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ORIGINAL PAPER

Detection of lamprey in Southernmost South America by environmental DNA (eDNA) and molecular evidence for a new species

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Abstract

Lampreys are jawless fishes belonging to the order Petromyzontiformes. *Geotria australis* is the sole representative lamprey species of the Geotriidae family and is widely distributed around South America, Australia, New Zealand, and sub-Antarctic Islands. In South America, the presence and distribution of *G. australis* are well characterized in Western Patagonia, in rivers flowing into the Pacific Ocean. In contrast, there is scarce information about the presence of this species in Eastern Patagonia, in rivers flowing into the Atlantic Ocean. Here, we provide the first report on the distribution of lamprey at the extreme south of Patagonia and suggest the occurrence of a new lamprey species. We developed an environmental DNA (eDNA) method to detect *G. australis* from water samples and obtained positive results in five basins flowing into the Atlantic Ocean and one river basin flowing into the Beagle Channel. Lampreys were captured from two eDNA-positive basins and used for genetic analysis. An 875 bp-sequence of the cytochrome b mitochondrial gene was obtained, and a phylogenetic analysis was carried out with this sequence and those available in GenBank, revealing Argentinean lamprey reported here, as a sister species of *G. australis* from Chile, Australia, and New Zealand. Also, the genetic distance values between lamprey reported here and *G. australis* were consistent with the genetic distances between species of different genera. Our results suggest that the Argentinean lamprey corresponds to a new specific taxon that could represent a new monotypic genus in Geotriidae.

Keywords Sub-antarctic fishes · Patagonia · Tierra del fuego · Freshwater fishes · Native species · Petromyzontiformes · Environmental DNA

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Introduction

Lampreys are jawless fish belonging to the order Petromyzontiformes, which comprise 40–45 species, depending on the author (Renaud 2011; Maitland et al. 2015; Potter et al. 2015). Lampreys are widely distributed in both hemispheres and are generally found in cold waters (Renaud 2011). In the Southern Hemisphere, only the Mordaciidae and Geotriidae families have been reported. Mordaciidae family includes three species: Chilean lamprey (*Mordacia lapicida*), short-headed lamprey (*M. mordax*) and precocious lamprey (*M. praecox*); while a unique species represents Geotriidae family: pouched lamprey (*Geotria australis*), widely distributed around Australia, New Zealand, South American Patagonia and sub-Antarctic Islands (McDowall 2002).

In South America, the distribution and life history of lampreys have been documented in rivers flowing into the Pacific Ocean, finding *G. australis* and *M. lapicida* in Western Patagonia (Chile). The latter is recognized as an endemic species of this region (Neira 1984; McDowall 1988; Habit et al. 2007). On the other hand, little is known about the presence and distribution of lampreys in Eastern Patagonia (Argentina), in rivers flowing into the Atlantic Ocean (Fig. 1a). In this regard, few studies describe some aspects of the morphology and life history of lampreys in this region, and only the presence of *G. australis* has been reported (Neira et al. 1988; Azpelicueta et al. 2001). Although *G. australis* presence has been reported in Western and Eastern Patagonia, several studies have described morphological differences between both populations (Neira et al. 1988; Renaud 2011; Potter et al. 2015). Besides, a cluster analysis based on body measurements from *G. australis* in larval stage performed by Neira et al. (1988) separated the Argentinian population from those of Chile, New Zealand, Tasmania, and Australia.

Argentinean section of Tierra del Fuego Island (TDF; 52° S–55° S) is located at the extreme south of Patagonia with rivers flowing into the Atlantic Ocean to the East, and into the Beagle Channel to the South (Fig. 1b). Freshwater environments of TDF have the lowest native fish biodiversity of all Patagonia (Cussac et al. 2009), with only two native species: small puyen and big puyen (*Galaxias maculatus* and *G. platei*, respectively) (Cussac et al. 2016). On the other hand, several exotic salmonids such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), chinook salmon (*O. tshawytscha*), and coho salmon (*O. kisutch*) have been introduced and established in TDF (Pascual et al. 2007; Chalde et al. 2019; Nardi et al. 2019). These exotic species might compete for space and food with native species and are considered a possible threat for native ecosystems (Pascual et al. 2002, 2007).

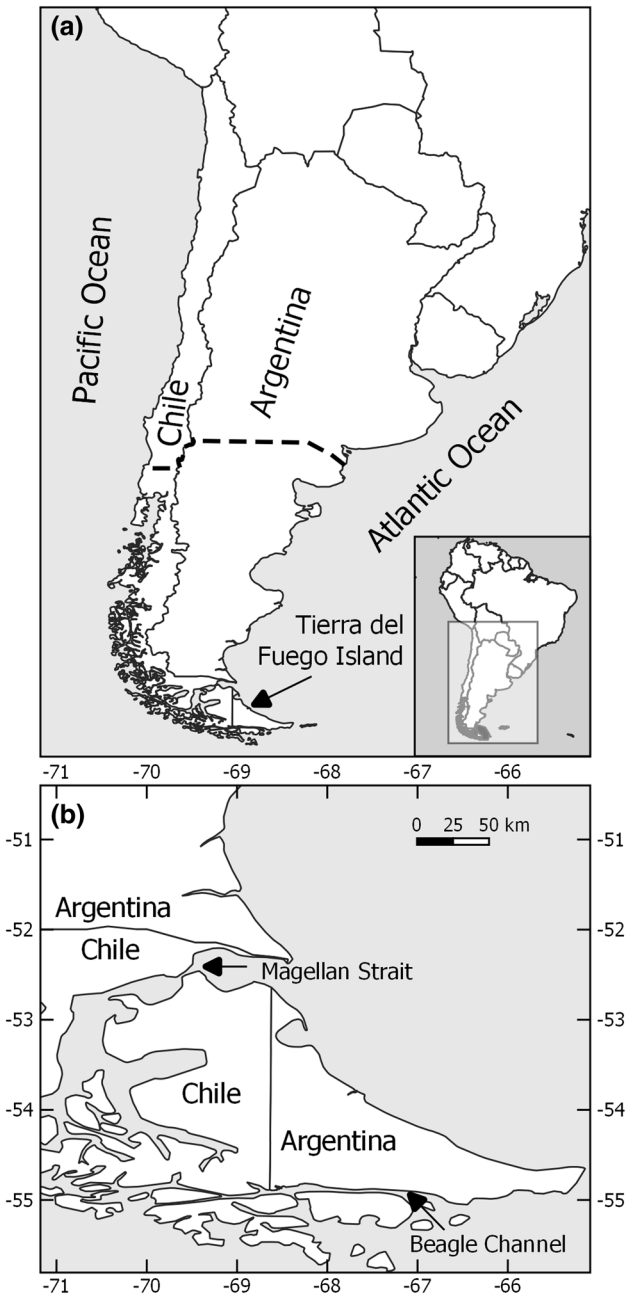


Fig. 1 Study area showing **a** Northern limit of Patagonia Region (dashed line) and, **b** Tierra del Fuego Island at the extreme south of Patagonia. Map created using QGIS 3.0.3 software

The knowledge of species presence and distribution is critical to developing management and conservation actions (Stem et al. 2005). However, one major limitation when determining the distribution of species by using conventional fishing gear (e.g., nets, traps, electrofishing, angler records) is the low detection rate of uncommon or endangered species because they are rare in the wild by definition, and also because detection requires taxonomic skills to identify them (Laramie et al. 2015; Mizumoto et al. 2017).

Environmental DNA (eDNA) is a relatively new approach used to detect the presence of species, even at low densities. Using this method, it is possible to detect species without actually seeing or catching them (Taberlet et al. 2018). The eDNA-based method has been successfully applied in several groups of aquatic species, such as amphibians (Evans et al. 2016), reptiles (Raemy and Ursenbacher 2018), and also fish (Roy et al. 2018). In this regard, several studies have used eDNA-based methods to detect different species of lampreys from water samples (Knebelsberger et al. 2014; Carim et al. 2016; Gingera et al. 2016; Bracken et al. 2019). Besides, since eDNA-based methods are a non-invasive tool, several works have been focused on the detection of endangered species using this approach (Carim et al. 2016; Mizumoto et al. 2017; Fernandez et al. 2018).

This study aims to gain insight into the presence and distribution of lampreys in Southernmost Patagonia, in rivers flowing into the Atlantic Ocean, where lampreys have been poorly studied, and in rivers flowing into the Beagle Channel, where the presence of lampreys have never been reported before. This work also expects to open the discussion on the possibility that *G. australis* is not the only lamprey species in Argentinean Patagonia, as previously reported.

Materials and methods

Bibliographic review

Due to the scarce information on the presence and distribution of lampreys in Patagonia, a bibliographic search was performed to review the available data in this region. An extensive literature search was carried out using Google Scholar covering all full publications in English and Spanish language between 1900 and 2018. A search strategy including a combination of keywords 'lamprey distribution Patagonia', 'lamprey distribution South America', and 'freshwater fishes Patagonia' was applied. All publications and their references were analyzed. Only publications where the lamprey captures and specific location were documented, were summarized. Neither personal communications nor unpublished data were considered as reliable records of lamprey.

Lamprey detection by eDNA

Primers design and validation

Two sets of primers were designed on the mitochondrial genome of *G. australis* using Primer 3.0 software (Koressaar and Remm 2007; Untergasser et al. 2012). For this, a sequence reported by Ren et al. (2016) obtained from an individual captured at the Oreti River, New Zealand, was used (Accession Number NC_029404). A set of primers was designed on the cytochrome b gene (cyt b) (10,431–11,606 bp) and the additional set on the cytochrome oxidase subunit I (COI) gene (1–1554 bp) (Table 1). To assess the reliability and specificity of the primers, an in silico and in vitro validation was performed, according to Nardi et al. (2019) (Online Resource 1). In brief, the in silico analysis was performed using the primer BLAST tool of the NCBI (www.ncbi.nlm.nih.gov//tools/primer-blast) and included all the species reported at the area: big and small puyen, chinook salmon, coho salmon, rainbow trout, brown trout, and brook trout (Cussac et al. 2016; Chalde et al. 2019; Nardi et al. 2019). Although not observed since reported by Moreno and Jara (1984), the galaxiids fish *Aplochiton taeniatus* and *A. zebra* were also included in the in silico analysis. For in vitro validation, total DNA was obtained from two lampreys (Lamprey 1 and 2) captured in Grande river basin (Menéndez River, TDF) in April 2015 and used as target DNA. Total DNA was also extracted from all the observed co-occurring non-target species (all the mentioned above, except for *Aplochiton spp*). Total DNA was extracted from muscle tissue using a DNeasy Blood and Tissue Kit (Qiagen, Germany), following the manufacturer's protocol. A dilution of 35×10^{-4} ng μL^{-1} was used for target and non-target templates in the specificity analysis, and four controls were established to check for cross-amplification. The amplification reactions were performed in triplicate by Real-Time PCR using a Step One Real-Time PCR System equipment (Applied Biosystems, USA) in a total volume of

Table 1 Set of primers designed on *Geotria australis* sequence reported by Ren et al. (2016), obtained from an individual captured at the Oreti River, New Zealand (Accession Number NC_029404)

Target gene	Forward primer 5′–3′	Reverse primer 5′–3′	Amplicon length (bp)
Cytochrome b (cyt b)	CCTACATACATCTCAACAA	GGTATTCTACTGGTTCAC	119
Cytochrome c oxidase subunit I (COI)	CCTCGTTCGTTGATTATTCTCC	GTTGGCTTAGTTCTGCTCGAAT	125

The cyt b primers did not amplify DNA of lampreys from Eastern Patagonia. The COI set of primers was further used for eDNA amplification

15 µL; including 1 × iTaq SYBR Green Supermix (Bio-Rad, USA); 0.85 µM of each primer and 1 µL of each template. Cycling conditions included a first denaturation step at 95 °C for 10 min and 40 cycles of a denaturation step at 95 °C for 15 s and an annealing/extension step at 60 °C for 1 min, followed by a melting curve from 60 °C to 95 °C at 0.3 °C increments. A cycle threshold (C_t) analysis was performed, and PCR products were visualized in 2% Low EEO agarose gels. The sequences obtained in the positive and specificity control were verified at the Macrogen Korea sequencing service.

Water sampling and eDNA extraction

A total of 21 environmental water samples were collected during early spring 2017 (September–October) with sterile bottles (1 L per site). Sample sites included seven basins flowing into the Atlantic Ocean and five basins flowing into the Beagle Channel (Fig. 2, Table 2). All of these rivers are located on the Argentinean section of the Island of TDF, except for Gallegos basin, which is the more austral basin in the continental region of Argentina. Water samples were maintained at 4–8 °C and filtered within the next 24 h using disposable sterile filter units of cellulose nitrate of 0.45-µm pore size until the full volume passed through. Filters were stored individually at − 80 °C until DNA extraction. eDNA was extracted using QiaShredder and Qiagen's DNeasy Blood & Tissue Kits (Qiagen GmbH, Germany) following the manufacturer's protocol with minor modifications, according to Goldberg et al. (2011). The extractions were done under sterile conditions in a laboratory unit exposed periodically to UV-light and where no other tissue samples were manipulated. One liter of milliQ water was filtrated, the eDNA extracted and further included in all analyses to confirm that contamination did not take place during filtration or eDNA extraction.

eDNA samples analysis

Environmental samples were analyzed for the possible presence of inhibitors of the PCR reaction, according to Goldberg et al. (2016). In brief, 1 µL of three different dilutions (1:10; 1:20 and 1:40) of the eDNA samples and a foreign DNA, known as "internal positive control" (IPC), were mixed in a tube. Then a PCR assay was performed to amplify the IPC. Samples were considered inhibition-free when the amplification plot of the IPC in the presence of the eDNA was comparable to that observed in the positive control (IPC without eDNA). Since eDNA samples were highly diluted (up to 1:40) to avoid inhibition, an amplification control was performed, according to Nardi et al. (2019). In brief, a fragment of 169 bp of the ribulose-1,5-bisphosphate carboxylase (rcbl) gene of diatoms inhabiting the area was used as amplification control. Only dilutions with no signs of inhibition and positive for the amplification control were further used for lamprey presence/non-detection analysis.

Lamprey detection by eDNA

For environmental samples amplification, the COI set of primers was used. A five-point standard curve was performed with DNA of target lamprey as template, including a serial dilution from 35×10^{-1} to 35×10^{-5} ng µL^{-1} to estimate the amplification efficiency of the primer pair. Standard curve fitted the equation $y = -3x + 27.336$; $R^2 = 0.992$. The efficiency of primers was 85–87%. Conditions of Real-Time PCR reaction were as previously described for this set of primers. Six replicates were performed per sample, including a negative water control in each run. One microliter of the diluted environmental sample was added as template. Amplification of at least two of the replicates, accompanied by a melting curve consistent with our target DNA ($T_m = 77.68$–78.13 °C) and sequencing at the Macrogen Korea service, was required to identify a positive detection of lamprey. Environmental sequences were edited and aligned using BioEdit Sequence Alignment Editor (Hall 1999).

Lamprey capture by electrofishing

In order to capture lampreys for genetic analysis, electrofishing surveys were performed in April 2018 in three eDNA-positive river basins: Gallegos, Grande, and Lapataia (Fig. 3). These basins were selected in order to represent the northern, central, and southern regions of the sampled area. For morphometric analysis, lampreys were captured by electrofishing in January 2019 and 2020 from Grande River. Gallegos basin is located in the southern region of the Santa Cruz province. It crosses Chilean and Argentine territory from the Andean mountain range at the west and the Atlantic Ocean at the east (51° 35′ 59″ S). It occupies an area of 19,306 km^2 with a mean annual flow of 39.1 m^3 s^{-1} and a mean annual air temperature of 6 °C (Diaz et al. 2017). Grande basin is located in the northern region of Tierra del Fuego Province. It also crosses Chilean and Argentine territory from the Andean mountain range at west to the Atlantic Ocean at the east (53° 47′ 17″ S). It occupies an area of 8580 km^2 with a mean annual flow of 45 m^3 s^{-1} and a mean annual air temperature of 5.6 °C (Iturraspe and Urciuolo 2007). Lapataia basin is born in Chilean glaciers located in Andean mountains, crosses the Acigami Lake of 22 km^2, and drains for 2 km across the Argentine territory, flowing into the Beagle Channel (54° 50′ 41″ S). It occupies an area of 540 km^2 with a mean annual flow of 18.7 m^3 s^{-1} and a mean annual air temperature of 5.4 °C (Niemeyer 1982; Iturraspe and Urciuolo 2007). We sampled a total of 61 sites in autumn

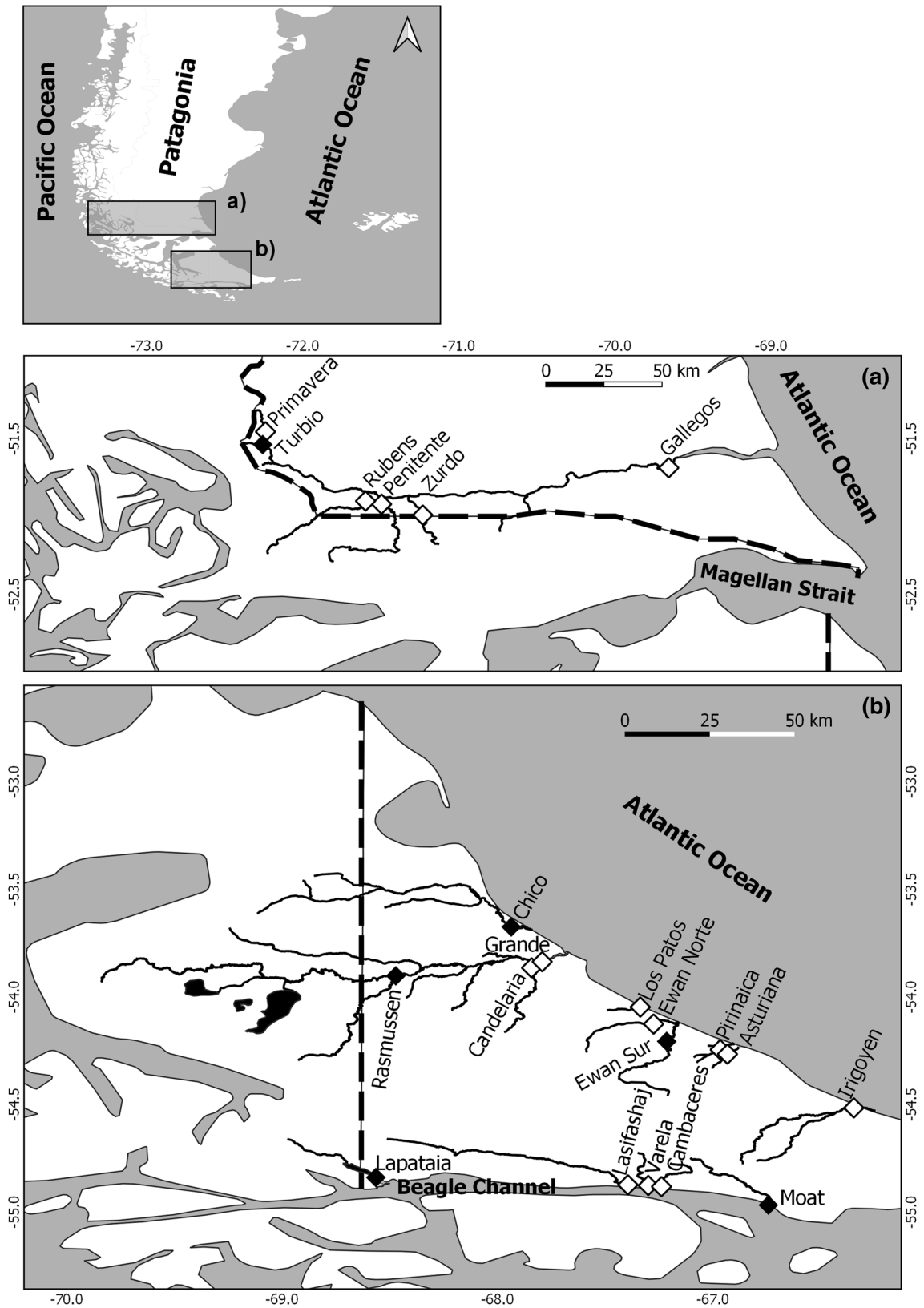


Fig. 2 Estimated presence/non-detection of lamprey in the study area based on the detection of environmental DNA (eDNA) in **a** Gallegos basin, Santa Cruz Province, and **b** Tierra del Fuego basins. Black and white squares indicate the presence and non-detection of lamprey, respectively. Dashed lines indicate the Argentine–Chilean border

Table 2 Results of the environmental DNA-based method for detection of lamprey

Basin	River	Outflowing	At the sample site		Distance from mouth (km)	Altitude (masl)	eDNA detection
			Latitude	Longitude			
Gallegos	Primavera	Atlantic	51° 31′ 32″ S	72° 16′ 03″ W	293	260	–
	Turbio	Atlantic	51° 32′ 36″ S	72° 14′ 01″ W	288	238	✓
	Rubens	Atlantic	51° 54′ 11″ S	71° 36′ 15″ W	205	115	–
	Penitente	Atlantic	51° 59′ 36″ S	71° 29′ 43″ W	216	127	–
	Zurdo	Atlantic	51° 59′ 36″ S	71° 13′ 55″ W	189	136	–
	Gallegos	Atlantic	51° 41′16″S	69° 39′ 20″ W	13	17	–
Chico	Chico	Atlantic	53° 40′ 25″ S	67° 56′ 07″ W	10	6	✓
Grande	Rasmussen	Atlantic	53° 54′ 04″ S	68° 28′ 17″ W	77	51	✓
	Candelaria	Atlantic	53° 51′ 49″ S	67° 50′ 28″ W	23	10	–
	Grande	Atlantic	53° 50′ 10″ S	67° 47′ 34″ W	15	6	–
Los Patos	Los Patos	Atlantic	54° 02′ 48″ S	67° 20′ 12″ W	2	7	–
Ewan	Ewan Norte	Atlantic	54° 07′ 33″ S	67° 16′ 29″ W	25	19	–
	Ewan Sur	Atlantic	54° 12′ 22″ S	67° 12′ 56″ W	20	25	✓
Ladrillero	Pirinaica	Atlantic	54° 14′ 50″ S	66° 57′ 58″ W	11	36	–
	Asturiana	Atlantic	54° 15′ 24″ S	66° 56′ 28″ W	13	42	–
Irigoyen	Irigoyen	Atlantic	54° 30′ 53″ S	66° 17′ 38″ W	0.4	4	–
Moat	Moat	Beagle Channel	54° 58′ 04″ S	66° 44′ 28″ W	0.8	10	✓
Cambaceres	Cambaceres	Beagle Channel	54° 52′ 54″ S	67° 14′ 20″ W	0.8	10	–
Varela	Varela	Beagle Channel	54° 52′ 31″ S	67° 18′ 08″ W	2	7	–
Lasifashaj	Lasifashaj	Beagle Channel	54° 52′ 22″ S	67° 23′ 38″ W	3	8	–
Lapataia	Lapataia	Beagle Channel	54° 50′ 32″ S	68° 33′ 51″ W	0.5	6	✓

(April). Among these, 23 sites corresponded to seven rivers of Gallegos basin; 19 sites corresponded to seven rivers of Grande basin, and the last 19 sites corresponded to Lapataia basin. All sites were sampled with a backpack electrofishing gear (LR-24 Electrofisher, Smith Root Inc, USA) set up at 75 Hz and 25% duty cycle used to produce 450-V, 75 Amps standard pulse (pulse width − 3 ms, 60 pulses s^{-1}).

Lamprey DNA extraction and PCR amplification

Total DNA from 14 lampreys was successfully extracted from muscle tissue using a DNeasy Blood and Tissue Kit (Qiagen, Germany), following the manufacturer's protocol. A PCR assay was used to amplify 1,100 bp of the mitochondrial cyt b gene, using the primers "Geotria496L" and "Phe1612H" for Geotriidae family reported by Lang et al. (2009). A reaction mixture containing 1 µL of total DNA (10 ng μL^{-1}), 1 unit of Go Taq DNA polymerase (Promega, USA), 1 × Go Taq polymerase buffer, dNTPs (0.2 mM of each) and forward and reverse primers (0.2 and 0.9 nM, respectively), was prepared. Cycling conditions were as follows: an initial denaturation of 5 min at 95 °C, 40 cycles of 30 s of denaturation at 94 °C, 40 s of annealing at 51 °C, and 80 s of extension at 72 °C, with a final extension of 5 min at 72 °C. PCRs were performed in a 2720 Thermal Cycler (Applied Biosystems, USA). Amplification products of the expected size were purified using a gel extraction kit (PuriPrep-GP; INBIO Highway, Argentina) and sequenced at Macrogen Korea service using the same primers as the amplification.

Phylogenetic analysis and genetic distances

The 14 cyt b gene sequences obtained were edited, contigs were assembled and aligned using BioEdit Sequence Alignment Editor (Hall 1999). The cyt b sequences from four individuals obtained in this study were deposited in GenBank (AN MK408981-4). Also, a sequence similarity search was performed using the Basic Local Alignment Search Tool (BLAST). Then, a matrix was constructed, including 41 sequences (875 bp) of the cyt b gene. From these, 38 sequences belonged to lamprey species reported by Lang et al. (2009); one sequence belonged to *G. australis* from New Zealand (AN NC_029404); one sequence corresponded to the cyt b sequence obtained in this study (Argentinean lamprey; AN MK408981); and one sequence was used as outgroup (*Myxine glutinosa;* AN AJ278504). From this matrix, a phylogenetic analysis was performed through three methods: Maximum Likelihood (ML), Bayesian Inferences (BI), and Maximum Parsimony (MP). ML analysis was carried out with the online

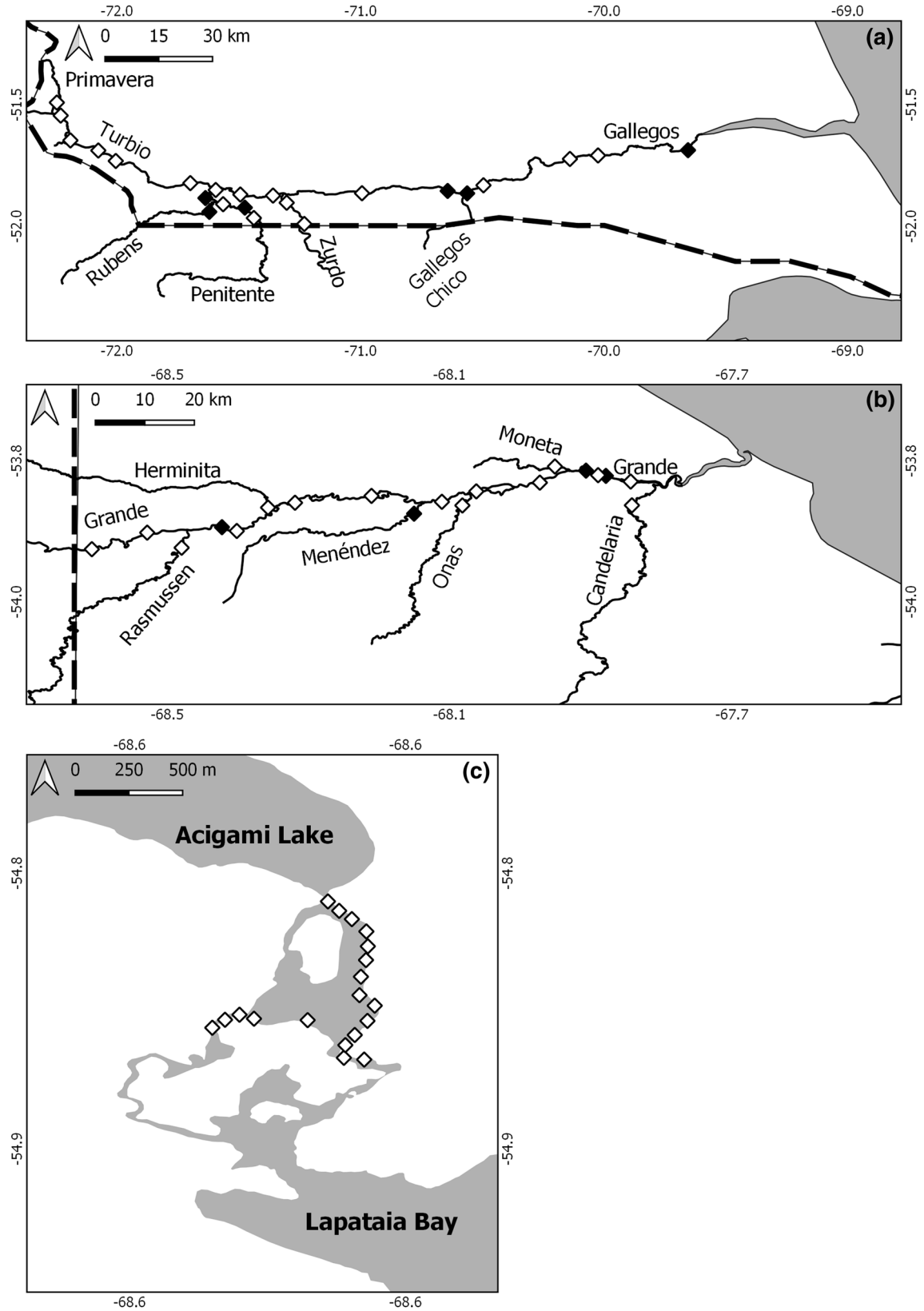


Fig. 3 Electrofishing survey sites at **a** Gallegos, **b** Grande, and **c** Lapataia basins. Black and white squares represent positive and negative sites for lamprey detection, respectively. Dashed lines indicate the Argentine–Chilean border

resource PhyML 3.0 (Guindon et al. 2010). The substitution model was determined by the Smart Model Selection (SMS) option (Lefort et al. 2017) through the Akaike Information Criterion (AIC). For branch support, bootstrap was performed with 1000 replicates. BI was performed on MrBayes 3.2.7 software (Ronquist et al. 2012). The substitution model was determined with jModelTest 3.7 (Posada 2008). The parameters set in MrBayes were 30,000,000 generations, nst = 6 and rates = invgamma; all other parameters were left on default settings. The branch support was expressed as a posterior probability in percentage. MP analysis was carried out with the software MEGA7 (Kumar et al. 2016) implemented with bootstrap, 1000 replicates, as branch support.

The genetic distances (Kimura 2-parameter distance) were estimated for the cyt b gene with the software MEGA7 (Kumar et al. 2016) and expressed as the proportion (p) of nucleotide sites at which two sequences are different for the intraspecific and interspecific level. For this analysis, bootstrap was implemented as the variance estimation method with 500 replicates. For the intraspecific level, the haplotypes distinguished among the 14 sequences from the Gallegos and Grande basins, were compared. For the interspecific level, the genetic distances were investigated at two instances: (a) among species from the same genus, and (b) among species from different genera. For the former analysis, a bibliographic revision was used; for the latter, one sequence per genus from the phylogenetic analysis was used. All the geographical representatives for *Geotria* (one from this work, Chile, New Zealand, and Australia), were included.

Morphometric analysis

After electrofishing, lampreys were transported alive to the aquarium facilities of the *Centro Austral de Investigaciones Científicas*. The total length (TL) and body weight (BW) were recorded to the nearest 0.1 mm and 0.01 g, respectively, and then used to calculate the condition factor with the equation $CF = \frac{BW}{TL^3} \times 10^6$ (Ricker 1975). The developmental stage of lampreys was assigned following the seven metamorphosing stages of *G. australis* proposed by Potter et al. (1980). Besides, a total of 11 morphometric measurements were made following Bird and Potter (1979) (See Fig. 5 for details) and used to compare with the measurements of *G. australis* from Donnelly and Warren Rivers in south-western Australia reported by Potter et al. (1980).

Results

Overview of the presence of lampreys in Patagonia

Two diadromous lampreys have been described for Chilean Patagonia: *G. australis* and *M. lapicida* (Neira 1984; Renaud 2011). Neira (1984), reported a distribution map that shows the presence of both species along Chile from 33° S to 41° S. The author also mentions the possible presence of *G. australis* on the South Pacific basins of the Chilean section of TDF. However, in this regard, we only found a study that reports the capture of one specimen of *G. australis* in a brook of the Chilean section of Tierra del Fuego (Norman 1937). The author gave no additional information on the location of the capture, nor if the brook was from a Pacific or Atlantic basin.

The first description of lampreys captured in Argentinian Patagonia was reported in Gallegos Basin by Eigenmann (1909), who mentioned the difficulties of finding lampreys in rivers of Patagonia. There is a more recent report on the presence of *G. australis* in the same basin at the estuary of the Gallegos River (Torres et al. 2006); however, no information on the number, size, or life stage of the individuals is given.

Ferriz et al. (1998) reviewed all publications of Patagonian fishes of Argentina, finding only one (unpublished) study performed specifically on lampreys (Nani 1950). In 2001, Azpelicueta et al. published a study on juveniles *G. australis* in Argentina, reporting the presence of this species in Negro and Chubut river basins. More recently, Aigo et al. (2008) and Cussac et al. (2016) made an extensive and interesting revision on the distribution of freshwater fishes of Patagonia reporting the presence of *G. australis* in four major basins of the region (Chubut, Gallegos, Negro, and Santa Cruz). Thus, taking into account only references where lampreys were captured and data on locations were provided, the presence of lampreys has been previously confirmed only in these four basins of Eastern Patagonia, extending from 41° S to 51° S (Table 3). Among these reports, that of Chubut River belongs to a juvenile individual found in a house water-pipe system in Chubut city (Table 3; Azpelicueta et al. 2001).

Primers validation for lamprey detection

The in silico analysis confirmed that both sets of primers showed specific alignment with *G. australis* mitochondrion genome (Accession Number NC_029404, New Zealand, Ren et al. 2016). Besides, no significant partial alignment was observed with any of the probable co-occurring species in the area. In vitro validation was achieved by Real-Time PCR using the target DNA (Lamprey 1 and 2, captured in Grande basin in 2015) and DNA of the species that have been reported at the area, as templates. Although several conditions were tested, it was not possible to obtain a positive result with the set of primers designed to amplify the cyt b gene. In contrast, a 125 bp amplicon was obtained with the COI set of primers. Amplification was only observed when the template/s included the target DNA (+ C and Specificity Control); while no amplification was observed when

Table 3 Streams with historical records on lampreys (reported as *Geotria australis*) captured in Argentina

Stream data[a]	Data on captures	Reference
Negro River Latitude at the mouth: 41° 01′ 23″ S Mean annual caudal: 972 m^3/s	Summer and Winter/near the river mouth/Sandy beach/larval stage	Mac Donagh (1936)
	Summer and Winter/448 km from the river mouth/14 specimens/buried about 10–60 cm in the muddy bottom/ juvenile stage/collected by hand	Azpelicueta et al. (2001)
	Spring/688 km and 250 km from the river mouth/low abundance/ larval stage/collected by electrofishing, seine net	Alvear et al. (2007)
Limay River (tributary of Negro River) Latitude at the mouth: 41° 01′ 23″ S Mean annual caudal: 736 m^3/s	693 km from the mouth/137 specimens/larval stage/Collected by electrofishing or by shovel and dip-nets	Neira (1988)
	Autumn/690 km from the river mouth/Sand and gravel/Collected by electrofishing	Baigún et al. (2002)
Chubut River Latitude at the mouth: 43° 20′ 27″ S Mean annual caudal: 35 m^3/s	One specimen/juvenile stage	Azpelicueta et al. (2001)
Santa Cruz River Latitude at the mouth: 51° 35′ 43″ S Mean annual caudal: 698 m^3/s	Autumn/195 and 55 km from the river mouth	Pascual et al. (2005)
Gallegos River and tributaries Latitude at the mouth: 51° 35′ 43″ S Mean annual caudal: 34 m^3/s	One adult and several individuals at larval stage[b]	Eigenmann (1909)

Data on captures include season/distance from the river mouth/number/habitat/developmental stage/fishing gear used. Not all data were available in all references

[a]Data from https://www.argentina.gob.ar

[b]Reported as *Geotria macrostoma galleguensis* and *Exomegas gill*

all templates except for the target DNA were added to the reaction (non-Specific Control) (see Online Resource 1 for details). No significant interference at the Ct values was observed when all the DNAs were present, in comparison to the Ct values obtained in the + C. The amplicons of the positive and specificity controls were sequenced, obtaining a 95% certainty of identity with *G. australis* COI gene. The COI set of primers was further used for the amplification of the environmental samples.

eDNA-based lamprey distribution map and environmental sequences analysis

Lamprey DNA was documented from 6 of the 21 sites sampled (Fig. 2; Table 2). The six positive sites corresponded to one tributary of the Gallegos basin in Santa Cruz Province and five river basins of TDF (Fig. 2; Table 2). An alignment was performed between the positive environmental amplicons, the positive amplification controls (genomic DNA of lamprey 1 and 2 as templates), and the COI gene sequence of *G. australis* (AN NC_029404; 1–1,554 bp). As a result, the environmental amplicons were a perfect match with lamprey 1 and 2 DNA, while these sequences were slightly different from the COI gene of *G. australis* with a 95–96% certainty of identity (Online Resource 2).

Phylogenetic analysis and genetic distances

For the genetic analysis, eight lampreys from Gallegos basin and six lampreys from Grande basin were captured, while no lampreys were captured in Lapataia River (Fig. 3, Table 4). Fourteen cyt b sequences were obtained, and the consensus sequence matched with *G. australis* genome (AN NC_029404; New Zealand), showing 85% certainty of identity. The phylogenetic analysis by the ML, MP, and IB methods resulted in trees with similar topologies. In the three of them, the cyt b sequences of lamprey captured in this study (Argentinean lamprey) conforms a clade with *G. australis* with a high support value (Fig. 4).

We were able to distinguish five haplotypes (H) among the 14 cyt b sequences. Haplotype 1 (H1) was the most frequent ($N = 10$) and was reported in both basins. A single sequence represented each of the additional haplotypes (H2–H5). The genetic distances for the cyt b sequences among haplotypes (intraspecific level) were 0.10–0.40% (Table 5a), and among the *G. australis* sequences were 0.20–1.90% (Table 5b). The genetic distances registered for the comparison between Argentinean lamprey and *G. australis* was 17.60% (Table 5b). After the bibliographic revision for lamprey species of the same genus, we observed a genetic distance of 0.20–5.70% (Table 6). Moreover, in the case of lamprey species from different genera, the registered value was 3.20–33.30% (Table 7).

Table 4 Sites location, length and developmental stage of the lamprey captured in Gallegos and Grande basins

Basin	River	Latitude	Longitude	Distance from mouth (km)	Length (mm)	*n*	Developmental stage[a]	Date of capture
Gallegos	Penitente	51° 55′ 11″ S	71° 29′ 43″ W	216	120–128	2	Juvenile	April 2018
	Rubens	51° 54′ 01″ S	71° 36′ 15″ W	205	31	1	Larval	April 2018
	Gallegos_1	51° 52′ 10″ S	71° 29′ 31″ W	203	35	1	Larval	April 2018
	Gallegos_2	51°53′37″S	71°35′52″W	185	38	1	Larval	April 2018
	Gallegos Chico	51° 51′ 43″ S	70° 33′ 59″ W	111	400	1	Adult	April 2018
	Gallegos3	51°41′16″S	69°39′20″W	13	30–35	2	Larval	April 2018
Grande	Menendez	53° 51′ 42″ S	68° 08′ 23″ W	40	111–113	3	Larval/Juvenile	April 2015
	Grande_1	53° 53′ 49″ S	68° 26′ 34″ W	72	62	1	Larval	April 2018
	Grande_2	53.82′ 09″ S	67° 88′ 25″ W	23	97	1	Juvenile	April 2018
	Grande_3	53° 48′ 42″ S	67° 53′ 56″ W	19	58–117	4	Larval/Juvenile	April 2018
	Grande	53° 49′ 11 ″S	67° 52′ 58″ W	19	110–118	2	Juvenile	January 2019
	Grande	53° 49′ 16″ S	67° 52′ 57″ W	19	92–104	2	Juvenile	January 2020

[a]In accordance with Docker (2015)

Morphometric analysis

We captured four individuals from Grande River that showed the key characters corresponding to developmental stages 4–5 described by Potter et al. (1980) (Table 4): (1) Eye with dark pupil and gray/silver iris; (2) Lateral and transverse lips of oral aperture fused; (3) Body with a grayish sheen; and (4) Much of ventral surface silver. The morphometric measurements and the comparison of the results obtained here with data reported by Potter et al. (1980) are shown in Fig. 5. The most notorious differences were the higher length and weight, the shorter branchial length, and the shorter gap between dorsal fins.

Discussion

Our bibliographic review revealed three main issues in relation to lampreys in Eastern Patagonia: (1) that *G. australis* is the only lamprey species reported; (2) that there are reliable reports on their presence in only three rivers basins (not considering the report on Chubut river that corresponds to a unique individual found in a water-pipe system), and (3) that the study of lampreys in this region has not been widely addressed since we found only one published study specifically on lampreys (Azpelicueta et al. 2001).

The eDNA-based method developed here allowed us to identify lamprey-positive basins in southernmost South America. However, questions arose about the identity of the COI environmental amplicons, since the sequences were slightly different from the COI gene of *G. australis*. For this reason, we decided to capture lampreys for genetic analysis, using the cyt b mitochondrial marker since there are a large number of available sequences in the databases. Besides, Mateus et al. (2013) have demonstrated that this marker is a powerful tool for lamprey species identification, even in cases of cryptic species. The cyt b sequences of lamprey reported here, were quite different compared to *G. australis* genome (82–85% certainty of identity), while no such difference was observed with the COI environmental amplicon (95% certainty of identity with *G. australis* genome). However, we must consider that this amplicon was only 80–90 bp. Also, the COI gene consists of several conserved regions alternate with variable regions (Kunal and Kumar 2013), and the fragment amplified in this study coincide with a conserved region.

Phylogenetic analysis revealed that Argentinean lamprey corresponds to a sister species to *G. australis*. Moreover, if we consider the percentage of genetic variation between Argentinean lamprey and *G. australis*, the value is surprisingly high (17.6%), even higher than expected among species of the same genus (Table 6). This result is more consistent with the genetic distances between species of different genera, with a mean value of 22.4%, as it can be observed from Table 7. The genetic distance analysis among the haplotypes of Argentinean lamprey agrees with an intraspecific variation if we compare it with the intraspecific values for *G. australis* from Chile, New Zealand, and Australia (Table 5a, b). Potter et al. (2015), suggested that *Geotria* would comprise at least two closely-related species rather than a single species. Our results suggest that the Argentinean lamprey may correspond not only to a new specific taxon but also that could represent a new monotypic genus in Geotriidae.

While this is the first report on genetic differences between lampreys from Argentina and *G. australis* from Chile and Australasia; several studies have previously reported morphological differences between them (Neira et al. 1988; Renaud 2011; Potter et al. 2015). In this study,

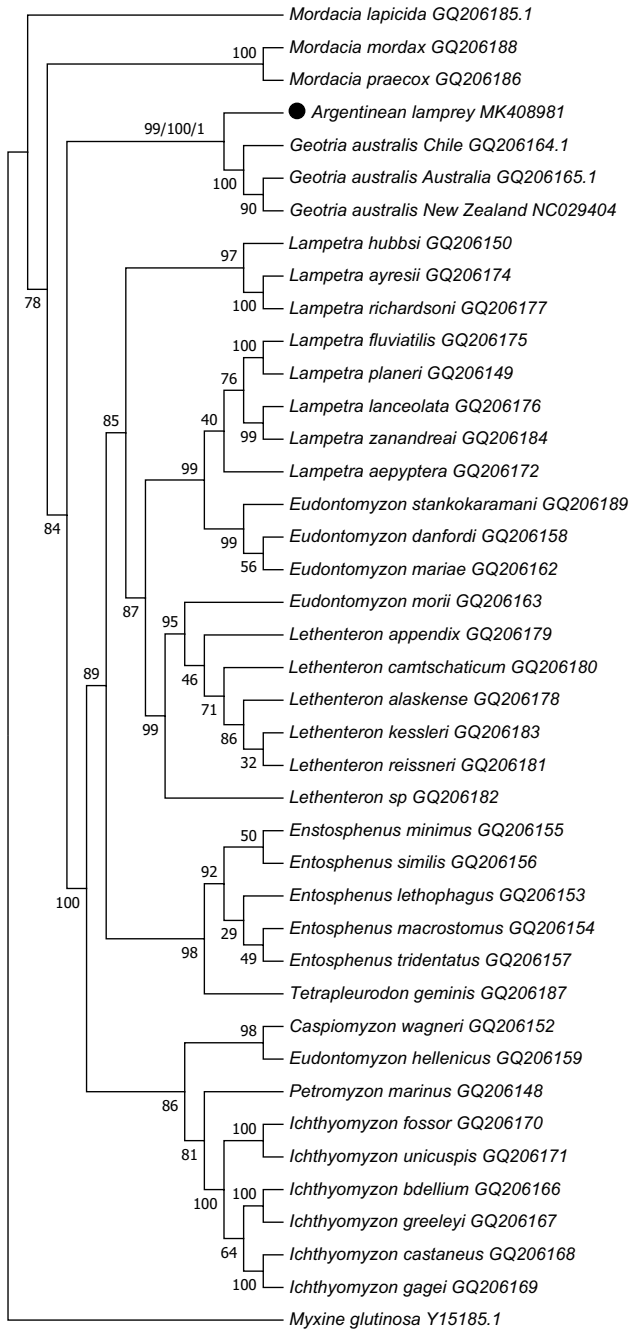


Fig. 4 Phylogenetic tree reconstruction of 41 taxa and 875 characters (cytochrome b gene) based on the Maximum Likelihood analysis. Maximum parsimony (MP) and Bayesian Inference (BI) yielded similar topologies. Bootstrap support values are indicated in the nodes. *Myxine glutinosa* was used as outgroup. Support values of MP (bootstrap support) and BI (posterior probability) are shown for the clade (*Geotria australis* + Argentinean lamprey; black dot)

we were able to analyze the morphology of only four lampreys in metamorphosis phase captured in Grande River in 2019 and 2020, since those captured in 2018 were not kept properly for the morphometric analysis. We found differences in some of the morphometric characteristics among Argentinean lampreys and data on juvenile *G. australis* reported by Potter et al. (1980) (Fig. 5). The most notorious differences were the higher length and weight of Argentinean lampreys. These results are in accordance with Azpelicueta et al. (2001), who reported lengths of 98.5 ± 9.1 mm (mean ± SD; $n = 13$) in lampreys in juvenile stage captured in Negro River (Argentina), being these values also higher than those reported by Potter et al. (1980). Additionally, Neira (1984) mentions that 85–90 mm is the size in which *G. australis* begins its metamorphosis, while we collected lampreys at larval stage that were 113 mm in length in Grande basin (Table 4).

Regarding morphological differences, we believe that more specimens are needed to reach conclusions since we were not able to obtain a large number of lampreys in the field surveys. The lack of success in capturing lampreys has been previously reported for Argentinean Patagonia. In this sense, several authors have mentioned the difficulty of capturing individuals and the low abundance of lampreys in rivers flowing into the Atlantic Ocean (Azpelicueta et al. 2001; Alvear et al. 2007; Cussac et al. 2016). Maybe other fishing gears should be tested since electrofishing has proven not being successful for lamprey capture, even in an eDNA-positive basin (Lapataia River).

Neira et al. (1988) reported several morphological differences between *Geotria* larvae from Argentina (Limay River) and *G. australis* larvae from Australia and Chile and suggested that gene exchange between them may be limited. Authors also suggested that *Geotria* from the Pacific and Atlantic coasts must show different migratory patterns, probably based on different currents and that these differences may have originated up to 6.5 million. years ago when a southern extension of the Andes cordillera between the tip of South America and the Antarctic peninsula restricted flow between the adjacent oceans. This hypothesis on the different currents and migration patterns is supported by the fact that *M. lapicida* does not extend from Chile around the base of South America into the rivers of Argentina (Hubbs and Potter 1971).

Finally, it is worth mentioning that *G. australis* is the sole lamprey species previously reported for Gallegos basin. However, all the individuals captured in this study from this basin were grouped as Argentinean lamprey, and the molecular evidence indicates that this lamprey corresponds to a new species, different from *G. australis*. Taking these data together, we believe that the species assignment of lampreys from rivers of Northern Patagonia, where *G. australis* is reported, should be revised.

Table 5 (a) Genetic divergences (Kimura 2-parameter distance) between the haplotypes (H) of Argentinean lamprey (below diagonal) and its standard deviation (above diagonal). (b) Genetic divergences (Uncorrected *p* distances) between all the *Geotria australis* considered in this study and one sequence of Argentinean lamprey (MK408981) (below diagonal) and its standard deviation (above diagonal)

(a)	1	2	3	4	5
1 Argentinean lamprey—H1		0.001	0.001	0.001	0.001
2 Argentinean lamprey—H2	0.001		0.001	0.002	0.002
3 Argentinean lamprey—H3	0.001	0.002		0.001	0.002
4 Argentinean lamprey—H4	0.002	0.003	0.001		0.002
5 Argentinean lamprey—H5	0.002	0.003	0.003	0.004	

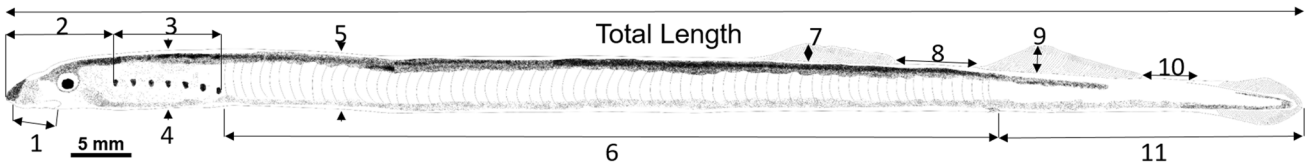
(b)	1	2	3	4
1 *G. australis* Chile		0.004	0.004	0.016
2 *G. australis* New Zealand	0.019		0.002	0.017
3 *G. australis* Australia	0.016	0.002		0.017
4 Argentinean lamprey	0.176	0.176	0.176	

Table 6 Genetic divergences between species of the same genus compiled from the bibliography

Compared species	Genetic divergence (%)	Source
Lampetra planeri/L. lusitanica	0.8–1.2	Mateus et al. (2013)
Lampetra planeri/L. auremensis	0.3–0.9	Mateus et al. (2013)
Lampetra planeri/L. alavariensis	0.4–1.1	Mateus et al. (2013)
L. pacifica/L. richardsoni	2.8–3.2	Reid et al. (2011)
Lampetra spp./any known species	2.3–5.7	Boguski et al. (2012)
Eudontomyzon hellenicus/E. graecus	0.8	Lang et al. (2009), Mateus et al. (2013)
Lethenteron kessleri/L. reissneri	0.2	Lang et al. (2009), Mateus et al. (2013)
Lethenteron appendix/L. alaskense	0.9	Lang et al. (2009), Mateus et al. (2013)

Table 7 Genetic divergences (Kimura 2-parameter distance) between lamprey species from different genera (below diagonal) and its standard deviation (above diagonal)

		1	2	3	4	5	6	7	8	9	10
1	*Entosphenus minimus* GQ206155		0.021	0.022	0.017	0.011	0.011	0.022	0.015	0.006	0.015
2	*Geotria australis* GQ206165.1	0.288		0.016	0.024	0.022	0.023	0.023	0.022	0.022	0.024
3	*Argentinean lamprey* MK408981	0.283	0.176		0.024	0.023	0.022	0.022	0.022	0.022	0.023
4	*Ichthyomyzon bdellium* GQ206166	0.193	0.323	0.311		0.017	0.016	0.022	0.015	0.017	0.017
5	*Lampetra aepyptera* GQ206172	0.104	0.308	0.292	0.194		0.009	0.021	0.014	0.012	0.015
6	*Lethenteron alaskense* GQ206178	0.098	0.298	0.288	0.168	0.072		0.022	0.014	0.012	0.015
7	*Mordacia lapicida* GQ206185.1	0.306	0.322	0.304	0.333	0.302	0.310		0.022	0.015	0.023
8	*Petromyzon marinus* GQ206148	0.148	0.304	0.285	0.144	0.156	0.146	0.309		0.014	0.014
9	*Tetrapleurodon geminis* GQ206187	0.032	0.294	0.271	0.185	0.114	0.106	0.297	0.152		0.015
10	*Caspiomyzon wagneri* GQ206152	0.148	0.314	0.284	0.189	0.166	0.157	0.322	0.134	0.151	



Measurement	Lamprey 1	Lamprey 2	Lamprey 3	Lamprey 4	Mean ± SD	Potter et al. (1980) ± 95 % IC
Total Length (mm)*	118.3	110.8	92.3	104.4	106.5 ± 11.0	87 - 94
Weight (g)*	1.67	1.45	0.85	1.27	1.31 ± 0.35	0.73 - 0.92
Condition Factor	1.01	1.07	1.08	1.12	1.07 ± 0.05	0.95 - 1.2
1. Disc	1.98	2.08	2.42	2.29	2.19 ± 0.02	1.9 - 2.3
2. Prebranchial length	8.20	8.90	9.54	8.52	8.79 ± 0.58	7.6 - 8.9
3. Branchial length*	7.60	8.10	8.62	8.58	8.23 ± 0.48	9.5 - 10.7
4. Branchial depth	5.00	5.30	4.96	4.87	5.03 ± 0.19	4.6 - 5.1
5. Trunk depth	4.70	5.20	4.72	4.82	4.86 ± 0.23	4.7 - 5.1
6. Trunk length	60.90	62.20	56.90	58.29	59.57 ± 2.41	57.5 - 59.5
7. Height of 1st dorsal fin	1.08	1.46	0.77	0.86	1.04 ± 0.31	0.75 - 1.45
8. Gap between dorsal fins*	7.70	7.00	8.02	6.69	7.35 ± 0.1	7.8 - 8.8
9. Height of 2nd dorsal fin	1.80	2.80	2.17	1.11	1.97 ± 0.71	1.4 - 2.2
10. Gap between 2nd dorsal and caudal fins	4.50	3.80	3.03	3.93	3.82 ± 0.60	3.0 - 4.4
11. Tail length	23.90	23.40	24.37	23.98	23.91 ± 0.40	23.0 - 24.7

Fig. 5 Total length (mm), weight (g), condition factor and body proportions (%TL) of lampreys captured in Grande River, and range of measurement data of *Geotria australis* from Donnelly and Warren Rivers in south-western Australia. All data correspond to individuals in developmental stages 4–5 following Potter et al. (1980). Asterisks show the most notorious differences between the morphometric measurements of lampreys captured in Grande River and Australia

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All sampling procedures and animal manipulation followed the local regulation by the Institutional Committee for the Care and Use of Laboratory Animals of National University of San Martín, https://www.unsam.edu.ar/investigacion/cicuae.asp.

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