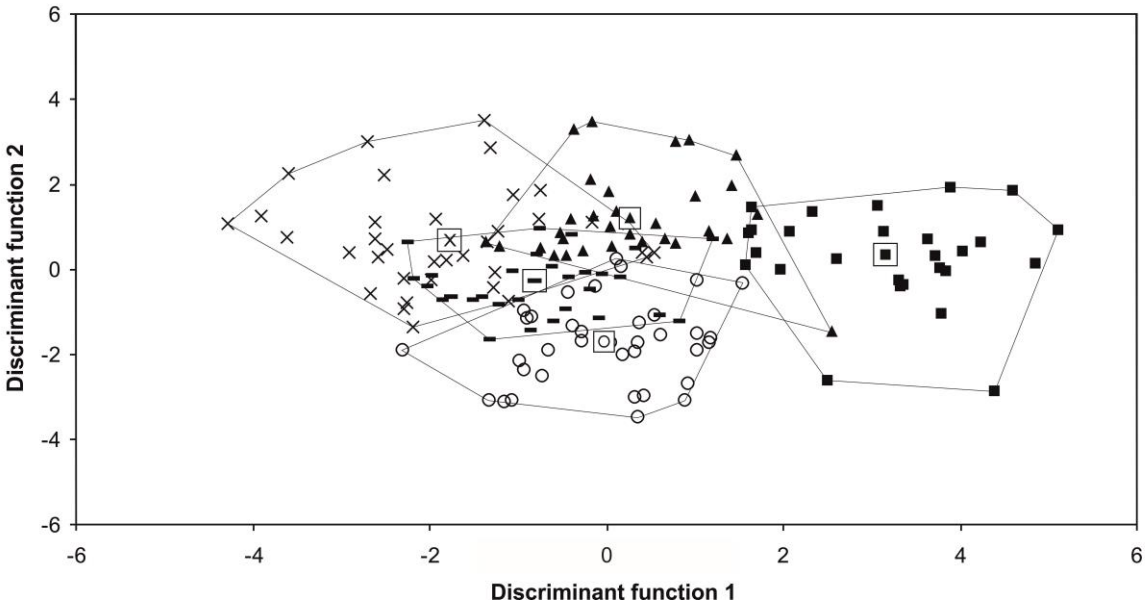


Figure 3 - Scatter plot of the discriminant Z scores, group centroids (squares) and outline polygons for the five examined groups of brook lampreys based on the morphometric characters, according to the first two discriminant functions. Symbols for groups: ○, Esmoriz; ■, Lis; ▲, Ribeiras do Oeste; •, Nabão; ×, Sado.

Table 4 - Eigenvalues and percentage of variance of the four discriminant functions attained in the stepwise discriminant analysis.

Function	Eigenvalue	% of variance	Cumulative %
1	2.621	55.1	55.1
2	1.125	23.7	78.8
3	0.612	12.9	91.7
4	0.395	8.3	100.0

The dentition is variable between populations. In total, 144 specimens were accurately analysed for teeth number, type and arrangement. In all analyzed specimens, there are three lateral circumoral teeth (endolaterals) on either side of the oral disc, which formula varies greatly between populations. In Lis and Ribeiras do Oeste the typical *L. planeri* formula 2-3-2 is the most common, whereas in the described species *L. alavariensis* (river Esmoriz), *L. auremensis* (river Nabão) and *L. lusitanica* (river Sado) the most common formula is 2-2-2. In *L. auremensis* this formula is present in all analyzed specimens except one, which has 2-2-2 on one side and 2-3-2 on the other side of the disc (Fig. 4, Table 6 and Appendix). The supraoral lamina bears

two unicuspid teeth separated by a toothless bridge. The infraoral lamina bears 5-9 cusps (Table 6), the marginal teeth usually enlarged and in several cases divided to form bicuspids. Exolaterals and posterials are absent. The anterior field is also variable between populations, both in the number of rows as in the number, type and arrangement of teeth. The number of rows varies between 1 and 2, the first row with 3-8 teeth. In general, teeth in the anterial field are all unicuspid, but in some specimens some teeth are bicuspid.

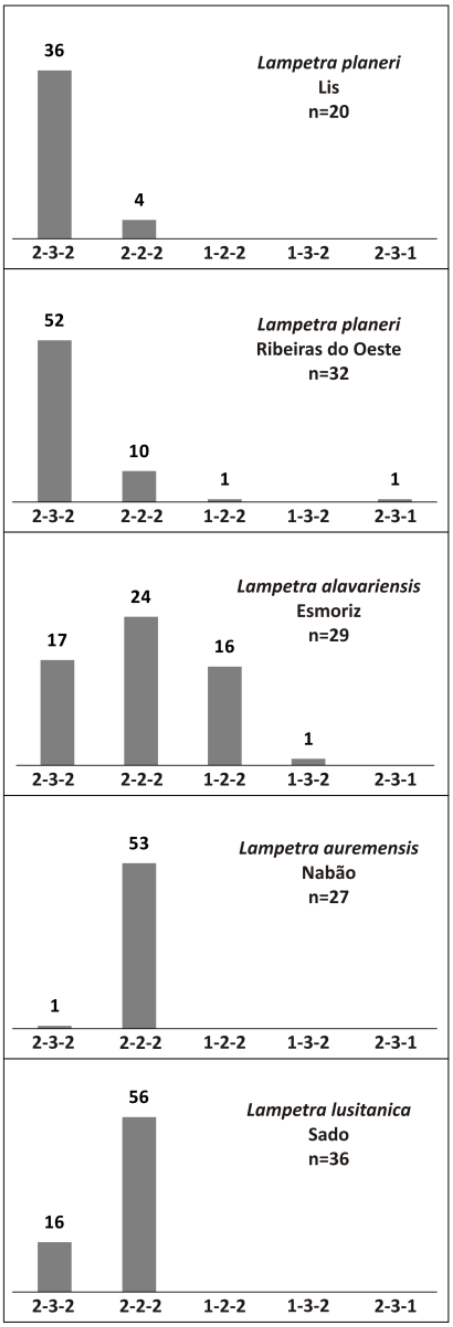


Figure 4 - Endolateral formula counts for the analysed populations. As endolaterals occur on both sides of the oral disc they have twice as many counts as the number of analysed individuals.

Table 5 - Classification results attained with the stepwise discriminant analysis cross-validation for morphometric characters. The table must be read horizontally.

Group	n	Percent correct	Number of individuals classified into group				
			L. alavariensis	***L. planeri* (Lis)**	***L. planeri* (Ribeiras do Oeste)**	***L. auremensis***	***L. lusitanica***
L. alavariensis	36	77.8	28	0	3	4	1
L. planeri (Lis)	27	85.2	1	23	3	0	0
L. planeri (Ribeiras do Oeste)	31	83.9	0	2	26	3	0
L. auremensis	31	64.5	4	0	3	20	4
L. lusitanica	38	71.1	1	0	6	4	27

Table 6 - Type and arrangement of endolaterals on each side of the oral disc and number of cusps in the infraoral lamina. Numbers of the endolateral formula reflect the type of endolateral teeth as follows: 1, unicuspid; 2, bicuspid; 3, tricuspid. R, right; L, left

Characters		Group				
		***L. alavariensis* (n=29)**	***L. planeri* (Lis) (n=20)**	***L. planeri* (Ribeiras do Oeste) (n=32)**	***L. auremensis* (n=27)**	***L. lusitanica* (n=36)**
LC						
R	L					
2-2-2	2-2-2	8 (28%)	2 (10%)	3 (10%)	26 (96%)	23 (64%)
2-3-2	2-3-2	6 (21%)	18 (90%)	24 (75%)		3 (8%)
2-3-2	2-2-2	3 (10%)		1 (3%)		4 (11%)
2-2-2	2-3-2	2 (7%)		2 (6%)	1 (4%)	6 (17%)
1-2-2	1-2-2	6 (21%)				
1-2-2	1-3-2	1 (3%)				
2-2-2	1-2-2	2 (7%)				
1-2-2	2-2-2	1 (3%)		1 (3%)		
2-3-2	2-3-1			1 (3%)		
IL						
9 cusps			4	6		
8 cusps		1	3	12		2
7 cusps		19	11	10	22	14
6 cusps		6	1	2	1	3
5 cusps		3	1	2	4	17

Discussion

Morphological differentiation

The data analyses on the morphometric characters assayed here indicate that the populations are significantly different (see Table 5), suggesting that morphometric variables are suitable for population discrimination and taxonomy of brook lampreys.

Our results identified the cephalic region as the most important morphological region to discriminate brook lamprey populations, as seven of the 10 discriminant variables are from this anatomic region (see Table 3 and Fig. 2). Also, the highest discriminatory power is given by variables from the cephalic region, like the disc length (d), preocular length (d-O) and eye diameter (O), as shown by the values of the potency index (see Table 3). Our results are in agreement with Almeida *et al*. (2008), who also identified the head as the most important morphological region to discriminate populations of sea lamprey larvae in Portuguese rivers.

According to Renaud (2011) the taxonomy of lampreys is based primarily on the dentition in the adult. Hardisty (1986) reported that *L. planeri* typically has 2-3-2 as an endolateral formula, and that variants such as 2-2-1, 2-2-2, 2-3-1, 2-3-3, and 1-2-1 have occasionally been recorded. Our results indicate that there is great variability in the dentition of the analyzed specimens, with most individuals of *Lampetra lusitanica*, *L. auremensis* and *L. alavariensis* presenting endolateral formulae not common in *L. planeri* (see Figs 4 and 5 and Table 6). Also, *L. lusitanica* and *L. auremensis* have in general one row of anterials, unlike the two rows reported for *L. planeri* by Renaud (2011).

The number of trunk myomeres was significantly different between populations, but there was overlap. The numbers observed in our study are within the limits reported for *L. planeri* by Potter & Osborne (1975), who compared data from different parts of Europe. A progressively greater number of trunk myomeres was found to the north, a pattern which has been previously observed in other lamprey species (e.g. Yamazaki & Goto 1997; Holčík & Delić 2000) and may therefore reflect environmental influence. The low number of trunk myomeres found in Ribeiras do

Oeste was surprising, considering that this population is genetically (Mateus *et al*. 2011b) and morphologically in other respects (e.g. dentition, this study) close to other *L. planeri* populations, and was therefore not considered a cryptic species. This is probably due to the fact that this character, despite being broadly used in the taxonomy of lampreys (e.g. Naseka *et al*. 2009; Reid *et al*. 2011), may be influenced by ecological factors (e.g. latitude and temperature during the first stages of the larval development, references above), and should therefore be cautiously used in lamprey taxonomy.

Discrete taxonomic entities in the Iberian Peninsula

In a previous study using mtDNA variation, we suggested the existence of a complex of incipient or cryptic species in the Iberian Peninsula that might have evolved in allopatry (Mateus *et al*. 2011b). The combination of the molecular and morphological data supports the description of the three cryptic lamprey species in Portugal, *Lampetra lusitanica*, *L. auremensis* and *L. alavariensis*, which evolved in allopatry and constitute divergent evolutionary lineages.

Results obtained from molecular analyses in Mateus *et al*. (2011b) suggested the past occurrence of repeated landlocking of anadromous forms, leading to the loss of migratory behaviour. In that study we identified four allopatric evolutionary lineages: one including the samples from Sado basin, here described as *Lampetra lusitanica* (Fig. 6c); another including the individuals from river Nabão, here described as *L. auremensis* (Fig. 6b); a third including the populations from Esmoriz and Águeda rivers, here described as *L. alavariensis* (Fig. 6a); and a last lineage with a wider distribution from Tagus river basin in the south to the northern Spanish river Deva. Populations from this last phylogenetic lineage remain as *L. planeri* because a genetic survey across Europe revealed that these were embedded in a widespread lineage across central and northern Europe (Espanhol *et al*. 2007; Mateus *et al*. 2011b), where *L. planeri* was originally described (Bloch 1784). This lineage is apparently the only one that still includes the migratory form, *L. fluviatilis*, and postglacial sea dispersal by the anadromous form, followed by demographic expansion and establishment of

freshwater resident populations apparently explain its widespread distribution (Espanhol *et al*. 2007; Mateus *et al*. 2011b).

Mitochondrial DNA sequences have been used extensively in taxonomy, as they enable researchers to resolve relationships between closely related taxa as well as to construct higher level phylogenies (Tautz *et al*. 2003). For both analysed genes in Mateus *et al*. (2011b) (cyt *b* and ATPase 6/8; 2002 bp), divergence between *L. lusitanica* and *L. planeri* ranged from 1.2 to 1.7% (mean ± SD = 1.5 ± 0.3%), between *L. auremensis* and *L. planeri* ranged from 0.5 to 1.2% (mean ± SD = 0.8 ± 0.2%), and between *L. alavariensis* and *L. planeri* ranged from 0.5 to 1.2% (mean ± SD = 0.8 ± 0.2%). Distances were calculated using the Kimura 2-parameter distance method, in MEGA V4 (Tamura *et al*. 2007). For comparison purposes, and because in most lamprey studies intra and inter-species genetic divergence has been calculated using the cyt *b* gene, we further calculated sequence divergence between the three new cryptic species and *L. planeri* for cyt *b* gene alone (1173 bp). In this gene, *L. lusitanica* differs from *L. planeri* from 0.8 to 1.2% (mean ± SD = 1.0 ± 0.2%), *L. auremensis* from *L. planeri* from 0.3 to 0.9% (mean ± SD = 0.5 ± 0.2%), and *L. alavariensis* from *L. planeri* from 0.4 to 1.1% (mean ± SD = 0.7 ± 0.2%).

Comparing the genetic distances exhibited between species of vertebrates based on the cyt *b* gene, Johns & Avise (1998) concluded that 90% of putative sister species show sequence divergences greater than 2% (see also Avise & Walker 1999). Sequence divergence in cyt *b* between some lamprey species is near or above this value, for instance Reid *et al*. (2011) calculated a 2.85 to 3.20% sequence divergence between *L. pacifica* Vladykov, 1973 and *L. richardsoni* Vladykov and Follett, 1965 within the Columbia Basin and Boguski *et al*. (2012) found that four *Lampetra* sp. populations in Oregon and California present a genetic divergence between 2.3 and 5.7% from any known species, and up to 8.0% from each other, suggesting that these populations may represent undescribed cryptic species. Many lamprey species, however, present lower levels of sequence divergence between them, showing levels that are in accordance with our results. For instance, cyt *b* sequence differs by 0.8% between the freshwater resident *Eudontomyzon hellenicus* Vladykov, Renaud, Kott and Economidis, 1982 and *Eudontomyzon graecus* Renaud and Economidis, 2010 from

Greece, by 0.2% between the freshwater resident *Lethenteron kessleri* (Anikin 1905) and *Lethenteron reissneri* (Dybowski 1869) from Russia, and by 0.9% between the freshwater resident *Lethenteron appendix* (DeKay 1842) and *Lethenteron alaskense* Vladykov and Kott, 1978 from Tennessee and Alaska, respectively (calculated from GenBank data provided on Lang *et al*. 2009).

Each of the evolutionary lineages attained in Mateus *et al*. (2011b, and here described as new cryptic species) are well supported and each have several diagnostic synapomorphies in the two analysed mitochondrial genes (4 in *L. alavariensis*, 3 in *L. auremensis* and 17 in *L. lusitanica*) (see Appendix and on-line supplementary information). *Lampetra lusitanica* was the first to diverge. Before the establishment of the exorheic network in the Plio-Pleistocene, most river systems drained to a large number of inland lakes. Since the uplifting of the Arrábida Chain in the Late Miocene and probably the posterior establishment of the Cascais and Setúbal canyons, Tagus and Sado basins have remained independent basins (see Mateus *et al*. 2011b). The divergence *L. auremensis* is probably related to events from the Late Miocene that extended through the Pliocene. Different tectonic movements (subsidence and uplift) of both banks produced distinct systems with particular characteristics. The dissimilarity of ecological conditions between the tributaries of both banks may have promoted the isolation and differentiation of populations within the Tagus river basin. The differentiation of the populations from the Esmoriz and Vouga rivers (*L. alavariensis*) was surprising because paleogeological evidence and previous phylogeographic studies with other freshwater fishes suggested recent connections between these basins and the adjacent Douro and Mondego drainages. We postulated that this high differentiation suggests limited dispersal capabilities of lampreys in these continuous freshwater systems (see Mateus *et al*. 2011b). Considering these new data, *L. planeri* is distributed in Portugal from river Tagus in the South to river Douro in the North, except in rivers Esmoriz, Vouga and Nabão (Fig. 7d).

Molecular evidence in several animal taxa has revealed that many already endangered species are cryptic species complexes (e.g. Ravaoarimanana *et al*. 2004; Stuart *et al*. 2006), making them a collection of even more critically endangered species with fewer numbers and smaller distributions (Bickford *et al*. 2007). Preventing

habitat loss is perhaps the greatest challenge for the conservation of global biodiversity, and prioritizing habitats for conservation often relies on estimation of species richness and endemism. The discovery of geographical and habitat-related patterns in distribution of cryptic species can therefore reveal new pockets of endemism and diversity that might warrant reconsideration of protection for particular habitats or sites (Bickford *et al*. 2007). In the near future it is expected that the total number of lamprey species will be updated based not only on morphology but also on molecular data, which will contribute to the conservation of overall lamprey diversity.

Systematics (according to Nelson, 2006)

Phylum: Chordata

Subphylum: Vertebrata

Superclass: Petromyzontomorphi

Class: Petromyzontida

Order: Petromyzontiformes

Family: Petromyzontidae Bonaparte, 1831

Genus: *Lampetra* Bonnaterre, 1788

Lampetra alavariensis sp. nov. (Figs 5a, 6a)

Holotype: MB05-002866, female, Ribeira de Mangas, Carvalheira de Maceda, Ovar (40º55'27.30" N; 8º37'19.20" W), Esmoriz drainage, Portugal. 127.6 mm Tl. Coll. C.S. Mateus and C.M. Alexandre. 09.XII.2009.

Paratypes: MB05-002867, 2 specimens, type locality. Coll. C.S. Mateus and C.M. Alexandre. 09.XII.2009.

Non-type material: MB05-002868, 4 specimens, river Águeda, Falgoselhe, Águeda (40º34'06.27" N; 8º21'19.58" W), Vouga drainage, Portugal. Coll. C.S. Mateus and C.M. Alexandre. 10.XII.2009.

Diagnosis: Diagnostic differences at two mitochondrial DNA genes were found: cytochrome *b* (cyt *b*) and ATPase (subunits 6 and 8) (ATPase 6/8) genes (Mateus *et al*. 2011b). This species is characterized by five private haplotypes (EMBL-Bank accession numbers: AJ937946-49 and FN641849) and four synapomorphies relative to *L. planeri*, *L. auremensis* and *L. lusitanica*, three in cyt *b* and one in ATPase 6/8 (base positions and substitutions: cyt *b*-132: T > C; cyt *b*-502: T > C; cyt *b*-630: T > C; ATPase 6/8-321: C > T) (see supplementary information -SI- 1 and 2).

Description: *Lampetra alavariensis* sp. nov. is a small freshwater non-parasitic lamprey. In the 36 analysed specimens, including the holotype (Fig. 6a), total length varies from 109.1 to 152.3 mm. Body proportions (as % of Tl) are as follows: disc length, 3.8 to 5.1; preocular length, 4.7 to 6.0; eye diameter, 1.3 to 1.6; postocular length, 2.7 to 3.2; prenostril length, 3.0 to 4.3; head depth, 4.2 to 4.9; interocular distance, 3.7 to 4.4; head width, 3.6 to 4.9; prebranchial length, 9.0 to 10.5; branchial length, 9.7 to 10.8; head length, 18.9 to 21.3; predorsal distance, 45.8 to 50.1; distance between disc and base of second dorsal fin, 63.9 to 67.6; dorsal part of caudal fin length, 32.4 to 36.1; first dorsal fin length, 12.1 to 16.7; second dorsal fin length, 21.1 to 25.1; body depth, 5.6 to 6.8; postbranchial length, 78.7 to 81.1. Trunk myomeres vary from 58 to 63, with a mode of 61. The supraoral lamina bears 2 unicuspid teeth separated by a bridge. The infraoral lamina bears 5-8 cusps (Table 6), the marginal teeth usually enlarged. In most cases (62%), division of at least one marginal cusp to form bicuspids occurred. The endolateral row on each side of disc consists of three teeth exhibiting great variability (Fig. 4; Table 6). The most common endolateral formula is 2-2-2 (occurred on both sides in eight individuals), followed by the formulae 2-3-2 and 1-2-2 (each occurred on both sides in six individuals). In one individual the formula 1-3-2 occurred on one side (Table 6). Exolaterals and posterials are absent. The anterior field has 2 rows of anterials, the first row with 6-8 unicuspid teeth (mostly 7).

Caudal fin shape is spade-like in 32 individuals (97%) and rounded in one (3%).

Coloration and pigmentation pattern: Live specimens of *Lampetra alavariensis* sp. nov. in the immature adult stage are brownish in the dorsal and lateral regions and become progressively whitish to the ventral region (although not perceptible in the

holotype picture, Fig. 6a). Branchial region is unpigmented. Lateral line neuromasts pigmented. The caudal fin is moderately pigmented in almost all cases, especially in the ventral lobe. Specimens preserved in 10% formalin become pale, predominantly yellowish.

Geographic distribution: *Lampetra alavariensis* sp. nov. is endemic to Portugal, inhabiting the north-western Portuguese drainages Esmoriz and Vouga (Fig. 7a). The population from Vouga drainage was assigned to the new taxon through molecular markers analysis (Mateus *et al*. 2011b).

Etymology: The specific epithet refers to the Portuguese district where the species occur, Aveiro (Alavarium in Latin).

Common name: Lampreia da Costa de Prata; Costa de Prata lamprey.

Conservation: In the last version of the Portuguese Red List of Threatened Vertebrates, *Lampetra planeri*, that included populations here described as *L. alavariensis*, was given a status of Critically Endangered according to the following IUCN (2001) criteria: B1ab (ii, iii, iv) (Cabral *et al*. 2005). The main threats to this new species depend on the watershed: the watersheds of the river Vouga are heterogeneous in terms of threats affecting freshwater organisms; in general, industrial pollution, channel and bank regulation and construction of weirs are the main threats. Urban pressure is particularly problematic in the Esmoriz basin, where residential zones are often very close to the watersheds.

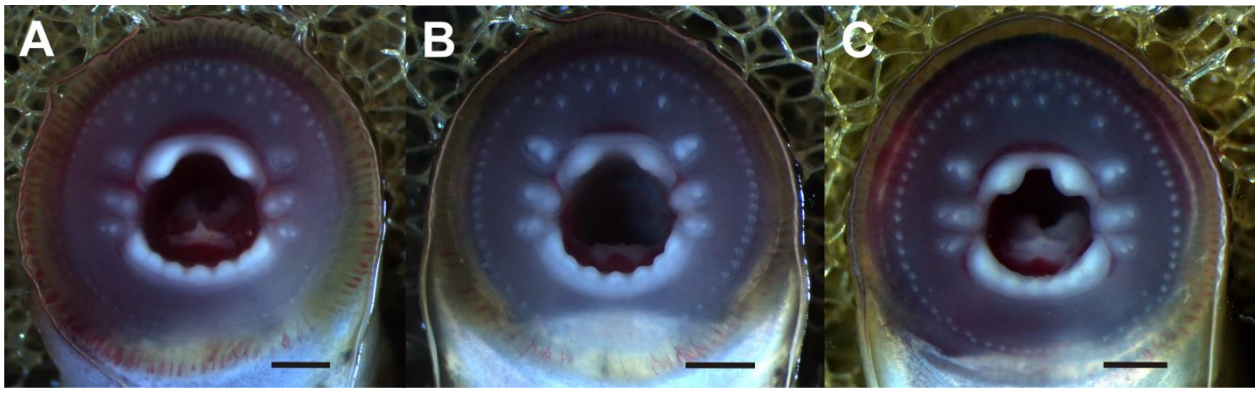


Figure 5 - Oral disc of the holotype of (A) *Lampetra alavariensis* sp. nov. (MB05-002866; Tl, 127.6 mm; immature adult; live specimen), (B) *Lampetra auremensis* sp. nov. (MB05-002869; Tl, 121.0 mm; immature adult; live specimen) and (C) *Lampetra lusitanica* sp. nov. (MB05-002871; Tl, 132.8 mm; immature adult; live specimen). Bar = 1 mm.

Figure 6 - Lateral view of the holotype of (A) *Lampetra alavariensis* sp. nov. (MB05-002866; Tl, 127.6 mm; immature adult; live specimen), (B) *Lampetra auremensis* sp. nov. (MB05-002869; Tl, 121.0 mm; immature adult; live specimen) and (C) *Lampetra lusitanica* sp. nov. (MB05-002871; Tl, 132.8 mm; immature adult; live specimen).

Lampetra auremensis sp. nov. (Figs 5b, 6b)

Holotype: MB05-002869, female, Ribeira do Olival, Caxarias, Ourém (39º42'15.60'' N; 8º32'06.84'' W), Tagus drainage, Portugal. 121.0 mm Tl. Coll. C.S. Mateus and C.M. Alexandre. 05.I.2012.

Paratypes: MB05-002870, 3 specimens, type locality. Coll. C.S. Mateus and C.M. Alexandre. 17.XII.2009.

Diagnosis: Endolateral formula 2-2-2 *vs*. 2-3-2; rounded caudal fin vs. spade-like caudal fin; diagnostic two mitochondrial DNA genes were differences at found: cytochrome *b* (cyt *b*) and ATPase (subunits 6 and 8) (ATPase 6/8) genes (Mateus *et al*. 2011b). This species is characterized by six private haplotypes FN641833-34, (EMBL-Bank numbers: accession FN641852-53, FR669668 and HF546517) and three synapomorphies relative to *L. planeri*, *L. alavariensis* and *L. lusitanica*, one in cyt *b* and two in ATPase 6/8 (base positions and substitutions: cyt *b*-357: T > C; ATPase 6/8-308: C > T; ATPase 6/8-338: C > T) (see SI 1 and 2).

Description: *Lampetra auremensis* sp. nov. is a small freshwater non-parasitic lamprey. In the 31 analysed specimens, including the holotype (Fig. 6b), total length varies from 101.4 to 129.3 mm. Body proportions (as % of Tl) are as follows: disc length, 3.6 to 4.6; preocular length, 4.6 to 5.7; eye diameter, 1.4 to 1.7; postocular length, 2.9 to 3.3; prenostril length, 3.2 to 4.3; head depth, 4.1 to 4.8; interocular distance, 3.7 to 4.3; head width, 3.5 to 4.6; prebranchial length, 9.1 to 10.6; branchial length, 9.4 to 10.7; head length, 19.1 to 21; predorsal distance, 46.5 to 51.1; distance between disc and base of second dorsal fin, 64.0 to 67.8; dorsal part of caudal fin length, 32.2 to 36.0; first dorsal fin length, 14.3 to 17.4; second dorsal fin length, 20.6 to 25.3; body depth, 5.7 to 6.5; postbranchial length, 79.1 to 80.9. Trunk myomeres vary from 58 to 62, with a mode of 60. The supraoral lamina bears 2 unicuspid teeth separated by a bridge. The infraoral lamina bears 5-7 cusps, the marginal teeth usually enlarged. In several cases (33%), division of at least one marginal cusp to form bicuspids occurred. The endolateral row on each side of disc consists of three teeth. The most common endolateral formula is 2-2-2 which occurred on both sides in 26 individuals; in one individual the formula 2-3-2 occurred in one side (Table 6). Exolaterals and posterials are absent. The anterior field has 1-2 rows of anterials, usually 1, with 3-7 unicuspid teeth (mostly 4).

Caudal fin shape is rounded in 20 individuals (62.5%) and spade-like in 12 (37.5%).

Coloration and pigmentation pattern: Live specimens of *Lampetra auremensis* sp. nov. in the immature adult stage are mostly greenish, and sometimes brownish or greyish in the dorsal and upper lateral regions and whitish in the lower lateral and ventral region. Branchial region is unpigmented. Lateral line neuromasts pigmented. Specimens preserved in 10% formalin become pale, predominantly yellowish.

Geographic distribution: *Lampetra auremensis* sp. nov. is endemic to Portugal, inhabiting river Nabão, a tributary of the right bank of Tagus river basin (Fig. 7b).

Etymology: The specific epithet refers to the area where the species occur, in the region of Ourém, inspired in the name of the region in the XII century, Aurem.

Common name: Lampreia do Nabão; Nabão lamprey.

Conservation: In the last version of the Portuguese Red List of Threatened Vertebrates, *Lampetra planeri*, that included populations here described as *L. auremensis*, was given a status of Critically Endangered according to the following IUCN (2001) criteria: B1ab (ii, iii, iv) (Cabral *et al*. 2005). The new species has a very restricted distribution, being confined to a tributary of the right bank of Tagus river basin (see Fig. 7b). This extremely reduced distributional range will require special conservation and management. The main threats in the area where it occurs are domestic pollution and channel and bank regulation.

Lampetra lusitanica sp. nov. (Figs 5c, 6c)

Holotype: MB05-002871, female, Ribeira da Marateca, Landeira, Vendas Novas (38º35'39.46'' N; 8º38'43.86'' W), Sado drainage, Portugal, 132.8 mm Tl. Coll. C.S. Mateus and C.M. Alexandre. 05.I.2012.

Paratypes: MB05-002872, 22 specimens, type locality. Coll. C.S. Mateus and C.M. Alexandre. 28. XI.2009.

Diagnosis: Endolateral formula 2-2-2 *vs*. 2-3-2; diagnostic differences at two mitochondrial DNA genes were found: cytochrome *b* (cyt *b*) and ATPase (subunits 6 and 8) (ATPase 6/8) genes (Mateus *et al*. 2011b). This species is characterized by 14 private haplotypes (EMBL-Bank accession numbers: AJ937955-57, FN641835-40, FN641856-57, FR669669-71) and 17 synapomorphies relative to *L. planeri*, *L. alavariensis* and *L. auremensis*, seven in cyt *b* and 10 in ATPase 6/8 (base positions and substitutions: cyt *b*-51: T > A; cyt *b*-237: C > T; cyt *b*-576: C > T; cyt *b*-768: G > A; cyt *b*-846: T > C; cyt *b*-858: A > C; cyt *b*-1122: T > C; ATPase 6/8-129: C > T; ATPase 6/8-267: A > T; ATPase 6/8-330: A > G; ATPase 6/8-337: A > G; ATPase 6/8-348: C > T; ATPase 6/8-471: G > A; ATPase 6/8-474: A > G; ATPase 6/8-675: T > C; ATPase 6/8-735: C > T; ATPase 6/8-795: C > T) (see SI 1 and 2).

Figure 7 - Geographic distribution (■) of (A) *Lampetra alavariensis* sp. nov., (B) *Lampetra auremensis* sp. nov., (C) *Lampetra lusitanica* sp. nov. and (D) *Lampetra planeri* in Portugal.

Description: *Lampetra lusitanica* sp. nov. is a small freshwater non-parasitic lamprey. In the 38 analysed specimens, including the holotype (Fig. 6c), total length varies from 109.7 to 140.0 mm. Body proportions (as % of Tl) are as follows: disc length, 3.0 to 4.2; preocular length, 3.8 to 5.7; eye diameter, 1.3 to 1.9; postocular length, 2.6 to 3.2; prenostril length, 2.6 to 4.2; head depth, 3.6 to 5.2; interocular distance, 3.5 to 4.4; head width, 3.5 to 4.8; prebranchial length,7.8 to 10.4; branchial length, 9.3 to 11.1; head length, 17.5 to 21.4; predorsal distance, 45.8 to 49.6; distance between disc and base of second dorsal fin, 63.1 to 67.3; dorsal part of caudal fin length, 33.1 to 36.9; first dorsal fin length, 13.5 to 16.8; second dorsal fin length, 22.0 to 26.1; body depth, 5.5 to 6.5; postbranchial length, 78.9 to 82.5. Trunk myomeres vary from 57 to 62, with a mode of 60. The supraoral lamina bears 2 unicuspid teeth separated by a bridge. The infraoral lamina bears 5-8 cusps, the marginal teeth usually enlarged. In several cases (31%), division of at least one marginal cusp to form bicuspids occurred. The endolateral row on each side of disc consists of three teeth. The most common endolateral formula is 2-2-2, which occurred on both sides of 23 individuals. The formula 2-3-2 occurred in both sides (n=3) and on one side (n=10) of the oral disc (Table 6). Exolaterals and posterials are absent. The anterior field has 1-2 rows of anterials, the first row with 4-7 unicuspid teeth.

Caudal fin shape is spade-like in 36 individuals (90%) and rounded in 4 (10%).

Coloration and pigmentation pattern: Live specimens of *Lampetra lusitanica* sp. nov. in the immature adult stage are brownish, greyish or greenish in the dorsal and upper lateral regions and whitish in the lower lateral and ventral region. Branchial region is unpigmented. Lateral line neuromasts pigmented. In few individuals the dorsal and lateral aspects are mottled and the ventral aspect is whitish. Specimens preserved in 10% formalin become pale, predominantly yellowish.

Geographic distribution: *Lampetra lusitanica* sp. nov. is endemic to Portugal, inhabiting the southwestern Portuguese drainage Sado (Fig. 7c).

Etymology: The specific epithet refers to the country where the species occur, Portugal, as Lusitania is considered the ancestral origin of Portugal.

Common name: Lampreia do Sado; Sado lamprey.

Conservation: In the last version of the Portuguese Red List of Threatened Vertebrates, *Lampetra planeri*, that included populations here described as *L. lusitanica*, was given a status of Critically Endangered according to the following IUCN (2001) criteria: B1ab (ii, iii, iv) (Cabral *et al*. 2005). This new species is inherently at risk of extinction because it occurs in the southern limit of Lampetra distribution in Europe, the Sado basin (see Fig.7c) that suffers from both anthropogenic pressure and potential effects of climate change. The main threats to this species are diffused pollution from agriculture practices, water extraction and channel and bank regulation. The first two threats are especially significant because in this basin the available water is normally reduced, especially in the months with higher temperatures. Water extraction here exacerbates negative effects of pollution by diminishing the dilution capacity of the streams.

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Supplementary information

Table S1 - Nucleotide substitutions in the 1173 bp segment of the cytochrome *b* mtDNA gene in the 56 haplotypes (*H*) attained in Mateus *et al*. (2011b). Dots represent matches with nucleotides present in haplotype 3 (*L. planeri*). Synapomorphies are marked in grey. Asterisks represent homoplasies.

	H	30	39	51	59	96	97	132	135	196	237	243	318	357	373	390	432	489	492	502	565	576	586	612	630	657	714	723	748	768	819	825	846	858	879	885	915	927	972	1024	1082	1083	1122
L. planeri	H3	A	A	T	G	G	G	T	G	G	C	G	C	T	G	T	C	T	T	T	T	C	A	C	T	A	T	A	G	G	G	A	T	A	T	C	T	C	C	C	T	G	T
	H6	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H30	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H31	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
	H34	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.
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	H39	.	.	.	.	A	A	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
	H40	.	.	.	.	A	A	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
	H41	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
	H42	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H43	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	G	.	.
	H44	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.
	H46	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H55	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
	H56	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
	H57	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H58	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H59	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
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	H62	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H64	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H65	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	C	.	.	.	.	.	.
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	H69	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.
	H72	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
	H73	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H74	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.
	H75	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H76	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H77	.	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H82	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
L. lusitanica	H35	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	G	C	C	.	.	C	A	.	.	.	.	C
	H36	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	G	C	C	.	.	C	A	.	.	.	.	C
	H37	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H49	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H50	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H51	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H52	.	C	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H53	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H54	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H70	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H71	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	G	C	C	.	.	C	A	.	.	.	.	C
	H79	G	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H80	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
	H81	.	.	A	A	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
L. auremensis	H47	.	.	.	.	A	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.
	H48	.	.	.	.	A	.	.	.	.	.	.	T	C	.	.	.	.	C	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.
	H66	.	.	.	.	A	.	.	.	.	.	.	T	C	.	.	.	.	.	C*	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.
	H67	.	.	.	.	A	.	.	.	.	.	.	T	C	.	.	.	.	.	C*	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.
	H78	.	.	.	.	A	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.
L. alavariensis	H26	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H27	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	C	.	T	T	.	.	.
	H28	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	C	G	.	.	A	.	.	.	.	.	.	.	C	.	T	T	.	.	.
	H29	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	C	.	T	T	.	.	.
	H63	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.

Table S2 - Nucleotide substitutions in the 829 bp segment of the ATPase (subunits 6 and 8) mtDNA gene in the 56 haplotypes (*H*) attained in Mateus *et al*. (2011b). Dots represent matches with nucleotides present in haplotype 3 (*L. planeri*). Synapomorphies are marked in grey. Asterisks represent homoplasies.

	H	*Nucleotide position*																																							
		2	38	106	116	129	137	190	267	308	321	330	337	338	349	358	367	376	386	399	411	432	435	438	447	460	471	474	606	648	675	708	720	735	768	776	783	793	805	814	819
L. planeri	H3	A	C	C	G	C	A	T	A	C	C	A	A	C	T	C	A	A	C	C	C	C	A	T	T	A	G	A	C	C	T	G	A	C	A	T	A	C	T	A	T
	H6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H30	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
	H31	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H34	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H38	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	C
	H39	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H40	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
	H41	.	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	H43	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	H56	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	H59	.	.	T	.	.	.	.	.	.	T*	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
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	H68	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
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	H75	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
	H76	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
	H77	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H82	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. lusitanica	H35	.	.	.	.	T	.	.	T	.	.	G	G	.	C	T	.	.	.	.	.	.	C	C	.	.	A	G	.	T	C	.	.	T	.	.	G	T	.	.	.
	H36	.	.	.	.	T	.	.	T	.	.	G	G	.	C	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H37	.	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H49	T	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H50	.	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	C	.	.
	H51	T	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	C	.	.
	H52	T	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H53	T	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	C	G	A	G	T	T	C	.	C	T	.	.	G	T	.	.	.
	H54	T	.	.	.	T	G	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H70	.	.	.	.	T	.	.	T	.	T*	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H71	.	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H79	.	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H80	.	.	.	.	T	G	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
	H81	.	.	.	.	T	.	.	T	.	.	G	G	.	.	T	.	.	.	.	.	.	C	C	.	.	A	G	T	T	C	.	.	T	.	.	G	T	.	.	.
L. auremensis	H47	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	G	.	.	.	.
	H48	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	G	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	G	.	.	.	.
	H66	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.
	H67	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
	H78	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	G	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	G	.	.	.	.
L. alavariensis	H26	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.
	H27	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.
	H28	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.
	H29	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.
	H63	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.

Paper V | European lamprey species: new insights on postglacial colonization processes and gene flow using microsatellite loci

Catarina S. Mateus[1,2,3,*], Pedro R. Almeida[1,2], Natacha Mesquita[3], Bernardo R. Quintella[2,4] & M. Judite Alves[3]

[1]Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, Largo dos Colegiais 2, 7004-516 Évora, Portugal.

[2]Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

[3]Museu Nacional de História Natural e da Ciência & Centro de Biologia Ambiental, Universidade de Lisboa, Rua da Escola Politécnica 56/58, 1250-102 Lisboa, Portugal.

[4]Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

*E-mail: csmateus@fc.ul.pt

Abstract

The ice ages are known to be the most dominant palaeoclimatic feature occurring on Earth, producing severe climatic oscillations. The Pleistocene ice ages, together with recent processes, considerably shaped the distribution and population structure of several species. Lampreys constitute excellent models to study the colonization of freshwater systems, as they commonly appear in pairs of closely related species of anadromous *versus* freshwater resident adults, thus having the ability to colonize unexplored habitats, through the anadromous species, and establish freshwater resident derivates. We used microsatellite loci to investigate the spatial structure, patterns of gene flow and migration routes of *Lampetra* populations in Europe. *Lampetra* in Europe is represented by the migratory *L. fluviatilis* and four resident species, *L. planeri*, *L. alavariensis*, *L. auremensis* and *L. lusitanica*, the last three endemic to the Iberian Peninsula. We found that in this southern glacial refugium almost all sampled populations represent a distinct genetic cluster, showing high levels of allopatric differentiation, reflecting long periods of isolation. The more recently colonized populations from central and northern Europe are less divergent among them, as result of their more recent common ancestor. They are represented by fewer genetic clusters and lower diversity, and there is evidence of strong recent gene flow among the migratory populations. The Iberian population of *L. fluviatilis* probably feeds on the Tagus estuary and adjacent coastal area, as no evidence for recent gene flow with other conspecific populations was found. This population showed strong evidence of past reduction in population size. We found no signal of hybridization between sympatric populations of *L. fluviatilis* and *L. planeri* from the Iberian Peninsula, and population structure analysis revealed that they constitute two distinct genetic clusters, indicating that these species constitute two distinct taxa that diverged very recently, as previous results from genomic analysis.

Keywords: *Lampetra*, glacial refugium, colonization patterns, hybridization, microsatellites.

Introduction

The Quaternary climatic oscillations and geographic restrictions imposed by the impassable glaciated areas are thought to have had major effects on the evolution and dispersal of numerous different species (e.g., Taberlet *et al.* 1998; Lorenzini & Lovari 2006). It is now clear that most fauna and flora presently distributed across Europe were isolated in southern refugia during the glacials, many in the Mediterranean peninsulas of Iberia, Italy and the Balkans (Hewitt 1999). After the glacials, and as the climate warmed rapidly, founder populations at the northern limits of the southern refugial range expanded rapidly northwards, into the new available habitats, leading to a reduction from southern to northern Europe in the extent of the number of species, subspecific division and allelic variation. While most northern expansions were driven extinct by subsequent ice ages, populations in southern areas could survive several ice ages, as the great variation in topography, climate and habitat in the south of Europe provided great opportunities for a species to find nearby suitable habitats throughout the climatic cycles (Hewitt 1999).

Freshwater fishes tend to show particularly deep phylogeographic structure as they do not normally disperse between river basins, and thus the distribution of their lineages tends to reflect the history of river drainages instead of contemporary dispersal (Gómez & Lunt 2006). Given the repeated cycles of geographical isolation and bottlenecking of northern fishes during glacial advances alternating with expansion and recolonization of newly formed habitats during glacial retreats, it is expected that bursts of speciation events at northern latitudes must have occurred in recent evolutionary times (Bernatchez & Wilson 1998). Recently deglaciated regions were relatively inaccessible to freshwater fishes; they were, however, easily reached by anadromous fishes. These fish breed in fresh water, having ample opportunity to colonize these unexploited systems and establish freshwater isolates (Bell & Andrews 1997). In most genera, lampreys occur in pairs of closely related species with divergent life histories: a parasitic, anadromous form and a non-parasitic, freshwater resident; these pairs are called “paired species”, and the non-parasitic (brook) species have apparently evolved from a form similar to that of an extant parasitic one (Hubbs 1925, 1940; Zanandrea 1959). In some cases more than one non-parasitic species has derived

from a single parasitic species; these are called “satellite species” (Vladykov & Kott 1979). For this reason, lampreys constitute excellent systems to study the postglacial colonization processes and emergence of freshwater derivates by the founder anadromous forms.

The parasitic European river lamprey (*Lampetra fluviatilis*) and the cryptic brook lampreys *Lampetra planeri*, *Lampetra alavariensis*, *Lampetra auremensis* and *Lampetra lusitanica* from the Iberian Peninsula are one example of such satellite species. These species occur in European watersheds, where their range extends from southern Norway to the western Mediterranean and the Iberian Peninsula in the south; the last three are endemic to the Iberian Peninsula. In this region, *L. planeri* is found in several river basins, *L. alavariensis*, *L. auremensis* and *L. lusitanica* are confined to Esmoriz and Vouga basins, Nabão sub-basin and Sado basin, respectively, and the anadromous *L. fluviatilis* occurs in Tagus river basin only (Mateus *et al.* 2012; Mateus *et al.* 2013a). The current distribution of the extant Iberian lamprey lineages is largely allopatric and the genetic divergence between them is consistent with extended periods of isolation during survival in separate glacial refugia throughout the ice ages (Espanhol *et al.* 2007; Mateus *et al.* 2011). Studies using mtDNA revealed that the complex of cryptic species from the Iberian Peninsula is an interesting example of the repeated emergence of resident forms from ancestral migratory ones, i.e., these brook lampreys were independently established at different times and in different locations from the same presumed migratory ancestor (*L. fluviatilis*-type). Whereas the three brook lampreys *L. alavariensis*, *L. auremensis* and *L. lusitanica* are well supported monophyletic groups, divergent from the present-day *L. fluviatilis*, *L. planeri* share haplotypes with the parasitic form, and populations from across Europe are embedded in the same genetic clade, implying that their emergence was more recent (Espanhol *et al.* 2007; Mateus *et al.* 2011). The taxonomy of *L. fluviatilis* and *L. planeri* has thus been considered problematic, as studies using different markers have revealed lack of differentiation between species (e.g., Schreiber & Engelhorn 1998; Espanhol *et al.* 2007; Blank *et al.* 2008), leaving open two possible scenarios: a very recent divergence event or a single species with phenotypic plasticity. The recent study of Mateus *et al.* (2013b), using genome-wide sequencing in sympatric populations of these species in

the Iberian Peninsula, represented an important step forward in this long-standing question, as it successfully identified fixed allelic differences between the two forms, corroborating their classification as distinct taxonomic units.

Hence, the European river lamprey and its related brook lampreys constitute an excellent model to study the effects of the ice ages and the successful postglacial colonization processes driven by an anadromous form, which led to the repeated establishment of independent freshwater resident isolates. In particular, lamprey satellite species from the Iberian Peninsula, where there is evidence of different species emerging in different locations and in different timings, are particular interesting. Also, the Iberian Peninsula is a region of prime interest to investigate these events, due to its role as refugium during the Pleistocene ice ages.

To further investigate the patterns of dispersal and signals of ancestral polymorphism derived from postglacial colonization events, and contemporary gene flow among and within species, we analyzed 10 polymorphic microsatellite loci in the paired *L. fluviatilis* and *L. planeri* across their distributional range, and in three cryptic sister species recently described for the Iberian Peninsula. Microsatellite loci constitute excellent markers to study contemporary relationships between closely related populations, as they are capable of detecting fine-scale divergence.

Materials and methods

Sampling, microsatellite amplification and genotyping

A total of 415 specimens from 10 sites were used in the analysis, with sample sizes ranging from 29 to 52 (Fig. 1; Table 1). Sampled species were the paired European brook and river lampreys (*Lampetra planeri* and *Lampetra fluviatilis*, respectively), and the three recently described Iberian brook lampreys *Lampetra alavariensis*, *Lampetra auremensis* and *Lampetra lusitanica* (Mateus *et al.* 2013a). Only one species was present in each sampling site, with the exception of the Sorraia River in the Tagus Basin (central Portugal), where *L. fluviatilis* and *L. planeri* are found in sympatry. When treated together, populations from Belgium, Germany and Finland are hereinafter

referred to as “northern populations” and populations from the Iberian Peninsula as “southern populations”. All rivers sampled in the Iberian Peninsula drain to the Atlantic Ocean, rivers Warche (river Meuse basin) and Schaale (river Elbe basin) drain to the North Sea, and rivers Beke (river Warnow basin) and Lestijoki drain to the Baltic Sea (Fig. 1).

Total genomic DNA was extracted following a standard phenol-chloroform protocol (Sambrook *et al.* 1989) and stored at -20ºC. DNA concentration was measured using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer and standardized to 50 ng μl^{-1} per sample.

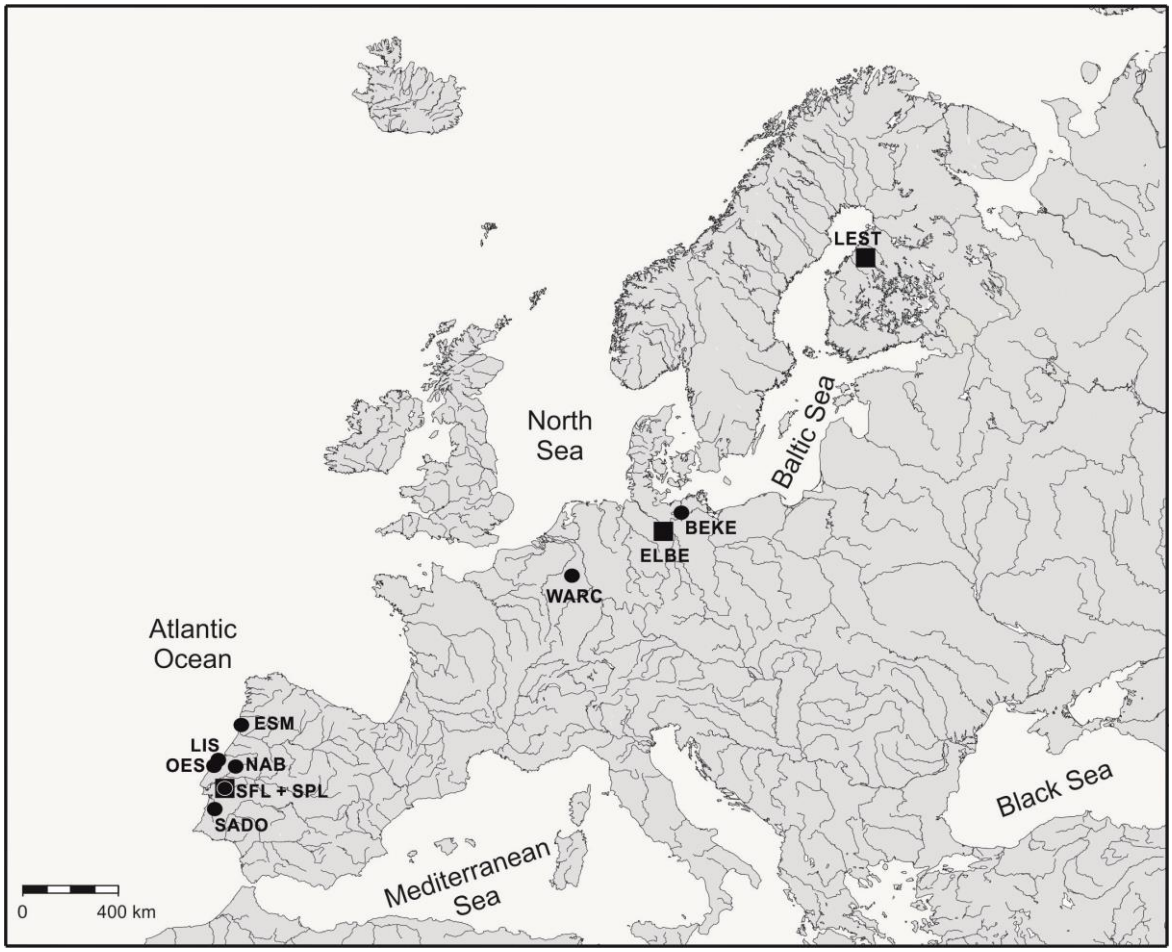

Figure 1 - Sampling sites of *Lampetra* populations in Europe. Squares represent migratory species (*L. fluviatilis*) and circles represent resident species (*L. planeri*, *L. alavariensis*, *L. auremensis* and *L. lusitanica*). See table 1 for details about species and sampling sites.

Table 1 - Locations, sizes (*n*) and specimens' details of *Lampetra* samples included in the study.

Country	Basin	River	Acronym	*n*	Species	Life stage
Finland	Lestijoki	Lestijoki	LEST	29	*L. fluv*	Adults
Germany	Warnow	Beke	BEKE	30	*L. plan*	Larvae
Germany	Elbe	Schaale	ELBE	40	*L. fluv*	Larvae
Belgium	Meuse	Warche	WARC	35	*L. plan*	Adults
Portugal	Esmoriz	Esmoriz	ESM	33	*L. alavar*	Adults
Portugal	Lis	Lis	LIS	33	*L. plan*	Adults
Portugal	Ribeiras do Oeste	Ribeira de S. Pedro	OES	31	*L. plan*	Adults
Portugal	Tagus	Nabão	NAB	35	*L. aurem*	Adults
Portugal	Tagus	Sorraia*	SPL	52	*L. plan*	Adults
Portugal	Tagus	Sorraia*	SFL	46	*L. fluv*	Adults and juveniles
Portugal	Sado	Marateca	SADO	51	*L. lusit*	Adults

L. plan, *L. planeri*; *L. fluv*, *L. fluviatilis; L. alavar, L. alavariensis; L. aurem, L. auremensis; L. lusit, L. lusitanica*
*Location where the paired *L. fluviatilis* and *L. planeri* occur in sympatry

Microsatellite loci were amplified using primers developed for lampreys, using the protocols described in the bibliography and further optimized to the target species. Initially, 49 primer sets were screened, and only those producing unambiguously determined bands and revealing polymorphic loci were selected for further analyses. In total, individuals were genotyped for 10 microsatellite loci using the primer sets Iun 2, Iun 5, Iun 7, Iun 10 and Iun 14 developed for *Ichthyomyzon unicuspis* and *Ichthyomyzon fossor* (McFarlane & Docker 2009), the primer sets Lspn 010-2, Lspn 019c, Lspn 044 and Lspn 094 developed for *Lethenteron* sp. N (Takeshima *et al.* 2005) and the primer set Pma*μ* 5 developed for *Petromyzon marinus* (Bryan *et al.* 2003). The reverse primers were 5'-labelled with 6-FAM, NED, PET or VIC (Applied Biosystems®) fluorescent dyes. Primer sets were grouped into multiplex reactions, and polymerase chain reactions (PCR) were set up in 12 µl volumes containing 2 µL of 50 ng μl^{-1} genomic DNA, 1.0 to 3.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM for each primer, 1 unit of DreamTaq™ DNA Polymerase (Fermentas) and 1× DreamTaq™ Buffer. PCR

conditions were as follows: initial denaturation at 94 °C for 1 min, followed by 25 cycles of 30 sec at 94 °C, annealing for 30 sec at temperatures ranging from 55 to 60 °C and 30 sec at 72 °C, and a final extension of 7 min at 72 °C. A number of sets of difficult amplification were completed using a Multiplex PCR Kit (Qiagen®) with 5 µl Qiagen Multiplex PCR master Mix, 3 µl RNase-free water, 1 µl Primer Mix (2 µM each primer) and 1 µl of 50 ng μl^{-1} of genomic DNA, using the following protocol: initial activation step at 95 ºC for 15 min, followed by 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 57 ºC for 90 sec and extension at 72 ºC for 60 sec, and a final extension of 30 min at 60 ºC. The PCR reactions were conducted on a Bio-Rad® thermal cycler.

Samples were genotyped in an ABI PRISM® 310 Genetic Analyzer and fragments were sized with GeneScan™-500 LIZ™ Size Standard. Allele sizes were determined using the software GeneMapper® 3.7 (Applied Biosystems®).

Data analysis

Microsatellite loci were first tested for null alleles, stuttering and large allele dropout using the software MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004). Each microsatellite locus was tested for Hardy–Weinberg equilibrium as implemented in ARLEQUIN 3.11 (Excoffier *et al.* 2005). Genetic diversity was measured as the mean allelic richness (AR), observed heterozygosity (Ho), unbiased expected heterozygosity (He, *sensu* Nei 1978) and mean number of alleles across loci (MNA), inferred using GENETIX 4.05.2 (Belkhir *et al.*1996-2004), with the exception of allelic richness, which was calculated and corrected for sample dimension by rarefaction using HP-Rare (Kalinowski 2005).

The genetic differentiation among samples was assessed through pairwise F_{ST} using Weir & Cockerham's (1984) estimator, and significance was assessed with 10^4 permutations, as implemented in GENETIX. The distribution of genetic variation was assessed among and within the 11 samples, the sympatric *L. fluviatilis* and *L. planeri*, and the genetic clusters attained with population structure analysis, through analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992). These analyses were performed in ARLEQUIN, using the allelic frequencies as genetic distance, and 10^4 permutations.

The Bayesian model-based clustering approach implemented in STRUCTURE 2.2 (Pritchard *et al.* 2000) was used to assemble individuals from the 11 samples into groups (genetic clusters). Runs were performed under the admixture model with correlated allelic frequencies and with number of groups (K) set between 1 and 12. For each K, 20 simulations were performed with a burn-in period of 10^5, followed by 10^5 Markov steps. Using the same parameters, two additional structure analyses were performed, one including solely the eight samples of *L. planeri* and *L. fluviatilis* (K between 1 and 9), and the other including the four samples from the North (K between 1 and 5). This allows to detect further structure in these populations, if present, that otherwise would be hidden due to the high differentiation among the five species, and between the northern and southern samples. The optimal K, and clustering achieved, was inferred using the protocol defined by Evanno *et al.* (2005) as implemented in STRUCTURE HARVESTER 0.6.93 (Earl & VonHoldt 2012), and taking into account the biological meaning of the clusters. The software DISTRUCT 1.1 (Rosenberg 2004) was used for the graphical display of population clusters.

Patterns of differentiation were visualized by principal coordinates analysis (PCoA), a multivariate technique that allows to find and plot the major patterns within a multivariate dataset, like multiple loci and multiple samples. This analysis was computed using GenAlEx 6.5 (Peakall & Smouse 2006, 2012).

The software NewHybrids 1.1 (Anderson & Thompson 2002) was used for the detection and classification of putative hybrids between sympatric populations of *L. fluviatilis* and *L. planeri* from Portugal. NewHybrids uses a Bayesian approach to identify different categories of hybrid individuals through the computation of the posterior probability that individuals fall into different hybrid (F_1, F_2 and backcrosses) or pure parental categories. It uses the allele frequencies of multilocus genotypes and a Markov Chain Monte Carlo procedure. Simulations were performed with a burn-in period of 10^5, followed by a sampling period of 10^5 Markov steps. A threshold of posterior probability > 50% was set up to classify an individual as belonging to a certain category.

Estimates of recent migration rates (*m*) between migratory populations were inferred using a Bayesian assignment test-based method in the program BAYESASS

3.0.1 (Wilson & Rannala 2003). BAYESASS estimates migration rates over the last two generations using a Markov chain Monte Carlo procedure and does not assume that populations are in migration-drift or Hardy–Weinberg equilibrium. Because BAYESASS focuses on contemporary migration rates, estimates are unaffected by the colonization processes. A total of 10^7 MCMC iterations (discarding the first 10^6 iterations as burn-in) were performed, and samples were collected every 2000 iterations. Delta values for migration rate, allele frequencies, and inbreeding values coefficients were set at 0.20, 0.40 and 0.60, respectively.

Further, to test the assignment of individuals to their sampling sites, the software GeneClass2 2.0.h (Cornuet *et al.* 1999) was used, including a likelihood-based method in which individuals are assigned to the locality in which the individual's genotype is most likely to occur. The Bayesian statistical approach of Rannala & Mountain (1997) was implemented.

Demographic signatures of recent bottlenecks were tested using the heterozygosity excess method implemented in BOTTLENECK 1.2.02 (Piry *et al.* 1999) under three different mutational models: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM). Significant deviations from mutational-drift equilibrium were tested using the Wilcoxon sign rank test with 10^5 simulations, and the distribution of allele frequency classes was examined for a deviation from the normal L-shaped distribution (Luikart *et al.* 1998). Past reductions in population size were also evaluated using the *M* ratio ($M = k/r$) statistic test as implemented in M_P_VAL (Garza & Williamson 2001), where in, *k* is the number of alleles present at a given microsatellite locus and *r* is the overall range in allele size. In recently reduced populations *M* is expected to be smaller than in populations at equilibrium, since the loss in any allele will contribute to a reduction in *k*, whereas only a loss of the smallest or largest alleles will contribute to a reduction in *r*, and thus *k* is expected to decrease more quickly than *r*. Significant reductions in population size were considered if less than 5% of the replicates are below the observed *M* value. Following Garza & Williamson (2001), we used the default settings for the two-phase mutation model (TPM) $p_s = 0.9$, $\Delta_g = 3.5$ and three values of θ ($\theta = 4$, $\theta = 10$ and $\theta =$

20). Populations LIS and OES were not included in the demographic analysis because they present only one polymorphic locus.

Results

Genetic diversity and differentiation

Summary statistics of the genetic diversity indices for each locus and sample are provided in Table S1 of the Online Supplementary Information. The total number of alleles per locus across populations varied from two, at the loci Iun7 and Lspn010-2, to 13 at the locus Iun14. Twelve private alleles were found, three of which in NAB, three in SPL and other three in SADO. The remaining three were found in BEKE (*n*=2) and SFL (*n*=1) (Table S1, Supplementary information). The mean number of alleles (MNA) across loci ranged from 1.1 (LIS and OES) to 3.8 (ELBE), mean allelic richness (AR) from 1.08 (LIS) to 2.62 in (ELBE), and expected heterozygosity (He) from 0.0239 (LIS) to 0.4417 (NAB) (Table S1, Supplementary information).

Signs of null alleles were detected with MICRO-CHECKER for limited situations across loci and populations: Pmaμ 5 in SPL and NAB; Iun 10 in SPL and BEKE; Iun 5 in ELBE and BEKE; and Iun 14 in SPL and BEKE. Significant null alleles' signature is related with heterozygote deficit and therefore with deviations from Hardy–Weinberg equilibrium, as seen in Table S1 (Supplementary information) considering the results for departures from Hardy–Weinberg equilibrium.

A considerable level of genetic differentiation among samples was observed (average F_{ST}=0.498, P<0.001) with pairwise F_{ST} values ranging from 0.0114 (ELBE-LEST) to 0.8915 (OES-ESM), all being significant (P<0.001 for all pairs, with the exception of ELBE-LEST where P<0.05) (Table 2).

Table 2 - Pairwise estimates of genetic differentiation (F_{ST}) among sites (above diagonal) and corresponding *P* values (below diagonal).

	LEST	BEKE	ELBE	WARC	ESM	LIS	OES	NAB	SPL	SFL	SADO
LEST	-	0.0519	0.0114	0.2849	0.5035	0.6642	0.6736	0.3820	0.2602	0.1463	0.4557
BEKE	< 0.001	-	0.0530	0.3963	0.5301	0.6959	0.7104	0.3989	0.2919	0.2378	0.5243
ELBE	0.04	< 0.001	-	0.2486	0.4644	0.6269	0.6402	0.3712	0.2330	0.1100	0.4127
WARC	< 0.001	< 0.001	< 0.001	-	0.5692	0.7047	0.6974	0.4464	0.3408	0.3704	0.6056
ESM	< 0.001	< 0.001	< 0.001	< 0.001	-	0.8877	0.8915	0.5472	0.3697	0.5131	0.7529
LIS	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	0.6166	0.5820	0.5656	0.6897	0.8396
OES	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	0.5839	0.5887	0.6996	0.8291
NAB	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	0.3909	0.4423	0.6273
SPL	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	0.3167	0.4931
SFL	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	0.3989
SADO	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-

AMOVA analysis among the 11 samples indicated that 48.34% of genetic variance occurred among samples (*P*<0.001), and variation within samples accounted for 51.66% (*P*<0.001); AMOVA between the sympatric paired *L. fluviatilis*/*L. planeri* in Sorraia revealed that 31.64% of the variance was significantly (*P*<0.001) explained among species; and AMOVA among the eight genetic groups attained with STRUCTURE (see below and Fig. 2A) indicated that the majority of variance occurs among groups (46.79%) and within samples (48.89%), whereas variance among samples within groups is low (4.32%) (*P*<0.001 for all three levels).

Population structure and admixture

The number of genetic groups represented in our samples and the level of admixture among them was assessed with STRUCTURE. This analysis revealed that the 11 samples are grouped in eight distinct genetic clusters: 1) LEST+BEKE+ELBE, 2) WARC, 3) ESM, 4) LIS+OES, 5) NAB, 6) SPL, 7) SFL and 8) SADO (Fig. 2A and Table 3). The first group exhibits strong evidence of admixture with the resident *L. planeri* from Belgium (WARC) and *L. fluviatilis* from Portugal (SFL), with a greater number of admixed individuals in the anadromous populations (LEST and ELBE). Most individuals of group 2 (WARC) are distinct and constitute a distinct genetic cluster with high proportion of membership (0.949). This is also the case in other groups comprised of resident

species, namely, ESM, LIS+OES, NAB and SADO. The sympatric paired *L. planeri* and *L. fluviatilis* from Portugal (SPL and SFL) constitute two distinct genetic clusters (6 and 7, respectively) that present a few admixed individuals between them. SPL also shows some evidence of admixture with ESM (Fig. 2A and Table 3). When STRUCTURE was run only for the eight samples of *L. planeri* and *L. fluviatilis* (Fig. 2B, K=5), and for the four northern samples (Fig. 2C, K=2), no additional genetic clusters were achieved, indicating that there is no hidden structure caused by the high differentiation among samples, and that the genetic cluster that groups the northern populations (LEST+BEKE+ELBE) is well supported.

The principal coordinates analysis (PCoA), revealed the existence of mainly six distinct clusters, NAB, SADO, ESM, LIS+OES, SPL+WARC, BEKE+LEST+ELBE+SFL (Fig. 3). These results are congruent with STRUCTURE, with the exception that it groups SPL with WARC, and SFL with the northern cluster, while they were four distinct clusters in STRUCTURE.

Individual assignment tests were applied to further investigate the genetic distinctiveness of the populations. In four populations of resident species (WARC, ESM, NAB and SADO) 100% of the individuals were assigned to their correct source population (Table 4), which is in agreement with STRUCTURE analysis. Samples from northern Europe were the ones with more individuals assigned to other populations; *L. fluviatilis* from Finland and Germany (LEST and ELBE, respectively) and the resident *L. planeri* from Germany (BEKE) had individuals assigned among the three populations, in agreement with STRUCTURE, that groups the three populations. The sympatric *L. fluviatilis* and *L. planeri* (SFL and SPL) had almost all individuals assigned correctly (96% and 98%), and small percentages (4% and 2%) assigned between them. LIS and OES showed 73% and 97%, respectively, of correctly assigned individuals, and the remaining were assigned also among each other (Table 4). This last result is consistent with the STRUCTURE and PCoA analyses, which revealed a close genetic relation between these two populations (Fig. 2 and Fig. 3).

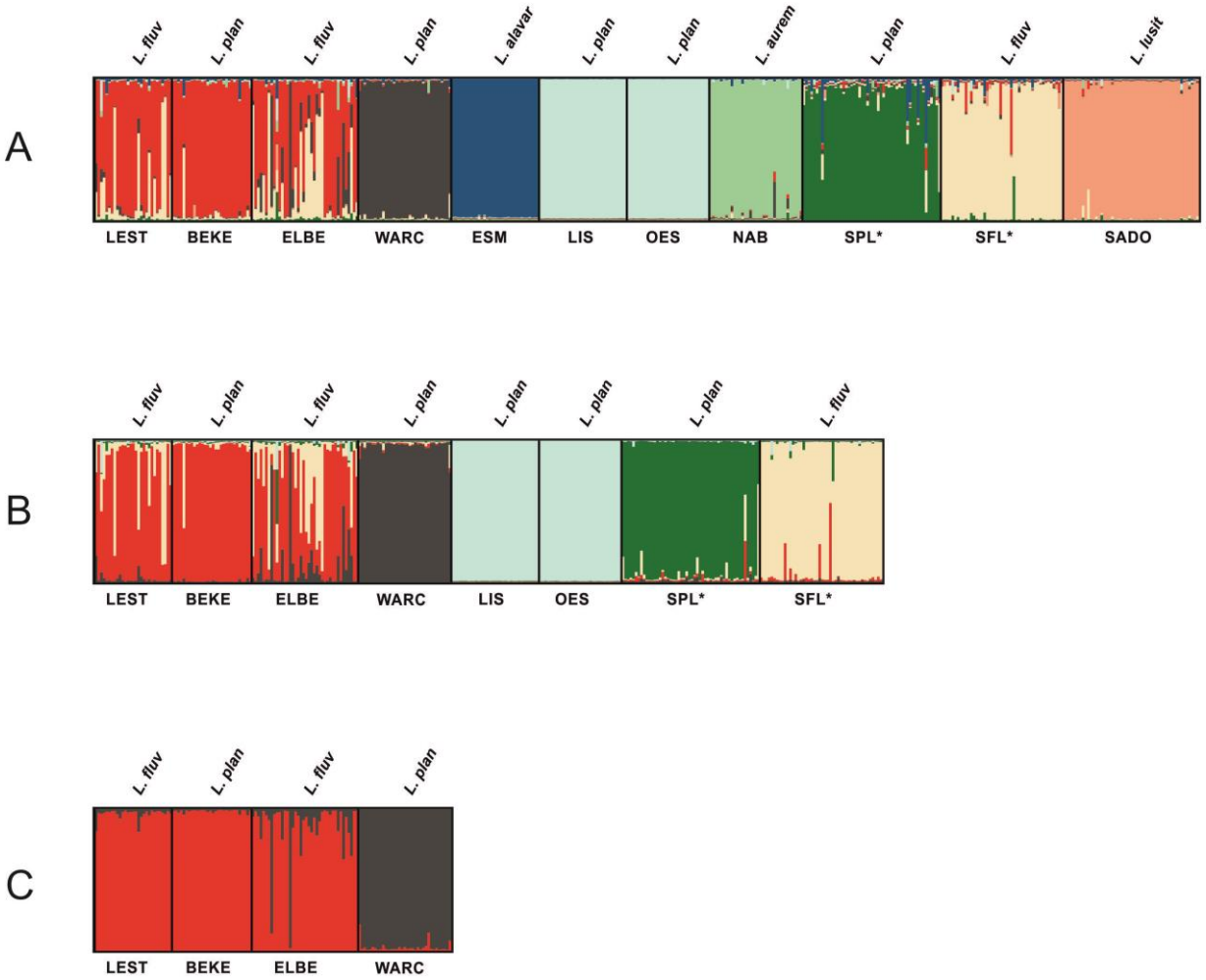

Figure 2 - Most likely population structure, computed under the admixture model with correlated allelic frequencies in STRUCTURE, considering A) all the 11 samples, K=8; B) the populations of *Lampetra planeri* and *Lampetra fluviatilis*, K=5; and C) the four northern populations, K=2. Each individual is represented by a vertical bar. In accordance with Fig. 1 and Table 1, sampled locations below plot and corresponding *Lampetra* species above.

Table 3 - STRUCTURE analysis for the 11 samples. Proportion of membership of each pre-defined population in each of the eight genetic clusters.

Population	Inferred clusters							
	1	2	3	4	5	6	7	8
LEST	0.735	0.028	0.014	0.013	0.007	0.010	0.188	0.005
BEKE	0.916	0.008	0.017	0.006	0.008	0.006	0.032	0.007
ELBE	0.551	0.136	0.016	0.012	0.014	0.018	0.243	0.011
WARC	0.009	0.949	0.009	0.003	0.006	0.005	0.014	0.004
ESM	0.003	0.003	0.977	0.003	0.003	0.004	0.005	0.003
LIS	0.003	0.002	0.003	0.981	0.003	0.003	0.003	0.002
OES	0.002	0.002	0.002	0.983	0.003	0.002	0.002	0.003
NAB	0.008	0.017	0.009	0.007	0.944	0.006	0.005	0.003
SPL	0.015	0.012	0.052	0.006	0.009	0.861	0.039	0.006
SFL	0.035	0.009	0.016	0.007	0.004	0.016	0.901	0.012
SADO	0.007	0.004	0.005	0.006	0.004	0.005	0.013	0.957

Clusters: 1, LEST+BEKE+ELBE; 2, WARC; 3, ESM; 4, LIS+OES; 5, NAB; 6, SPL; 7, SFL; 8, SADO

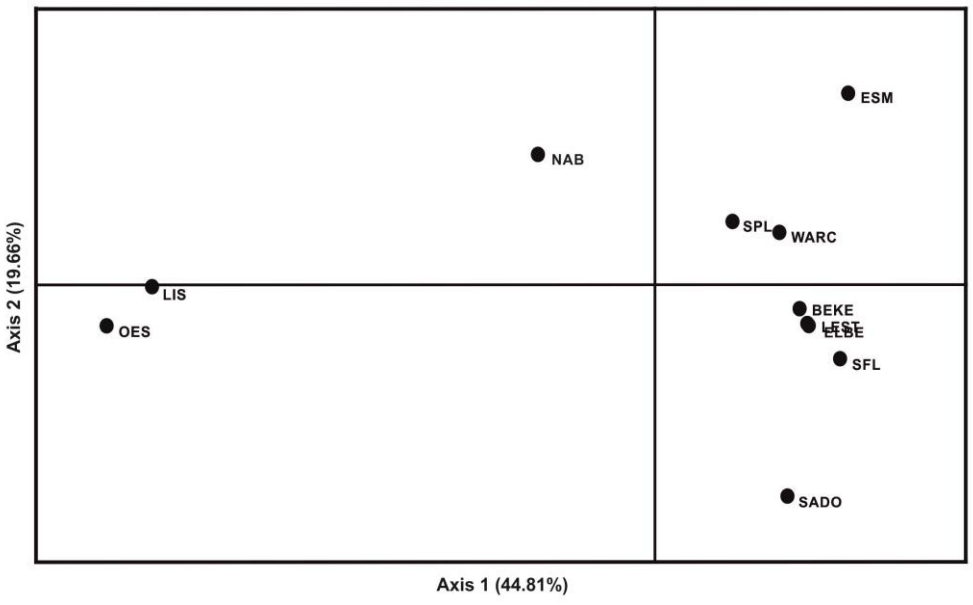

Figure 3 - Principal coordinates analysis plot (PCoA) computed by GenAlEx. The percentage of variation explained by each axis is shown. Sample's acronyms as in Fig. 1 and Table 1.

Table 4 - Assignment tests performed with GeneClass2. Values represent the percentage of individuals from each studied sample assigned to each of the sampled populations based on the Bayesian method.

	Assigned population										
	LEST	*BEKE*	*ELBE*	*WARC*	*ESM*	*LIS*	*OES*	*NAB*	*SPL*	*SFL*	*SADO*
LEST	**80**	3	17	0	0	0	0	0	0	0	0
BEKE	3	**94**	3	0	0	0	0	0	0	0	0
ELBE	10	15	**68**	5	0	0	0	0	0	2	0
WARC	0	0	0	**100**	0	0	0	0	0	0	0
ESM	0	0	0	0	**100**	0	0	0	0	0	0
LIS	0	0	0	0	0	**73**	27	0	0	0	0
OES	0	0	0	0	0	3	**97**	0	0	0	0
NAB	0	0	0	0	0	0	0	**100**	0	0	0
SPL	0	0	0	0	0	0	0	0	**98**	2	0
SFL	0	0	0	0	0	0	0	0	4	**96**	0
SADO	0	0	0	0	0	0	0	0	0	0	**100**

Each row contains the samples from one sampled locality and the columns indicate the localities to which the samples were assigned (*i.e.*, in which their genotypes had the highest likelihood of occurring).
Diagonal values are in bold and represent the proportion of individuals assigned to the population in which they were sampled

Putative hybrids between sympatric *L. fluviatilis* and *L. planeri*

The existence of putative hybrids between the sympatric paired *L. planeri* and *L. fluviatilis* from Portugal (SPL and SFL, respectively) was investigated with NewHybrids. In this analysis, each individual was assigned a posterior probability (*p*) of belonging to one of the six different genotype classes resulting from two generations. From the 52 samples of *L. planeri*, 49 (94%) were classified as being pure *L. planeri* using the posterior probability threshold of 0.5, 20 of which showing $p > 0.99$ and 25 showing $0.8 < p < 0.99$ (Fig. 4). For this species only one individual was classified as hybrid (F_2; second generation hybrid) with posterior probability of 0.664 and one individual was classified as being pure *L. fluviatilis* (posterior probability = 0.537) (Table 5). All the 46 individuals of *L. fluviatilis* were identified as such (pure *L. fluviatilis*), from which 40 exhibit $p > 0.99$ and 5 showing $0.8 < p < 0.99$. No F_1 or backcross hybrids were found in any of the species (Table 5).

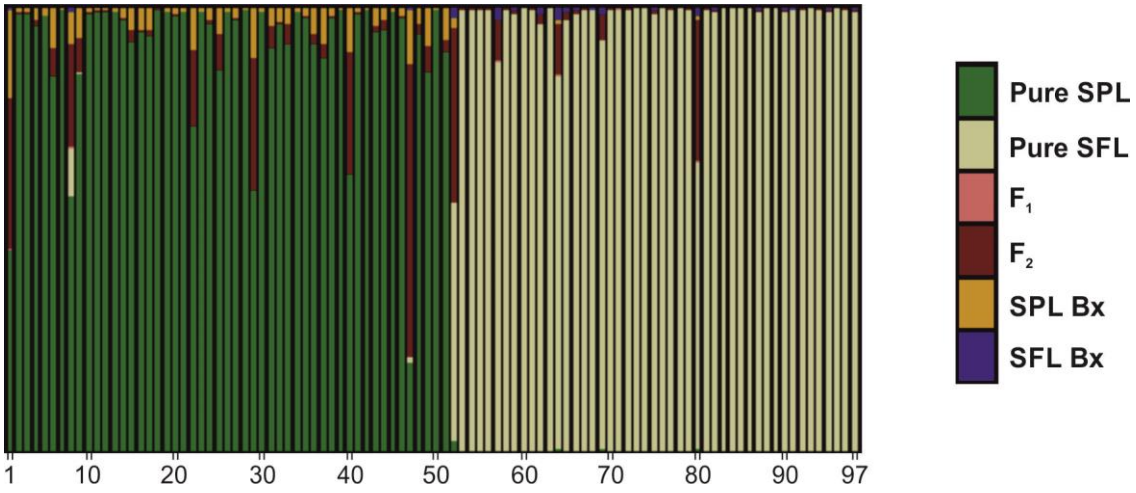

Figure 4 - Estimated posterior probabilities that each individual from the sympatric populations of *Lampetra planeri* (SPL; *n*=52) and *Lampetra fluviatilis* (SFL; *n*=46) belongs to each of the six different genotype frequency categories that arise from two generations of potential interbreeding (parental species, F_1, F_2 and backcrosses), computed by NewHybrids. Each individual is represented by a vertical bar. For the individuals identified as belonging to a certain hybrid category, posterior probability values are detailed in Table 5.

Table 5 - Hybridization analysis for the sympatric *L. fluviatilis* (SFL) and *L. planeri* (SPL) from Portugal. Estimated posterior probabilities of belonging to one of the six genotype frequency classes (pure parental, F_1, F_2 or backcrosses) for the individuals showing some evidence of hybridization. An individual is identified as belonging to a certain class if the posterior probability of falling into that class is above 0.5. Specimens are numbered as in Figure 4.

Species	Specimen	Pure SPL	Pure SFL	F_1	F_2	SPL Bx	SFL Bx
L. planeri (SPL) *n*=52	1	0.452	-	-	0.342	0.200	-
	8	0.577	0.106	-	0.233	0.075	-
	22	0.733	-	-	0.171	0.096	-
	29	0.587	-	-	0.301	0.113	-
	40	0.625	-	-	0.274	0.099	-
	47*	0.198	0.013	-	0.664	0.120	-
	52	0.023	0.537	-	0.394	0.023	0.022
L. fluviatilis (SFL) *n*=46	80	-	0.649	-	0.319	0.009	0.018

Pure SPL, pure *Lampetra planeri*; Pure SFL, pure *Lampetra fluviatilis*; F_1, first generation hybrid; F_2, second generation hybrid; SPL Bx, *L. planeri* backcross (pure *L. planeri* mating with F_1); SFL Bx, *L. fluviatilis* backcross (pure *L. fluviatilis* mating with F_1).
Grey shading indicates the class the individuals were classified into; *Individual identified as hybrid.

Migration rate among populations

Recent migration rates (*m*) among samples were estimated using BAYESASS. This analysis was performed for the three anadromous populations included in the study, i.e., *L. fluviatilis* from Portugal (SFL), from Germany (ELBE) and from Finland (LEST). The proportion of individuals derived from their own location was high in SFL (*m*=0.979) and in LEST (*m*=0.968), and relatively low in ELBE (*m*= 0.705) (Fig. 5). Accordingly, a relatively high proportion of immigrants (*m*=0.286) was detected from LEST into ELBE. SFL is the most isolated population, with the highest proportion of non-immigrants (*m*=0.979) and low migration rates (*m*≤0.02) in both directions (Fig. 5).

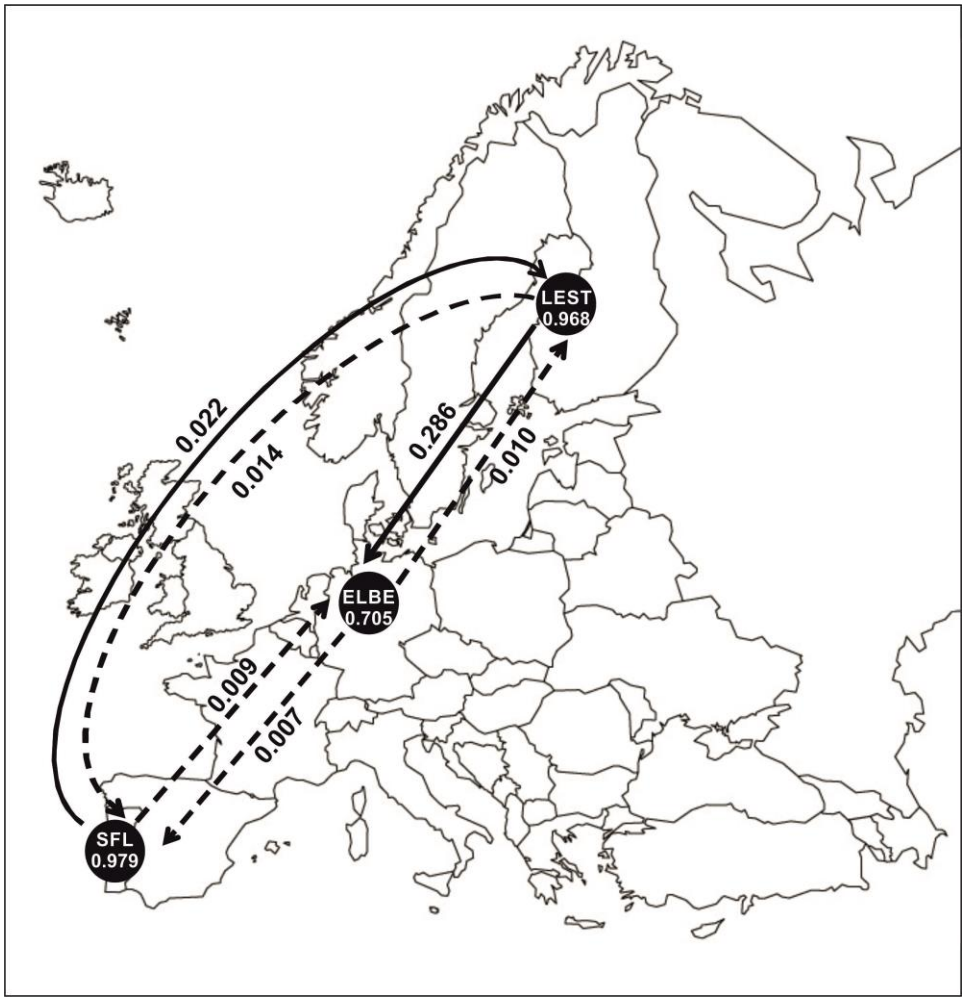

Figure 5 - Recent migration rates (*m*) between migratory populations estimated using BayesAss. Within circles, acronyms represent samples as in Fig. 1 and Table 1, and numbers denote the proportion of non-immigrants within populations. Arrows indicate direction of gene flow among populations and respective *m* value. Dashed arrows represent values of *m* lower than 0.02.

Demographic history

Bottleneck analysis revealed consistent signs for recent contraction of population size only in the WARC population, which showed significant (P<0.05) heterozygote excess according to the three mutational models tested, and a shift in the distribution of allele frequency classes from the expected L-shaped distribution (Table 6). NAB population also presents significant (P<0.01) heterozygote excess according to IAM, but no deviation from the expected L-shaped distribution (Table 6). Using the *M* ratio statistic test, we found strong evidence of past reduction in population size for the migratory *L. fluviatilis* from Portugal (SFL), as the *M* ratio was significantly smaller than the equilibrium expectation (P<0.05) for all the prebottleneck θ values considered. None of the remaining populations presented signs of reduction in population size (Table 6).

Table 6 - Demographic analysis. *P* values for one-tailed heterozygote excess (grey shading indicates significant *P* values), deviation of allele frequency classes from a normal L-shaped distribution and *M* ratio tests.

	IAM	TPM	SMM	L-shape	*M* ratio value
LEST	0.326	0.820	0.993	no deviation	0.72
BEKE	0.578	0.963	0.994	no deviation	0.75
ELBE	0.248	0.590	0.936	no deviation	0.71
WARC	0.014	0.020	0.037	deviation	0.79
ESM	0.156	0.563	0.906	no deviation	0.83
NAB	0.007	0.064	0.082	no deviation	0.75
SPL	0.097	0.216	0.784	no deviation	0.78
SFL	0.326	0.674	0.976	no deviation	0.66†
SADO	0.422	0.422	0.578	no deviation	0.82

IAM, infinite allele model; TPM, two-phase model; SMM, stepwise mutation model

*The populations LIS and OES were not included in the analysis as they only have one polymorphic locus (Lspn 094) (Table S1, Supplementary information).

†Location with *M* ratio value significantly smaller than the equilibrium expectation (P < 0.05) for all the prebottleneck θ values considered (4, 10 and 20).

Discussion

Genetic diversity, population structure and postglacial dispersal

The colonization processes that took place after the glacial periods, when populations from the southern Mediterranean peninsulas expanded north across Europe shaped, together with recent processes, the genetic structure of current taxa. Southern populations isolated in refugia and sub-refugia accumulated variation through the ice ages, and the founders that rapidly moved northward during interglacials only represented a subsample of the southern diversity (Hewitt 1996). In this study, this pattern is clearly seen by the higher number of allopatric *Lampetra* species in the Iberian Peninsula, compared with northern Europe. Also, southern samples have the majority of private alleles, which is representative of their higher genetic diversity, also revealed previously by mitochondrial DNA (mtDNA) (Espanhol *et al.* 2007; Mateus *et al.* 2011). STRUCTURE and principal coordinates analyses also corroborate the above scenario (see Figs. 2 and 3), as they revealed higher number of genetic groups in the Iberian Peninsula (including the recently described *Lampetra* species, that evolved in allopatry) than in the northern latitudes. This study revealed that anadromous populations from central and northern Europe have high proportions of membership from the population of *L. fluviatilis* from the Iberian Peninsula (the SFL genetic cluster); because contemporary gene flow from south to the north and vice-versa is happening in very small proportions (see Fig. 5), this signal seems to be due to ancestral polymorphism, as a result of the colonization process. This pattern is in agreement with the findings of Espanhol *et al.* (2007) and Pereira & Almada (2013), which revealed a star-like haplotype network for the mitochondrial genes ATP6 and ATP8, where all specimens from the Tagus population (here SFL) display the ancestral haplotype, which is consistent with a scenario of dispersal and expansion. STRUCTURE analysis for the 11 samples revealed the existence of eight genetic clusters that are strongly related to geography, grouping northern populations in the same cluster, except the resident *L. planeri* from Belgium. Grouping of Iberian populations reflects in general their specific status, with the exception of LIS and OES that were grouped together, but not grouped with the other population of *L. planeri* from Portugal (SPL).

Those two populations present very low levels of genetic diversity, having one single polymorphic locus and none private alleles. In general, the alleles present in those populations are rather common, most of the times having a frequency of more than 50% in other populations. The differentiation of those populations from SPL seems, therefore, to reflect this lack of diversity, which statistically makes them unique. Those populations may be facing a genetic bottleneck, but this analysis could not be performed due to the existence of a single polymorphic locus.

The repeated emergence of resident forms from ancestral migratory ones in different locations and times is a phenomenon very well known in lampreys (Hubbs 1925, 1940; Zanandrea 1959; Vladykov & Kott 1979), and promotes varying degrees of reproductive isolation between founder and derived species. As previously observed with mtDNA, the three brook lampreys *L. alavariensis*, *L. auremensis* and *L. lusitanica* are well supported species, highly divergent from the present-day *L. fluviatilis*. The brook *L. planeri*, however, does not present such high differentiation from *L. fluviatilis*, as previously found by the sharing of mtDNA haplotypes with the parasitic form, evidence that their emergence was more recent (Espanhol *et al.* 2007; Mateus *et al.* 2011). Considering *L. planeri* and its ancestor *L. fluviatilis*-type, there is further partitioning in colonization processes, as populations of both forms from southern Europe are more divergent than populations from northern Europe, the last presenting strong signals of hybridization resulting from a more recent colonization process.

Populations in Central and Northern Europe

In opposition to the southern species and populations, which were grouped in several genetic clusters due to their high levels of differentiation, northern populations were grouped in the same genetic cluster as result of their more recent common ancestral. The exception was the resident *L. planeri* from river Warche in Belgium, which constitutes a single genetic cluster. This population seems to be facing a genetic bottleneck (see table 6), which explains the relatively low number of fixed alleles found in each locus (maximum of three, see Table S1 Supplementary information), that can reflect the isolation of this population; it is located very upstream in the river basin,

with several important obstacles downstream (M. Ovidio pers. comm.) isolating it from other populations and thus preventing gene flow. Regarding long-term isolation, glaciations have been considered an important factor in brook lamprey evolution, both in Europe and North America (Hardisty & Potter 1971c; Hardisty 1986). These periods favour the abandonment of anadromous habits due to blocking of migratory routes. For example, the blocking of the Gulf of Bothnia by ice at the height of the last glaciation would have prevented access to this area by anadromous lampreys of the *L. fluviatilis* group from the rivers of the eastern Baltic, thus producing conditions favourable for the emergence of *L. planeri* populations (Hardisty 1986). This scenario would imply the existence of additional smaller refugia in central and northern Europe, where resident populations could have survived over the glacial period. Several cryptic northern refugia have been hypothesized, one of which in the Belgian Ardennes. These northern refugia would have been in areas of sheltered topography that provided suitable stable microclimates (Stewart & Lister 2001). River Warche is located in the Ardennes, and we thus hypothesize that the population from river Warche may represent an independently evolved population that survived during glaciations in the Belgian Ardennes refugium. Another possible explanation is that the two observed northern genetic clusters represent different postglacial recolonization routes by the founder population. For testing these hypotheses and for a better understanding of the migratory routes of European lampreys following the glacial periods, further studies with mtDNA, and including more northern populations, should be employed.

Gene flow among species and populations

The three new species *Lampetra alavariensis*, *Lampetra auremensis* and *Lampetra lusitanica* endemic from Portugal constitute solid distinct genetic groups, with no signal of ongoing gene flow with any of the samples included in this study, supporting the mtDNA data (Mateus *et al.* 2011).

Sympatric *L. fluviatilis* and *L. planeri* present significant genetic differentiation and almost no signal of hybridization. The significant differences between species was reinforced by the AMOVA results that indicate that 31.64% of the variance is

significantly (P<0.001) explained between species. These results are in agreement with our previous work using restriction site-associated DNA (RAD) sequencing (Mateus *et al.* 2013b) that suggests that these species are two distinct taxa that diverged recently.

Results attained for northern populations of *L. fluviatilis* and *L. planeri*, however, indicate that these species are grouped in a single cluster (STRUCTURE analysis) and may be experiencing or have experienced until recently gene flow. A number of studies in central and northern populations have suggested this scenario, reporting cases of communal spawning (Huggins & Thompson 1970; Lasne *et al.* 2010), and the production of viable offspring through artificial hybridization (Enequist 1937; Hume *et al.* 2013). This scenario is most likely explained by the postglacial colonization of northern habitats by a southern *L. fluviatilis*-type and consequently later appearance of the northern populations, as explained above. Having this into account, one must be aware that differentiation of resident *versus* anadromous populations may be an undergoing process in many locations, or in other lamprey paired species, as suggested by (Docker *et al.* 2012) for the paired silver (*Ichthyomyzon unicuspis*) and northern brook (*Ichthyomyzon fossor*) lampreys. These authors could not find significant genetic differences between the two species where they occur in sympatry, suggesting the existence of ongoing gene flow between them.

BAYESASS revealed high recent gene flow between the migratory northern populations ELBE and LEST, which is corroborated by the assignment tests, where 17% of the individuals from LEST were assigned to ELBE and 10% of the individuals from ELBE were assigned to LEST. In contrast, the migratory *L. fluviatilis* from the Iberian Peninsula seems to behave more like a resident species, showing almost absence of ongoing gene flow with northern populations and high degree of isolation and differentiation. This result, together with the relatively small size of the individuals may reveal reduced levels of mobility during the parasitic adult phase probably associated with its permanence in the large Tagus estuary (c. 300 km^2) and adjacent coastal area. *L. fluviatilis* migrants can be separated on the basis of size into “typical” and “praecox” forms, whose mean lengths are approximately 30 and 24 cm, and mean weights about 53 and 22 g, respectively (Abou-Seedo & Potter 1979). The size difference between the typical and praecox forms is thought to be due to differences in the time spent feeding

in the sea, the last reducing their marine feeding phase by at least 1 year (Abou-Seedo & Potter 1979). The population from the Tagus river resembles these smaller praecox forms; it has in average 26 cm total length and 33 g weight, and one of the individuals was as small as 20 cm of total length and 19 g weight (unpublished data). For instance, Kemp *et al.* (2010) registered values of 80.7 g and 36.3 cm for this species in north-east England. The southern population, inhabiting exclusively the lower part of Tagus river basin, shows strong evidence of past reduction in population size (this study), and the low number of individuals caught in the last years is representative of the rareness of this population (Mateus *et al.* 2012).

For a better understanding of the contemporary patterns of gene flow in more recently established northern populations, recent migration routes among and within northern *L. fluviatilis* and *L. planeri* populations should be further investigated.

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Supplementary information

Table S1 Measures of genetic diversity assayed at ten microsatellite DNA loci for each sampled location. Sample acronyms correspond to locations as in Fig. 1 and Table 1. Number of alleles per locus (Na) with number of private alleles in parentheses, mean allelic richness (AR), unbiased expected heterozygosity (He), observed heterozygosity (Ho), significance of departure from Hardy–Weinberg Equilibrium (HWE), mean number of alleles across loci (MNA) and number of polymorphic loci in each location (P). Grey shading indicates loci where MICRO-CHECKER detected signs of null alleles and relation with deviations from Hardy–Weinberg equilibrium. NS, non-significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; *n*, sample size; †, private allele with frequency >50%

Sample	LEST	BEKE	ELBE	WARC	ESM	LIS	OES	NAB	SPL	SFL	SADO	Overall
	n=29	*n*=30	*n*=40	*n*=35	*n*=33	*n*=33	*n*=31	*n*=35	*n*=52	*n*=46	*n*=51	
Locus												
Iun 2												
Allelic range (bp)	123-129	123-129	123-129	123-126	123	123	123	120-123	120-126	123-126	123-126	120-129
Na	3	3	3	2	1	1	1	2	3	2	2	4
AR	1.9755	2.0493	1.8841	1.9397	1.0000	1.0000	1.0000	1.9978	2.8435	1.5660	1.4096	
He	0.2716	0.2672	0.2434	0.3578	0.0000	0.0000	0.0000	0.4969	0.5937	0.1421	0.0942	
Ho	0.3103	0.3000	0.2750	0.4000	0.0000	0.0000	0.0000	0.5143	0.4600	0.1522	0.0588	
HWE	NS	NS	NS	NS	-	-	-	NS	NS	NS	NS	
Iun 5												
Allelic range (bp)	246-267	246-312	246-297	246-258	255-282	252	252	252	252-258	252-282	249-252	246-312
Na	5	6 (1)	7	3	2	1	1	1	3	4	2 (1†)	9

AR	3.0616	3.7347	3.8711	2.3257	1.2821	1.0000	1.0000	1.0000	1.8593	2.2939	1.9978	
He	0.5793	0.6836	0.6452	0.4352	0.0597	0.0000	0.0000	0.0000	0.2417	0.2871	0.4980	
Ho	0.5556	0.3793	0.4545	0.3429	0.0606	0.0000	0.0000	0.0000	0.2745	0.2273	0.6078	
HWE	NS	***	***	NS	NS	-	-	-	NS	NS	NS	
Iun 7												
Allelic range (bp)	179-181	179	179-181	179-181	179	179	179	179-181	179-181	179-181	179	179-181
Na	2	1	2	2	1	1	1	2	2	2	1	2
AR	1.5414	1.0000	1.8503	1.9937	1.0000	1.0000	1.0000	1.8416	1.9975	1.8255	1.0000	
He	0.1307	0.0000	0.2755	0.4737	0.0000	0.0000	0.0000	0.2687	0.4957	0.2609	0.0000	
Ho	0.1379	0.0000	0.2162	0.6286	0.0000	0.0000	0.0000	0.3143	0.7885	0.2609	0.0000	
HWE	NS	-	NS	NS	-	-	-	NS	***	NS	-	
Iun 10												
Allelic range (bp)	137-188	179-185	137-191	137-191	185	188	188	182-185	173-191	137-188	125-188	125-191
Na	5	3	6	3	1	1	1	2	6 (1)	5	5 (1†)	8
AR	3.6206	2.2174	4.0753	2.8050	1.0000	1.0000	1.0000	1.9986	4.1921	3.3064	1.8967	
He	0.6836	0.3181	0.7034	0.6195	0.0000	0.0000	0.0000	0.5035	0.7633	0.6467	0.1888	
Ho	0.6207	0.1000	0.6389	0.5143	0.0000	0.0000	0.0000	0.3824	0.3137	0.5870	0.1429	

HWE	NS	***	NS	NS	-	-	-	NS	***	NS	*	
Iun 14												
Allelic range (bp)	371-425	373-425	371-425	395	395-425	379	379	375-415	369-425	371-425	373-451	369-451
Na	6	5	7	1	3	1	1	4	7 (2)	5	2 (1)	13
AR	3.8329	3.5045	3.4953	1.0000	2.1686	1.0000	1.0000	3.0652	3.3168	3.4413	1.4096	
He	0.6370	0.6763	0.5642	0.0000	0.3506	0.0000	0.0000	0.6220	0.6306	0.6276	0.0942	
Ho	0.7586	0.4667	0.5500	0.0000	0.3030	0.0000	0.0000	0.6765	0.4118	0.5870	0.0980	
HWE	NS	*	NS	-	NS	-	-	NS	**	NS	NS	
Lspn 010-2												
Allelic range (bp)	208	208	208	208	208	208	208	204-208	204-208	208	208	204-208
Na	1	1	1	1	1	1	1	2	2	1	1	2
AR	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.9951	1.1839	1.0000	1.0000	
He	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.4807	0.0381	0.0000	0.0000	
Ho	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5429	0.0385	0.0000	0.0000	
HWE	-	-	-	-	-	-	-	NS	NS	-	-	
Lspn 019c												
Allelic range (bp)	136-144	136-146	136-144	142-146	142-144	136	136	136-146	142-144	142-144	142	136-146

Na	3	4	3	2	2	1	1	3	2	2	1	4
AR	1.8590	2.5249	2.0643	1.1429	1.9610	1.0000	1.0000	2.8309	1.5256	1.9752	1.0000	
He	0.1936	0.3915	0.2520	0.0286	0.3883	0.0000	0.0000	0.6166	0.1291	0.4193	0.0000	
Ho	0.1379	0.4000	0.2778	0.0286	0.5152	0.0000	0.0000	0.7143	0.1373	0.5870	0.0000	
HWE	NS	NS	NS	NS	NS	-	-	NS	NS	**	-	
Lspn 044												
Allelic range (bp)	212-216	212-216	212-216	212-216	212	214	214	196-214	212-216	212-216	214-216	196-216
Na	3	3	3	3	1	1	1	3 (1)	3	3	2	4
AR	2.9342	2.8917	2.8354	2.7933	1.0000	1.0000	1.0000	2.4190	2.7569	2.6567	1.9118	
He	0.6515	0.6169	0.6021	0.5946	0.0000	0.0000	0.0000	0.4542	0.5551	0.4852	0.3302	
Ho	0.6552	0.5333	0.5000	0.7143	0.0000	0.0000	0.0000	0.4286	0.6346	0.4783	0.4118	
HWE	NS	NS	NS	NS	-	-	-	NS	NS	NS	NS	
Lspn 094												
Allelic range (bp)	202-206	202-206	202-206	202-204	202	200-202	200-202	202-208	198-204	180-206	200-202	180-208
Na	3	3	3	2	1	2	2	4 (1)	3	5 (1)	2	7
AR	2.5715	1.4746	2.3603	1.9508	1.0000	1.7953	1.9045	3.5982	1.7968	2.8371	1.9970	
He	0.4428	0.0977	0.4091	0.3731	0.0000	0.2392	0.3173	0.7058	0.1944	0.4687	0.4925	

Ho	0.4138	0.1000	0.4054	0.3714	0.0000	0.2727	0.3226	0.6786	0.2115	0.5000	0.4510	
HWE	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS	
Pma*μ* 5												
Allelic range (bp)	243-249	243-251	245-249	245-249	245-247	247	247	241-249	243-249	245-249	245	241-251
Na	4	5 (1)	3	2	2	1	1	4 (1)	4	2	1	6
AR	2.4618	2.5726	2.7716	1.9989	1.8866	1.0000	1.0000	2.2191	3.1922	1.2066	1.0000	
He	0.4785	0.4452	0.6049	0.5056	0.3021	0.0000	0.0000	0.2682	0.6338	0.0430	0.0000	
Ho	0.4828	0.5667	0.5526	0.4857	0.3636	0.0000	0.0000	0.1765	0.3333	0.0435	0.0000	
HWE	NS	NS	NS	NS	NS	-	-	*	***	NS	-	
All loci												
MNA	3.5	3.4 (2)	3.8	2.1	1.5	1.1	1.1	2.7 (3)	3.5 (3)	3.1 (1)	1.9 (3)	
P	9	8	9	8	4	1	1	9	10	9	6	
AR	2.49	2.30	2.62	1.90	1.33	1.08	1.09	2.30	2.47	2.21	1.46	
He	0.4069	0.3497	0.4300	0.3388	0.1101	0.0239	0.0317	0.4417	0.4275	0.3381	0.1698	
Ho	0.4073	0.2846	0.3870	0.3486	0.1242	0.0273	0.0323	0.4428	0.3604	0.3423	0.1770	

Chapter 4

Lamprey species pairs: real species or morphs of a single species?

Mateus CS, Stange M, Berner D, Roesti M, Quintella BR, Alves MJ, Almeida PR, Salzburger W (2013) Strong genome-wide divergence between sympatric European river and brook lampreys. *Current Biology*, **23**, R649-R650.

Paper VI | Strong Genome-wide Divergence between Sympatric European River and Brook Lampreys

Catarina S. Mateus[1,2,3,4,6,*], Madlen Stange[1,6], Daniel Berner[1], Marius Roesti[1], Bernardo R. Quintella[2,5], M. Judite Alves[4], Pedro R. Almeida[2,3], & Walter Salzburger[1,*]

[1]Zoological Institute, University of Basel, Vesalgasse 1, CH-4051 Basel, Switzerland.

[2]Center of Oceanography, Faculty of Sciences of the University of Lisbon, Campo Grande, 1749-016 Lisbon, Portugal.

[3]Department of Biology, School of Sciences and Technology, University of Évora, Largo dos Colegiais 2, 7004-516, Évora, Portugal.

[4]National Museum of Natural History and Science & Center for Environmental Biology, University of Lisbon, Rua da Escola Politécnica 56/58, 1250-102 Lisbon, Portugal.

[5]Department of Animal Biology, Faculty of Sciences of the University of Lisbon, Campo Grande, 1749-016 Lisbon, Portugal.

[6]These authors contributed equally to this work

[*]E-mail: csmateus@fc.ul.pt (CSM), walter.salzburger@unibas.ch (WS)

Lampreys, together with hagfishes, are the only extant representatives of jawless vertebrates and thus of prime interest for the study of vertebrate evolution (Smith *et al*. 2013). Most lamprey genera occur in two forms with divergent life histories: a parasitic, anadromous and a non-parasitic, freshwater resident form (Hubbs 1925, 1940; Enequist 1937; Zanandrea 1959; Espanhol *et al*. 2007; Lasne *et al*. 2010; Docker *et al*. 2012). The taxonomic status of such 'paired species' is disputed, however. While indistinguishable at larval stages, but clearly distinct as adults, they cannot be differentiated with available genetic data (Espanhol *et al*. 2007; Docker *et al*. 2012), which has fuelled speculations that the two forms may in fact represent products of phenotypic plasticity within a single species. Here, we use restriction site-associated DNA sequencing (RADseq) to examine the genetic population structure of sympatric European river (*Lampetra fluviatilis* L., 1758) and brook (*Lampetra planeri* Bloch, 1784) lampreys. We find strong genetic differentiation and identify numerous fixed and diagnostic single nucleotide polymorphisms (SNPs) between the two species, 12 of which can be unequivocally assigned to specific genes.

Lampreys - often referred to as cyclostomes because of their circular mouth - commonly occur as species pairs with distinct post-larval life histories. The so-called brook lampreys spend their entire life in fresh water, whereas their parasitic counterparts, the river lampreys, spend most of their adult life in the ocean or in estuaries and return to fresh water only for reproduction (Hubbs 1925, 1940; Enequist 1937; Zanandrea 1959; Espanhol *et al*. 2007; Lasne *et al*. 2010; Docker *et al*. 2012). Whether these two forms are real species or are products of phenotypic plasticity in a single species has puzzled biologists for decades (Hubbs 1925, 1940; Enequist 1937). In the adult stage, river lampreys are much larger and morphologically distinct from brook lampreys, which is why they have been described as distinct species. On the other hand, the larvae of the two forms are indistinguishable, the adults co-occur on breeding grounds and often spawn in common nests (Lasne *et al*. 2010), and they produce viable offspring when crossed artificially (Enequist 1937), lending support to the plasticity hypothesis. Importantly, no genetic evidence is available to date that would suggest their separation (e.g., Espanhol *et al*. 2007; Docker *et al*. 2012). Sympatric European *L. fluviatilis* and *L. planeri* even share mitochondrial haplotypes,

which was suggested to reflect ongoing gene flow or, alternatively, incomplete sorting of ancestral polymorphisms (Espanhol *et al*. 2007).

To address this 'paired species' conundrum in lampreys, we examined one pair in detail by means of Illumina-sequenced RAD. We considered 17 specimens of *L. fluviatilis* (Figure 1A) and 18 specimens of *L. planeri* (Figure 1B) collected from a common spawning site in the Sorraia River, a tributary of the Tagus River in Portugal, the southern limit of their distribution (see Supplemental Information published with the online version of this article). Sequences from one individual were used to build a pseudo-reference genome spanning 39,865 RAD loci (3.79 Mb), against which all individuals were aligned. Screening the alignments recovered 8,826 polymorphic RAD loci, yielding a total of 14,691 informative SNPs.

Global F_{ST} based on all SNPs between the two sympatric lampreys was no less than 0.37, suggesting strong genome-wide genetic differentiation despite the shared mitochondrial DNA haplotypes reported earlier for the exact same system (Espanhol *et al*. 2007). Likewise, a genetic assignment test using Structure unambiguously separated the surveyed individuals into two distinct clusters (Figure 1C). The same result was obtained when the SNPs were analyzed in a phylogenetic context (Figure 1D). We thus provide the first genetic evidence for the taxonomic validity of the two European lamprey species *L. fluviatilis* and *L. planeri*. At the same time, we highlight the power of next generation sequencing technologies to resolve old questions in biology. Our data further agree with the assumption that resident lampreys are derived from migratory ones (Hubbs 1925, 1940). The genome scan revealed much greater genetic diversity in *L. fluviatilis* than in *L. planeri*. For instance, *L. fluviatilis* displayed a 42% higher density of private SNPs than *L. planeri* (7,399 *versus* 5,198; binomial $P < 0.001$; see also branch-lengths in Figure 1D). In addition, the greater genetic diversity in the migratory species might also reflect the larger effective population size and less restricted gene flow. By contrast, we expect resident species to be more prone to genetic bottlenecks and genetic drift due to their reduced mobility.

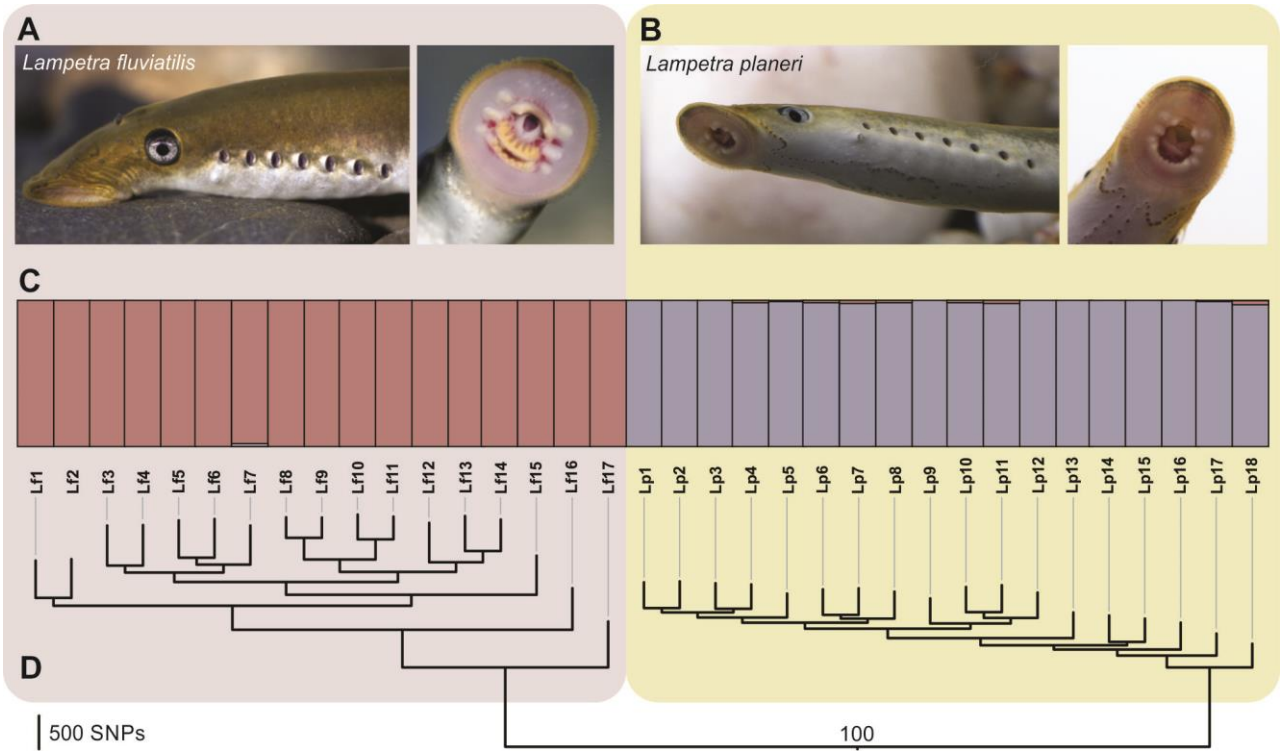

Figure 1 – Genetic divergence in a lamprey species pair.
The European river lamprey (*Lampetra fluviatilis*) (A) and the European brook lamprey (*Lampetra planeri*) (B) are morphologically distinct in the adult stage. (C) A Bayesian population assignment test with Structure and a subsequent evaluation with Structure Harvester revealed the existence of two clusters (K = 2) in our SNP dataset, corresponding to the two sympatric species *L. fluviatilis* and *L. planeri*. Each bar represents the assignment probability (0 to 1) of a single specimen to one of these two clusters (color coded in red and purple, respectively). (D) Phylogeny of the 35 lamprey specimens from the Sorraia River in Portugal based on 14,691 SNPs and maximum parsimony in PAUP* (heuristic search with stepwise addition, TBR branch swapping and allowing polymorphisms). The specimens are grouped into two clades, which exactly match the two species *L. fluviatilis* and *L. planeri* (the bootstrap value for the basal branch is provided).

To gain insight into genes potentially underlying the divergence between the sympatric lampreys, we screened the marker data for loci fixed for different alleles between the two species (F_{ST} = 1), identifying 166 such distinctive SNPs. Making use of the recently published genome of the sea lamprey (Smith *et al*. 2013), a distant relative of the species under investigation, we subjected these loci to reciprocal BLAST searches. This allowed us to link 12 of these loci to annotated genes. Interestingly, most of the genes showing fixed allelic differences between the two lampreys are related to functions that have previously been implicated in the adaptation to a migratory *versus* resident life-style in lampreys and bony fishes. For instance, fixed

differences were found in the vasotocin gene, a major player in saltwater-freshwater osmoregulation and also involved in life history divergence (Balment *et al*. 2006), and in the gonadotropin-releasing hormone (GnRH), a key gene in gonadal development and differentiation (Sower & Kawauchi 2001). We also found fixed genetic differences in four genes related to immune functions, three axial patterning genes, a pineal-gland-specific opsin, a sodium channel gene, and a tyrosine phosphatase gene. These genes are likely to contribute to ecologically based reproductive isolation in this lamprey system, paving the way for subsequent functional and evolutionary analyses. A more detailed discussion of the species-distinctive loci and their possible ecological role is provided in the Supplemental Information, along with a screen for large-scale genomic divergence between males and females in *L. planeri*.

In summary, we show that the sympatric lampreys *L. fluviatilis* and *L. planeri* are genetically highly distinct, and that the regions of strongest divergence contain several candidate genes for adaptation to a migratory *versus* resident life-style.

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Supplemental Information

Figure S1 - Analysis of genomic divergence between males and females based on sex-specific read coverage across RAD loci in the lamprey *L. planeri* (A) and in threespine stickleback (*Gasterosteus aculeatus*) (B).
The existence of a relatively large genomic region highly differentiated between males and females will cause RAD loci within these regions to show sex-biased read coverage [details given in S1]. In a male-heterogametic system, for instance, read coverage for X-linked loci will be twofold higher in females than males as compared to autosomal loci for which read coverage between the sexes should be equal. The reason is that Y-linked sequences align poorly to their X-counterpart. Exactly this situation is found in stickleback: while most data points lie within the region predicted for autosomal loci (shown as yellow line in the plot), an additional cluster is visible along the line predicted for X-linked loci (green line; the expectation for W-linked loci in a female-heterogametic system is shown as blue line). By contrast, no deviation from the autosomal expectation is evident in *L. planeri*, indicating the absence of physically extensive genomic differentiation between males and females. Hence, if sex determination in this lamprey species is genetically based, the underlying system evolved without major chromosome divergence. Alternatively, sex determination might be under strong environmental influence, as generally assumed to occur in lampreys [S2–S4].

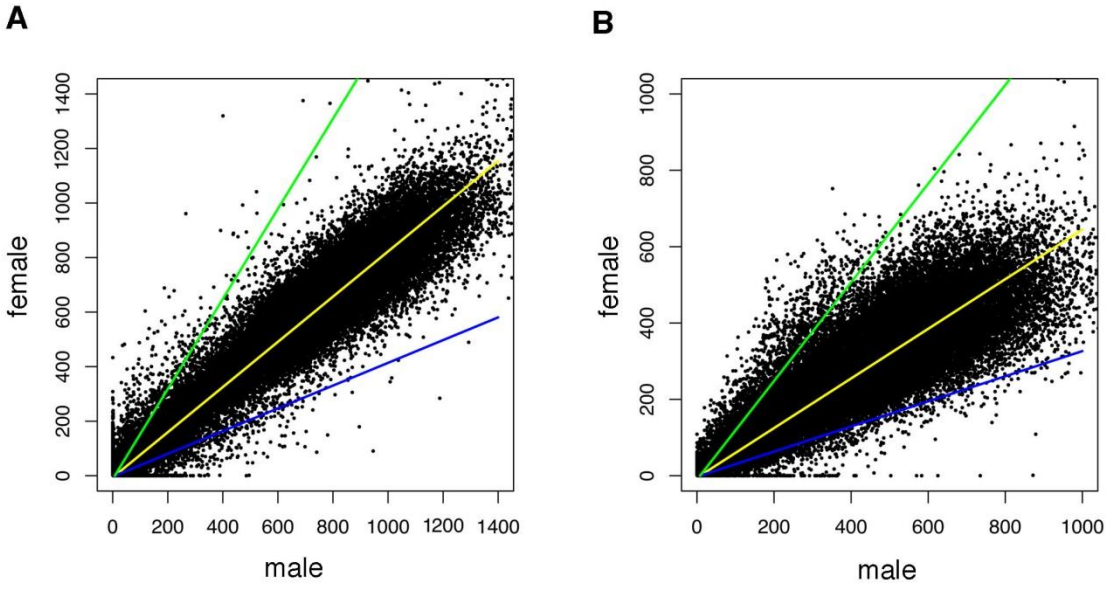

Table S1 - Genes and gene families attained after BLAST of the SNPs with F_{ST}=1.

Gene/Gene family	Function	References
Neurohypophysial gene (vasotocin)	Osmoregulation	[S5-S8]
Gonadotrophin-releasing hormone 2 precursor (GnRH2)	Gonadal maturation and migratory behavior	[S8-S11]
Pineal gland-specific opsin gene (P opsin)	Photoreception	[S12-S17]
Mannose-binding lectin-associated serine protease-1 (MASP-1) gene	Immunity	[S18, S19]
Ikaros-like genes (IKLF2)	Immunity	[S20, S21]
Variable lymphocyte receptor (VLR) gene	Immunity	[S22-S25]
CD45 gene (PTPRC, Protein tyrosine phosphatase, receptor type C)	Immunity	[S26, S27]
Homeobox genes (HoxW10a, Hox7, Emx)	Axial patterning and segmental identity	[S28-S31]
Voltage-gated sodium channel gene	Conduction of electrical signaling in nerves and muscles	[S32, S33]
Protein tyrosine phosphatase receptor type A precursor (PTPRA) gene	Regulation of cellular processes	[S5, S34]

Sampling

We collected, by electric fishing, 17 juvenile specimens of the anadromous *L. fluviatilis* at the start of their downstream trophic migration in January of two consecutive years (2009 and 2010), and 18 adult specimens of the resident *L. planeri* during the breeding season between late November 2009 and January 2010. All samples were collected in the Sorraia River, a tributary of the left bank of the Tagus River basin, where both species occur in sympatry. On the Iberian Peninsula, Tagus is the only river where the anadromous *L. fluviatilis* is known to occur, and it represents the southern range limit of both species [S35]. Tissue samples were preserved in 100% ethanol and deposited in the zoological collection 'Museu Bocage' of the Museu Nacional de História Natural e da Ciência (MUHNAC) (Lisbon, Portugal). Sampling was performed under the permission of the Instituto da Conservação da Natureza e das Florestas.

Restriction-site associated DNA (RAD) library preparation

RAD library preparation followed the protocol of Baird *et al.* [S36] and further modifications [S37, S38]. Briefly, DNA was extracted with the "DNeasy Blood & Tissue Kit" (Qiagen) following the manufacturer's protocol. Genomic DNA from each individual was digested with the *Sbf*1 restriction enzyme. Each digest was then 5-mer barcoded for sample identification, and the 35 total samples were multiplexed into a single library. Final PCR enrichment was performed in 8 separate reactions to reduce amplification bias. Finally, the library was single-end sequenced with 100 cycles in a single Illumina HiSeq 2000 genome analyzer lane at D-BSSE Basel. Illumina reads are available from the Sequence Read Archive (SRA) at NCBI under the accession number PRJNA206554.

Marker generation

The reads were first quality-filtered and demultiplexed according to the individual barcodes. Using sequence data from the one individual with the highest read number, the reads were clustered by tolerating a maximum of two mismatches. For each cluster (representing a RAD locus), the consensus sequence was derived, and the unique consensus sequences were concatenated to form a 3.79 MB pseudo-reference genome. These steps were carried out using Stacks v0.9996 [S39]. Next, data from each of the 35 individuals were aligned against the pseudo-reference genome using Novoalign v2.08.03 (http://www.novocraft.com), tolerating approximately six high-quality mismatches (-t Flag 180). We enforced unique alignment, thereby avoiding that distinct loci in the pseudo-genome actually derived from the same locus in the true genome because of substantial polymorphism. The alignments were then converted to *bam* format using Samtools v0.1.18 [S40]. Next, each RAD locus was genotyped at the whole-haplotype level. We here called a homozygous genotype when the dominant haplotype occurred in at least 18 copies and the second most frequent haplotype occurred less than six times. A heterozygote was called when the two most frequent haplotypes occurred in at least 18 copies each. A locus not matching these criteria received a haploid genotype based on the dominant haplotype if that haplotype

occurred in at least six copies, or were scored as missing data otherwise. As genotyping used fixed coverage thresholds, loci with excessive read coverage were down-sampled at random to 70x before genotyping (average read coverage per individual and RAD locus was 114.2, sd = 59.8). Finally we combined the consensus sequences of all individuals to screen each RAD locus for SNPs. To exclude polymorphisms with low information content and technical artifacts [S41], SNPs displaying a minor allele frequency of 0.06 or lower were excluded from the data set. The resulting SNP panel for analysis included 34,267 SNPs. Genotyping and SNP calling was carried out using the R language [S42], benefiting from the bioconductor packages Biostrings and Rsamtools.

Population genetic and phylogenetic analyses

Prior to the analyses of genetic differentiation we eliminated SNPs with insufficient representation across individuals (threshold: 15 nucleotides from each population). The SNPs were used to calculate the haplotype-based fixation index (F_{ST}) [see S38] between the two samples. We then used Structure 2.3.4 [S43] to determine the number of genetic clusters (*K*) in our dataset and to estimate, for each individual, the assignment probability to these clusters. First, structure was run for 100,000 generations, with a burnin of 10,000 generation, and applying the admixture model for $K = 1$ to $K = 5$ and three independent replicates for each *K*. Using Structure Harvester [S44], we found that the most likely number of *K* was 2. We then repeated the Structure analysis for $K = 2$, running it for 500,000 generations (Figure 1C) and applying a burnin of 50,000. PAUP* [S45] was used to perform a phylogenetic analysis with the SNP dataset under maximum parsimony applying a heuristic search (stepwise addition and TBR branch swapping and allowing polymorphisms). Confidence assessment was performed with a bootstrap analysis and 1000 replicates. The resulting tree (Figure 1D) had a length of 22,632 steps. We also performed a neighbor-joining tree search (not shown), which produced a highly similar topology.

Screening fixed polymorphisms for candidate genes

For the 166 SNPs fixed for different alleles (F_{ST} = 1) between the samples, a homology search was first completed by performing a BLAST [S46] search on the NCBI public database. BLAST hits were then further mapped to annotated genes in the Ensembl database [S47] making use of the recently released genome of the sea lamprey (*Petromyzon marinus*) [S48]. The hits were then confirmed by a reciprocal BLAST search, i.e. blasting the respective sea lamprey contig against all RAD tags. In total, we could link twelve RAD loci to annotated genes (Table S1). We found fixed differences in vasotocin, which is involved in many aspects of fish physiology and behavior, including circadian and seasonal biology, metabolism, reproduction and osmoregulation [S5-S8]; in the gonadotropin-releasing hormone 2 (GnRH2), a key gene in gonadal development and differentiation, and regulation of the reproductive and migratory behavior, by controlling secretion of pituitary hormones [S8-S11]; in the non-visual pineal gland-specific opsin gene (P opsin), which is key in photoreception in lamprey larvae, controlling the changes in body coloration and metamorphosis, and in adults through control of sexual maturation [S12-S17]. We found four genes implicated with immune functions: a mannose-binding lectin-associated serine protease (MASP), the ikaros factor-like 2 gene (IKFL2), variable lymphocyte receptor (VLR), and the protein tyrosine phosphatase receptor type C (PTPRC or CD45) [see S18-S27]. We also found hits with three homeobox genes (HoxW10a, Hox7, Emx), which are known to play important roles in the specification and patterning of different regions along the body axes [S28-S31]. The Emx gene, in particular, is known to play a major role in forebrain development. Hits were also found with the voltage-gated sodium channel gene, known to play an essential role in physiology through the initiation and propagation of action potentials in neurons and other electrically excitable cells such as myocytes and endocrine cells [S32, S33], and finally, in the protein tyrosine phosphatase receptor type A precursor (PTPRA). The protein encoded by PTPRA is a member of the protein tyrosine phosphatase (PTPase) family. PTPases are known to be involved in the regulation of a variety of cellular processes including cell activation, growth and differentiation, mitotic cycle, and oncogenic transformation [S5, S34].

Genomic screen for large sex-specific regions

We here used a subsample of five females and seven males from the resident species *L. planeri*. This included all lamprey individuals for which sex was known (note that *L. fluviatilis* were sampled as migrating juveniles, precluding the phenotypic identification of sex). The full alignments of these 12 individuals were used to screen visually for the presence of a major sex-linked genomic region. For this, the total number of reads was counted separately across all males and all females at each of the 38,308 total RAD loci. For each locus, the total female count was then plotted against the total male count. The rationale was that RAD loci in sex-specific regions should exhibit systematic read coverage bias between males and females relative to loci in autosomal regions, because of differential alignment success to the reference sequence [for details see S1]. This approach should thus allow detecting at least large-scale differentiation between males and females visually. For comparison, we performed an analogous investigation with exactly the same sample size using RAD data from threespine stickleback [S1], a species with a major XY chromosomal system [S48].

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Chapter 5

General Discussion and Conclusions

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The knowledge about the phylogeny, phylogeography and systematics of lampreys has evolved much in the past years with the advent of relatively inexpensive and rapid DNA sequencing. Molecular tools, coupled with information from other types of data such as morphology, physiology and ecology, are essential to better understand the origin, evolution, and relationships among taxa.

Cryptic speciation and regions of endemism

Speciation is not always accompanied by morphological change; some species are characterized by genetic diagnostic synapomorphies but share conservative body forms. The recognition of such cryptic species has increased exponentially over the past two decades mainly due to the increasing availability of DNA sequences (Bickford *et al.* 2007), and is fundamental for the effective protection of evolutionary valid taxa in conservation planning programs (Cook *et al.* 2008).

The oldest fossil lamprey known to date, *Priscomyzon riniensis* from the Devonian period of South Africa, looks strikingly similar to modern lampreys, and this finding depicts lampreys as a group of "ancient specialists that have persisted as such and survived a subsequent 360 million years" (Gess *et al.* 2006). This highly conserved morphology is probably the reason for the relatively reduced number of recognized lamprey species, when taking into account their ancestry. Few are the morphological characters used to distinguish species and genera of lampreys, and most are limited to the adult phase of the life cycle. For this reason, the use of molecular data to address taxonomic issues is of extreme importance, and several recent studies employed this tool in lamprey systematics (e.g., Yamazaki *et al.* 2006; Reid *et al.* 2011; Mateus *et al.* 2013a). The identification of three cryptic species of the genus *Lampetra* endemic to Portugal in the course of this study (Mateus *et al.* 2013a; paper IV) reinforces the importance of the use of genetic markers to unveil lamprey biodiversity. It followed the evolutionary species concept of Wiley (1978), according to which "a species is a lineage of ancestral descendant populations which maintains its identity from other

such lineages and which has its own evolutionary tendencies and historical fate". These species were described following a combination of molecular and morphological data; they constitute well supported monophyletic clades with a number of diagnostic synapomorphies in two mitochondrial genes, and each has likely evolved in allopatry. These species have dramatically small distributional ranges, especially *Lampetra auremensis*, inhabiting river Nabão sub-basin, a tributary of Tagus basin (Mateus *et al.* 2013a; paper IV). Cryptic speciation is commonly found in species with restricted distributions, like freshwater macroinvertebrates and fish (Cook *et al.* 2008). In lampreys, there are a number of studies suggesting the existence of cryptic resident species; for instance, Boguski *et al.* (2012) suggested the existence of four undescribed *Lampetra* brook species on the west coast of North America, that are morphologically cryptic but show high genetic divergence; a similar situation is observed for the northern and southern groups of the brook *Lethenteron reissneri* (*L.* sp. N and *L.* sp. S., respectively), from Japan (Yamazaki & Goto 1996, 1997; Yamazaki *et al.* 2003).

The geographical distribution of genetic diversity in many species of animals often reveals that their current distribution was strongly influenced by the Pleistocene ice ages and subsequent postglacial colonization (Hewitt 1999). These palaeoclimatic events caused a reduction in genetic diversity from southern to northern Europe at three levels: the number of species, the extent of subspecific division and the allelic variation, explained by the dramatic loss of variation that occurred during postglacial range expansion from southern glacial refugia (Hewitt 1999). Hence, southern regions, such as the Iberian Peninsula, support high biological diversity and are rich in endemisms (Gómez & Lunt 2006). Regarding lampreys, a first study by Espanhol *et al.* (2007) identified unique mtDNA lineages in the Iberian Peninsula, suggesting refugial persistence and subsequent accumulation of variation over several ice ages. In the present study, we looked deeper into the Iberian populations, further identifying several evolutionary lineages recognized first as distinct ESUs, Evolutionary Significant Units *sensu* Moritz (1994) (Mateus *et al*. 2011a; paper III), and later as a complex of cryptic species (Mateus *et al.* 2013a; paper IV). *Lampetra lusitanica*, endemic to Sado basin, was the first to diverge, dating back to the late Miocene with the uplifting of the Arrábida Chain and the subsequent split of Tagus and Sado basins; the divergence of

Lampetra auremensis, endemic to Nabão sub-basin, is also related with geomorphological events starting in the late Miocene, namely different tectonic movements (subsidence and uplift) of the right and left banks of Tagus basin, that produced distinct isolated systems with particular characteristics; the differentiation of *Lampetra alavariensis*, endemic to Vouga and Esmoriz basins, however, could not still be assigned to any geomorphological event (see Mateus *et al.* 2011a).

The uniqueness of the Iberian Peninsula was further confirmed (paper V) using nuclear markers (ten microsatellite loci), where we found that each new species constitutes a distinct genetic group, showing no evidence of recent gene flow among them or with *L. planeri*. Even in the species with broader distribution, *L. fluviatilis* and *L. planeri*, populations from the south are differentiated from northern populations. We further observed that the more recently founded populations from northern Europe are less divergent among them, and that they are represented by fewer genetic clusters, as result of their more recent common ancestor (paper V). A reduced genetic diversity from south to north was also found by Boguski *et al.* (2012) in *Lampetra* species from western North America. Most populations from south of the Columbia River were genetically divergent, and the authors suggest that extended periods of isolation may have influenced gene connectivity among the most genetically divergent *Lampetra* lineages. When testing for recent migration between populations of the migratory *L. fluviatilis*, we found that there is no strong signal of migration between the population from Portugal and populations from northern Europe, but a strong signal of recent gene flow was detected between northern migratory populations (paper V). Also, we found that several individuals from these northern localities have high proportions of membership from the population from Portugal, probably due to ancestral polymorphism, as a result of the colonization process that took place during the interglacials from a southern *L. fluviatilis*-type ancestor. These results are in agreement with results of Espanhol *et al.* (2007) and Pereira & Almada (2013), which revealed a star-like haplotype network for the genes ATP6 and 8, with all specimens from the Tagus population displaying the ancestral haplotype, which is consistent with a scenario of dispersal and expansion.

The lamprey paired species enigma

The question about the taxonomic validity of members of paired species arose well before the advent of molecular tools (Enequist 1937; Zanandrea 1959). This type of data, coupled with previous knowledge, would be expected to give new insights on the issue. The problem is that the use of single genetic markers, often of low resolution, did not allow excluding alternative scenarios, and several are the factors that can contribute to these ambiguities, such as convergent evolution, incomplete lineage sorting, ancestral polymorphism and hybridization. There are several studies using molecular markers to unravel the lamprey paired species question, but until recently the used markers did not appear to provide sufficient resolution for closely related lamprey taxa, such as species pairs, to conclude whether the lack of reciprocal monophyly is result of shared ancestral polymorphisms or, alternatively, of contemporary gene flow (e.g., Schreiber & Engelhorn 1998; Espanhol *et al.* 2007; Blank *et al.* 2008). This uncertainty has significant conservation implications, as following metamorphosis resident and migratory lampreys have different habitat requirements and vulnerabilities. Also, assuming that migratory lampreys mediate gene flow among otherwise isolated brook lamprey populations, loss of the parasitic species would lead to a loss of genetic diversity in small, isolated brook lamprey populations and to greater levels of local extinction (Docker 2009).

In our study using mtDNA (Mateus *et al.* 2011a; paper III) the definition of conservation units was based on the monophyletic evolutionary lineages attained, what would include *L. planeri* and *L. fluviatilis* in the same unit, but because the conservation measures to be applied in the different adult phase are distinct, we suggested that populations of the migratory *L. fluviatilis* should constitute a separate ESU, that should include populations from across Europe. This option was supported by our posterior studies using both microsatellite markers (paper V) and genomics (Mateus *et al.* 2013b; chapter VI). Those studies revealed significant and diagnostic differences between the migratory and the resident forms, corroborating their classification as distinct taxonomic units, and have significant impact in lamprey research and conservation. We identified a total of 166 single nucleotide polymorphisms (SNPs) fixed for different alleles (F_{ST} = 1) between the two species, 12

of which could be assigned to annotated genes. These genes encode the vasotocin, which is involved in many aspects of fish physiology and behavior, including circadian and seasonal biology, metabolism, reproduction and osmoregulation; the gonadotropin-releasing hormone 2 (GnRH2), a key gene in gonadal development and differentiation, and regulation of the reproductive and migratory behavior, by controlling secretion of pituitary hormones; the non-visual pineal gland-specific opsin gene (P opsin), which is key in photoreception in lamprey larvae, controlling the changes in body coloration and metamorphosis, and in adults through control of sexual maturation. We found four genes implicated with immune functions: a mannose-binding lectin-associated serine protease (MASP), the ikaros factor-like 2 gene (IKFL2), variable lymphocyte receptor (VLR), and the protein tyrosine phosphatase receptor type C (PTPRC or CD45). We also found hits with three homeobox genes (HoxW10a, Hox7, Emx), which are known to play important roles in the specification and patterning of different regions along the body axes. Hits were also found with the voltage-gated sodium channel gene, known to play an essential role in physiology through the initiation and propagation of action potentials in neurons and other electrically excitable cells such as myocytes and endocrine cells, and finally, in the protein tyrosine phosphatase receptor type A precursor (PTPRA). The protein encoded by PTPRA is a member of the protein tyrosine phosphatase (PTPase) family. PTPases are known to be involved in the regulation of a variety of cellular processes including cell activation, growth and differentiation, mitotic cycle, and oncogenic transformation (see Mateus *et al.* 2013b; paper VI and references herein).

These findings are essential to understand the mode of speciation in paired and satellite lamprey species, as well as the ecological factors that may have determined the emergence of non-parasitic derivates. The repeated origin of brook lampreys from river lampreys seems to have occurred independently in different lamprey genera and in different locations (Hubbs 1925, 1940), with paired or satellite species occurring in most lamprey genera worldwide (Salewski 2003). As the ecological characters are the same in all species pairs, it must be assumed that the environmental conditions which led to assortative mating must have been similar in every population in which non-parasitic lampreys evolved (Salewski 2003). These events of parallel evolution, in which

the same trait evolves in parallel, and independently in separate populations experiencing similar environments, provides evidence that ecological selective forces were likely responsible for promoting speciation, i.e., ecological speciation (Schluter & Nagel 1995; Schluter 2001). Hubbs (1940) postulated that the evolution of freshwater resident lampreys from migratory ones is correlated with life in small streams, where a suitable food supply in the way of large fish is scarce or seasonal. Hardisty (1986) discussed that the evolution of both forms might be the result of a trade-off between high fecundity and high predation risk in the feeding phase of the parasitic form against lower fecundity and lower predation risk due to the shorter adult life of the non-parasitic form. This author also suggested that changes in the environment, in particular the formation of new barriers to migration or the reduced availability of host fishes might promote a complete abandonment of adult feeding. There is a strong disruptive selection on habitat choice and use by paired/satellite species in lampreys because sympatric lampreys have the potential to choose between either a whole life in streams or one in a marine environment through anadromous migration for the last years of their life. Consequently, the anadromous forms grow bigger, which affects mate choice and leads to disruptive sexual selection against hybrids with intermediate body size (Salewski 2003). Adult body sizes differ considerably between members of paired species, and this has been considered an important isolating mechanism in paired lamprey species (Hardisty & Potter 1971). The efficiency of the spawning act is dependent on a precise positioning of the genital regions of the two sexes, and thus effective fertilization is only likely when lampreys are of similar body lengths (Hardisty & Potter 1971). In summary, brook lampreys must have evolved by ecological speciation in small streams and upland tributaries with scarce trophic resources or adverse conditions to migrate, and avoiding the predation risk associated with a marine phase; the shortening in the duration of the adult phase, complete abandonment of adult feeding and subsequent reduction in adult body size kept them reproductively isolated from the migratory form due to size-based assortative mating, even when occurring in sympatry. Some of the candidate genes identified during this study (Mateus *et al.* 2013b; paper VI) seem to be related with the traits hypothesized to be under selection. For instance, the vasotocin is involved in osmoregulation, and thus in the ability to have an anadromous lifestyle, the gonadotropin-releasing

hormone 2 (GnRH2), in involved in gonadal development and differentiation, and in the regulation of the reproductive and migratory behavior, which differ both in timing and intensity between species, and the four genes implicated with immune functions may be related with an anadromous adult phase when exposure to contaminants and parasites is higher.

Our results contrast with findings in similar paired systems, like several species of salmonid fishes with alternative migratory tactics, where genetic divergence generally does not occur between sympatric anadromous and freshwater resident morphs (reviewed in Dodson *et al.* 2013). It is apparent that alternative migratory tactics in salmonids originate from common gene pools, i.e., they co-exist within populations, and all individuals may potentially adopt any of the alternative phenotypes. Body size is the most commonly reported liability trait controlling the decision to migrate in salmonids. Genetic divergence between phenotypes, however, has been reported in few cases, generally associated with spatial and temporal segregation of spawning activities (reviewed in Dodson *et al.* 2013).

Further analysis, ideally using the same approaches, should be performed in other lamprey pairs. Differences in the times when each nonparasitic species evolved and in the degree of reproductive isolation, population size, and strength of selection pressures may result in differences in the degree of morphological and genetic differentiation between pairs (Docker 2009). For instance, recently Docker *et al.* (2012) suggested that there is gene flow between the paired silver (*Ichthyomyzon unicuspis*) and northern brook (*Ichthyomyzon fossor*) lampreys. The authors analysed mtDNA and microsatellite markers, but the low number of genotyped nuclear loci (only three) may not have been sufficient to detect differentiation. In contrast, Yamazaki & Goto (1998) found one diagnostic allozyme allele between the sympatric paired *Lethenteron japonicum* and *Lethenteron kessleri*, suggesting that they are reproductively isolated. It appears that different species pairs, and even the same pair in different locations, are at different stages of speciation. In fact, in our study using microsatellites (paper V) we found significant genetic differentiation between sympatric brook and river lamprey from Portugal, but low differentiation in the same pair in populations from locations in

northern Europe, probably due to their more recent common ancestor following expansion.

Threats and conservation

Most lamprey species worldwide are of conservation concern, with freshwater-resident species with restricted ranges being the most threatened (Renaud 1997). The main threats faced by the ammocoetes are in general those related with the direct impact in the riverbed (such as dredging) and in the water quality, whereas for the juveniles and adults it varies depending on the life cycle. For anadromous species, the severe reduction in the available habitat caused by the construction of insurmountable obstacles is one of the most drastic and widespread threats, as it blocks the access to suitable spawning grounds during the reproductive migration. The habitat loss in Iberian rivers was calculated by Mateus *et al.* (2012), who concluded that at least 80% of the river stretches previously used by anadromous lampreys inhabiting that region (i.e. *Petromyzon marinus* and *Lampetra fluviatilis*) were lost. Other threats like pollution and habitat destruction affect both migratory and freshwater resident species to a similar extent. In Portugal and Spain the sea lamprey faces an additional threat; it is extensively explored during the reproductive season, having high commercial value due to its gastronomic importance. The overfishing of these upstream migrants compromises the long term survival of already endangered populations.

It is urgent to protect threatened species, especially endemic freshwater resident species with extremely reduced distributions, such as *Lampetra alavariensis*, *Lampetra auremensis* and *Lampetra lusitanica*, that were proposed to be classified as *Critically Endangered* (Mateus *et al.* 2013a; paper IV). The reduced distribution of these species, together with several threats mostly caused by anthropogenic activities, place these species as extremely threatened. Also, the migratory *L. fluviatilis* from Portugal (Tagus basin) requires further studies and management efforts; unlike its global conservation status (*Least Concern*), it is classified as *Critically Endangered* in Portugal and is already extinct in Spain. The Tagus population represents the species'

southern limit of distribution, and is especially vulnerable to potential effects of climate change. Furthermore, it seems that individuals from this basin are not migrating to central or northern Europe, or vice-versa, constituting a somewhat isolated population (paper V). Also, its distribution in Tagus river basin is limited to lower stretches by the impassable dams (Mateus *et al.* 2012).

Management and conservation measures to protect *Lampetra* species in the Iberian Peninsula are required, and the first efforts to be made should focus on the preservation and rehabilitation of habitats through, for instance, the reestablishment of the longitudinal continuity, cleaning of the most polluted stretches, and reestablishment of riparian vegetation, having into account the specific characteristics of each basin. Specific river stretches to be intervened should be selected based on the study of Ferreira *et al.* (2013), who draw a map with the probability of occurrence of *Lampetra* sp. in Portugal and classified the respective stretches with different conservation priorities.

Future Research

This dissertation allowed us to better understand the phylogeography, morphological variation, patterns of colonization and gene flow of the genus *Lampetra* in Europe, and it also raised a number of questions for future research.

The realization that there are numerous fixed and diagnostic single nucleotide polymorphisms (SNPs) between the paired *L. fluviatilis* and *L. planeri* from Portugal (Mateus *et al.* 2013b; paper VI), together with the data we attained with microsatellites (paper V), suggesting that the northern *L. fluviatilis* and *L. planeri* may be experiencing gene flow, indicates that further studies in other populations of the same pair, as well as other lamprey paired species, are needed. The analysis of populations at different stages of differentiation should extend our understanding on the speciation process.

There is an increasing number of studies about the genetic basis of ecological speciation, and the genes that underlie differences in phenotypic traits. We identified that the regions of strongest divergence between the two species contain several

candidate genes for adaptation to a migratory *versus* resident life-style in lamprey and fish species (Mateus *et al.* 2013b; paper VI). The further quantification of the gene expression for the identified genes in both species along their life cycle will allow a better understanding about the genes involved in the sympatric speciation, through ecological pressures, of these paired species.

Following our results on the postglacial colonization patterns of *Lampetra* in the Iberian Peninsula, and the realization that *L. fluviatilis* from Portugal might be isolated from northern populations, the ecology and migratory habits of this species in Portugal should be further investigated; whether individuals of this population migrate to the sea, or instead remain in the estuary and adjacent coastal areas, will give further insights about its isolation from northern populations, or the possibility that gene flow is happening in one direction only, with individuals from central and northern Europe entering Tagus river basin.

After the work by Ferreira *et al.* (2013), where a number of river stretches were identified as of conservation priority, for all the six species inhabiting the Iberian Peninsula, and especially for the recently described ones, further data on the microhabitat preferences will support the conservation measures to be applied to each basin. It is also important to investigate the tributaries used by the rare *L. fluviatilis*, to be able to plan the priority reestablishment of the longitudinal continuity in that particular river stretches. Due to its rareness, studies with juveniles and adults of the Iberian population are difficult to conduct (Mateus *et al.* 2012; paper II), but the identification of several diagnostic SNPs will allow, for the first time, to distinguish larvae of *L. planeri* and *L. fluviatilis*. With this new tool we will be able to study the distribution of larvae of both species, their proportions when in sympatry, and specific habitat preferences. This represents an important step forward in lamprey paired species ecology, as studies conducted to date on larvae could only be applied to the genus, despite marked differences in post metamorphic ecology. The incapacity to distinguish larvae has precluded a more detailed knowledge about the potential segregation of larvae when both species occur in sympatry.

Following the realization that *L. planeri*, and not only *P. marinus*, occurs in the northern Spanish region Asturias (Mateus *et al.* 2011b; paper I; Perea *et al.* 2011), an extensive sampling campaign in that region and neighbour regions like Galicia and

Cantabria should be performed, in order to detect the possible presence of additional *L. planeri* populations, and maybe *L. fluviatilis*, in that regions. Finally, it is crucial to include the three new cryptic species in the national Red List, after an accurate prospection of their distribution and main threats.

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Contactos:
Universidade de Évora
Instituto de Investigação e Formação Avançada - IIFA
Palácio do Vimioso | Largo Marquês de Marialva, Apart. 94
7002-554 Évora | Portugal
Tel: (+351) 266 706 581
Fax: (+351) 266 744 677
email: iifa@uevora.pt