environments

Brief Report

The Presence of Lampreys in the Tyrrhenian Rivers of the Campania Region (Southern Italy): A New Record of the Sea Lamprey *Petromyzon marinus* (Linnaeus 1758)

Caterina M. Antognazza [1,*], Alberto Gentile [2], Giuseppe Crosa [1] and Serena Zaccara [1]

[1] Department of Theoretical and Applied Sciences, University of Insubria, Via J.H. Dunant 3, 21100 Varese, Italy; giuseppe.crosa@uninsubria.it (G.C.); serena.zaccara@uninsubria.it (S.Z.)
[2] Omnia Ecosystem Srl, Via Roma 54, 84086 Roccapiemonte, Italy; gentile.alberto79@gmail.com
* Correspondence: caterina.antognazza@uninsubria.it; Tel.: +39-0332-421422

Abstract: The southern Italian peninsula has been suggested to be an important European district for lampreys' genetic diversity. All lamprey species ever described throughout the Italian peninsula are protected within European legislation and listed in Annex II of the EU Habitats Directive (92/43/EEC) and Annex III of the Bern Convention (82/72/CEE) as species of conservation concern, and the Habitats Directive ensures the designation of "sites of community interest" (SICs) for threatened species. During a survey to collect preliminary data on lampreys' presence in the Cilento, Vallo di Daino, and Alburni National Park (PNCV) located in the Campania region, where 28 sites of community interest (SICs) have been established by the EU Habitats Directive (92/43/EEC), two specimens of sea lamprey (*Petromyzon marinus*, Linnaeus, 1758) were detected for the first time. The specimens were genetically characterized through the sequencing of the mtDNA control region locus. The study highlighted the significant importance of the Campania region for lampreys, which, concerning *Lampetra* sp., was found to have peculiar genetic characteristics and unique alleles that have not been described elsewhere. Furthermore, the recognition of the sea lamprey, *P. marinus*, emphasized the value of this area, especially in terms of laying the groundwork for future habitat protection strategies.

Keywords: conservation; control region; *Lampetra* sp.; mtDNA; *Petromyzon marinus*; southern Italy

Citation: Antognazza, C.M.; Gentile, A.; Crosa, G.; Zaccara, S. The Presence of Lampreys in the Tyrrhenian Rivers of the Campania Region (Southern Italy): A New Record of the Sea Lamprey *Petromyzon marinus* (Linnaeus 1758). *Environments* **2023**, *10*, 125. https://doi.org/10.3390/environments10070125

Academic Editor: Yiannis G. Matsinos

Received: 1 June 2023
Revised: 6 July 2023
Accepted: 17 July 2023
Published: 18 July 2023

1. Introduction

Of the 41–45 recognized species of lamprey [1–3], only 4 have been found in the Italian peninsula: the stream lamprey, *Lampetra planeri* (Bloch, 1784); the river lamprey, *Lampetra fluviatilis* (Linnaeus, 1758); the sea lamprey, *Petromyzon marinus* (Linnaeus, 1758); and the Po brook lamprey, *Lampetra zanandreai* (Vladykov, 1955). All species are protected within European legislation and listed in Annex II of the EU Habitats Directive (92/43/EEC) and Annex III of the Bern Convention (82/72/CEE) as species of conservation concern [4]. The Habitats Directive ensures the conservation of a wide range of endemic, rare, or threatened species, with European Union Member States required to designate "sites of community interest" (SICs) [5].

There are two sedentary species: *L. zanandreai*, restricted to the north of the Po River, and *L. planeri*, identifiable in the basins south of the Apennine chain, which guarantees its reproductive isolation [6], while *P. marinus* and *L. fluviatilis* are anadromous species that complete the life history migrating from the sea habitat to the upstream of rivers [7], a complex life cycle that is exposed to more environmental pressures because of the need to use both marine and freshwater ecosystems. In addition, both species are parasitic, allowing them to spread thanks to passive transport on hosts, often capable of long migration [8], and promoting genetic homogeneity and a lack of natal homing, especially for *P. marinus* [8,9].

L. planeri is considered a "sister" species of *L. fluviatilis*, morphologically similar in the larval and juvenile stages but with marked biological differences in adults, the former confined to freshwater and the latter anadromous (Zanandrea, 1959). Despite these biological differences, there has been much discussion in the literature about whether these two types are unique species or simply different life forms of a single species [10]. Indeed, little or no genetic divergence at the mitochondrial level [11] and gene flow observations support the idea that this *Lampetra* species is in fact a single species [12].

Specific to the Italian peninsula, structured populations of the anadromous *L. fluviatilis* are thought to be present in the south, specifically in the Bussento River (Campania region) [13], but without any certain record, and some reproductive populations have been described in the Magra River (Liguria) [14]; thus, *L. fluviatilis* in Italy is classified as regionally extinct [15]. The southern Italian peninsula has been suggested to be an important European district for *Lampetra* sp. genetics, and further investigations are desirable to potentially clarify the ambiguous status of this species pair [10] and verify the presence of stable populations.

P. marinus used to be widely distributed in both the Tyrrhenian and Adriatic sides since the mid-twentieth century [16]. Currently, stable presence is restricted to the Ligurian Sea, close to Magra River mouth [17], and sporadic records have been recently documented in both the Tyrrhenian Sea (Lazio region) [18] and Adriatic Sea [19], increasing interest in further investigations into the southern Italian peninsula. It is, thus, currently listed in the IUCN Italian Red list of threatened vertebrate species as critically endangered [20].

In the Campania region (Southern Italy), several national and regional parks are present, in which designated conservation areas and fauna biomonitoring are periodically actualized. More specifically, in the Cilento, Vallo di Daino, and Alburni National Park (PNCV) located in the Campania region, 28 sites of community interest (SICs) have been established by the EU Habitats Directive (92/43/EEC) [4] in order to maintain or restore the natural habitats, protecting threatened, endemic, or rare species, such as lampreys. Nonetheless, there is a lack of information of the real status of lamprey populations. Therefore, the aim of this study was to collect preliminary data on lampreys' presence in three Campanian rivers, flowing in the national parks and genetically characterized through the sequencing of the mtDNA control region locus.

2. Materials and Methods

A total of 33 adult lampreys have been successfully recorded and collected during a sampling campaign performed between November 2020 and January 2021 in Sele and Bussento basins. In detail, for Sele basin, there were sampling sites on Sele River and one site on Calore River, which is an important tributary of Sele basin, and in Bussento River, there were two sites (Table 1; Figure 1). A portion of the tail of each individual was stored in 98% ethanol until processing. Extraction of genomic DNA was completed through a proteinase K (EUROCLONE, Pero, MI, Italy) digestion, followed by sodium chloride extraction and ethanol precipitation [21]. A total of 599 base pairs (bp) of the control region (non-coding region I according to [22]), were amplified using the primer pair LampFor (ACACCCAGAAACAGCAACAAA) and LampRev (GCTGGTTTACAAGACCAGTGC) [9]. Polymerase chain reaction (PCR) amplifications were performed with Multiplex PCR kit (QIAGEN Italia, Milan, Italy) in 10 µL reaction volume containing approximately 10 ng of template DNA and 0.2 µM primer pair.

Table 1. Sampling locations. River, basin, site code, number of individuals sampled (*n*), successfully sequencing individuals (*n*), geographic coordinates, and haplotype distribution are indicated.

						Haplotype Distribution							
						***Lampetra* sp. ***							***P. marinus***
River	**Basin**	**Site Code**	***n***	**Geographic Coordinate**	***n***	**Lamp1**	**Lamp2**	**Lamp3**	**Lamp4**	**Lamp5**	**Lamp6**	**Lamp7**	**PMVG8 ¥**
Calore	Sele	Cl6	6	40.541464°; 15.062329°	6		1	1	1	1	1	1	
Sele	Sele	Sl1bis	2	40.785339°; 15.239226°	2	2							
		Sl1	8	40.741105°; 15.241834°	8	7	1						
		Sl4	2	40.621727°; 15.184806°	1	1							
		Sl5	8	40.537989°; 15.031528°	7	3		2					2
Bussento	Bussento	BU3	2	40.133772°; 15.543976°	2		1		1				
		BU5	5	40.075422°; 15.506300°	5		2			3			
		Tot	33		31	13	5	3	2	4	1	1	2

* GenBank accession number: OR095182-OR095188. ¥ GenBank accession number: EF565737 [11].

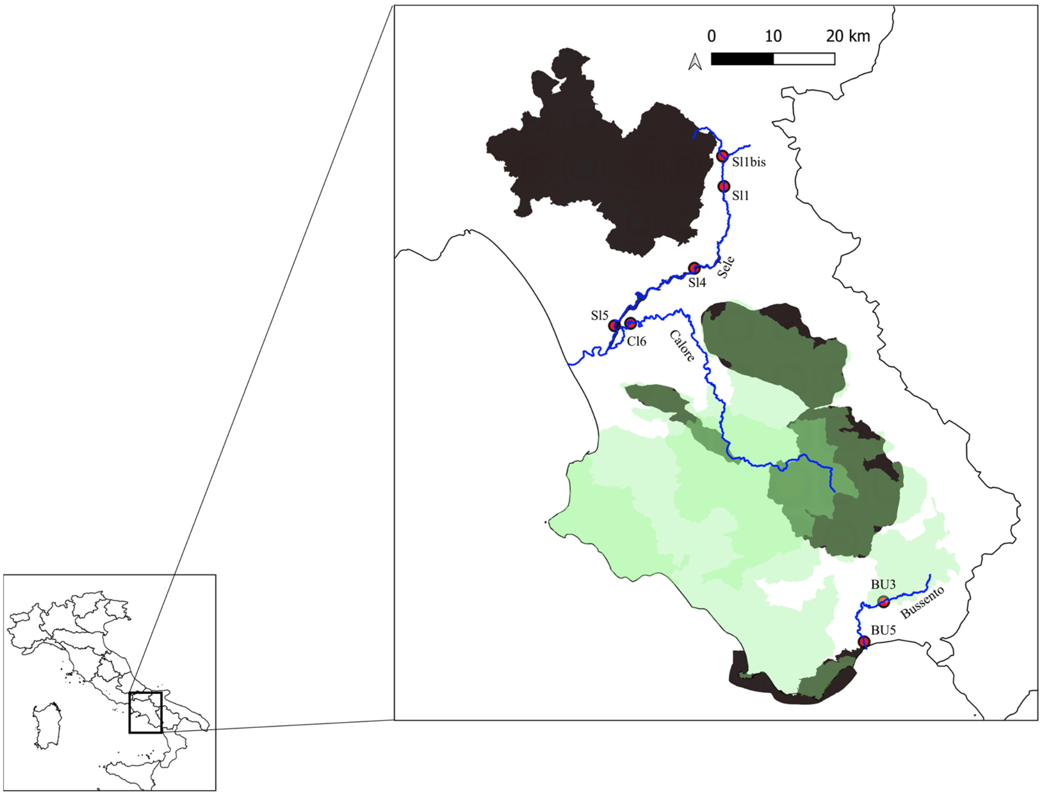

Figure 1. Map of sampling sites on Bussento River, Sele River, and Calore River. Site code refers to Table 1. Green background indicates Cilento, Vallo di Daino, and Alburni National Park (PNCV). Dark areas indicate SICs and ZPSs (Special Protection Zones) included in the sampled basins.

Thermal cycling was performed as follows: denaturation of 15 min at 95 °C, followed by 30 cycles of 94 °C for 60 s, 60 s at 55 °C of annealing temperature, the extension step at 72 °C for 60 s, and the final elongation at 72 °C for 10 min. PCR products were purified using the EuroSAP kit (PCR Enzymatic clean-up kit- EUROCLONE, Pero, MI, Italy) and sequenced in both directions at Macrogen Inc (Milano, Italy).

Sequences were manually aligned using BioEdit v. 7.1.3.0 [23] to eliminate ambiguities and check polymorphic sites. DNA polymorphism indices, like haplotype (H) and nucleotide (π) diversity, were calculated using DnaSP v. 5 [24].

The taxonomic attribution was performed through the genetic affinity with sequences from GenBank database; sequences selected using the BLASTn technique (https://blast.ncbi.nlm.nih.gov (8 May 2023)), with percentage of identity $\geq$ 99%, and published in a peer review study, have been included in the multiple alignment (Supplementary Table S1). The uncertainty and the still ongoing debate about the phylogenetic relationship and species status of the two paired species, *L. planeri* and *L. fluviatilis*, led to a lack of clear nomenclature for haplotypes deposited in Genbank. Therefore, since the aim of the study was not to contribute toward disentangling this issue, no final taxonomic classification has been made in case of the presence of these two species. Relationships among haplotypes were analyzed with a parsimony network estimated by the software TCS v. 1 [25].

3. Results and Discussion

Sequencing was successful in 31 individuals, while it failed for 2 individuals collected at two stations on the Sele River (samples Sl4_1 and Sl5_7) (Table 1). Two individuals caught in the most downstream station in the Sele River (SI5 site) were classified as *P. marinus* (Sl5_6 and Sl5_8) due to a complete overlapping of the principal and widely distributed European haplotype PMVG8 (GenBank Accession No. EF565737), detected in rivers from Germany to Portugal [9], and recently described in the Adriatic Sea [19]. This haplotype retrieved in the Sele River is thus included in the star-like structure grouping the European sea lamprey, as described in Petetta [19].

All 29 lampreys blasted in Genbank resulted in sequences more similar to *L. fluviatilis* haplotypes, according to the BLAST technique, and they showed seven unique haplotypes with a haplotype diversity (H) of 0.76 and nucleotide diversity (π) of 0.024 (Table 1). Five variable sites were detected, and an indel of 39 bp was detected in the two most represented haplotypes (Lamp1 and Lamp2). This indel has been treated as a transversion following Pereira [26]. The new sequences of the control region were deposited in GenBank database under the following accession numbers: OR095182-OR05188.

The geographic distribution of haplotypes evidenced by Lamp1, the most frequent in this study, is localized exclusively in the Sele River and not in its tributary (Calore River) or in the Bussento basin. On the contrary, Lamp2 was detected in four localities: in the Calore, Sele, and Bussento Rivers (Table 1; Figure 2). In the Sele River, three haplotypes were recorded, reporting values of genetic variability (H = 0.67 and π = 0.11) lower than the values recorded in the Calore River (H = 0.8 and π = 0.0028), in which six haplotypes were displayed, two of which were unique to this river (Table 1). In the Bussento River, three haplotypes were recorded (Lamp2, Lamp4, and Lamp5), showing comparable values of genetic variability found in the Sele River (H = 0.67 and π = 0.0011).

The relationships among haplotypes are represented in the TCS network (Figure 2). The haplotypes Lamp3 and Lamp4 were linked to the central haplotype Lamp5 by one mutational step, while the two haplotypes Lamp6 and Lamp7, private in the Calore River, are both separated by two mutational steps (Figure 2). In general, the observed Italian haplotypes are in a star-like structure around haplotype Lamp5 and grouping separately from Genbank haplotypes, where haplotype LPLAGI14 [26] acts as a link. The haplotypes displaying the 39 bp indel (Lamp1 and Lamp2) were connected to haplotype Lamp3 by one and two mutational steps, respectively, and three mutational steps separated them from the other haplotypes selected by the BLAST technique in Genbank (Figure 2).

These results reported, based on the sequencing of the mitochondrial control region, the presence of *P. marinus* in the Campania rivers for the first time. Despite the low number of individuals, these two specimens represent the only individuals of *P. marinus* analyzed in rivers flowing in the Tyrrhenian Sea. Only two additional individuals have ever been genetically identified in an Italian waterbody, but offshore, on the Adriatic side [19]. The presence of stable populations is yet to be determined, and considering their complex lifecycles, national legislation should also take into account that their vulnerability is likely to increase with climate change due to predicted hydrological alterations that result from more unpredictable precipitation patterns [27]. The implementation of genetic studies on more specimens is necessary to draw robust inferences from the genetic population of *P. marinus* in the Tyrrhenian Sea. Indeed, the detection and protection of *P. marinus* populations and spawning sites would promote the conservation of this species, not only in the Italian peninsula, but also across the Mediterranean Sea.

The analyses based on the mitochondrial control region revealed a hotspot of genetic diversity of *Lampetra* sp. In the Campania region, in particular in the Calore River, thus suggesting a good state of conservation and suitable management action in the Campanian SICs adopted to date.

Further studies expanding into other basins are necessary to assess the actual status of *Lampetra* sp. in order to establish the potential presence of stable populations and to shed more light on the presence of *P. marinus*. The collection and inclusion of more populations in the Campania region and the addition of more genetic markers might also help gain knowledge about the genetic diversity of *L. fluviatilis* and its sister species, *L. planeri*, which was not possible to clarify in this study.

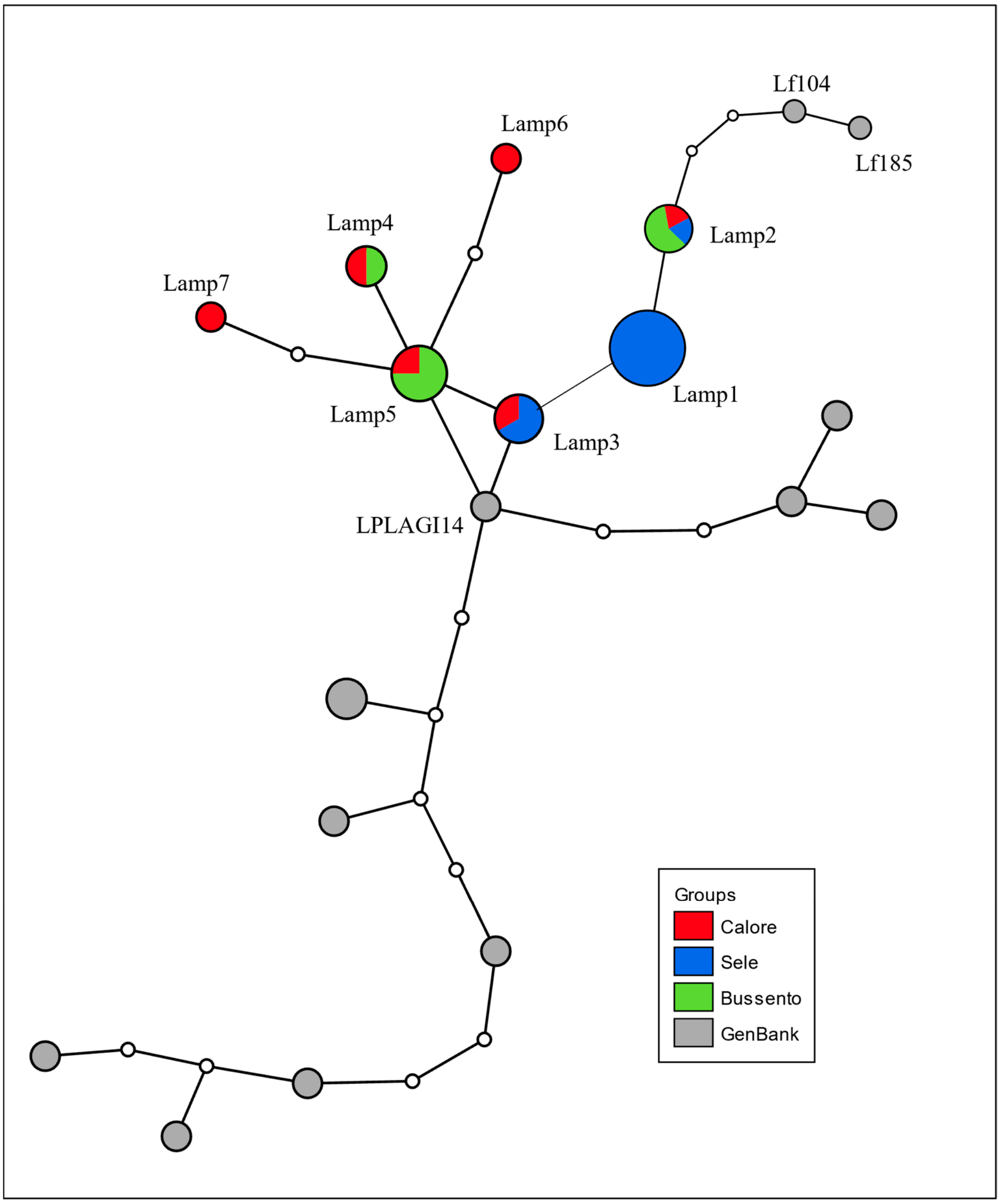

Figure 2. Minimum spanning network of *Lampetra* sp. haplotypes based on mtDNA control region (599 bp in length) and GenBank sequences based on BLASTn match (*cf* Supplementary Table S1). Each circle represents one haplotype, and the sizes of circles are proportional to the number of individuals sharing the same haplotype. White small circles represent missing haplotypes (mutational steps).

4. Conclusions

In conclusion, this study highlighted the significant importance of the Bussento River and Sele basin for lampreys, which, concerning *Lampetra* sp., were found to have peculiar genetic characteristics and unique alleles never described elsewhere. Furthermore, the recognition of the sea lamprey, *P. marinus*, in the Sele River emphasized the value of this area, especially in terms of laying the groundwork for future habitat protection strategies so that the presence of lampreys in these basins and, in general in southern Italy, is favored.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/environments10070125/s1, Table S1: List of sequences of *Lampetra fluviatilis* selected from GenBank. Accession number, voucher, origin, and referenced study are indicated [10,22,26].

Author Contributions: Conceptualization, S.Z. and A.G.; methodology, C.M.A. and S.Z.; software, C.M.A.; validation, C.M.A., S.Z., A.G. and G.C.; formal analysis, C.M.A.; resources, A.G. and S.Z.; data curation, C.M.A. and S.Z.; writing—original draft preparation, C.M.A.; writing—review and editing, C.M.A., S.Z., A.G. and G.C.; visualization, C.M.A., S.Z., A.G. and G.C.; supervision, S.Z.; project administration, S.Z., A.G. and G.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data used in this study not provided as a Supplementary Materials or deposited on GenBank database are available upon reasonable request to the corresponding author.

Acknowledgments: The authors would like to thank for their collaboration the Directorate General for Agricultural, Food and Forestry Policies of the Campania Region and the Food Safety Coordination Department of the Southern Experimental Zooprophylactic Institute of Portici for their technical–administrative collaboration. The authors would also like to thank the Cilento, Vallo di Diano, and Alburni National Park Authority for the authorizations granted.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Docker, M. Bill Beamish's contributions to lamprey research and recent advances in the field. *Guelph Ichthyol. Rev.* **2006**, *7*, 1.
2. Potter, I.C.; Gill, H.S.; Renaud, C.B.; Haouchar, D. The taxonomy, phylogeny and distribution of lampreys. In *Lampreys: Biology, Conservation and Control*; Docker, M., Ed.; Springer: Berlin/Heidelberg, Germany, 2015; pp. 35–73.
3. Riva-Rossi, C.A.-O.; Barrasso, D.A.-O.; Baker, C.; Quiroga, A.P.; Baigún, C.; Basso, N.G. Revalidation of the Argentinian pouched lamprey *Geotria macrostoma* (Burmeister, 1868) with molecular and morphological evidence. *PLoS ONE* **2020**, *15*, e023379. [CrossRef] [PubMed]
4. Directive, H. Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. *Off. J. Eur. Union* **1992**, *206*, 50.
5. La Posta, A.; Duprè, E.; Maggiore, A.M.; Tartaglini, N. La Rete Natura 2000 in Italia: Un patrimonio di biodiversità da gestire/conservare e monitorare. *Plant Sociol.* **2007**, *44* (Suppl. S1), 49–55.
6. Tagliavini, J.; Gandolfi, G.; La Fata, I.; Zerunian, S. Caratterizzazione di lamprede di bacini dei versanti tirrenico e adriatico dell'Italia centrale con analisi del DNA mitocondriale. *Biol. Ambient.* **2007**, *21*, 113–117.
7. Morais, P.; Daverat, F. History of fish migration research. In *An Introduction to Fish Migration*, 1st ed.; Taylor & Francis Group: Boca Raton, FL, USA, 2016; p. 11.
8. Silva, S.; Vieira-Lanero, R.; Barca, S.; Cobo, F. Densities and biomass of larval sea lamprey populations (*Petromyzon marinus* Linnaeus, 1758) in north-western Spain and data comparisons with other European regions. *Mar. Freshw. Res.* **2016**, *68*, 116–122. [CrossRef]
9. Almada, V.C.; Pereira, A.; Robalo, J.; Fonseca, J.; Levy, A.; Maia, C.; Valente, A. Mitochondrial DNA fails to reveal genetic structure in sea-lampreys along European shores. *Mol. Phylogenet Evol.* **2008**, *46*, 391–396. [CrossRef]
10. De Cahsan, B.; Nagel, R.; Schedina, I.; King, J.J.; Bianco, P.G.; Tiedemann, R.; Ketmaier, V. Phylogeography of the European brook lamprey (*Lampetra planeri*) and the European river lamprey (*Lampetra fluviatilis*) species pair based on mitochondrial data. *J. Fish Biol.* **2020**, *96*, 905–912. [CrossRef] [PubMed]
11. Espanhol, R.; Almeida, P.R.; Alves, M.J. Evolutionary history of lamprey paired species *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch) as inferred from mitochondrial DNA variation. *Mol. Ecol.* **2007**, *16*, 1909–1924. [CrossRef] [PubMed]
12. Rougemont, Q.; Gaigher, A.; Lasne, E.; Côte, J.; Coke, M.; Besnard, A.-L.; Launey, S.; Evanno, G. Low reproductive isolation and highly variable levels of gene flow reveal limited progress towards speciation between European river and brook lampreys. *J. Evol. Biol.* **2015**, *28*, 2248–2263. [CrossRef]
13. Soto, E.; Bianco, P.G. I pesci e loro conservazione in aree protette dell'Italia centrale e meridionale. *Ital. J. Freshw. Ichthyol.* **2017**, *1*, 73.

14. Ciuffardi, L.; Bassani, I.; Pini, D.; Balduzzi, A.; Arillo, A. Segnalazione di presenza della lampreda di fiume (*Lampetra fluviatilis*) nel bacino spezzino del Magra-Vara. *XII Congr. Naz. AIIAD San Sepolcro. Abstr.* **2010**, *1*, 55.
15. Freyhof, J. Lampetra fluviatilis. In *The IUCN Red List of Threatened Species 2011*; IUCN: Gland, Switzerland, 2012.
16. Zerunian, S. *Pesci Delle Acque Interne d'Italia*; Quaderni di conservazione della natura; Ministero dell'Ambiente: Istituto nazionale fauna selvatica: Roma, Italy, 2004; Volume 20.
17. Ciuffardi, L.; Monaci, E.; Balduzzi, A.; Mori, M.; Arillo, A. Stato di conservazione della popolazione di Lampreda di mare nel bacino del Magra-Vara (*Provincia della* Spezia). *Biol. Ambient.* **2007**, *21*, 107.
18. Ferri, V.; Crescia, P.; Soccini, C.; Olini, A.; Celletti, S. First occurrence of Sea Lamprey, *Petrmyzon marinus* Linnaeus 1758 (*Agnatha*, *Petromyzontiformes*, *Petromyzontidae*) in the River Mignone (Northern Lazio). *Nat. Hist. Sci.* **2021**, *8*, 29. [CrossRef]
19. Petetta, A.; Righi, T.; Splendiani, A.; Virgili, M.; Giovannotti, M.; Lucchetti, A.; Barucchi, V.C. A new mtDNA control region haplotype from sea lamprey (*Petromyzon marinus* Linnaeus, 1758: Petromyzontiformes) collected off the Italian Adriatic coast. *Mar. Biodivers* **2019**, *49*, 2917–2920. [CrossRef]
20. Rondinini, C.; Battistoni, A.; Peronace, V.; Teofili, C. *Lista Rossa IUCN dei Vertebrati Italiani*; Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare: Roma, Italy, 2013.
21. Aljanabi, S.M.; Martinez, I. Universal and rapid salt-extraction of high-quality genomic DNA for PCR-based techniques. *Nucleic Acids Res.* **1997**, *25*, 4692–4693. [CrossRef]
22. Blank, M.; Jürss, K.; Bastrop, R. A mitochondrial multigene approach contributing to the systematics of the brook and river lampreys and the phylogenetic position of *Eudontomyzon mariae*. *Can. J. Fish. Aquat. Sci.* **2008**, *65*, 2780–2790. [CrossRef]
23. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
24. Librado, P.; Rozas, J. DnaSP: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **2009**, *25*, 1451–1452. [CrossRef]
25. Clement, M.; Posada, D.; Crandall, K. TCS: A computer program to estimate gene genealogies. *Mol. Ecol.* **2000**, *9*, 1657–1660. [CrossRef] [PubMed]
26. Pereira, A.M.; Robalo, J.I.; Freyhof, J.; Maia, C.; Fonseca, J.P.; Valente, A.; Almada, V.C. Phylogeographical analysis reveals multiple conservation units in brook lampreys *Lampetra planeri* of Portuguese streams. *J. Fish Biol.* **2010**, *77*, 361–371. [CrossRef] [PubMed]
27. Guo, Z.; Andreou, D.; Britton, J.R. Sea Lamprey *Petromyzon marinus* Biology and Management Across Their Native and Invasive Ranges: Promoting Conservation by Knowledge Transfer. *Rev. Fish. Sci. Aquac.* **2017**, *25*, 84–99. [CrossRef]