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SHORT COMMUNICATION

Putting European lampreys into perspective: A global-scale multilocus phylogeny with a proposal for a generic structure of the Petromyzontidae

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Abstract

Previous studies on the phylogenetic relationships between lamprey species relied either on a low number of morphological characters related to the feeding apparatus, or on a low number of molecular mitochondrial DNA markers. Here, we apply a multilocus approach to assess the phylogenetic relationships of northern hemisphere lampreys, with a special emphasis on the 17 European species. The study comprises two mitochondrial (*cytochrome c oxidase subunit 1* gene—DNA barcodes, and *cytochrome b* gene) and two nuclear (*internal transcribed spacers I* and *II*) markers to investigate species' phylogenetic affinities. The phylogeny obtained with mitochondrial markers revealed a clear and highly supported separation of all northern hemisphere lampreys. Among those, our multilocus results show several polyphyletic genera, stressing the need for a taxonomic revision in a near future. *Lampetra morii* (Berg, 1931) from East Asia, often included in *Eudontomyzon*, is placed in the genus *Lethenteron*. *Lampetra richardsoni* Vladykov & Follett, 1965 and *Entosphenus hubbsi* (Vladykov & Kott, 1976) should be placed in a new genus, as well as the southern populations of *Lethenteron camtschaticum* (Tilesius, 1811) and *Lethenteron reissneri* (Dybowski, 1869). Considering European species, our results argue for a taxonomic revision of *Eudontomyzon*, with emphasis on *Eudontomyzon vladykovi* Oliva & Zanandrea, 1959.

KEYWORDS
Europe, mitochondrial markers, nuclear markers, taxonomic revision

Resumen

Estudios anteriores sobre las relaciones filogenéticas entre especies de lampreas, se basan en un escaso número de caracteres morfológicos relacionados con las estructuras de alimentación, o en un bajo número de marcadores moleculares de ADN mitocondrial. Aquí aplicamos un enfoque multilocus para evaluar las relaciones filogenéticas de las lampreas del hemisferio norte, con especial énfasis en 17 especies

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europeas. Este estudio comprende dos marcadores mitocondriales (*citocromo oxidasa c subunidad 1* —ADN barcodes, y *citocromo oxidasa b*) y dos marcadores nucleares (*espaciadores transcritos internos I y II* (ITS)) para investigar la afinidad filogenética de las especies. La filogenia obtenida con losmarcadores mitocondriales reveló una separación clara y altamente apoyada de todas las lampreas del hemisferio norte. Entre ellas, nuestros resultados multilocus muestran varios géneros polifiléticos, lo que resalta la necesidad de una revisión taxonómica en el futuro. *Lampetra morii* (Berg, 1931) de Asia oriental, a menudo incluida en *Eudontomyzon*, se sitúa en el género *Lethenteron*. *Lampetra richardsoni* Vladykov & Follett, 1965 y *Entosphenus hubbsi* (Vladykov & Kott, 1976) deben colocarse en un nuevo género, así como las poblaciones meridionales de *Lethenteron camtschaticum* (Tilesius, 1811) y *Lethenteron reissneri* (Dybowski, 1869). Considerando las especies europeas, nuestros resultados abogan por una revisión taxonómica de *Eudontomyzon*, con énfasis en *Eudontomyzon vladykovi* Oliva & Zanandrea, 1959.

1 | INTRODUCTION

Lampreys are an ancient vertebrate group, with a general anatomy conserved for at least 360 million years (Gess et al., 2006). This group's life cycle includes a filter feeding larval phase that lasts for several years, while buried in freshwater sediment (e.g., Potter et al., 2015; Renaud, 2011). After this growth period, metamorphosis takes place and individuals acquire teeth on the oral disk, functional eyes and, in certain species, osmoregulatory ability, allowing them to enter marine waters. In some species, juveniles migrate to the sea or to a different freshwater body, and a trophic phase begins. The animals then feed by parasitism or predation for some time, while growing and sexually maturing. After this period, they migrate back to the rivers, where they spawn and end their semelparous life (e.g., Potter et al., 2015; Renaud, 2011). This life cycle is usually considered the ancestral state in lampreys (Docker, 2009). Other lampreys lack a trophic phase and spawning follows metamorphosis in the freshwater body where larval growth took place. These species are called non-trophic and, together with their ancestral trophic species, form species pairs or species complexes. Several genera include one trophic and one or several non-trophic species. In this situation, the non-trophic species are called satellite species (Vladykov, & Kott, 1979). When the non-trophic satellite species evolved recently, one expects a similar morphology between trophic and non-trophic species. This phenomenon has been observed in many lamprey genera (Potter et al., 2015). However, when divergence is old, the non-trophic species may present a morphology quite distinct from the ancestor, as the non-functional feeding apparatus could have degenerated (Docker, 2009). Therefore, traditional morphology-based phylogenetic analyses have only considered the relationships between trophic species, assuming these as proxies for their respective satellite ones (Gill et al., 2003).

Lampreys occupy wide geographic areas, displaying an anti-tropical distribution (Renaud, 2011). They are currently restricted to the regions above 20° northern and southern latitudes (Renaud, 2011). In the southern hemisphere, there are two families of lampreys: Geotriidae, with two predatory species (Riva-Rossi et al., 2020); and Mordaciidae, with three species, two of which are trophic. In the northern hemisphere, the family Petromyzontidae contains approximately 40 species, 15 of which with a trophic life cycle (Potter et al., 2015).

A morphology-based phylogeny of trophic lamprey species was published by Gill et al. (2003) using several characters: mouth structure, number and type of teeth and oral laminas, number and shape of velar tentacles, eye position, relative position of dorsal fins, and position of the cloaca relative to the dorsal fins. This work revealed a basal polytomy, failing to resolve the relationship between the families Geotriidae, Mordaciidae, and Petromyzontidae (Figure 1 left). The latter was shown to be monophyletic and its basal dichotomy revealed two sister groups, one grouping *Ichthyomyzon* and *Petromyzon* (both North American genera, but the anadromous *Petromyzon* also occurs in Europe) and the other one clustering the remaining northern hemisphere lamprey species. In the latter group, the most basal genus was *Caspiomyzon* (confined to the Caspian Sea), followed by *Tetrapleurodon* (a Mexican Pacific genus) and *Entosphenus* (a Pacific genus, present in both, North America and Asia). The remaining species belong to three distinct genera: *Lampetra* (a West Palearctic and North American genus), its sister genus *Eudontomyzon* (European with one Asian species), and *Lethenteron* (Europe, Arctic, and Asia).

The phylogenetic relationships between the aforementioned trophic species were also assessed in molecular studies. Using the mitochondrial *cytochrome b* gene, Potter et al. (2015) revealed a somewhat different topology: the basal polytomy was resolved, with Mordaciidae as the most ancient family and sister to the Geotriidae + Petromyzontidae group (Figure 1 right); contrasting results were also found for within the group *Lethenteron* + *Eudontomyzon* + *Lampetra*, with the *cytb* phylogeny raising questions about the taxonomic validity/congruence of the genus *Eudontomyzon* and *Lampetra*.

The contrasting tree topologies obtained with morphological and molecular data may be related to methodological limitations.

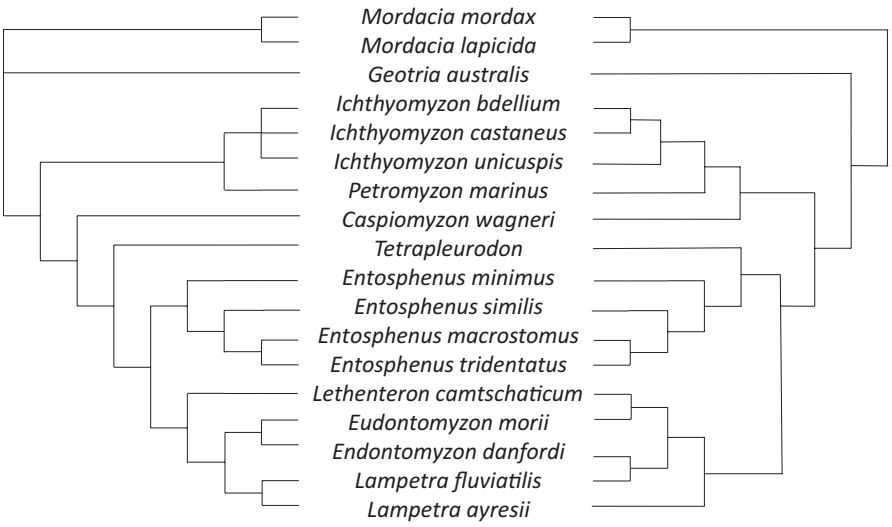

FIGURE 1 Relationships between trophic lamprey species using morphological characters (on the left) and genetic data (on the right), after Potter et al. (2015)

Morphological studies rely on few characters, mostly associated with the feeding apparatus (Docker et al., 1999), likely to reflect homoplasy. Lampreys are thus still a challenge for taxonomists and a good example for cryptic taxa exhibiting only few characters that can be measured or counted (no fin rays, no scales, and no ossified structures). Furthermore, lampreys show strong allometric growth after metamorphosis (Krappe, 2004) limiting morphometric studies to individuals in exactly the same phase of life. Krappe (2004) reports growth of the disk and shrinkage of the body and tail between 5%–15% after metamorphosis for different body proportions in *Lampetra planeri* . Therefore, the traditional taxonomy in lampreys is mainly based on their dentition and number of myomeres. On the other hand, molecular studies conducted so far used only mitochondrial genes (Potter et al., 2015). Hence, a comprehensive study with a broad database, including non-trophic species and additional molecular markers (with both nuclear and mitochondrial genes), is still lacking, despite its relevance in clarifying the phylogenetic relationships among the different families of lampreys.

Here, we apply a multilocus approach to assess the phylogenetic relationships of northern hemisphere lampreys, with a special emphasis on European species. This study comprises mitochondrial marker sequences (*cytochrome c oxidase subunit 1* gene [*COI*]—DNA barcodes, and *cytochrome b* gene [*cytb*]) and nuclear markers (*internal transcribed spacers I* and *II* [*ITS-1* and *ITS-2*]), the spacer DNA regions situated between the nuclear ribosomal RNA genes. A total of 17 European species were investigated. This is the first work to include mitochondrial and nuclear markers in a wide number of lamprey species with trophic and non-trophic lifecycles, shedding light into taxonomical/genetic discordances found in previous studies and providing insight into a fully comprehensive phylogeny of the group.

2 | MATERIALS AND METHODS

DNA sequences were obtained from 37 individuals from 17 described and one putative undescribed European species (Barbieri et al., 2015) (Table 1). Lampreys were caught by electrofishing or collected by hand net following local laws and regulations. Specimens, mainly larvae, were attributed to each species based on their sampling location and preserved in ethanol 96° for molecular analyses. Whenever possible, samples were taken from individuals near the species-type locality. Given the diversity of type localities attributed to *Eudontomyzon vladykovi*, individuals from two sampling points were included in this study. Two samples of the Mexican *Tetrapleurodon geminis* , were also included for topological stability. Sample tissue was deposited in ISPA-IU Genetics laboratory.

Total DNA was obtained using REDExtract-N-Amp kit (Sigma-Aldrich) following the manufacturer's instructions. Amplifications were conducted in 20 μl total-reaction volume with 10 μl of REDExtract-N-ampl PCR reaction mix (Sigma–Aldrich), 0.8 μl of each primer (10 μM), 4.4 μl of sigma-water, and 4 μl of template DNA. *COI* was amplified using the primers COI-F1(5′-TCA ACC ACC CAC AAA GAC ATT GGC AC-3′ (Ivanova et al., 2007) and COI-2R (5′-ACT TCA GGG TGA CCG AAG AAT CAG AA-3′; adapted from Ward et al., 2005). The amplicon size was 698 bp (alignment size 604 bp). *Cyt b* was amplified using the primers Cytb494L (3′-AGC CTT CTC TTC AGT TAT ACA CAT TTG TCG-3′) and Phe1612H (5′-CTT CAG TGC TCT GCT TTA ATG-3′) (Lang et al., 2009). The amplicon size was 1187 bp (alignment size 894 bp). *ITS-1* was amplified using the primers Kp2 (5′-AAA AAG CTT CCG TAG GTG AAC CTG CG-3′) and 5.8S (5′-AGC TTG CTG CGT TCT TCA TCG A-3′; Phillips et al., 1995). The amplicon size was 433 bp (alignment size 340 bp). *ITS-2* was amplified using the primers 5.8SR (5′-CTA CGC CTG TCT GAG TGT C-3′) and 28S (5′-ATA TGC TTA AAT TCA GCG GG-3′) (Phillips et al., 1995). The amplicon size was 393 bp (alignment size 293 bp). PCR followed usual conditions, with 55°C as annealing temperature for all fragments. Sequencing and purification were performed in Macrogen Europe.

Chromatograms were inspected and edited using CodonCode Aligner (CodonCode Corporation, www.codoncode.com). GenBank accession numbers of obtained sequences (from MW917204 to MW917227, MW925712 to MW925743, MW937280 to MW937308, and MW937309 to MW937342) are listed in Table 1 and alignments presented in supplementary material.

TABLE 1 Lamprey species (trophic species in red, non-trophic species in black), distributions ([EUR]—Europe; [ENA]—Eastern North America; [WNA]—Western North America; [AS]—Asia; [AUS]—Australia; [SA]—South America), specimen vouchers and GenBank accession numbers of samples used in this study (accession numbers marked with [gb] are available GenBank sequences)

Taxon and distribution	Sampling point	Specimen voucher	GenBank Accession numbers			
			COI	*Cyt b*	*ITS-1*	*ITS-2*
Lampetra fluviatilis (Linnaeus, 1758) [EUR]	Neva River, Russia	LFLUST9	MW917226	MW925737		MW937338
		LFLUST25			MW937304	
Lampetra planeri (Bloch, 1784) [EUR]	Rhine River, Germany	LPLAOB10		MW925739		MW937339
		LPLAOB16		MW925740	MW937306	
			KM373659[gb]			
Lampetra auremensis Mateus, Alves, Quintella & Almeida, 2013 [EUR]	Ribeira do Olival, Portugal	LTJN35	MW917223	MW925734	MW937298	MW937331
Lampetra alavariensis Mateus, Alves, Quintella & Almeida, 2013 [EUR]	Vouga River, Portugal	LPVG8	MW917221	MW925732	MW937296	MW937329
		LPVG9	MW917222	MW925733	MW937297	MW937330
Lampetra lusitanica Mateus, Alves, Quintella & Almeida, 2013[EUR]	Sado River, Portugal	LPSAD12	MW917227	MW925738		
		LPSAD2			MW937305	MW937338
Lampetra zanandreai (Vladykov, 1955)[EUR]	Po River, Italy	LAMPZ25		MW925730	MW937295	
		LAMPZ26		MW925731		
			KJ554015[gb]			
Lampetra soljani Tutman, Freyhof, Dulčić, Glamuzina & Geiger, 2017[EUR]	Krupa River, Bosnia and Herzegovina	EUKR1		MW925741		MW937326
		EUKR2		MW925742	MW937294	MW937327
		EUKR3		MW925743		MW937328
			KJ554074[gb], KJ553990[gb], KJ553874[gb], KJ553819[gb], KJ553778[gb], KJ553756[gb], KJ553665[gb]			
Lampetra lanceolata Kux & Steiner, 1972 [EUR]	Iyidere River, Turkey	LLANC1	MW917211	MW925720		MW937316
		LLANC2	MW917212	MW925721	MW937286	MW937317
Petromyzon marinus Linnaeus, 1758 [EUR; ENA]	Guadiana River, Portugal	PMG1			MW937307	MW937340
			NC_001626[gb]	NC_001626[gb]		
Lampetra ninae (Naseka, Tuniyev & Renaud, 2009) [EUR]	Kakhatskali River, Georgia	LENI3	MW917224	MW925735	MW937301	MW937334
		LENI4	MW917225	MW925736	MW937302	MW937335
	Tskaltsminda Creek, Georgia	LENI5			MW937303	MW937336
		LENI1			MW937299	MW937332
		LENI2			MW937300	MW937333
Eudontomyzon danfordi Regan, 1911[EUR]	Mureş River, Romania	EUDA10	MW917206	MW925714	MW937282	MW937311
		EUDA8		MW925715		

(Continues)

TABLE 1 (Continued)

Taxon and distribution	Sampling point	Specimen voucher	GenBank Accession numbers			
			COI	*Cyt b*	*ITS-1*	*ITS-2*
Eudontomyzon mariae (Berg, 1931) EUR	Don River, Russia	EUMA33	MW917213	MW925722	MW937287	MW937318
		EUMA34	MW917214	MW925723	MW937288	MW937319
Eudontomyzon vladykovi Oliva & Zanandrea, 1959 EUR	Sava River, Danube, Croatia	EUVL4	MW917220	MW925729	MW937293	MW937325
Eudontomyzon vladykovi Oliva & Zanandrea, 1959 EUR	Drava River, Croatia	EUVL2	MW917219	MW925728	MW937292	MW937324
Eudontomyzon stankokaramani Karaman, 1974 EUR	Ohrid Lake, Macedonia	EUST1	MW917217	MW925726	MW937291	MW937322
		EUST2	MW917218	MW925727		MW937323
Eudontomyzon sp. Almopaios EUR	Aridaios River, Greece	EU1	MW917215	MW925724	MW937289	MW937320
		EU2	MW917216	MW925725	MW937290	MW937321
Caspiomyzon wagneri (Kessler, 1870) EUR; AS	Kura River, Azerbaijan	CWK1	MW917204	MW925712, MW925713	MW937280, MW937281	MW937309, MW937310
		CWK2	MW917205			
Caspiomyzon hellenicus (Vladykov, Renaud, Kott & Economidis, 1982) EUR	Prosotsani, Strymon drainage, Greece	EUHE1				MW937315
		EUHE3	MW917209	MW925718		MW937314
		EUHE4	MW917210	MW925719	MW937285	
Caspiomyzon graecus (Renaud & Economidis, 2010) EUR	Kembi spring, Greece	EUGR3	MW917207	MW925716	MW937283	MW937312
		EUGR4	MW917208	MW925717	MW937284	MW937313
Lethenteron camstchaticum (Tilesius, 1811) EUR; AS; ENA; WNA			KF701113[gb], KC353468[gb], KJ866208[gb], KJ866209[gb]	KF701113[gb], KC353468[gb], KJ866208[gb], KJ866209[gb]		
Tetrapleurodon geminis Álvarez, 1964 WNA	Celio River, Mexico	LMEX3827			MW937308	MW937341
		LMEX3826				MW937342
			JN028424[gb], JN028425[gb]	GQ206187[gb]		
Entosphenus hubbsi (Vladykov & Kott, 1976) WNA			HQ55730[gb], JN025327[gb]	GQ206150[gb]		
Entosphenus lethophagus(Hubbs, 1971) WNA			HQ579097[gb], KX389870[gb]	GQ206153[gb]		
Entosphenus similis Vladykov & Kott, 1979 WNA			JN025330[gb], JN025331[gb]	GQ206156[gb]		
Entosphenus tridentatus(Richardson, 1836) WNA; AS			KF918874[gb], KF929845[gb]	GQ206157[gb], GU120749[gb], GU120750[gb], GU120753[gb], GU120798[gb], GU120810[gb]		
Ichthyomyzon bdellium(Jordan, 1885) ENA			JN026861[gb], JN026859[gb]	GQ206166[gb]		
Ichthyomyzon castaneus Girard, 1858 ENA			EU524088[gb], EU524087[gb], JN026867[gb]	GQ206168[gb]		

(Continues)

TABLE 1 (Continued)

Taxon and distribution	Sampling point	Specimen voucher	GenBank Accession numbers			
			COI	*Cyt b*	*ITS-1*	*ITS-2*
Ichthyomyzon fossor Reighard & Cummins, 1916 [ENA]			KM267716[gb]	KM267716[gb]		
Ichthyomyzon gagei Hubbs & Trautman, 1937[ENA]			JN026886[gb], JN026888[gb], HQ557147[gb], JN026892[gb], JN026884[gb]	GQ206169[gb]		
Ichthyomyzon unicuspisHubbs & Trautman, 1937 [ENA]			KM267717[gb]	KM267717[gb]		
Ichthyomyzon greeleyi Hubbs & Trautman, 1937 [ENA]			JN026897[gb], HQ557146[gb]	GQ206167[gb]		
Lampetra ayresii(Günther, 1870) [ENA]			JQ354155[gb]	GQ206174[gb], GU120807[gb], GU120867[gb]		
Lampetra aepypyera (Abbott, 1860)[ENA]			KP742974[gb]	KP742974[gb]		
*Lampetra richardsoni*Vladykov & Follett, 1965 [WNA]			JN026960[gb]	GQ206177[gb], GU120728[gb], GU120758[gb], GU120802[gb]		
Lethenteron kessleri(Anikin, 1905)[AS]			AB198748[gb]–AB198752[gb], JN027078[gb]	GQ206183[gb], KJ684774[gb], KJ684724[gb]–KJ684731[gb]		
*Lethenteron alaskense*Vladykov & Kott, 1978 [WNA]			JN027060[gb]–JN027062[gb]	GQ206178[gb]		
Lethenteron appendix(DeKay, 1842)[ENA]			KM267719[gb]	KM267719[gb]		
Lethenteron reissneri(Dybowski, 1869) [AS]			KC353466[gb], AB565771[gb]	KC353466[gb], AB565771[gb]		
Eudontomyzon morii(Berg, 1931)[AS]			KM267718[gb]	KM267718[gb]		
Geotria australis Gray, 1851 [AUS; SA]			KT185629[gb]	KT185629[gb]		
Geotria macrostoma(Burmeister, 1868) [SA]			MT478622[gb], MT478624[gb], MT478625[gb], MT478632[gb], MT478636[gb], MT478642[gb]–MT478644[gb]	MT478645[gb]–MT478652[gb]		
*Mordacia praecox*Potter, 1968 [AUS]			KJ669535[gb]	GQ206186[gb]		
Mordacia lapicida(Gray, 1851) [SA]			JN027251[gb]	GQ206185[gb]		

Whenever published sequences were available, they were added to the dataset and used in the analyses (Table 1—a total of 65 *COI* sequences available from 28 species and 55 *cyt b* sequences available from 25 species).

The mitochondrial database was extended by including *cytb* and *COI* sequences for 23 additional taxa available in GenBank: *Entosphenus hubbsi*, *Entosphenus lethophagus*, *Entosphenus similis*, *Entosphenus tridentatus*, *Eudontomyzon morii*, *Geotria australis*, *Geotria macrostoma*, *Ichthyomyzon bdellium*, *Ichthyomyzon castanaeus*, *Ichthyomyzon fossor*, *Ichthyomyzon gagei*, *Ichthyomyzon unicuspis*, *Ichthyomyzon greeleyi*, *Lampetra ayresii*, *Lampetra aepypyera*, *Lampetra richardsoni*, *Lethenteron camtschaticum*, *Lethenteron kessleri*, *Lethenteron alaskense*, *Lethenteron appendix*, *Lethenteron reissneri*, *Mordacia praecox*, and *Mordacia lapicida* (see Table 1 for details). Sequences were selected using the following criteria: when mitogenomes were available, their *cytb* and *COI* were selected from the same individual; when mitogenomes were not available, only longer sequences (>500 bp) were used; when several longer sequences were available, all haplotypes were used; sequences with ambiguities were discarded whenever other sequences were available. We concatenated sequences from the same individual, whenever possible.

Maximum Composite Likelihood genetic distances between sequences and net distances between taxa were estimated using Mega v.7 (Kumar et al., 2016). Phylogenetic analyses were performed on all markers independently (see Alignments S1–S3 for European species and S4 and S5 for all lamprey species), on a mitochondrial alignment (concatenating *cytb* and *COI*—see alignments S6 for European species and S7 for all lamprey species), and on a full concatenation of all markers (Alignment S8 for European species). Given their physical proximity, *ITS-1* and *ITS-2* were considered a single marker. Maximum parsimony-based (MP) phylogenetic relationships were estimated using PAUP (Swofford, 2001), with 100 heuristic searches using random additions of sequences and implementing the TBR algorithm. Branch support values for each node were tested by bootstrap analysis, with 1000 resamplings (Felsenstein, 1985). Maximum likelihood (ML) phylogenetic trees were inferred using RAxML BlackBox (Stamatakis et al., 2008). Branch support was tested by 100 rapid bootstrap inferences. Bayesian analysis (BA) was performed using MrBayes 3.2.6 (Ronquist et al., 2012) with two independent runs of four simultaneous Markov Monte Carlo chains (MCMC) for four million generations (sampling every 100 generations). Separate partitions were used for each mitochondrial protein-coding gene, while also partitioning the first and second codon positions from third codon positions, each with independent GTR models.

In addition, we also estimated the phylogeny of European species and their divergence times using BEAST v. 1.8.4 (Drummond et al., 2012), with two separate site and clock models, one for both mitochondrial and another for both nuclear markers. A lognormal relaxed clock was assumed for both regions. We applied an a priori rate of 0.01 substitutions/site/MY for the mitochondrial markers, as previously used for lampreys by Docker et al. (1999) and consistent with Kuraku and Kuratani (2006), while clock rates for the nuclear markers were left with a uniform prior, due to the lack of any suitable rate estimate. We used a Yule speciation prior for both partitions and ran four independent analyses for one billion generations, logging parameters every 10,000 generations. We used a burn-in of 10%, combined the four analyses using LogCombiner v.1.10.4. (Drummond et al., 2012), and checked for convergence of all parameters by inspecting Effective Sample Size (ESS) values, using Tracer v.1.7.2. Convergence was considered acceptable when ESS values were >300. The maximum clade credibility tree was constructed with TreeAnnotator v.1.10.4 (Drummond et al., 2012).

3 | RESULTS

The phylogeny obtained using the mitochondrial dataset (895 bp *cytb* + 604 bp *COI*) revealed a clear separation of northern hemisphere lampreys from southern ones with high support (Figure 2 and Figures S1 and S2). Northern hemisphere lampreys formed two basal clades. The first comprised *Caspiomyzon wagneri* and its two Greek satellite species, *Caspiomyzon graecus* and *Caspiomyzon hellenicus*, together with their sister group *Petromyzon* + *Ichthyomyzon* (T clade in Figure 2). In the second basal clade, neither *Lethenteron*, *Lampetra* nor *Eudontomyzon* were found to be monophyletic. The representatives of the genus *Lethenteron* appeared in two subclades (clades U and V in Figure 2), and the species *Le. camtschaticum* in both: one clustering most of the species of this genus (V clade in Figure 2) and a second in a basal position (U clade in Figure 2). The representatives of the genus *Lampetra* appeared in four clades, one of them comprising all European *Lampetra* species (Z clade in Figure 2). *Lampetra richardsoni* and *La. ayresii* and *La. aepytera* were excluded from this clade and recovered as basal to subclades comprising the majority of *Eudontomyzon* and *Lampetra* species. All *Eudontomyzon* species clustered in a monophyletic group X clade in Figure 2, except *Eu. morii*, which clusters with *Lethenteron* and *Lampetra* of the V clade in Figure 2.

The phylogenetic relationships among European lamprey species inferred with the concatenated dataset (2151 bp long, from *COI*, *cytb*, *ITS-1*, and *ITS-2*) also recovered well-supported subclades for *Eudontomyzon* and *Lampetra* (Figure 3). In contrast, *C. graecus* and *C. hellenicus* did not cluster with *C. wagneri*, and *Petromyzon marinus* had a different position from the mitochondrial-based tree (cf. Figures 2 and 3).

Within *Lampetra* clade, the different analyses yielded a well-supported subclade including *Lampetra zanandreai*, *Lampetra lanceolata*, *Lampetra soljani*, and *Lampetra ninae*. This subclade is grouped with the other European *Lampetra*, when using both the complete and the mitochondrial datasets (Figures 2 and 3), but not with the concatenated nuclear markers alone (Figures S3–S5). The genetic distances between these clades are presented in Table 2.

According to the calibrated species tree (Figure 4), *Eudontomyzon* and *Lampetra* separated from each other around three mya. The group comprising *P. marinus*, *C. wagneri*, and *C. graecus* and *C. hellenicus* separated from the rest of analyzed species around 16 mya.

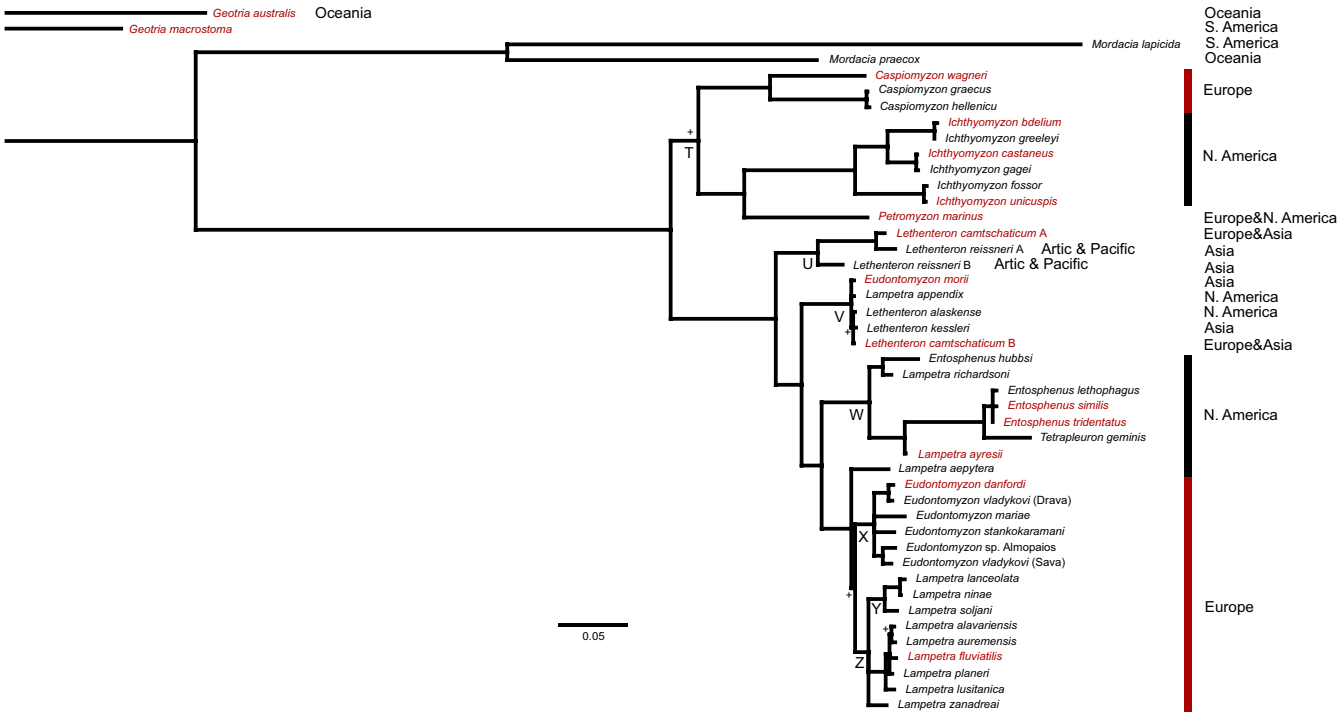

FIGURE 2 Bayesian analysis (BA) phylogenetic tree based calculated with concatenated *COI* and *cytb* markers from all lampreys included in this study, estimated using Mr. Bayes. All depicted bifurcating nodes had a posterior probability >0.99, except nodes with symbol (0.75< + <0.90). Scale bar indicates nucleotide substitutions per site. Trophic species are indicated in red. Letters T–Z indicate clades referred to in the text

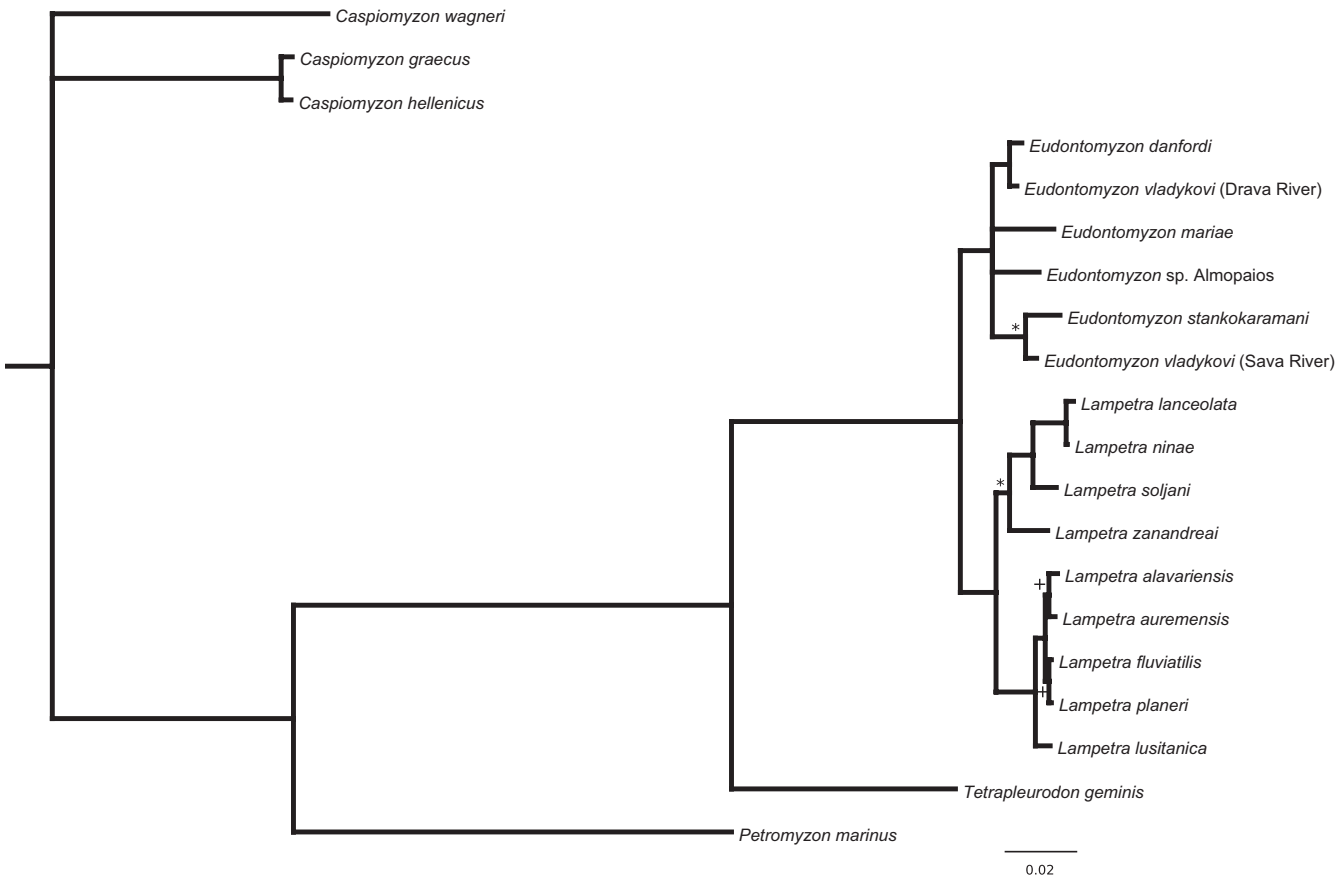

FIGURE 3 Maximum credibility species tree obtained using *BEAST, based on two underlying genealogies: mitochondrial markers (COI and *cytb*) and nuclear markers (ITS-1 and ITS-2, considered as a single linked marker given the physical proximity of both regions). All nodes have a posterior probability >0.99, except nodes with symbol (0.90< * <0.99; 0.75< + <0.90)

TABLE 2 Average Maximum Composite Likelihood estimates of molecular distance (% ± *SE*) within and between major European lamprey clades, using the *COI*, *cytb*, *ITS-1*, and *ITS-2* fragments, respectively

	Eudontomyzon	"Strict" European *Lampetra*[a]	*Lampetra zanandreai* + *Lampetra lanceolata* + *Lampetra ninae*	"Broad" European *Lampetra*[b]	*Caspiomyzon hellenicus* + *C. graecus*
Eudontomyzon	1.144 ± 0.301% 2.522 ± 0.400% 0.0814 ± 0.075% 0.1936 ± 0.1794%				
"Strict" European *Lampetra*[a]	2.145 ± 0.757% 3.067 ± 0.571% 0.666 ± 0.494% 0.10820 ± 0.608%	0.359 ± 0.185% 0.680 ± 0.172% 0.001 ± 0.007% 0.162 ± 0.152%			
Lampetra zanandreai + *Lampetra lanceolata* + *Lampetra ninae*	1.758 ± 0.637% 2.531 ± 0.518% 0.384 ± 0.330% 0.428 ± 0.348%	1.125 ± 0.459% 2.139 ± 0.442% 1.081 ± 0.657% 1.478 ± 0.704%	0.826 ± 0.291% 2.245 ± 0.416% 0.724 ± 0.380% 0.000 ± 0.000%		
"Broad" European *Lampetra*[b]	1.618 ± 0.609% 2.234 ± 0.447% 0.241 ± 0.130% 0.404 ± 0.223%			1.215 ± 0.369% 2.656 ± 0.413% 0.922 ± 0.393% 0.865 ± 0.455%	
Caspiomyzon hellenicus + *C. graecus*	8.800 ± 2.657% 21.307 ± 2.822% 12.073 ± 55.999% 9.659 ± 3.834%	9.764 ± 2.929% 23.55 ± 3.249% 12.85 ± 50.00% 11.15 ± 29.74%	9.135 ± 2.792% 22.29 ± 3.107% 12.91 ± 52.52% 10.42 ± 29.74%	9.081 ± 2.747% 22.40 ± 2.966% 12.58 ± 56.86% 10.04 ± 4.211%	0.601 ± 0.377% 0.564 ± 0.246% 0.000 ± 0.000% 0.000 ± 0.000%

Estimates were calculated using Mega v.7. The "strict" European Lampetra clade includes *Lampetra fluviatilis*, *Lampetra planeri*, *Lampetra alavariensis*, *Lampetra auremensis*, and *Lampetra lusitanica*. The "broad" European Lampetra clade also includes *Lampetra zanandreai*, *Lampetra lanceolata*, and *Lampetra ninae*.

[a]Includes *La. fluviatilis*, *La. planeri*, *La. alavariensis*, *La. auremensis*, and *La. lusitanica*.

[b]Includes *La. fluviatilis*, *La. planeri*, *La. alavariensis*, *La. auremensis*, *La. lusitanica*, *La. zanandreai*, *La. lanceolata*, and *La. ninae*.

Separation of *C. graecus* and *C. hellenicus* from *C. wagneri* took place around 7 mya (Figure 4, Table S1). The mean estimated *ITS* clock was 0.0034 substitutions/site/MY (95% HPD interval of 2.22×10^{-3} to 4.73×10^{-3}).

4 | DISCUSSION

4.1 | Inferences from the mitochondrial dataset

In this comprehensive lamprey phylogenetic study, the monophyly of northern hemisphere lampreys is clearly confirmed (Figure 2). One of our most striking results was the polyphyletic nature of four northern hemisphere genera of lampreys, resulting in the need for future taxonomic revision of *Lethenteron*, *Entosphenus*, *Lampetra*, and *Eudontomyzon*. The general topology of the phylogenetic tree resulting from the concatenated mitochondrial data is similar to that found by Lang et al. (2009) and Potter at al. (2015) using only the *cytb* gene. This was expected, as we used the same or similar sequences as used in those studies. Nevertheless, the inclusion of mitochondrial *COI* data provided better support for their conclusions and some differences concerning genus relationships.

The genus *Lethenteron* appeared in two well-separated clades. *Lethenteron camtschaticum* was polyphyletic when using the concatenated mitochondrial dataset (Figure 2). Considering that the type locality of this genus-type species (*Le. appendix*) is in USA, we argue that the taxa in clade V (northern populations of *Le. camtschaticum*, *Le. appendix*, *Le. alaskense*, *Le. kessleri* and "*Eu.*" *morii*) should be considered *Lethenteron*, while the southern populations of *Le. camtschaticum* and *Le. reissneri* (clade U) should be assigned to a new genus. In the work of Lang et al. (2009), *Le. reissneri* was shown to belong to *Lethenteron*. However, the samples included in that study were from the northern area of distribution of the species (in Russia), while those in our study (taken from complete mitochondrial genomes) are from the southern area (Japan). Lang et al. (2009) also included two undescribed *Lethenteron*: *Le.* sp. N, collected in Japan, grouped with other *Lethenteron* species; and *Le.* sp. S, sampled in South Korea, shown to belong to a new undescribed genus. The phylogenetic position of this new genus is similar to what was found in the present study for *Le. camtschaticum* and *Le. reissneri*, both from Japan. Both studies strongly suggest that *Lethenteron* is a northern genus, comprising species from Eastern and Western North America basins, Artic basins, and northwest Pacific drainages (including "*Eu.*" *morii*, which is here proposed to be a species of *Lethenteron*). Conversely, a new genus should be described to include southern Japan and South Korea lampreys.

Our mitochondrial results also revealed the polyphyletic nature of the genus *Entosphenus*: a clade comprising the type species

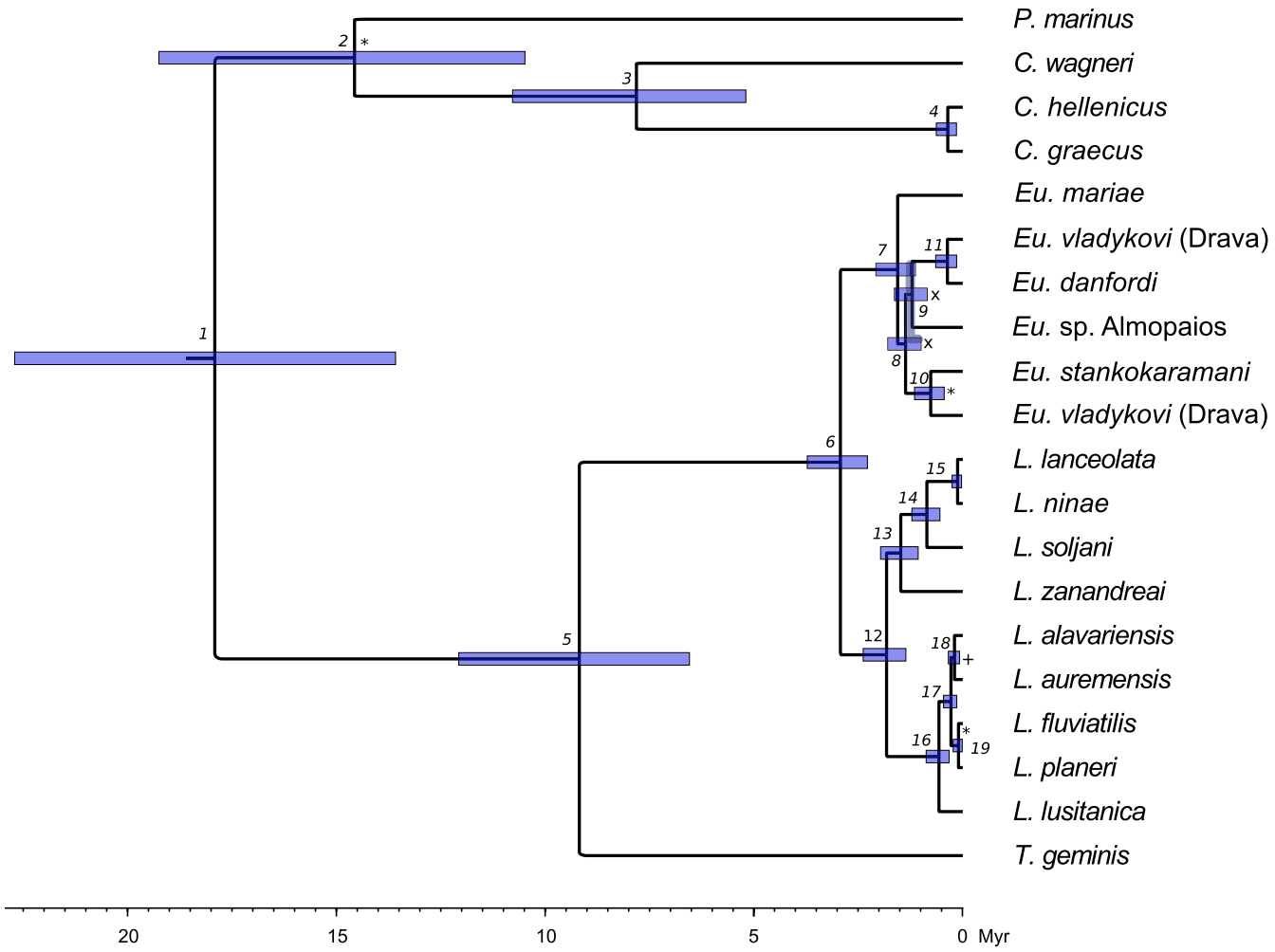

FIGURE 4 BA tree with time-calibrated maximum clade credibility from BEAST analysis, calculated with mitochondrial (COI and *cytb*) and nuclear (ITS-1 and ITS-2) data. Node bars depict 95% highest posterior density. All nodes have a posterior probability >0.99, except nodes with symbol (0.90< * <0.99; 0.8< + <0.9; 0.7< × <0.8). Node age and HPD 95% range for each numbered node is indicated in Table S1

En. tridentatus, *En. lethophagus*, and *En. similis*, and another clade, clustering *En. hubbsi* and *La. richardsoni*. These phylogenetic relationships had already been suggested by Lang et al. (2009) using only *cytb*. However, the two studies yielded distinct results concerning *La. ayresii*: in Lang et al. (2009), this species is sister to *La. richardsoni*, while in the present work, it is closer to the genus *Entosphenus* than to the *La. richardsoni* and *En. hubbsi* clade. Therefore, we suggest the description of a new genus encompassing *En. hubbsi* and *La. richardsoni*. The taxonomic clarification of *La. ayresii* requires further work using nuclear markers and / or genomic data. For the time being, we exclude *La. richardsoni* and also *La. ayresii* from *Lampetra* and encourage the description of a new genus for these species.

The present work corroborated additional conclusions of previous studies such as the suggestion of Lang et al. (2009) to only include the European species in the genus *Lampetra*, moving *Lampetra richarsoni*, *La. ayresii*, and *Lampetra aepyptera* to new genera. This later species, although close to European *Lampetra*, was already considered *Okelbergia aepyptera* by Lang et al. (2009).

4.2 | Inferences from the extended dataset (mitochondrial + nuclear markers)

Concerning the West Paleartic species, the present phylogenetic analysis with the extended dataset revealed the monophyly of *Eudontomyzon* and *Lampetra*, clustering them as sister genera (Figure 3). A similar result was reported both by our mitochondrial data (Figure 2) and by Lang et al. (2009). The inclusion of *La. ninae* in the European *Lampetra* clade had very good support when combining all fragments (Figure 3), and it is in agreement with Li (2014) and Tutman et al. (2017).

The genus *Lampetra* probably originated in the Lower Pleistocene (2.38–1.36 My; node 12 in Figure 4 and Table S1), an epoch characterized by oscillations between glacial and interglacial conditions. In glacial periods, a migratory form of *Lampetra* might have colonized southern rivers, whose temperatures were suitable for breeding and reproduction. Although presently *Lampetra fluviatilis* is restricted to western Mediterranean basins (Freyhof, 2011), it could

have been an abundant species in the Eastern Mediterranean and Black Sea in glacial periods, when the sea and river temperatures were lower. During interglacials, the distribution of the migratory *Lampetra* was probably restricted to northern locations, with more favorable temperatures, similar to present day conditions. Under such environmental conditions, the non-migratory southern populations, confined to freshwater systems, isolated from other populations and with smaller population sizes, could have diverged from their migratory ancestor by genetic drift. This process might have led to the formation of these relict species, confined to southern locations.

The sister–species relationship of *C. hellenicus* and *C. graecus* was previously reported by Lang et al. (2009) and is confirmed here with the mitochondrial and combined datasets (Figures 2 and 3). The combined mitochondrial and nuclear analysis did not confirm their cluster with *C. wagneri*, as suggested in previous studies and our extended mitochondrial analysis. However, as it does not contradict this relation, we support the two Greek species should be placed in *Caspiomyzon*.

Eudontomyzon is a European monophyletic genus and diverged from *Lampetra* in the upper Pliocene (between 3.5 and 2.3 My; node 6 in Table S1). It is characterized by the absence of an extant anadromous species: *Eudontomyzon danfordi* is the trophic species of this group, but is potamodromous (Talabishka et al., 2012). The colonization patterns for this genus, assuming the absence of any other anadromous species, could have followed the evolution of the main European freshwater bodies during the Plio-Pleistocene. Our multilocus approach reveals the existence of several groups within *Eudontomyzon*, which seem to correspond to geographic affinities: *Eu. danfordi*, the predatory species, is the sister species of *Eu. vladykovi* which is widespread in the Danube drainage; and *Eudontomyzon stankokaramani*, contrary to the results in Lang et al. (2009), does not occupy a basal position in this genus, but is instead the sister group of *Eu.* sp., which might be an undescribed species from the Sava river. These results argue for a taxonomic revision of this genus, with emphasis on *Eu. vladykovi*. Also the Greek *Eudontomyzon* sp. Almopaios might represent an undescribed species. Its inclusion in this genus has high support (Figures 2 and 3). However, the specific status of this population should be further analyzed, as divergence from its sister species/population is very low (0.45% for *cytb* and 0.8% for *COI*).

This study represents the most comprehensive work done so far on the phylogeny of European lampreys using mitochondrial and nuclear markers. Our results not only corroborate previous studies, they also raise several new pertinent questions and suggest taxonomic changes that should be addressed in the future. The inclusion of non-trophic species in this study enables us to clarify some taxonomic uncertainties, especially important when the divergence between them and their trophic species is old.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. BA phylogenetic tree calculated with coi marker from all lampreys included in this study.

Figure S2. BA phylogenetic tree calculated with cytb marker from all lampreys included in this study.

Figure S3. BA phylogenetic tree calculated with *COI* marker from European lampreys.

Figure S4. BA phylogenetic tree calculated with *cytb* marker from European lampreys.

Figure S5. BA phylogenetic tree calculated with concatenated *ITS-1* and *ITS-2* nuclear markers from European lampreys.

Table S1. Mean node age (My) and 95% highest posterior density (HPD) intervals estimated using mitochondrial (*COI* and *cytb*) and nuclear markers (*ITS-1* and *ITS-2*).

Alignment S1. *COI* sequence alignment of European lamprey species used in BA inference.

Alignment S2. *Cytb* sequence alignment of European lamprey species used in BA inference.

Alignment S3. *ITS-1* and *ITS-2* sequence alignment of European lamprey species used in BA inference.

Alignment S4. *COI* sequence alignment of all lamprey species included in this study in BA inference.

Alignment S5. *Cytb* sequence alignment of all lamprey species included in this study in BA inference.

Alignment S6. Concatenated *COI* and *Cytb* sequences alignment of European lamprey species used in BA inference.

Alignment S7. Concatenated *COI* and *Cytb* sequences alignment of all lamprey species included in this study in BA inference.

Alignment S8. Concatenated *COI*, *Cytb*¸ *ITS-1* and *ITS-2* sequences alignment of European lamprey species used in BA inference.

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