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

Catarina S. Mateus[1, 2, 3, 5], 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*

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

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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.

Contents

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, 1986*a*).

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 and 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 and

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 and Austin, 2009; Page and 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

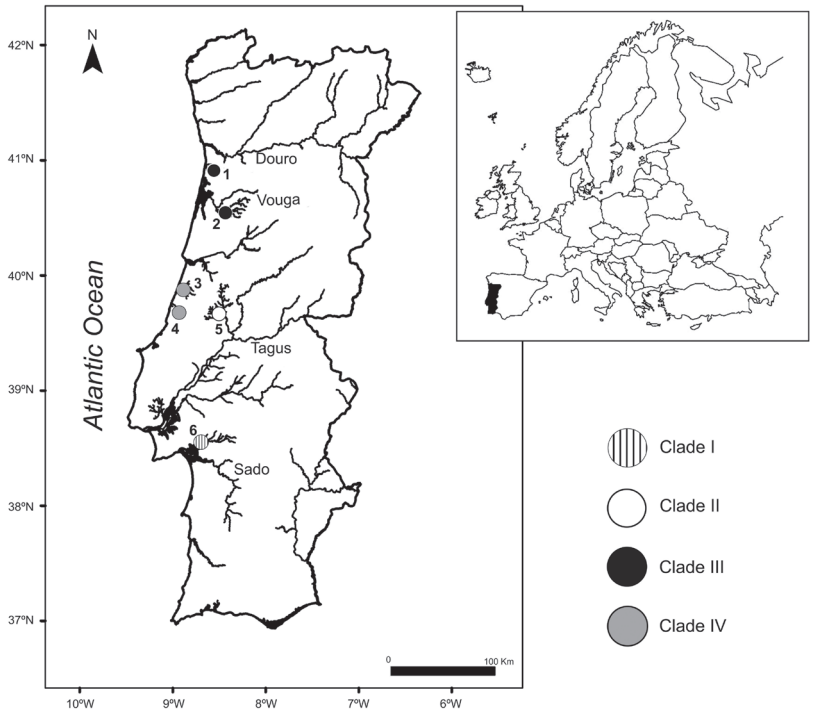

Fig. 1. Collection sites of brook lampreys in Portugal (*circles*). Circles are filled according to the clades recognized in Mateus *et al*. (2011b). Site locations: 1, river Esmoriz; 2, river Vouga; 3, river Lis; 4, Ribeiras do Oeste; 5, river Nabão; 6, river Sado.

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 and 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 and 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 and Kott, 1979; Vladykov *et al*., 1982; Holčík and Šorić, 2004; Renaud and 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 and Goto, 1996and, 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). 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 and 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 (40°55'27.30" N; 8°37'19.20" 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.,

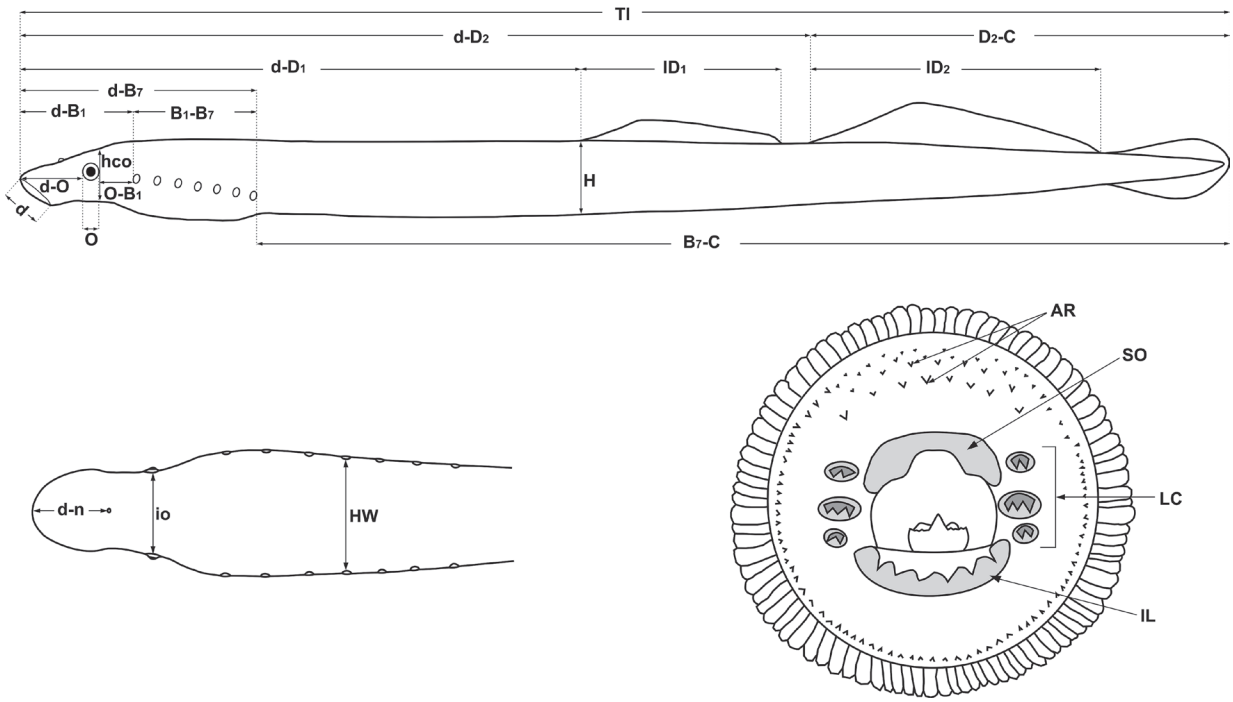

Fig. 2. Scheme of the morphometric measurements and meristic counts used to examine morphological variation of adult brook lampreys. Variables: Tl, total length; d, disc length; d-O, preocular length; O, eye diameter; O-B_1, postocular length; d-n, prenostril length; hco, head depth; io, interocular 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.

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

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

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 (1986*b*). The morphometric character H

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. *significant at the 0.1% level.

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

(body depth) was measured below the base of first dorsal fin, and not in the position presented in Holčík (1986*b*), to avoid measurement errors. We also added a character not present in Holčík (1986*b*), 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 and Follett (1967). All counts and measurements were made on the left side of the body following the procedure summarized by Holčík (1986*b*).

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 and MacCrimmon (1987):

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

where ACij is the adjusted character measurement i of the j specimen; OCij is the unadjusted character measurement i of the j specimen; β 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 (β) (Claytor and MacCrimmon, 1987).

Kruskal–Wallis was used to compare the number of trunk myomeres between groups. No significant

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

Table 3. Summary of discriminant loadings and potency index for adjusted morphometric characters. * Largest absolute correlation between each variable and any discriminant function.

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

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). 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 (χ^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).

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.

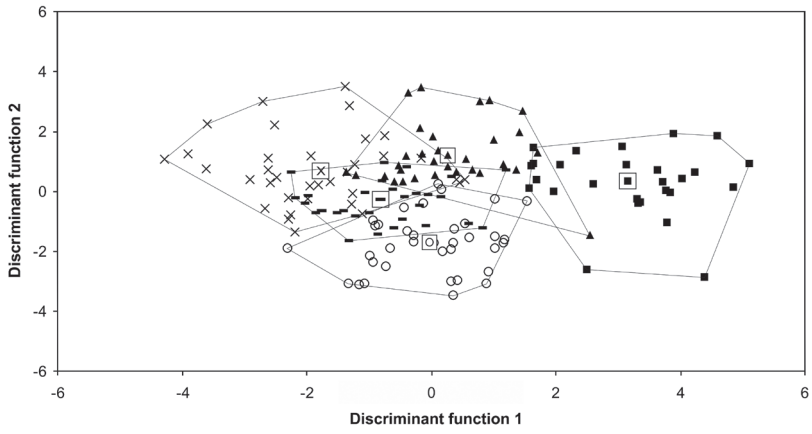

Fig. 3. Scatter plot of the discriminant Z scores, group centroids (*squares*) and outline polygons for the five examined groups of brook lampreys based on the morphometric characters, according to the first two discriminant functions. Symbols for groups: ○, Esmoriz; ■, Lis; ▲, Ribeiras do Oeste; –,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

Table 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 (LC) on each side of the oral disc and number of cusps in the infraoral lamina (IL). 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

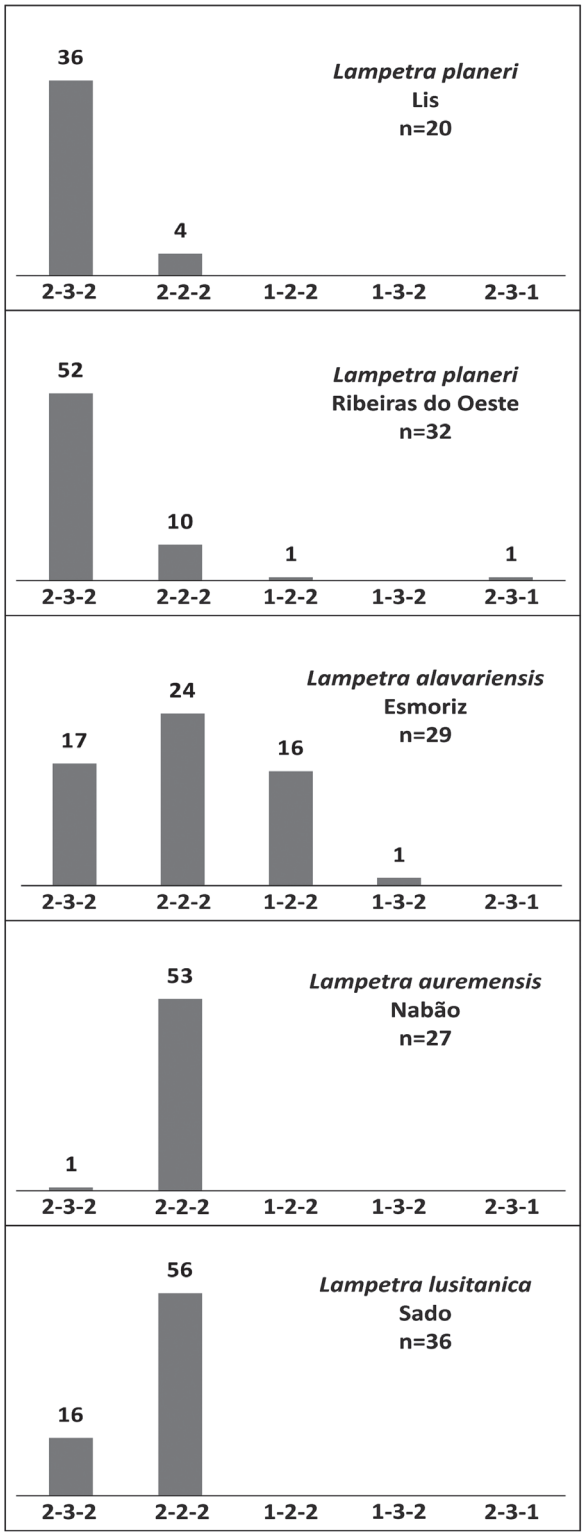

Fig. 4. Endolateral formula counts for the analysed populations. As endolaterals occur on both sides of the oral disc they have twice as many counts as the number of analysed individuals.

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 and 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 and Goto, 1997; Holčík and 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. 6*c*); another including the individuals from river Nabão, here described as *L. auremensis* (Fig. 6*b*); a third including the populations from Esmoriz and Águeda rivers, here described as *L. alavariensis* (Fig. 6*a*); 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 and Avise (1998) concluded that 90% of putative sister species show sequence divergences greater than 2% (see also Avise and 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.

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On-line Supplementary information (SI)

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.

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.

Appendix

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 5*a*, 6*a*)

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.

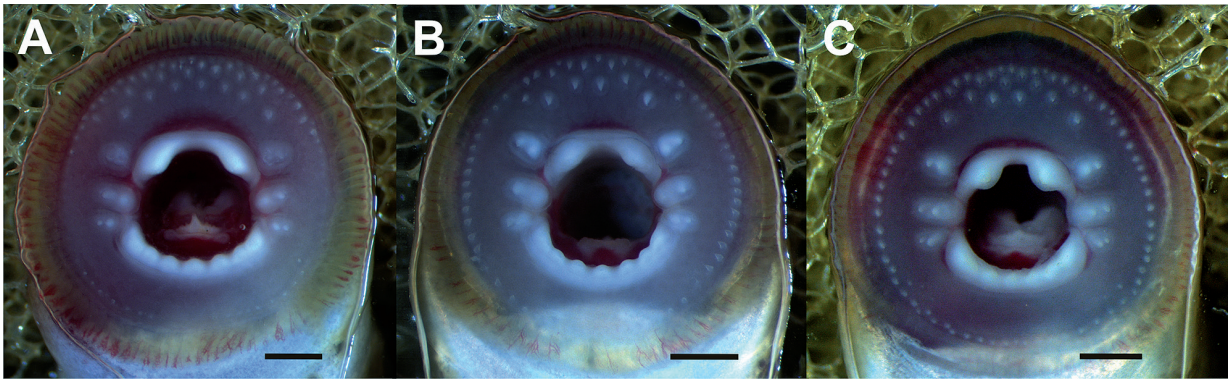

Fig. 5. Oral disc of the holotype of (A) *Lampetra alavariensis* sp. nov. (MB05-002866; Tl, 127.6 mm; immature adult; live specimen), (B) *Lampetra auremensis* sp. nov. (MB05-002869; Tl, 121.0 mm; immature adult; live specimen) and (C) *Lampetra lusitanica* sp. nov. (MB05-002871; Tl, 132.8 mm; immature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

Fig. 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).

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 online 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. 6*a*), 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. 6*a*). 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 northwestern Portuguese drainages Esmoriz and Vouga (Fig. 7*a*). 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.

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

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 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 six private haplotypes (EMBL-Bank accession numbers: FN641833-34, 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).

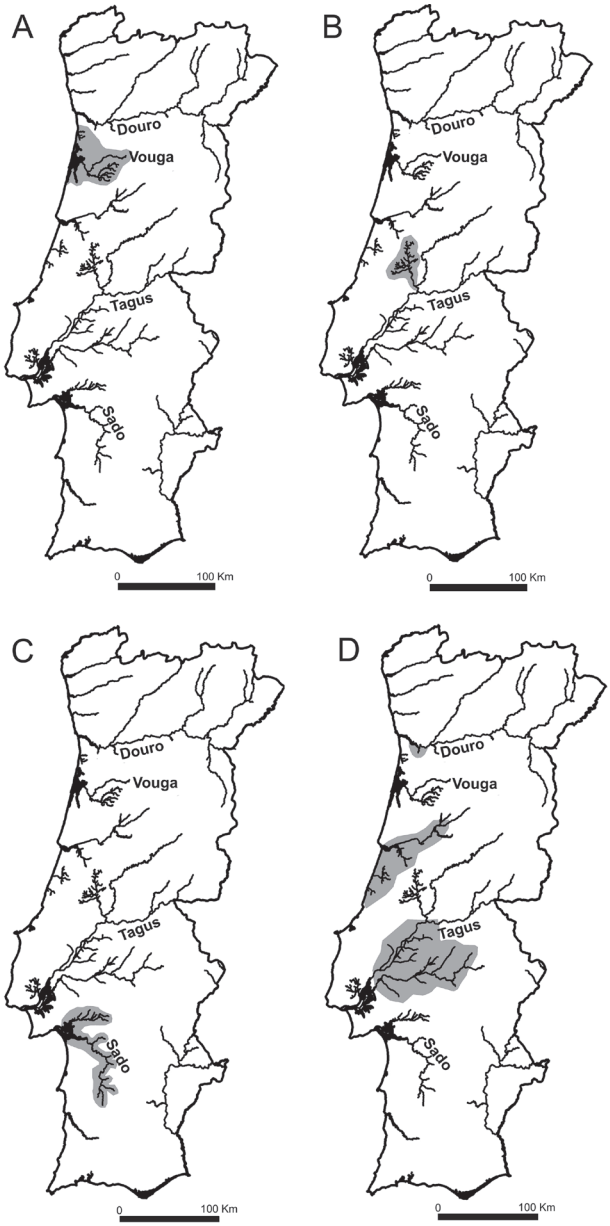

Fig. 7. Geographic distribution (■) of (A) *Lampetra alavariensis* sp. nov., (B) *Lampetra auremensis* sp. nov., (C) *Lampetra lusitanica* sp. nov. and (D) *Lampetra planeri* in Portugal.

Description: Lampetra auremensis sp. nov. is a small freshwater non-parasitic lamprey. In the 31 analysed specimens, including the holotype (Fig. 6*b*), 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. 7*b*).

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. 7*b*). 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).

Description: Lampetra lusitanica sp. nov. is a small freshwater non-parasitic lamprey. In the 38 analysed specimens, including the holotype (Fig. 6*c*), 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. 7*c*).

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.7*c*) 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.

	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	1112
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	.	.	.	.	.	.
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	H42	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H43	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	G	.	.
	H44	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.
	H46	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
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	H57	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	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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	H73	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
	H74	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.
	H75	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
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	H77	.	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
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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
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	H49	.	.	A	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	.	C	C	.	.	C	A	.	.	.	.	C
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L. auremensis	H47	.	.	.	.	A	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.
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L. alavariensis	H26	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
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	H29	.	.	.	.	A	.	C	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	C	.	T	T	.	.	.
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	H	Nucleotide position																																					
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			3	0	1	2	3	9	6	0	2	3	3	3	3	4	5	6	8	9	1	3	3	3	4	6	7	7	0	4	7	0	2	3	6	7	8	9	0	1	1
		2	8	6	6	9	7	0	7	8	1	0	7	8	9	8	7	6	6	9	1	2	5	8	7	0	1	4	6	8	5	8	0	5	8	6	3	5	4	4	9
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
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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	.	.	.
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	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	.	.	.
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	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	.	.	.	.
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L. alavariensis	H26	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.
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