UNIVERSITAT DE BARCELONA

Genetic variability of minnows and loaches in rivers and high mountain lakes from the Pyrenees and Italian Alps

Jongmo Suh

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Doctoral Thesis

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Doctoral Thesis

TESI DOCTORAL

Universitat de Barcelona
Facultat de Biologia
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Programa de Doctorat en Ecologia, Ciències Ambientals i Fisiologia Vegetal

Genetic variability of minnows and loaches in rivers and high mountain lakes from the Pyrenees and Italian Alps

Variabilitat genètica del veró i llops, llopets de riu i misgurns en rius i estanys d'alta muntanya dels Pirineus i dels Alps Italians

Memòria presentada per JONGMO SUH per optar
al grau de doctor per la Universitat de Barcelona

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Centre d'Estudis Avançats de Blanes (CEAB)
Consejo Superior de Investigaciones Científicas (CSIC)
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Vist i plau del codirector de la tesi	Vist i plau de la codirectora de la tesi	Vist i plau de la tutora de la tesi
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Investigador del CEAB	Investigadora del CEAB	Professora de la UB

청산별곡(靑山別曲)

살겠노라 살겠노라. 청산에 살겠노라.
머루랑 다래를 먹고 청산에 살겠노라.
얄리얄리 얄랑셩 얄라리 얄라

우는구나 우는구나 새야. 자고 일어나 우는구나 새야.
너보다 시름 많은 나도 자고 일어나 우노라.
얄리얄리 얄라셩 얄라리 얄라

가던 새 가던 새 보았느냐. 물 아래 가던 새 보았느냐.
이끼 묻은 쟁기를 가지고 물 아래 가던 새 보았느냐.
얄리얄리 얄라셩 얄라리 얄라

이럭저럭 하여 낮일랑 지내 왔건만
올 이도 갈 이도 없는 밤일랑 또 어찌 할 것인가.
얄리얄리 얄라셩 얄라리 얄라

어디다 던지는 돌인가 누구를 맞히려는 돌인가.
미워할 이도 사랑할 이도 없이 맞아서 우노라.
얄리얄리 얄라셩 얄라리 얄라

살겠노라 살겠노라. 바다에 살겠노라.
나문재, 굴, 조개를 먹고 바다에 살겠노라.
얄리얄리 얄라셩 얄라리 얄라

가다가 가다가 듣노라. 외딴 부엌 가다가 듣노라.
사슴이 장대에 올라서 해금을 켜는 것을 듣노라.
얄리얄리 얄라셩 얄라리 얄라

가다 보니 불룩한 술독에 독한 술을 빚는구나.
조롱박꽃 모양의 누룩이 매워 (나를) 붙잡으니 내 어찌 하리이까.
얄리얄리 얄라셩 얄라리 얄라

This work, by an unknown author from the Goryeo period (lasted from 918 to 1392 in the Korean Peninsula), depicts the passive and escapist lifestyle of Goryeo people. It can be characterized as folk literature or escapism literature, revealing an extreme tendency toward escaping reality and a denial of the actual circumstances.

Commonly found in Goryeo poetry is a prevailing theme of resignation, expressions of life's lamentations, and a vivid portrayal of the escape through alcohol. This reflects a sense of resignation and escapism prevalent in the literature of common people.

In the realm of resigned sentiments, one can sense a strong determination toward life, yet the aspiration for a clear resolution reflects both the humble expression of their ideal world and the escapist aspect of their reality.

PS. I found it difficult to provide an accurate translation, so I wrote the original text in Korean. You can use Google Translate App. to get a rough translation.

Acknowledgements

In October 2017, just before completing my master's degree in South Korea, I came to Spain, my first European experience, invited by Dr. Hyunbin Jo. Drawing on my experience in identifying zooplankton communities at brackish waters, I stayed for about a month, focusing on the identification of freshwater samples. While most of my time in the laboratory was spent in front of a microscope, even after working on preparations for my master's graduation in the evenings, the memories of winding down with a glass of wine remain unforgettable. I would like to take this opportunity to express my gratitude to Dr. Hyunbin Jo for providing me with the opportunity to study in CEAB.

Before returning to Korea, Dr. Marc Ventura proposed the opportunity to pursue a Ph.D. in CEAB, which was particularly appealing to me as someone with a strong interest in conservation ecology. The chance to participate in research contributing to the restoration of ecosystems in high mountain lakes destroyed by the invasion of alien species was incredibly attractive. Despite the new field of study, the distance of 10,000 km from my own country, and the challenges of living in a place where I couldn't speak their native language at all, I could make a positive decision without hesitation. This was driven by a profound attraction to the field I had long desired to explore.

Furthermore, the words "If something is difficult, challenging, or seems strange to you, always talk about it to me. We grown up from different cultural backgrounds, so there might be misunderstandings, but we can understand each other through communication," provided significant support to my decision. I am sincerely thankful to Dr. Marc Ventura, who has been helping and guiding me with the same dedication for several years, and to Dr. Teresa Buchaca, too.

In February 2018, after graduating with a master's degree in South Korea, I embarked on my first sampling trip in the Pyrenees in July 2018. Guided by

Prof. Kwang-hyeon Chang, who supervised my master's degree and consistently provided valuable advice during that period, I had gained confidence in sampling through various experiences. However, as I wasn't familiar with mountain climbing, the memory of my initial sampling in the Pyrenees remains vivid and powerful.

After finish the the first sampling, I stayed in the Pyrenees for a month, assisting local fishermen employed in the project LIFE LIMNOPIRINESU to eradicate the genus *Phoxinus* introduced in the lakes of the Pyrenean high mountain lakes. Participating in the removal of invasive species through electrofishing and netting not only motivated my future research but also allowed me to appreciate the value of the study. It provided me with a deeper understanding and passion for the ecosystems of high mountain lakes, an experience that was entirely new to me. I was very happy with colleagues such as Eloi, Neus, Isma and the rest of the fishermen who assisted in sampling and collaborated in the eradication of invasive species in the lakes of the Pyrenees.

In the summer of 2019, I settled to a new field by initiating a full-scale genetic analysis. I would like to express my endless gratitude to Jenny Caner. Who was a great teacher to me, who lacked basic knowledge because I majored in environmental engineering, not ecology. During my time at CEAB, I spent the majority in the Molecular Analysis Lab, and my most frequent collaborator was Jenny. Thanks to her guidance, from extraction to amplification in the lab and from result verification to analysis in the office, she generously shared all methods and experiences related to genetic analysis. This enabled me to take my first steps into research on molecular ecology. Additionally, I extend my gratitude to Andreu Albó and Dr. Federica Lucati, for their assistance.

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various tasks and translations. Thanks for his help in handling administrative tasks that are as difficult as doctoral thesis. And my sincere thanks also go to Dra. Mariàngels Puig, Dr. Danilo Buñay, Claudia Riera, Jesús Ortiz, and Fatima Chaoui and Nerina Gilbert whom just arrived.

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Especially, I sincerely appreciate allowing me to use the data and results from some colleagues. Mitochondrial DNA data from Elisenda Andrés, a student who had been doing her MSc and analysed some genes of *Phoxinus* before me at CEAB and I express my thanks for that.

Most notably, I extend deep thanks to Drs. Miguel Clavero, E. Aparicio, R. Rocaspana, Q. Pou-Rovira for providing the overall samples of Loach in Catalonia used in Chapter 3, as well as supplying data and analysis results related to the introduction history and characteristic changes. Thanks to them, I was able to successfully conclude one chapter of my thesis.

Dra. Marisol Felip from the University of Barcelona kindly provided assistance on many issues related to my doctoral degree and thesis. I appreciate her support and consideration throughout my doctoral program

as a mentor. And I am also grateful to the Catalan government. Thanks to the FI scholarship, I was able to study without economic concerns for the duration of three years.

And thank you again. to my surpervisor, Marc Ventura, who guided me and assisted in the writing of this thesis despite my lack of proficiency in both Catalan and English. Despite my limited language skills, your constant positive encouragement and support have led me to this point. You have guided me both mentally and intellectually. Though I still have much to learn, I will strive to become a better scientist through accumulating experiences.

Lastly, I would like to be greatful to my parents for enabling me to be here. Thanks to my father, Young bong Suh (서 영봉) and mother, Jin ae Lee (이 진애), who always take care of the family, I could pursue my studies abroad without worries. It was an endeavor that would have been impossible without the understanding and support of my parents. I am incredibly thankful. I will try to show as much goodness as you believe and support. I also extend my thanks to my younger sibling, Ye jin Suh (서 예진), who was there for our parents while I was abroad.

I am grateful to many others as well. When we meet in person, I will be greeting thank you once again.

Vull expressar el meu agraïment sincer a les moltes persones que m'han ajudat a completar els meus estudis de doctorat. Tot i que encara em falta molt per aprendre, faré tot el possible per ser un bon ecòleg.

제가 박사과정을 마치기까지 도움을 주신 많은 분들에게 진심으로 감사인사 드립니다. 아직 많이 부족하지만 좋은 생태학자가 되도록 노력하겠습니다.

Contents

General Abstract

The invasion of alien species is a major global threat to ecosystems, with significant impacts, especially on freshwater ecosystems. The species diversity in freshwater ecosystems has significantly declined worldwide, and in Europe, freshwater fish represent one of the animal groups with the highest number of invasive species. Due to human activities, restrictions on geographical distribution have disappeared, and fish are being introduced into new and distant areas for recreational and other economic benefits. The purpose of this thesis was to identify and assess the distribution of two invasive fish introduced by human activities, infer the spread process and introduction pathways. Through the use of various genetic analysis techniques, we aimed to characterize introduced individuals across a broad area and, beyond species identification, obtain information about inter-species hybridization and intra-species admixture.

The first chapter aimed to accurately identify the species and assess the distribution of the genus *Phoxinus* in high-altitude lakes in the Pyrenees and the Italian Alps. The goal was to understand the introduction pathways of these species in order to shed light on their presence and distribution. Mitochondrial DNA (COI and Cytb; mtDNA) was sequenced in 201 individuals sampled from 53 sites including alpine lakes and adjacent water bodies. This data was utilized for species identification through phylogenetic tree analysis and for inferring introduction pathways using haplotype networks. *P. dragarum* has been discovered in its native habitat, including the Garonne basin, and in lakes to the east of the Pyrenees, and rivers in Catalonia. *P. bigerri* was primarily found in its native range, the Adour basin, and lakes to the west of the Pyrenees, suggesting it has been introduced. Lower species diversity of genus *Phoxinus* was observed in areas where fishing is prohibited, indicating that most introductions are associated with the use of live bait by anglers. The regulation of fishing has been confirmed as an effective measure to prevent the introduction of invasive species.

The second chapter involved a more detailed using a total of 890 individuals from 62 locations, and nuclear DNA (microsatellite) sequencing was conducted. With the analyses of microsatellites (nuclear DNA; nDNA) we identified various lineages, including information on interspecies and intraspecies hybridization.

The nDNA results generally aligned with mtDNA, but some differences were noted. Individuals from Catalonia identified as *P. septimaniae* based on mtDNA were identified as *P. dragarum* or *P. bigerri* based on nDNA, suggesting that the individuals known as *P. septimaniae* in Catalonia are hybrids. Contrary to the assumption that there would be a high rate of intraspecies hybridization in isolated lake environments, a low rate was observed. An interesting observation was the recording of nuclear and mitochondrial three-way hybrids of *P. septemaniae* (mtDNA) and *P. dragarum x P. bigerri* (nDNA) in the River Tenes.

The third chapter focused on the introduced species of loaches in the Catalonia, particularly in lowland rivers with greater human activity and accessibility. Individuals of *Barbatula*, *Cobitis*, and *Misgurnus*/*Paramisgurnus* were collected from 21 locations and were sequenced for COI and RAG-1. These data were utilized for species identification through phylogenetic tree analysis and introduction pathway inference using haplotype networks. We identified at least five non-native fish species (*C. bilineata*, *M. anguillicaudatus*, *M, bipartitus*, *P. dabryanus*, and an undescribed *Barbatula* species) in the Catalonia, and most of them were spreading in their distribution. The characteristics of invasive species were evolving over time after introduction, and the established invasive species in Catalonia shifted from being large and long-lived fish species used for culture or game fishing to small, benthic fish species used as live bait or for ornamental purposes.

General Introduction

1. Impacts of invasive species on ecosystems

Freshwater ecosystems cover a rich diversity of species and habitats. Despite their relatively small distribution on the world's surface (less than 1%), current estimates suggest that freshwater ecosystems provide suitable habitat for at least 126,000 plant and animal species of the world (Balian, Segers, Martens, & Lévéque, 2008).

These freshwater ecosystems have experienced a significant decline (McLellan, Iyengar, Jeffries, & Oerlemans, 2014), attributed globally to factors such as habitat destruction, environmental pollution, water-level reductions, and invasive species (Collen et al., 2014). Other complex factors such as land-use changes, population growth, overfishing, and climate change have been identified as threats not only to freshwater ecosystems but also to terrestrial ecosystems' conservation (Brook, Sodhi, & Bradshaw, 2008; Jo et al., 2019; Stendera et al., 2012).

When organisms are moved from their known native range and introduced to new areas by humans, it also has been described as zoogeographic pollution (Cambray, 2003). This indicates that the impact of introduced species often causes serious harm to the ecosystem. As time progresses, barriers to the geographical distribution of species are gradually breaking down due to human activities (S. Su, Cassey, & Blackburn, 2016). Introduced species are increasingly moving from their native regions to relatively new areas for recreational, commercial, food and other economic purposes (Minchin, Cook, & Clark, 2013). Climate change and altered conditions can lead alien species to transform into invasive species, and over time, there is a tendency for them to dominate ecosystems (Baxter, Fausch, Murakami, & Chapman, 2004).

Interpreting the impact of these invasive species on native organisms and communities might be challenging because invaders can interact among themselves and affect native ecosystem in various ways (Simberloff, 2006). An exemplary case of invasive species includes freshwater fish. In Europe, freshwater fish are among the animal groups with the highest proportion of invasive species (Hulme, 2009). They are considered a threat to invasded

ecosystems, leading to significant changes in ecosystem composition and competition between native and invasive species for prey and habitat (Kiruba-Sankar et al., 2018).

Cases have been reported where invasive species alter the evolutionary pathway of native species through competitive exclusion, niche displacement, hybridization, introgression, predation, and ultimately extinction. Invasive species themselves also undergo adaptations and evolution in response to interactions with native species and adaptation to new environments (Mooney & Cleland, 2001). This ultimately leads to issues such as various ecological disruptions, decreasing ecosystem diversity and the destruction of native ecosystems.

1.1. **Case 1: Freshwater watersheds encompassing rivers and coastal areas.**

Invasive species are considered one of the five major threats to global aquatic biodiversity (Sala et al., 2000), particularly impacting freshwater habitats (Dudgeon et al., 2006; Geist, 2011). Due to the isolation of most freshwater habitats, the natural dispersal of aquatic organisms to new habitats occurs infrequently. As a result, aquatic communities tend to be more different to each other. So the rate of species movement through human pathways increases, amplifying the potential impact on biodiversity (Keller, Geist, Jeschke, & Kühn, 2011).

In particular, freshwater fish in Europe have been identified as one of the animal groups with the highest number of invasive species (Hulme, 2009). Previous studies on invasive species in aquatic ecosystems have consistently shown strong negative ecological outcomes (Miró, 2011; Reid et al., 2019; D. Schindler & Parker, 2002; Tiberti, Von Hardenberg, & Bogliani, 2014a; Ventura et al., 2017; Vinyoles et al., 2007; Vitule, Freire, & Simberloff, 2009). Similar to habitat destruction, the introduction of non-native fish species into native ecosystems can disrupt resource flows and have widespread impacts on interconnected ecosystems (Baxter et al., 2004). It may also pose a

significant risk in certain environments, such isolated pools in temporary rivers, which maintaining local freshwater biodiversity during periods of no flow (Bonada et al., 2020).

Invasion by mammals is more likely to contribute to the extinction of native species when involving intercontinental rather than intracontinental movement, whereas invasion by fish carries an equal likelihood of causing extinctions both intercontinental and intracontinental movement. Moreover, powerful cases of negative impacts on native species have been more frequently observed in freshwater systems than in marine systems (Ricciardi & Kipp, 2008). Furthermore, freshwater systems tend to show a higher proportion of invasive species leading to declines in native species, with, on average, 11% of all invasive species having a high impact in freshwater systems compared to 4% in marine systems (Ricciardi & MacIsaac, 2011).

The introduction of invasive fish species into freshwater areas occurs through various routes. According to the analysis of introduction pathways in South America, North America, Europe, Australia, and Africa, aquaculture is a major reason for the introduction of invasive species (Sultana & Hashim, 2015). Many species introduced by humans for social and economic benefits have escaped from aquaculture, establishing themselves as invasive species (Liu & Li, 2009). Introduced species have been found in many regions around the world, including Brazil (Britton & Orsi, 2012) and Indonesia (Muchlisin, 2012).

Therefore, preventing and reducing the impacts of biological invasions is a priority in freshwater management. It has been shown that eradicating or controlling established invasive species can contribute to the conservation of freshwater biodiversity (Bosch et al., 2019; Miró et al., 2020). In Chapter 3 of this study, we aim to provide fundamental information of several invasive species in the Catalonia by clarifying the origins of non-native populations, detect independent introduction events, and comprehend and identify invasive species.

1.2. **Case 2: High mountain lakes of all over the world have many ecosystem similarities**

High mountain lakes are located in remote headwaters, far from populated areas, and due to their distance from densely inhabited regions, they can support unique plant and animal communities. Therefore, they possess tremendous ecological and environmental value (Cole & Landres, 1996; Kernan, Ventura, et al., 2009). Many of these lakes are situated within protected areas, offering scenic, anthropological, biodiversity, and conservation interests. There exist varying levels of protection, ranging from basic conservation to highly protected areas (Knapp, 1996; Wiley, 2003).

Figure i-1. Pictures of Genus *Phoxinus* in high mountain lakes of the Pyrenees. Top right *Phoxinus bigerri* and bottom left *Phoxinus dragarum.*

High mountain lakes are often considered pristine, but they are facing threats due to global stressors such as climate change and industrial pollution. These pollutants include acid precipitation, persistent organic pollutants, and trace metals (Catalan et al., 2013; Davidson & Knapp, 2007). However, the most

significant direct anthropogenic stressors are the introduction of invasive fish through human activities (Pister, 2001; Wiley, 2003).

High mountain lakes are originally fishless due to natural barriers. This is because natural barriers make it difficult for fish to move from downstream and settle down. However, since the 19th century, these ecosystems worldwide have frequently seen the introduction of invasive fish species, primarily for the purpose of fishing. These introductions have been frequently reported in sites such as the mountain ranges of the Rocky Mountains and Sierra Nevada in western North America, Tatras, Alps, Pyrenees, Cantabric range and Sistemas Central and Ibéric in Europe, or the Canterbury high elevation plains in the South Island of New Zealand (Brancelj, Sisko, Brancelj, Jeran, & Jacimovic, 2000; Martinez-Solano, Barbadillo, & Lapena, 2003; Miró & Ventura, 2013; R. Pechlaner, 1984; D. E. Schindler, Geib, & Williams, 2000; Terrero, 1951; Toro, Granados, Robles, & Montes, 2006; Wiley, 2003; Wissinger, McIntosh, & Greig, 2006). Moreover, after the 1900s, minnows were introduced as live-bait for fishing in some mountainous areas (Figure i-1). For example, in the Nordic lakes of Scotland, Norway, Pyrenees and Alps (Maitland & Campbell, 1992; Miró, Buñay, & Ventura, 2015; Museth, Hesthagen, Sandlund, Thorstad, & Ugedal, 2007; Tiberti et al., 2020).

These lakes and other isolated systems can be considered naive to the impacts of various invasive species due to their evolutionary isolation, leading to a lack of experience with diverse invaders (Cox & Lima, 2006). Since the 1980s, numerous studies have shown that trout introduced into historically fishless lakes have become apex predators, causing several significant ecological changes, including resulting in the extinction of native fauna without the selection pressures to adapt to large aquatic predators (Knapp & Matthews, 2000; Osorio et al., 2022; Pope, 2008; Schabetsberger, Luger, Drozdowski, & Jagsch, 2009; Tiberti, von Hardenberg, & Bogliani, 2014b), drastic decline or elimination of amphibian and reptile populations (Knapp, 2005; Miró, Sabas, & Ventura, 2018; Orizaola & Braña, 2006; Pilliod et al., 2010; Pope, 2008; Tiberti & von Hardenberg, 2012). Changes in species composition and size structure of zooplankton and benthic macro-invertebrates (Brancelj et al.,

2000; Knapp, Matthews, & Sarnelle, 2001; Tiberti et al., 2014a), large-scale changes in ecosystem processes such as the planktonic food web or nutrient cycling (Magnea, Sciascia, Paparella, Tiberti, & Provenzale, 2013; Sarnelle & Knapp, 2005) and indirect impacts on surrounding habitats through resource depletion (Epanchin, Knapp, & Lawler, 2010).

Studies on the impact of fish introductions on the communities of large invertebrates and benthic organisms in high mountain lakes in the European Alps have been conducted. Through comparisons between lakes with introduced fish and those without, significant structural changes in pelagic zooplankton and littoral macroinvertebrates were revealed. These findings are consistent with previous research (Knapp & Matthews, 2000; Parker, Schindler, Donald, & Anderson, 2001; Schabetsberger et al., 2009). Additionally, unexplored impacts on aquatic algae trophic cascades (Sarnelle & Knapp, 2005) and impacts on nearby terrestrial habitats (Epanchin et al., 2010; Matthews, Knapp, & Pope, 2002; Pope, Piovia‐Scott, & Lawler, 2009) showed clear similarities in separate areas, supporting the ecological risk (Tiberti et al., 2014a).

2. High mountain lakes and efforts for conservation

The Pyrenees mountain range is a continuous highland biogeographical region located in southwestern Europe, spanning from the Atlantic to the Mediterranean (2°05′W–3°15′E, 43°18′–42°16′N). This region contains approximately 4500 alpine lakes and ponds, all of which provide suitable habitats for freshwater organisms (Miró, 2016). These lakes have been extensively investigated, especially in the past few decades, since the late 19th century. Research has predominantly focused on describing their primary environmental and ecological characteristics (Catalan et al., 2009; Kernan, Catalan, Ventura, & Curtis, 2009; Kernan, Ventura, et al., 2009; R. Thompson, Ventura, & Camarero, 2009), with a particular emphasis on investigating their role as sentinels of global change (Camarero & Catalan, 2012; Catalan et al., 2013).

Figure i-2. landscape of some high mountain lakes where genus *Phoxinus* introduced in Pyrenees. Clockwise, starting from the upper left corner: Lakes tres estanys, Lake El Cao, Lake Dellui, Lake Muntanyó d'Àrreu, Lake Naorte and Lake Llong.

The distribution of biological species in the vast mountainous terrain does not adhere to political borders but varies according to environmental features or gradients. Therefore, to conserve this ecosystem, a comprehensive examination of issues across the entire Pyrenees Mountain range is necessary, requiring collaboration among diverse countries. Currently, there are six different countries or regions responsible for environmental stewardship in the Pyrenees. Generally, the northern part belongs to France, the southern part comprises the Basque Country, Navarre, Aragon, and Catalonia regions

of Spain, and Andorra is situated to the southeast. Consequently, there is a diverse range of conservation agencies and environmental management policies across the entire range. In essence, research on the Pyrenees seeks to eliminate artificial borders, involving all nations and administrative bodies, to necessitate mountain-scale measures and provide a foundation for conservation policies (Miró, 2016; Ventura et al., 2017).

As an example, the restoration effort aimed to eradicate or intensively control introduced trout and minnows in eight mountain lakes located within the EU Natura 2000 network in the Pyrenees (five lakes in Aigüestortes and Estany de Sant Maurici National Park, threat the Alt Pirineu Natural Park) (Figure i-2). The goal was to restore these mountain lakes and reintroduce native aquatic fauna populations by eliminating or controlling non-native fish species. Similarly, the lakes in the Alps, another representative mountain range, share common characteristics. Previous research has revealed a contrast between the distribution of brook trout in the lakes of Gran Paradiso National Park (GPNP) and the institutional conservation goals of the national park. Consequently, GPNP executed an extensive eradication campaign within the Bioaquae (Biodiversity Improvement of Aquatic Alpine Ecosystems) project. The two projects, LIFE+ Bioaquae (www.bioaquae.eu) and LIFE+ LimnoPirineus (www.lifelimnopirineus.eu), were carried out in collaboration with fishermen and local authorities (Figure i-3). The former, starting in 2013 in the Italian Alps within Gran Paradiso National Park, successfully removed trout from four lakes and their surrounding streams, achieving fish removal from all lakes. The latter project, initiated in 2015, focused on removing brook trout, rainbow trout, brown trout, and genus *Phoxinus* from eight lakes and their adjacent streams (Ventura et al., 2017). Both projects used intensive gill netting, electrofishing and fyke nets, a valuable non-invasive method that has been successfully utilized in restoration programs without causing lethal effects on non-target species (Knapp & Matthews, 1998; Parker et al., 2001; Ventura et al., 2017; Vredenburg, 2004). This approach also provides sufficient assurances regarding concerns about the conservation of other species (Tiberti et al., 2014a).

Figure i-3. Eradicating genus *Phoxinus* using nets and electrofishing, and selling fish for their use as a live bait at Decathlon in France.

The mentioned examples, demonstrate the effectiveness of preventing new introductions and maintaining the conservation status of high mountain lakes. Additionally, through the mentioned projects, many lakes have successfully eradicated or reduced invasive species as of 2023, leading to the restoration of native ecosystems and biodiversity (Miró et al., 2020; Osorio et al., 2022).

3. The utility and application of genetic tools for conserving the ecosystem from invasive species

Risk assessment for invasive species is generally conducted to inform two types of risk management decisions. Firstly, it's about the risk of introduction or transportation of potentially invasive species, their vectors, or pathways

before establishment (leading to decisions to authorize, prohibit, or allow certain activities under specific conditions). Secondly, it includes allocating limited resources for controlling already established invasive species, involving a prompt response (Andersen, Adams, Hope, & Powell, 2004). To conduct risk assessment, a clear understanding of the current distribution of species is crucial, and for risk management, identifying the pathways of introduction is essential.

Genetic tools, including mitochondrial DNA and nuclear DNA, have been successfully utilized in numerous previous studies for species identification and understanding distribution status. In the case of the main target species of this study, the genus *Phoxinus*, ongoing research is being conducted, as evidenced by recent discoveries of new species (V Caputo Barucchi et al., 2022; Corral‐Lou et al., 2019; De Santis, Delmastro, Vanetti, Britton, & Zaccara, 2021; Denys et al., 2020; Palandačić, Witman, & Spikmans, 2022).

In addition, population biology studies of invasive species have clarified many fundamental issues in ecology and evolutionary biology. Detailed population biological research is expected to contribute to the effective control of widespread or long-established invasive species, potentially leading to the development of new control methods. Moreover, it can assist in policy establishment, particularly in focusing attention on the overall process of invasion, allowing for a better understanding of the introduction pathways with a higher likelihood of becoming problematic. Effectively managing invasive species involves one of the most efficient strategies are detecting them early and eradicating or, at the very least, controlling them before they spread (Simberloff, 2003).

Additionally, explicit analysis of the genetic structure of invasive species can further enable effective management of invasive species. Examples of potential applications of population genetics in invasive species research include predicting invasiveness to reduce the occurrence of new invasions, predicting the efficacy of alternative control efforts and improving management of invasive species within native communities (Sakai et al., 2001). Thus, genetic markers answer biological questions most efficiently and

also contribute to much broader investigations of evolutionary, population and conservation biology (Sunnucks, 2000).

The invasion mechanism can be explained through evolutionary genetics. Recent research findings suggest that the success of many invasive species may rely more on the ability to respond to natural selection than on broad physiological robustness or variability. Therefore, these studies emphasize the importance of genetic structure, and selection on it can lead to evolutionary adaptations and potential species divergence. These findings emphasize the utility of genomic approaches, contributing to the understanding of invasion mechanisms by analyzing gene expression, gene interactions, and genomic rearrangements associated with invasion events (Lee, 2002).

In conclusion, the use of various genetic tools is essential and beneficial for a range of purposes, from assessing the risk based on the identification and distribution of invasive species to devising strategies through understanding the introduction pathways. This thesis, leveraging genetic analyses using mitochondrial DNA in Chapters 1 and 3, as well as nuclear DNA in Chapters 2 and 3, aims to provide crucial and informative data. Ultimately, it seeks to contribute to the conservation of rehabilitated lakes by preventing potential recontamination situations caused by invasive species.

General Objectives

The purpose of this thesis was to identify and assess the distribution of some relevant invasive fish species introduced by human activities, infer the spread process and introduction pathways. Through the use of various genetic analysis techniques, we aimed to characterize introduced individuals across a broad area and, beyond species identification, obtain information about inter-species hybridization and intra-species admixture. The goal was to provide more specific insights into the invasion history of invasive fish species and raise awareness about the severity associated with it.

This study is broadly divided into Chapter 1 and 2, encompassing high mountain lakes, including those in the Pyrenees and Alps, along with their surrounding watersheds. Chapter 3 focused on the loaches of the river network in Catalonia.

1. The first objective consisted in describing the species of the genus *Phoxinus* that had been introduced in high mountain lakes of the Pyrenees and the Italian Alps, and the River Ebro and the inner Catalonia using mitochondrial DNA and trying to discover their possible origin (Chapter 1).

2. The second objective consisted in describing the introduction process of the species of the genus *Phoxinus* that had been introduced in high mountain lakes of the Pyrenees and the Italian Alps, and the inner Catalonia using nuclear DNA (microsatellites) (Chapter 2).

3. The third objective consisted in describing the process of introduction of loaches in Catalonia using mitochondrial and nuclear DNA and trying to discover their possible origin (Chapter 3).

4. The fourth objective aimed to explore how the process of fish invasion fish differ under different fishing regulations. Several study areas are protected as national or natural parks and protected areas due to their ecological and geological value, resulting in regulations varying at the national and regional

levels for conservation and management. For instance, some regions prohibited fishing entirely, others allowed fishing but banned the use of live bait, while there were areas where all types of fishing were permitted. (Chapter 1).

5.The analysis of mtDNA (COI, Cytb) results, along with nDNA (microsatellite) analysis, allows for an examination of species composition and various intra-species sub-lineages. Our fifth objective was to utilize isolated, relatively homogeneous high mountain lakes as scenarios for species hybridization or admixture and compare them to lowland rivers, sties with higher anthropogenic disturbances. This included situations where multiple lineages had been introduced into a single population. (Chapter 2).

6. The final objective was to evaluate how the irruption of new profiles among invasive species could hinder the design and implementation of prevention and rapid response management strategies to deal with biological invasions using the loaches as a case study (Chapter 3).

Part 1: Illuminating the invasive species (genus *Phoxinus*) in high mountain lakes using mitochondrial DNA and nuclear DNA.

Chapter 1

A genetic perspective on the invasion history of the European minnow (*Phoxinus* spp.) in the Pyrenees and the Italian Alps

1. Abstract

In order to prevent the introduction of species such as minnows (*Phoxinus* spp.) into high mountain lakes, it is necessary to determine the alien species being introduced and identify the potential routes of introduction. This study focuses on the genetic analysis of different *Phoxinus* populations from high mountain lakes and nearby streams from the Pyrenees and the Alps. We sequenced two mitochondrial fragments, cytochrome c oxidase subunit I (COI) and cytochrome b (Cytb), from 201 individuals belonging to 64 localities. We identified four species of the genus *Phoxinus*: *P. dragarum* has been found in its native Garonne basin as well as in high mountain lakes to the east of the Pyrenees, where it was introduced, and in rivers in Catalonia. *P. bigerri* has been primarily found in its native Adour basin and high mountain lakes to the west of the Pyrenees, where it was introduced, as also suggested by the regional discontinuity in the haplotype network. As for *P. septimaniae*, its suspected introduction from its native Rhone basin to high mountain lakes and lowland lake in Italy is indicated, but the introduction route to the Catalonia inner basin in Spain remains elusive. *P. csikii* was introduced to high mountain lakes in the Italian Alps, and most haplotypes matched those from the Rhone or Rhine basins rather than its native Danube basin. Furthermore, the confirmation of the coexistence of two or more species in three high mountain lakes and four rivers, along with the discovery of multiple haplotypes in many lakes, indicated multiple introductions. For *P. dragarum*, which had the largest sample size, relatively low genetic diversity was observed at the sites where fishing is prohibited which suggests that new introductions are less frequent than elsewhere as a likely consequence of reduced fishing activities.

2. Introduction

Freshwater species have suffered the largest decline on a global scale, as a result of anthropogenic factors such as habitat destruction, water pollution and the introduction of invasive species among others (Collen et al., 2014). Biological invasions have rapidly increased over the past century and are

considered a major threat to biodiversity and ecosystem preservation (Simberloff et al., 2013). In Europe, freshwater fish are one of the animal groups with the greatest number of invasive species (Hulme, 2009). The introduction of freshwater fish is closely related to human activities (Gido, Schaefer, & Pigg, 2004). It is particularly relevant to fishing for salmonids (Cambray, 2003), which has important negative ecological consequences (Vitule et al., 2009).

High mountain lakes are generally isolated from lower streams by topographical barriers that have prevented natural fish colonization (Knapp et al., 2001; Miró & Ventura, 2013; R Pechlaner, 1984). In these ecosystems, the introduction of fish is mainly related to recreational fishing and often promoted by many different national administrations (Pister, 2001; D. Schindler & Parker, 2002). In the Pyrenees and the Alps, the first documented introductions of trout took place during the Middle Ages, with the first documents mentioning the presence of trout already in the 14th or 15th century in the Pyrenees (Miró, 2011) or the Alps (R. Pechlaner, 1984), respectively. These first introductions of salmonids has become pervasive in the late 19 century, giving rise over time to collateral introductions of other fish species used mainly as live bait or forage fish, such as the European minnow (*Phoxinus* spp.) (Miró & Ventura, 2015), causing serious negative effects for the local ecosystem, especially when they become the dominant fish species in the lake (Teresa Buchaca et al., 2019; A. Miró et al., 2018; Osorio et al., 2022; Schabetsberger, Jersabek, & Brozek, 1995). These are relatively small cyprinids inhabiting naturally lowland rivers and lakes throughout northern Eurasia. They usually behave as surface fish, gregarious, generally active and exhibiting sexual dimorphism that becomes evident during the reproduction period (Denys et al., 2020).

Until recently, the European minnow was considered to be only one species, *P. phoxinus* (Linnaeus 1758). However, studies carried out at the beginning of this century began to propose the existence of several species within the genus *Phoxinus* (Figure 1-1). Currently, the genus is known to consist, at least, of 15 already recognized species that inhabit Eurasia (Denys et al., 2020; Fricke, Eschmeyer, & Van der Laan, 2018; Palandačić, Bravničar, Zupančič,

Šanda, & Snoj, 2015; Palandačić, Naseka, Ramler, & Ahnelt, 2017; Vucić, Jelić, Žutinić, Grandjean, & Jelić, 2018). In the Iberian Peninsula, the presence of three species has been described: *P. bigerri*, *P. septimaniae* Kottelat (2007), and *P. dragarum* Denys et al. (2020) (Corral‐Lou et al., 2019; Denys et al., 2020). Within Iberia, populations from Cantabria and Galicia, as well as western parts of the River Ebro, and the River Duero together with some rivers from western France were identified as *P. bigerri* Palandačić et al. (2017), while those found in rivers of the Catalan Mediterranean coast were identified as *P. septimaniae* and *P. dragarum*. Corral-Lou et al. (2019). According to the latter study the last two species do not appear to be native to the Iberian Peninsula, while *P. bigerri* might be native from some of the Cantabric basins only, and therefore introduced in the Ebro and Duero (Garcia-Raventós et al., 2020). In France, in addition to the three previous species, *P. phoxinus*, *P. csikii* Hankó, 1922 and *P. fayollarum* Denys, Dettaï, Persat, Daszkiewicz, Hautecoeur & Keith, 2020 are also present, being all six native from France, among other regions (Denys et al., 2020). In Italy, *P. lumaireul* (Schinz, 1840) is the only native species, although two non-native species (V Caputo Barucchi et al., 2022), *P. septimaniae* and *P. csikii*, are also present (De Santis et al., 2021).

In order to prevent the introduction of these alien species it is necessary to know which species have already been introduced and the possible routes of introduction. As such, the species of the genus *Phoxinus* are still being discovered and cases of hybridization within the genus are being reported (Angers & Schlosser, 2007). With this information one may suggest better prevention mechanisms at different stages, such as constricting pathways, intercepting movements at borders, and assessing risk for intentional imports (Simberloff et al., 2013). This study focuses on the genetic analysis, based on two mitochondrial fragments, of different populations of the genus *Phoxinus* from high mountain lakes and surrounding basins of the Pyrenees and high mountain lakes of the Italian Alps. We aimed to: (1) identify the *Phoxinus* species present in our study area and better delineate their geographic distribution; (2) ascertain whether such species can be considered native or introduced in the study area and elucidate their introduction routes; and (3) explore relationship among various fishing regulations and the risk of invasion.

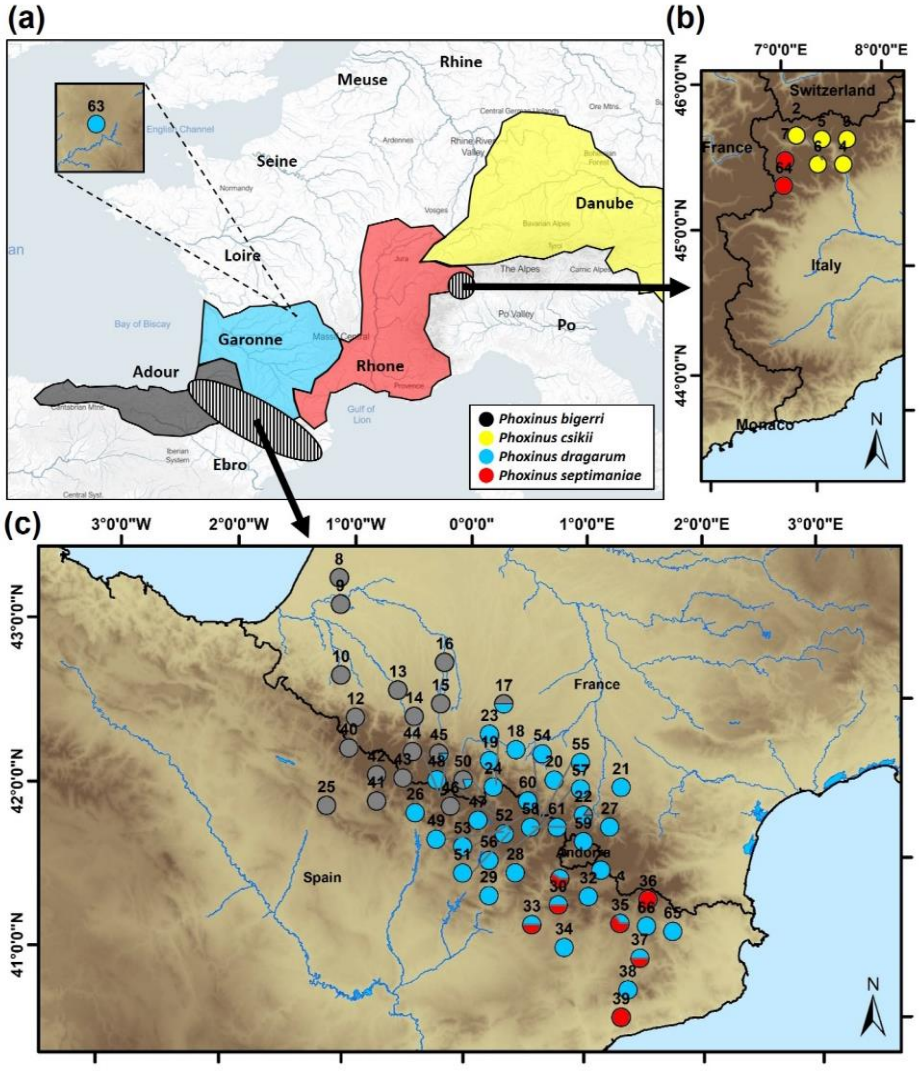


Figure 1-1. Map of the sampling sites of this study and the origin of genus *Phoxinus* by previous study (Corral - Lou, Perea, Aparicio, & Doadrio, 2019; Denys et al., 2020).

Country	Basin	Lake or River - Num. of sequences with COI/cytb (Site Num.)
France	Adour	Ruisseau de Cabanes 0/1 (8), Luy 0/1 (9), Lausset 1/1 (10), Le Gave d'aspe 1/0 (12), Gave de pau 1/1 (13), Canal de Branc 2/2 (14), Adour 2/2 (15, 16),
France	Garonne	Arrat-Devant 1/2 (17), Job 2/2 (18), Pique 2/2 (19), Salat 0/1 (20), Le Douctouyre 1/1 (21), Triouzoune 1/2 (63)
France	Lake of Pyrenees	É tang Mort 6/5 (22)
Italy	Lakes of Alps	Gran Lac 2/1 (2), Balena 2/1 (3), Lago Vernouille 2/1 (4) Lago Leita 2/1 (5), Lago Misérin 2/1 (6), Lago di Dres 1/1 (64)
Italy	Po	Lago di Ceresole 5/2 (7)
Spain	Aragon Riv.	Rio Gállego 2/2 (25), Río Bellós 2/2 (26),
Spain	Catalan Riv.	Noguera Pallaresa 6/4 (27-29), Segre 14/13 (30-33), El Llobregat 2/2 (34), Ter 5/4 (35, 36), Río Gurri 2/0 (37), Riera d'Avencó 2/1 (38), Tenes 3/1 (39), El Fluvià 2/0 (65), Vallfogona 2/2 (66)
Spain	Garonne	Garonne 5/5 (23, 24)
Spain	Lakes of Catalan Pyrenees	Xic de Travessani 12/5 (48), Estany de Llebreta 2/2 (49), Estany Llong 5/3 (50), Bassa Nord de l'Estanyet Dellui 5/2 (51), Estanyol Dellui 2/1 (52), Muntanyó d'À rreu 5/4 (53), Tres estanys de baix 5/1 (54), Tres estanys del mig 15/4 (55), Estany de Finestres 3/2 (56), Estany de Soliguera 7/1 (57), Estany Closell 12/6 (59), Estany de Naorte 4/5 (60), Estany de Rovinets 4/3 (61), Estany de Malniu 3/3 (62),
Spain	Lakes of Aragon Pyrenees	Ibón de Estanés 2/2 (40), Ibón de Asnos 2/2 (41), Ibón de Sabocos 2/2 (42), Ibón de Lapazosa 2/2 (43), Ibón de Urdiceto 2/2 (44), Ibón del Cau 5/4 (45), Ibon de Sen 2/4 (46), Ibones d'Angliós 2/2 (47)

Table 1-1. Country, basin and locality information of the sampled sites. After the name of each sampling site, we describe the number of analyzed sequences of each gene fragment (COI/Cytb) and the code of each locality in parentheses.

3. Materials and methods

3.1. Study area and sample collection

The sampling of genus *Phoxinus* consisted of 64 sites, including 24 Pyrenean high mountain lakes and 33 sites from nearby rivers, and 6 high mountain lakes and 1 reservoir from the Italian Alps (Figure 1-1, Table 1-1). The capture of fish individuals was carried out with fyke-nets or electrofishing depending on the site. Of all the sampled individuals, we selected a maximum of 30 individuals per site covering a gradient of shapes and sizes to ensure that we analyzed all the species present. Individuals were either fixed in absolute ethanol or frozen (- 20 ºC) until DNA extraction.

3.2. Molecular analyses

We analysed a total of 201 specimens of the genus *Phoxinus*. DNA was extracted from muscle tissue using the HOTShot technique (Montero - Pau,

Gómez, & Muñoz, 2008) or QIAmp DNeasy Blood and Tissue Kit (QIAGEN, Germany). To identify each individual to the species level, we sequenced two mitochondrial gene fragments, the cytochrome c oxidase subunit I (COI) and the cytochrome b (Cytb). For COI, a fragment of 652-658 base pairs (bp) was amplified using the primer sets HCO-2198 / LCO-1490 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994), jgLCO1490 / jgHCO2198 (Geller, Meyer, Parker, & Hawk, 2013), or FishF2, VF2T1/FishR2, FR1d (Ivanova, Zemlak, Hanner, & Hebert, 2007; R. D. Ward, Zemlak, Innes, Last, & Hebert, 2005). In the case of Cytb, a fragment of 1188-1190 bp was amplified using the primer pairs Gludg-L / H16460 (Palumbi, 1996; Perdices & Doadrio, 2001) or CytoF-Thr-R / GluF-CytoR (Zardoya & Doadrio, 1998).

Amplification conditions consisted of a total reaction volume of 25 μL containing 1x PCR buffer (SilverStar, Eurogentec), 1.5 mM MgCl2, 200 μM of each dNTP, 0.2 μM for each primer, 2 μL of DNA extract, 1 U Taq polymerase, and UV-sterilized mQ water. PCR conditions differed for each primer set. For the COI primers HCO-2198 / LCO-1490 we used a denaturation step of 3 min at 95 °C, 5 cycles of 95 °C (30 s), 45 °C (30 s) and 72 °C (60 s), followed by 30 cycles of 95 °C (30 s), 50 °C (30 s) and 72 °C (60 s), and a final elongation of 15 min at 72 °C; for jgLCO1490 / jgHCO2198 we used a denaturation step of 1 min at 95 °C, 5 cycles of 94 °C (40 s), 45 °C (40 s) and 72 °C (60 s), followed by 35 cycles of 94 °C (40 s), 51 °C (40 s) and 72 °C (60 s), and a final elongation of 1 min at 72 °C; for FishF2, VF2T1/FishR2 and FR1d we used a denaturation step of 2 min at 94 °C, 35 cycles of 94 °C (30 s), 52 °C (40 s) and 72 °C (60 s), and a final elongation of 10 min at 72 °C. For the Cytb primers Gludg-L / H16460 we used a denaturation step of 10 min at 95 °C, 40 cycles of 95 °C (60 s), 54 °C (60 s) and 72 °C (90 s), and a final elongation of 10 min at 72 °C; for CytoF-Thr-R / GluF-CytoR we used 5 cycles of 94 °C (60 s), 45 °C (60 s), and 72 °C (105 s), followed by 35 cycles of 94 °C (60 s), 50 °C (60 s) and 72 °C (105 s).

3.3. Phylogenetic analyses and haplotype networks

The obtained sequences were aligned using MEGA 7 (Kumar, Stecher, & Tamura, 2016). The alignments were checked by eye and corrected according to the translated amino-acid alignment, and sequence divergence (Kimura 2-parameter model) was calculated using the same software. To aid in species identification, the aligned sequences were compared with sequences from previously published studies, which were available in the GenBank database (NCBI). Haplotype and nucleotide diversities, as well as number of polymorphic sites, were calculated with DnaSP 6.12 (Rozas, Sánchez-DelBarrio, Messeguer, & Rozas, 2003) for each species and region (i.e. separating the native areas of each species from areas where they are introduced).

A phylogenetic tree was constructed by combining the results of our study with sequences downloaded from GenBank (Total 396 sequences of 570 bp for COI and 438 sequences of 1089 bp for Cytb.). *Oreoleuciscus potanini*, *Tribolodon hakonensis*, and *Rhynchocypris lagowskii* were used as outgroup species for the COI phylogenetic tree, while *Chondrostoma oxyrhynchum* was used as outgroup for the Cytb phylogenetic tree. To select the appropriate nucleotide-substitution model, we used jModelTest 2.1.3 (Posada, 2008). We then constructed two phylogenetic trees using both the Bayesian Inference (BI) method with MrBayes 3.2.7 (Ronquist et al., 2012) and the Maximum Likelihood (ML) method with PhyML (Guindon et al., 2010). In BI, two parallel runs of four Monte Carlo Markov chains were run for 3 million generations, trees were sampled every 100 generations, and the first 25% sampled trees were discarded as burn-in. In PhyML, the substitution model, i.e. a gamma model of rate heterogeneity and invariant sites (TIM1+I+G for COI, GTR+I+G for Cytb) was suggested by jModelTest, and branch support was evaluated by bootstrapping with 1000 replicates. Identification of each phylogenetic clade to the species level was based on previous studies conducted in similar research areas, particularly by Corral-Lou et al. (2019) and Palandačić, Kruckenhauser, Ahnelt, and Mikschi (2020).

We used Haploview (Salzburger, Ewing, & Von Haeseler, 2011), which transforms phylogenies into haplotype networks, to construct haplotype

networks for each species. The RAxML 7.7.1 maximum likelihood-based method (Stamatakis, 2006) was employed to estimate the phylogeny, using the optimal nucleotide-substitution model determined by JModelTest and applying 1000 bootstrap replicates.

4. Result

4.1. Species identification and genetic diversity

This study resulted in 186 COI and 129 Cytb newly generated sequences from high mountain lakes and connected basins of the Pyrenees and the Italian Alps. The phylogenetic analysis of our sequences together with those retrieved from GenBank identified 22 different clades which could be attributed to different species, including 14 previously described and 8 undescribed species (Figure 1-2). Both gene fragments resulted in very similar phylogenetic trees.

Our sequences consistently belonged to four species, *P. dragarum*, *P. bigerri*, *P. septimaniae* and *P. csikii*. The mean genetic distances (divergence) between all species of the genus were 5.36 ± 1.48 % for COI and 8.59 ± 1.23 % for Cytb. Since the nucleotide sequence of Cytb is longer than that of COI, it exhibited a clearer distinction between species. The closest species to those found in this study was *P. lumarieul* to *P. csikii* (2.30, 6.27 %), *P. phoxinus* to *P. septimaniae* (4.2, 7.40 %), *P. fayollarum* to *P. dragarum* (2.87, - %)and *P. dragarum* to *P. bigerri* (4.11, 7.15 %), in agreement with their original distribution (Figure 1-1). Regarding intraspecies distances, the values for *P. dragarum*, *P. bigerri*, *P. septimaniae* and *P. csikii* were 0.23 ± 0.05 %, 0.56 ± 0.16 %, 0.73 ± 0.18 %, and 0.46 ± 0.13 % for COI, and 0.60 ± 0.10 %, 1.45 ± 0.19 %, 0.56 ± 0.12 %, and 1.53 ± 0.22 % for Cytb, respectively. Both COI and Cytb showed high intraspecies diversity in *P. bigerri*. However, it was *P. septimaniae* in COI and *P. csikii* in Cytb that exhibited the highest intraspecies distance.

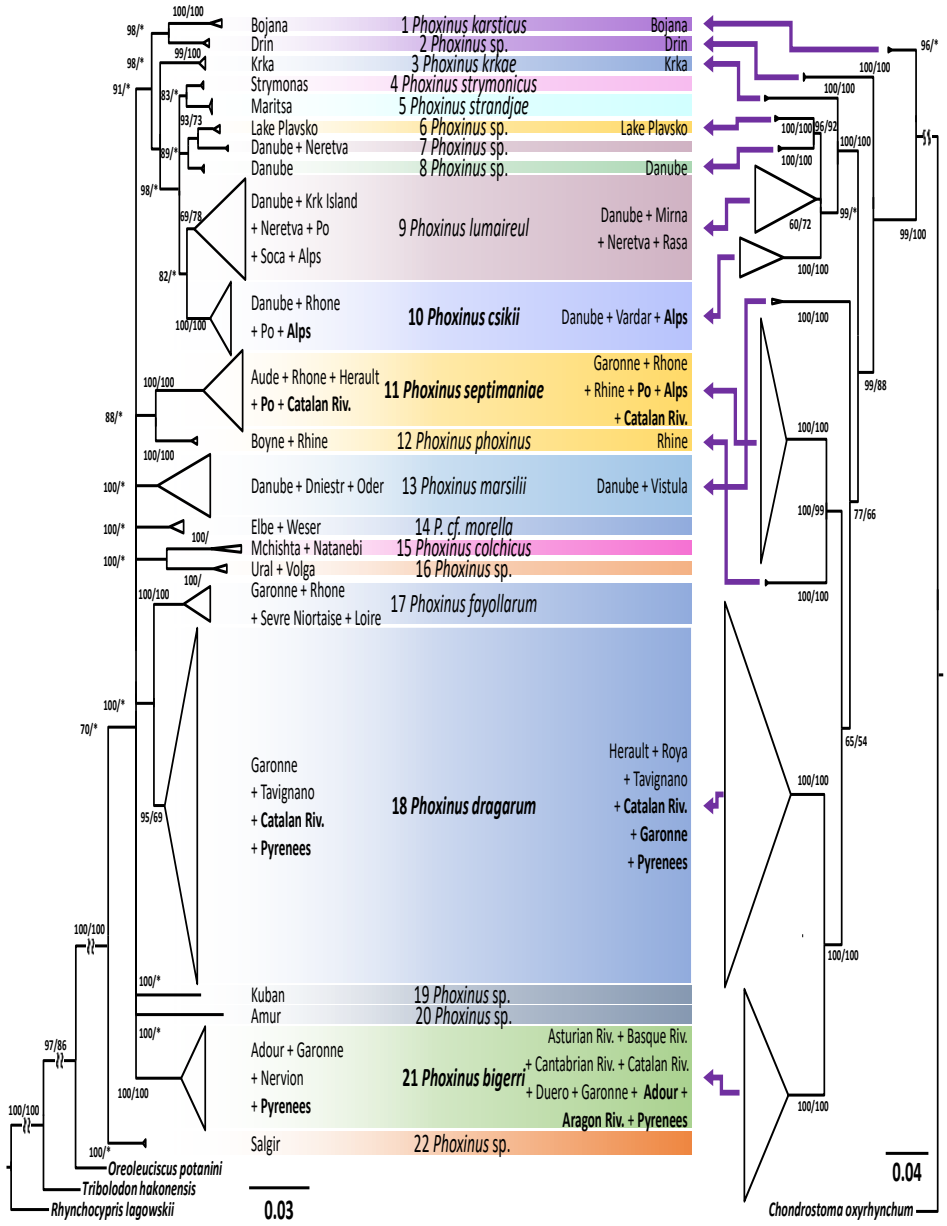


Figure 1-2. Phylogenetic reconstruction by Bayesian inference. The left tree is based on a fragment of the cytochrome c oxidase gene subunit I (COI) and the right tree is based on a fragment of the cytochrome b gene (Cytb). The values in front of each node are the significance resulting from Bayesian inference and maximum likelihood inference (%). The species and site names in bold are those with sequences obtained in this study. See Table 1-1, Table S1-1 and S1-2 for GenBank accession numbers.

Country	Basin	Site	Coexist species with COI	Coexist species with cytb
Spain	Catalan Pyrenees	Estany Llong (HML)	*P. bigerri, P. dragarum*	*P. bigerri, P. dragarum*
Spain	Aragon Pyrenees	Ibón del Cau (HML)	*P. bigerri, P. dragarum*	*P. bigerri, P. dragarum*
Spain	Catalan River	Segre - La Seu d'Urgell (LLR)	*P. dragarum, P. septimaniae*	*P. dragarum, P. septimaniae*
Spain	Catalan River	Segre - Ribera Salada (LLR)	*P. dragarum, P. septimaniae*	*P. dragarum, P. septimaniae*
Spain	Catalan River	Río Gurri (LLR)	*P. dragarum, P. septimaniae*	-
France	Pyrenees	Étang Mort (HML)	*P. bigerri, P. dragarum*	-
France	Garonne	Arrat-Devant (LLR)	-	*P. bigerri, P. dragarum*

Table 1-2. Sites where two *Phoxinus* species were found to coexist (LLR: lowland river, HML: high mountain lake).

In the lakes of the Pyrenees we found two species of Phoxinus: *P. dragarum* (lineage 18) was found in most of the Pyrenees although it was more common in the Central-Eastern Pyrenees, while *P. bigerri* (lineage 21) was found mainly in the western Pyrenees. In the lakes of the Italian Alps we found three species: *P. csikii* (lineage 10), *P. septimaniae* (lineage 11) and *P. lumaireul* (lineage 9), although the latter species was only present in sequences retrieved from GenBank. In the rivers of Catalonia, in addition to *P. dragarum*, we found extensive populations of *P. septimaniae* (Figure 1-2). Among all the sites we found coexistence of two species in seven sites (Table 1-2): we found both *P. dragarum* and *P. bigerri* in three high mountain lakes and in one river in France, and in three Catalan rivers we found coexistance of *P. dragarum* and *P. septimaniae*.

Table 1-3. presents genetic variability indices categorized by regional divisions for each species. An interesting observation is that, despite the limiting factor of sample size there are cases where introduced regions exhibit higher genetic diversity index than native habitats. *P. dragarum* which are known as native form Garonne basin, nucleotide diversity was the highest in there for both COI and Cytb, while haplotype diversity showed higher values in introduced areas, including high mountain lakes. Similarly, for *P. bigerri*, which is known to have its native habitat in Adour basin, it exhibited genetic diversity comparable to or even higher than that in high mountain lakes.

		P. dragarum										*P. bigerri*									
		cytb					COI					cytb					COI				
		N	H	H_d	P_i ($\times 10^{-4}$)	S	N	H	H_d	P_i ($\times 10^{-4}$)	S	N	H	H_d	P_i ($\times 10^{-4}$)	S	N	H	H_d	P_i ($\times 10^{-4}$)	S
Adour (France)	LLR											7	4	0.81	35.6	5	6	6	1	92.4	22
Garonne (France)	HML	2	2	1	18	1	4	4	1	35.2	7	1	-	-	-	-					
Garonne (France, Spain)	LLR	10	3	0.38	39.5	11	12	10	0.95	164.6	60	2	2	1	19.2	1					
Ebro (Aragon)	HML	3	2	0.67	12	1	3	2	0.67	18.4	3	15	5	0.7	47	8	16	10	0.93	87.3	33
Ebro (Aragon, Basque)	LLR	2	1	0	0	0	2	2	1	73.5	8	2	2	1	19.2	1	2	2	1	9.2	1
Ebro (Catalonia)	HML	63	8	0.27	6.2	8	32	21	0.97	55.4	50	4	2	0.67	12.8	1	2	2	1	36.7	4
Ebro (Catalonia)	LLR	14	3	0.47	9.1	2	9	7	0.92	36.7	12										
Catalonia Inner (Spain)	LLR	8	3	0.61	12.2	2	6	5	0.93	44.1	9										
Basque Inner (Spain)	LLR											3	1	0	0	0					

		P. septimaniae										*P. csikii*									
		cytb					COI					cytb					COI				
		N	H	H_d	P_i ($\times 10^{-4}$)	S	N	H	H_d	P_i ($\times 10^{-4}$)	S	N	H	H_d	P_i ($\times 10^{-4}$)	S	N	H	H_d	P_i ($\times 10^{-4}$)	S
Catalonia Inner (Spain)	LLR	4	3	0.83	18.5	2	4	2	0.5	19.4	4										
Ebro (Catalonia)	LLR	3	2	0.67	12.3	1	5	3	0.7	23.3	6										
Po (Italy)	HML	1	-	-	-	-	1	-	-	-	-	8	2	0.25	4.7	1	4	3	0.83	18.8	4
Po (Italy)	LLL	5	3	0.7	14.8	2	2	1	0	0	0										

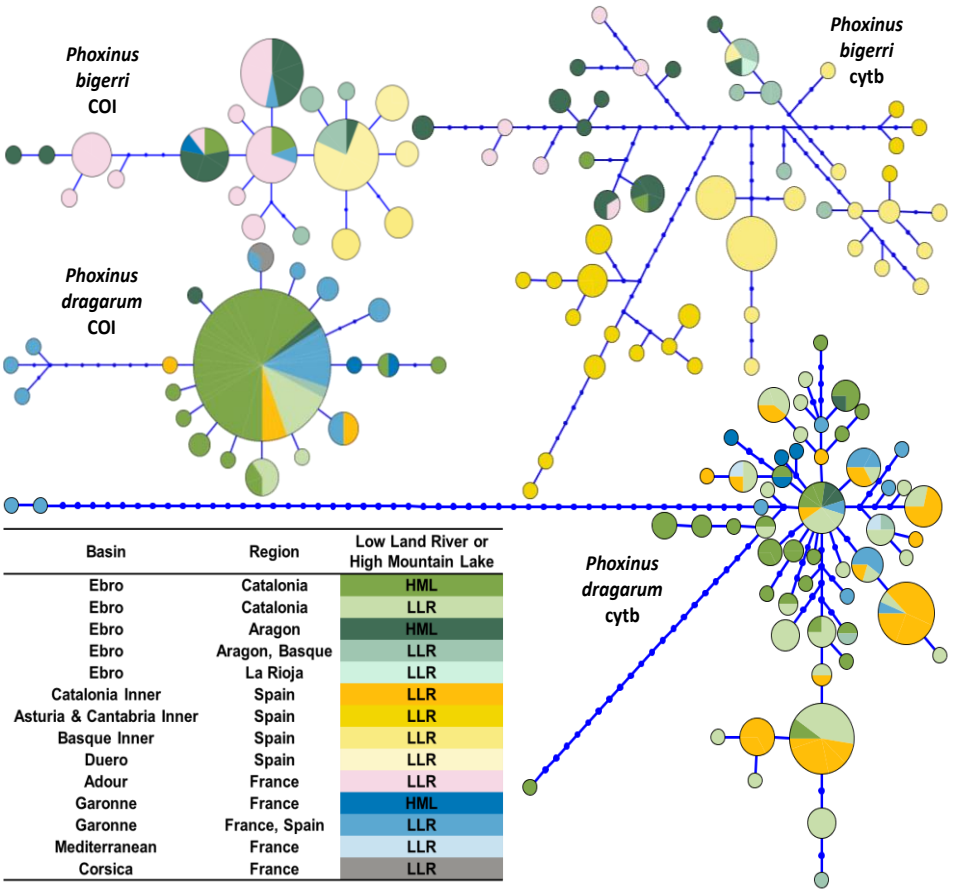
Table 1-3. Genetic variability indices for each *Phoxinus* spp. in each high mountain lake for two mitochondrial DNA gene fragments (Cytb and COI). N = number of individuals; H = number of haplotypes; S = number of polymorphic sites; H_d = haplotype diversity; and P_i = nucleotide diversity, LLR: lowland river, HML: high mountain lake.

4.2. Haplotype networks by each species

Eight different haplotype networks were generated for the four species from this study, for COI and Cytb (Figures 1-3 & 1-4). For all the species, the haplotype networks were quite different between the two gene fragments in agreement with the contrasting differences in their length and differences in the number of sequences available. The species with more analysed sequences was *P. dragarum* (Table 1-3). In this species, the COI haplotype network included 20 haplotypes of which 75 % were unique and 25 % were shared between at least two regions (Figure 1-3). There was one dominant haplotype with most of the sequences from the lakes of Pyrenees that was also present in the lowland

Catalan rivers, in the Ebro and the Garonne (the native basin of the species). For Cytb we found 56 haplotypes of which 71 % were unique and 29 % were

shared with two or more regions, a similar pattern to COI. Of these, 29% were shared between the area of origin and the rivers of inner Catalonia and Ebro, one was shared between the high mountain lakes and the rivers of inner Catalonia and one in the three areas. In high mountain lakes we found 10/24 haplotypes in the 17/16 lakes sampled (for COI/Cytb), with one lake with three haplotypes for COI (Lake Closell) and two lakes with four haplotypes (Lakes Mort and Muntanyó d'Àrreu) for Cytb. One haplotype was present in one lake from the Catalan Pyrenees and in a lake from France. The Lakes Finestres and Soliguera, the former draining to the latter, did not share any haplotypes.



Basin	Region	Low Land River or High Mountain Lake
Ebro	Catalonia	HML
Ebro	Catalonia	LLR
Ebro	Aragon	HML
Ebro	Aragon, Basque	LLR
Ebro	La Rioja	LLR
Catalonia Inner	Spain	LLR
Asturia & Cantabria Inner	Spain	LLR
Basque Inner	Spain	LLR
Duero	Spain	LLR
Adour	France	LLR
Garonne	France	HML
Garonne	France, Spain	LLR
Mediterranean	France	LLR
Corsica	France	LLR

Figure 1-3. Haplotype networks of *P. dragarum* and *P. bigerri* based on COI and Cytb; Basin - depending on the scale, a watershed is divided into regions or watersheds within a region are combined, Region - by the study, region is divided into region or country level., LLR: lowland river, HML: high mountain lake.

The *P. bigerri* haplotype network showed the most distinct spatial pattern in both COI and Cytb (Figure 1-3) and included 19 and 51 haplotypes for COI and Cytb respectively, of which 21 % / 6 % were shared between two or more regions and 79 % / 94 % were unique for COI and Cytb. The COI haplotype network is largely divided into two parts, one group contains haplotypes from rivers from the Ebro basin (from Aragon and the Basque Country) and the Duero basin, and the other group includes rivers from the Adour basin together with lakes from the Pyrenees. The Cytb haplotype network was similar to those of COI, but with an additional group from Cantabria, available only for this gene (Corral - Lou et al., 2019). In high mountain lakes we found 7/11 haplotypes, most of them in lakes from the Aragonese Pyrenees, with the exception of two haplotypes that were found in Lake Llong, in the Catalan Pyrenees, inside the area where fishing is forbidden since 1989. This is the only location where *P. bigerri* was found in Catalonia.

P. septimaniae also presented an important spatial segregation in the two haplotype networks (Figure 1-4), although they are not totally comparable for not having the same number of sequences (retrieved from GenBank). We found 28 haplotypes for COI and 16 for Cytb, of which both 25 % were shared between regions. In COI there were many sequences from the French Mediterranean and the Rhine (retrieved from the GenBank), while in Cytb there were many sequences from Catalan rivers. In these haplotype network we only found three haplotypes for both COI and Cytb, that were also present in the French Mediterranean rivers for COI, but not present in the native area for Cytb (Rhone and the French Mediterranean rivers). Within high mountain lakes we only found *P. septimaniae* in one of the lakes from the Italian Alps (Lake Dres, Gran Paradiso National Park). Most of the Italian haplotypes from this study were from the Ceresole Reservoir (Po, three/one haplotypes). Eight of the sequences downloaded from Genbank were also from eight high mountain lakes, with the two most common haplotypes also present in the Rhone and Rhine rivers.

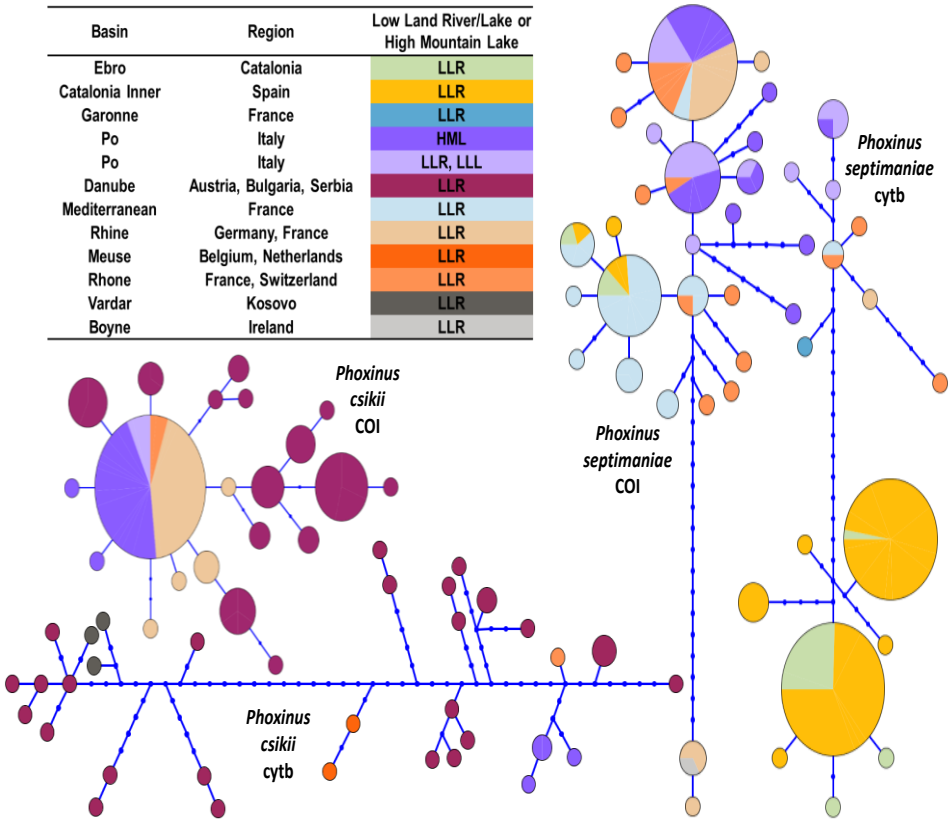


Figure 1-4. Haplotype networks of *P. csikii* and *P. septimaniae* based on COI and Cytb; Basin - depending on the scale, a watershed is divided into regions or watersheds within a region are combined, Region - by the study, region is divided into region or country level., LLR: lowland river, HML: high mountain lake.

Like the latter species, *P. cesikii* was also found in high mountain lakes of the Italian Alps, and was absent in the Pyrenees and the Iberian Peninsula (Figure 1-4). On the other hand, the haplotype network of COI showed that most sequences from the lakes of the Italian Alps belonged to a single haplotype. This haplotype was also present in the Rhine and Rhone rivers. We found 21 and 33 haplotypes for COI and Cytb respectively. Most of the haplotypes were from the Danube.

4.3. Genetic diversity

We compared the species diversity for each species separating the known native areas from those where they are introduced (Figure 1-5). In the latter case we also calculated the diversity in high mountain lakes separately, where introductions are most recent. We found a decreasing tendency in diversity from native areas through introduced areas to alpine lakes for most of the species, which was clearer for COI than for Cytb and for haplotype diversity rather than nucleotide diversity. In the case of *P. septimaniae*, we did not obtain sufficient alpine lake sequences for Cytb to be able to obtain the statistic.

We also calculated the mean number of *Phoxinus* species and intraspecific diversity (Figure 1-6) found in high mountain lakes depending on their fishing management strategy. We have found that in the lakes where the most

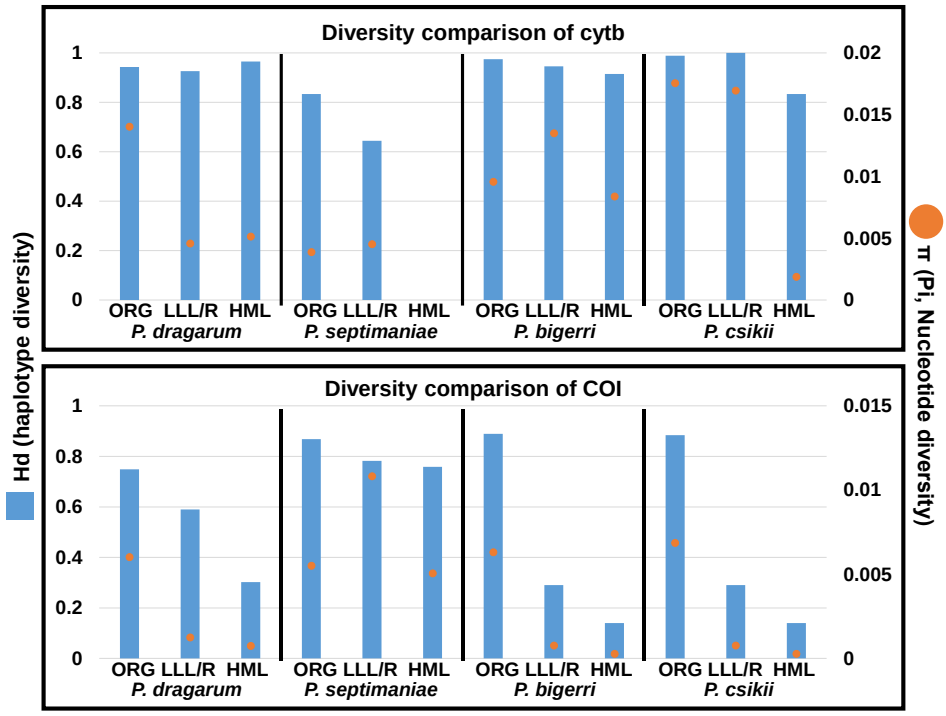


Figure 1-5. Mean genetic diversity of native and introduced habitats; Hd (haplotype diversity), π (Nucleotide diversity), ORG (Native), LLL/R (Low land lakes or river), HML (Alpine lakes). Both newly generated and GenBank sequences were included in the analysis.

active fishing practice is allowed there is the greatest number of species per lake (1.7 species per lake; Figure 1-6 (a)) and the highest diversity, while in the lakes where fishing is nowadays forbidden the diversity is low. This tendency is especially clear for *P. dragarum*, due to the higher available sample size.

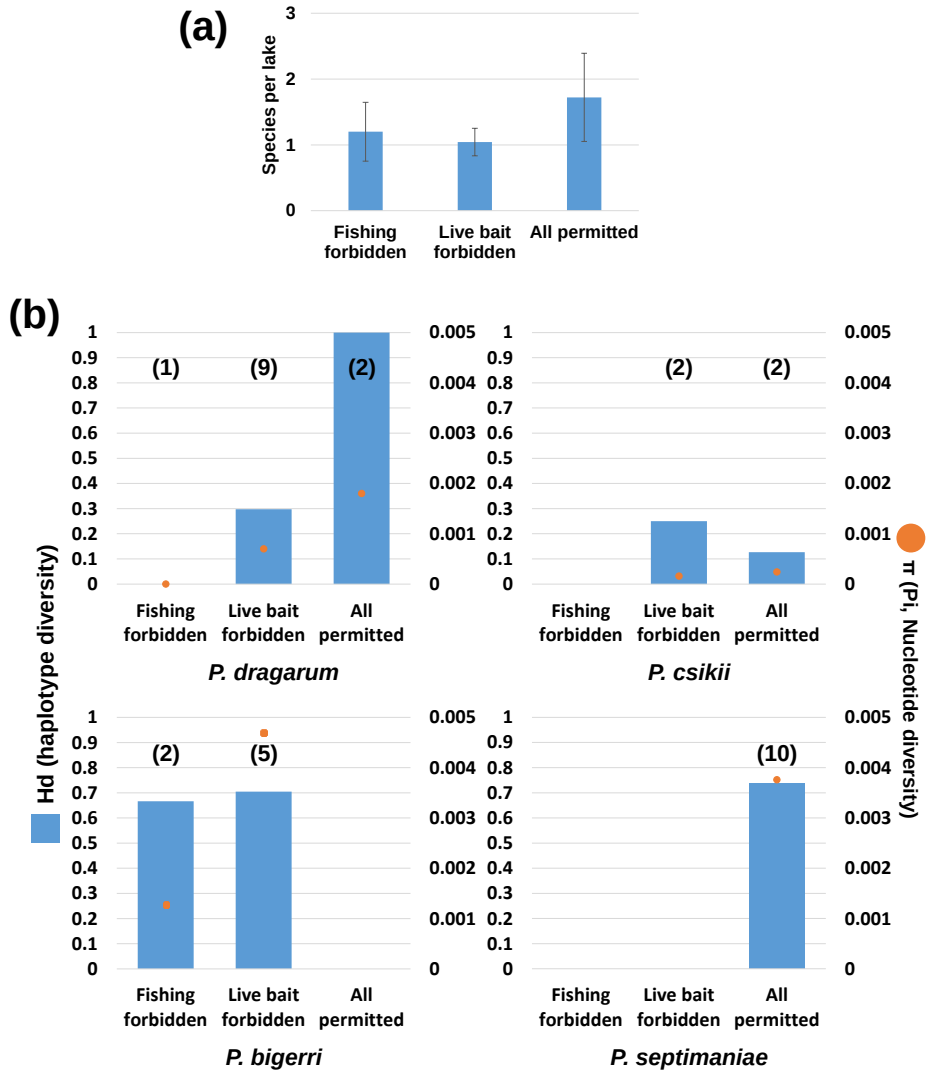


Figure 1-6. Mean number of species per each high mountain lake (a) and genetic diversity (b) of the different high mountain lakes subdivided according to the fishing management implemented in each lake; Hd (haplotype diversity), π (Nucleotide diversity). Both newly generated and GenBank sequences were included in the analysis.

5. Discussion

5.1. Invasive or native species?

The outcomes of the molecular studies on species identification and distribution of the genus *Phoxinus* has recently led to the discovery of new species, such as *P. dragarum* or *P. fayollarum*. And also, there are still undescribed species, like *P. cf. morella* (V Caputo Barucchi et al., 2022; Cruz et al., 2022; Denys et al., 2021; Kottelat, 2007; Palandačić et al., 2022; Reier, Kruckenhauser, Snoj, Trontelj, & Palandačić, 2022; Turan, Bayçelebi, Özuluğ, Gaygusuz, & Aksu, 2023). The use of *Phoxinus* spp. as live bait (Miró & Ventura, 2015) is favouring their translocation to different areas. Therefore, establishing the native areas is not easy.

According to Denys et al. (2020) all species of *Phoxinus* described from France are native, with *P. dragarum* native from the Garonne basin. In the Iberian Peninsula, *P. dragarum* has been found in Catalonia, including in the River Segre (an effluent of Ebro) and in the inner rivers of Catalonia (Corral‐Lou et al., 2019). The results of this study confirm the distribution presented by Corral-Lou et al. (2019), including a lack of spatial genetic pattern (Figure 1-3), which would not be expected in a species native of the area. In addition to these results, we have collected historical data from all Catalan rivers (data not shown; E. Aparicio Pers. Comm) which shows that the first detection of *Phoxinus* was relatively recent (i.e. after 1980), and has been spreading since this date. We suggest that this species has been introduced in Catalonia, and therefore should be considered allochthonous of the Iberian Peninsula. The much lower genotypic diversity in Catalonia also points towards this direction. For the same reason the other species found in the Catalan rivers, *P. septimaniae,* is also to be considered an introduced species. The very low haplotype diversity in the Catalan rivers (Figure 1-4) suggests that the introduction of *P. septimaniae* in Catalonia is relatively recent.

In contrast, *P. bigerri* has been described to be native from the Adour drainage together with the Iberian Cantabric drainages (Denys et al., 2020). This species has been described to be introduced in the Duoro basin (Garcia-Raventós et al., 2020) but of uncertain origin in the Ebro basin (Corral‐Lou

et al., 2019). The discontinuity of the spatial pattern in haplotype network and low genetic diversity of the sequenced individuals of the Ebro compared to those of Adour or the Cantabric, together with the absence of the species in the Segre suggest that *P. bigerri* can be considered to be introduced in the Ebro. Furthermore, historical fishing data (E. Aparicio; Pers. Comm.) also confirm that this species can be considered invasive in the Ebro. In Italy, the only known native *Phoxinus* species is *P. lumaireul* (De Santis et al., 2021), and therefore, *P. csikii* and *P. septimaniae*, which are currently present in lakes of the Alps, can be considered to be introduced.

5.2. Introduction routes in high mountain lakes

With the exception of few studies focussing on the introduction processes of *Phoxinus* in lakes of Norway (Museth et al., 2007) or high mountain lakes of the Pyrenees (Miró & Ventura, 2015) or the Alps (De Santis et al., 2021; Schabetsberger et al., 2023), most of the existing studies have focused on rivers. Nearly all high mountain lakes are naturally fishless (Moyle, Yoshiyama, & A, 1996; Ventura et al., 2017), and the introduction of minnows results in negative effects for native lake fauna (A. Miró et al., 2018; Osorio et al., 2022; Schabetsberger et al., 1995) and even on introduced lake trout (Museth, Borgstrøm, & Brittain, 2010; Tiberti et al., 2022). Therefore, all minnows introduced in these ecosystems (as is also the case of salmonids) should be viewed as invasive species. Due to the difficulty of morphological differentiation of the different species, that require sexually mature adults (Denys et al., 2020), this study focused on the use of genetic tools to describe the species introduced on the lakes and their possible invasion routes. We found that *P. dragarum* was the main introduced species in the lakes from the South-Eastern part of the Pyrenees and *P. bigerri* in the Western part of the mountain range. This distribution partially agrees with the distribution of the two species in the rivers of both France and the Iberian Peninsula. The clear spatial segregation of *P. bigerri* haplotypes (Figure 1-3) shows that most of the individuals introduced in the lakes of both Spanish and French Pyrenees have origin in the Adour region (France). Current fishing regulations in the Pyrenees are very different depending on the region (Figure 1-6 (b); see

below), ranging from fishing ban in the core area of Aigüestortes and Estany de Sant Maurici National Park, to live bait banning in the whole Spanish Pyrenees, to allowance of live bait fishing in all the French Pyrenees with the exception of the Eastern area (Oriental Pyrenees department). Therefore, it is likely that the introduction of *P. bigerri* from Adour area to the southern slope of the Pyrenees was conducted by angler used to fish with live bait in their area of origin (France). In contrast, the genetic information obtained with the haplotype network of *P. dragarum* is much less conclusive, and does not allow to identify the origin of the introduced individuals in high mountain lakes. However, the absence of *P. dragarum* in the stream sectors immediately below the mountain area in the Spanish Pyrenees (Figure 1-1) suggests that the same scenario of *P. bigerri* is possible.

In the Italian Alps we have found one single individual of *P. septimaniae* in one lake of Gran Paradiso National Park (Lake Dres), being the only individual of minnow present in the lake (the lake was restored in 2016 under LIFE BIOAQUAE; Tiberti and Splendiani (2019)). However, the same species was also found in eight high mountain lakes in a nearby area (south of the Aosta valley) by De Santis et al. (2021) and in the reservoir of Ceresole (De Santis et al., 2021; Palandačić et al., 2017), a lake with a very high haplotype diversity. Italian streams are naturally inhabited by *P. lumaireul*, endemic from Italy (De Santis et al., 2021). These authors did not find *P. septimaniae* or other species of minnows in the Western Po River (with the exception of one single individual of *P. csikii*). Since the haplotype found in Lake Dres is also present in Ceresole reservoir, it is most likely that the source of the introduction into this lake comes from the Ceresole reservoir. The haplotype network of this species for COI also shows that the two most common haplotypes found in Italian high mountain lakes are also present in the Rhone River, in one haplotype of the Mediterranean rivers of France (the two native areas of the species; Figure 1-1), and quite abundant in the Rhine. On the other hand, none of the haplotypes found in high mountain lakes have been found in the Danube (the native area of the species), but the dominant haplotype in COI has been also found to be abundant in Rhine and in one individual of the Rhone. in conjunction with the haplotype network of Cytb, it appears that, in the case of *P.csikii*, populations with similar genetic characteristics have been

preserved in isolated regions after introduction, without additional introductions.

5.3. Different introduction events

In the lakes of the Pyrenees and the Alps we have found different *Phoxinus* species within the same mountain range or in the same lake, and at the same time different haplotypes (genetically different individuals belonging to the same species) also within the same lake or in different lakes of the same mountain range. What tells us in both cases is that they probably came via different introduction routes, although it cannot be excluded that different species or genetically differentiated individuals of the same species may have been introduced all at once or by a single channel, for example one fisherman. In fact, in France, individuals of the genus *Phoxinus* can be bought in specialized fishing centres, although some fishermen also catch them in lowland rivers. Therefore, the most logical hypothesis is that the same fishermen introduce some individuals that are genetically very close. Additionally, the genetic mutation rate in the sequenced mitochondrial genes is low enough (Brower & Desalle, 1994) to disregard that new genetic mutations have developed during the years that the genus *Phoxinus* have been in the lakes (about 40-50 years at the most; Miró and Ventura (2015)).

5.4. Genetics of invasive alien species

Combining the results from this study together with those retrieved from the GenBank and separating the different localities between native and introduced (i.e. lowland rivers and high mountain lakes), we found that overall, there was a decrease in both haplotype and nucleotide diversities from the native area to the two introduced habitat types (Figure 1-5). There are some exceptions, which are likely a result of differences in the consistencies of the data (e.g. sample size is different for the different species and categories). This is in agreement with previous studies that found lower genetic variability in areas where a species has been introduced compared to

its native area, where it has been evolving for many years under different selection pressures, despite finding particular cases where the general rule does not apply (Barrett, 2015; Bock et al., 2015). We also find contrasting differences between the different species, which is likely a result of a different colonisation time, with more recently colonised habitats having lower genetic diversity (e.g. as is the case of high mountain lakes, compared to lowland rivers, where minnow introductions are older). The haplotypic variability of *P. septimaniae* in the rivers of Catalonia is lower than *P. dragarum,* or that of *P. csikii* is lower than that of *P. septimaniae* in the Italian Alps (Figure 1-3 & Figure 1-4), both cases likely a result of a more recent introduction of the less diverse lakes.

5.5. Effects of fishing regulations

Another interesting aspect is that there seems to be a relationship between the intensity of fishing in a geographic area and the number of species found in each lake or the genetic diversity within each species (Figure 1-6). The localities where we have found more than one species introduced into the same lake are the lakes of the French Pyrenees and the Italian Alps of the southern side of the Aosta Valley. These two areas, which do not have live bait fishing prohibited, are probably the most active areas for live bait fishing. On the other hand, in the lakes of the Catalan and Aragonese Pyrenees (peripheric area of Aigüestortes and Estany de Sant Maurici National Parc, Alt Pirineu Natural Park and Posets Maladeta Natural Park) and of the Mont Avic Natural Park, fishing with live bait is prohibited so in theory *Phoxinus* should not be found. Despite of that, we found different species or haplotypes within the same lake. On the other hand, in the strict area of the National Park of Aigüestortes and Estany de Sant Maurici and within the Gran Paradiso National Park, where fishing was banned in 1989 and 1950 respectively, we find the lowest incidence of introduced minnows, and only one *Phoxinus* lineage. These introductions took place before the fishing ban, or by very occasional illegal events. Overall, the level and intensity of fishing seems to be the key management strategy to regulate minnow introductions.

6. Conclusion

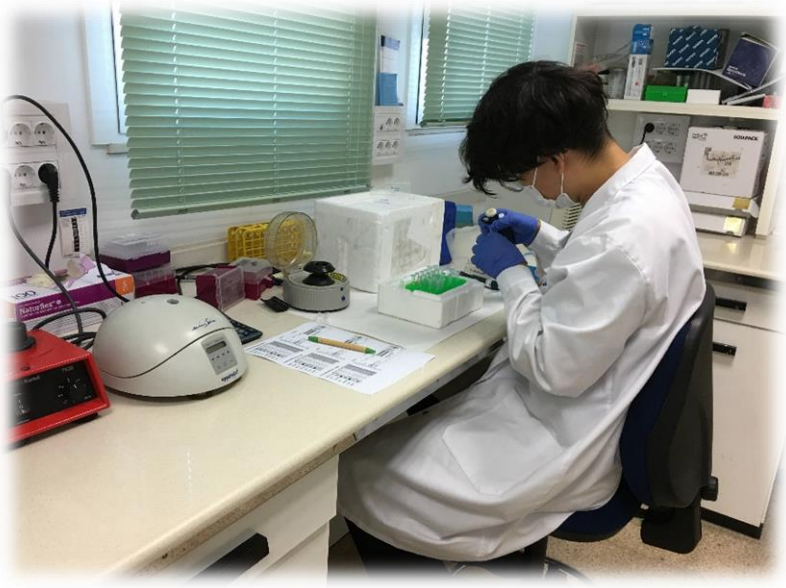
What can be done to prevent minnow dispersal? Ceasing institutional fish stocking campaigns, fishing bans and prohibition of using live-baits demonstrated to be effective conservation measures to slow down or stop fish invasions in mountain areas. In addition alien fish removal have the potential to reverse the invasion process through habitat restoration project (LIFE BIOAQUAE www.bioaquae.eu, LIFE LIMNOPIRINEUS www.lifelimnopirineus.eu/en, LIFE RESQUE ALPYR www.liferesquealpyr.eu). More detailed distribution data and taxonomic studies are also needed to improve knowledge on fish introductions and inform people and stakeholders about the spread of *Phoxinus* spp. Outside their native ranges, a conservation issue for native biota as well as for native minnows.

The results of this study illustrate that genetic analyses of introduced species can provide information on the dynamics of invasion, as they provide us important details about the provenience of introduced populations. However, the mitochondrial DNA analysis we performed can only estimate and cannot demonstrate whether the history of introduction to alpine lakes in Spain came from Catalan rivers or from France. The use of higher-resolution genetic tools such as microsatellite analysis could explain the origins of the diverse lineages discovered, providing useful data for conservation.

Part 1

Chapter 2

Patterns of genetic differentiation and admixture in four species of European minnows from the Italian Alps, the Pyrenees and lowland rivers



1. Abstract

Freshwater invasive fish species have had negative impacts on ecosystems and this can be even more serious in isolated, unique ecosystems such as high mountain lakes. In this study, using microsatellites, we analyzed approximately 900 samples of the genus *Phoxinus* from 62 sites located in high mountain lakes and nearby catchments from the Pyrenees and the Italian Alps, confirming the presence of four species *P. bigerri*, *P. csikii*, *P. dragarum* and *P. septimaniae*. The results from the mitochondrial marker used in Chapter 1 and the nuclear markers used in this study were combined to analyze interspecific hybridization and intraspecific admixture. The findings revealed the discovery of *P. dragarum* and *P. bigerri* in Italy, the presence of *P. bigerri* in Catalonia and *P. dragarum* in Aragon. We found mitochondrial/nuclear DNA hybrids (7% of the cases) including all the individuals identified as *P. septimaniae* in the rivers from Catalonia. We found a lower proportion of nuclear DNA hybrids (3%) including the individuals of river Tenes, that were both nuclear and mitochondrial hybrids of three species, *P. septimaniae, P. dragarum* and *P. bigerri*. We also found a certain proportion of intraspecific admixture between the different lineages, that was higher in high mountain lakes and rivers where they were introduced (mean of 2 and 1.7 lineages per site respectively) compared to native rivers (1.1). We did not find any evidence of downstream colonisation from lake sources. These results demonstrate that the invasion of non-indigenous species can undermine the uniqueness of native species, and provide a basis for a more elaborate estimation of introduction pathways compared to studies that used mitochondrial markers. Utilizing the inferred potential introduction pathways for management and conservation, along with continuous monitoring of distribution and genetic exchanges, will contribute to the countermeasure of invasive fish species.

2. Introduction

Freshwater ecosystems occupy only 1% of the Earth's surface, yet they are responsible for hosting 10% of known species and a third of vertebrate species.

Freshwaters are also hotspots for human activities that have led to widespread habitat degradation, pollution, flow regulation and water extraction, fisheries overexploitation, and alien species introductions (Dudgeon et al., 2006; Strayer & Dudgeon, 2010). Among them, biological invasions have rapidly increased over the past century and are considered a major threat to biodiversity and ecosystem preservation (Simberloff et al., 2013). In Europe, freshwater fish are one of the animal groups with the greatest number of invasive species (Hulme, 2009).

Such introductions, especially, have more critical consequences in particular regions. The introduction of fish into high mountain lakes, naturally devoid of fish due to topographical barriers and ecological characteristics (Knapp et al., 2001; Miró & Ventura, 2013; R Pechlaner, 1984), is mainly promoted by various national administrative agencies as part of recreational fishing (Pister, 2001; D. Schindler & Parker, 2002). Over time, these first introductions have led to secondary introductions of other fish species used as live bait, which have caused significant adverse effects on the local ecosystems, particularly when they become the dominant fish species in the lake (Teresa Buchaca et al., 2019; Miró, Sabás, & Ventura, 2018; Osorio et al., 2022). The genus *Phoxinus*, commonly used as live bait, is composed of small fishes, widespread in Eurasia from Spain to Korea. They are rheophilic and cryophilic inhabitants of streams and clear lakes (Denys et al., 2020).

The lack of research and stabilizing selection is reflected in the diversity of the genus *Phoxinus*. Over the last two decades, studies related to this genus have continued to expand, and it has become evident that the genus *Phoxinus* encompasses previously undetected taxonomic complexity (Denys et al., 2020; Kottelat & Freyhof, 2007; Palandačić et al., 2017). Most of the genetic analyses within the genus *Phoxinus* have been based on mitochondrial gene analysis, while nuclear data supporting species delimitation is either uncertain or still incomplete (Palandačić et al., 2020). In the Iberian Peninsula, the species known to be present are *P. bigerri* Kottelat 2007, *P. septimaniae* Kottelat 2007 and *P. dragarum* Denys, Dettai, Persat, Daszkiewicz, Hautecoeur & Keith, 2020 (Corral‐Lou et al., 2019; Denys et al., 2020). The native range of *P. bigerri* is known to include the Adour and the Loire basin in France, as well as the

eastern Cantabrian region in Spain, but not the River Ebro (see Chapter 1). In the case of *P. septimaniae,* it naturally extends from the Rhone basin and the French coastal Mediterranean catchments (Denys et al., 2020). *P. dragarum* is an endemic species in the Garonne basin and is known to have been introduced to Spain in the Ebro basin (Denys et al., 2020). In France, in addition to the three previously mentioned species, *P. phoxinus, P. csikii* Hankó, 1922, and *P. fayollarum* Denys, Dettaï, Persat, Daszkiewicz, Hautecoeur & Keith, 2020 are also found, all of which are native to France and various other regions (Denys et al., 2020). In Italy, *P. lumaireul* Schinz, 1840 is the sole native species (V. Caputo Barucchi et al., 2022), while the other two species found, *P. septimaniae* and *P. csikii*, are introduced (De Santis et al., 2021).

To figure out the pathways of invasive species introduction, prevent further harm and protect unique local ecosystems, a broader range of genetic analytical approaches and extensive genetic background information from various regions should be supported. Species within the *Phoxinus* genus are still being discovered, and cases of hybridization within this genus have been reported (Angers & Schlosser, 2007; Mee & Taylor, 2012). With this information, we can propose better prevention mechanisms at various stages, including restricting pathways, intercepting movements at borders and assessing risk for intentional imports (Simberloff et al., 2013). In effect, many introduced species have been successfully eradicated or managed to low densities (Simberloff, 2009). An effective early warning and rapid response system would be of significant assistance in eradicating invasive species. This can be further supported with approaches from related fields that are now possible, thanks to the ability to maintain many invasive species at low levels through biological, chemical or physical control methods (Simberloff, 2014).

In this context, this study primarily aimed at investigating the diversity of *Phoxinus* populations from high mountain lakes in the Pyrenees and Italian Alps, as well as their surrounding catchments, using microsatellites. We were able to perform species level differentiation as well as identify lineages and sublineages within species, which aided in exploring potential introduction pathways. The objective of the study was to (1) confirm a more detailed

geographic distribution of species by comparing and integrating nuclear microsatellites with mitochondrial genes (Chapter 1), (2) deduce more precise introduction pathways by identifying sub-lineages within the same species, and (3) use high mountain lakes as scenarios of possible species hybridisation or admixture and (4) raise awareness about the severity of risks posed by invasive species and contribute to the development of measures to prevent further spread.

3. Materials and Methods

3.1. Fish sampling

The sampling area was designed to encompass the distribution of the genus *Phoxinus*, spanning the Pyrenees and the Alps, including Spain, France and Italy. Combining new samples with those used in chapter 1, we analyzed up to 30 individuals per site from a total of 62 sampling sites, resulting in a collection of 890 individuals for nuclear DNA (nDNA) analysis (Table 2-1). As for the Pyrenees, we additionally collected samples from the border areas of Spain and France, as well as some rivers in Catalonia (Table 2-1). Individuals were either fixed in absolute ethanol or frozen (- 20 ºC) until DNA extraction. Evolutionary lineages were identified based on nDNA data, and species were confirmed using mitochondrial DNA (mtDNA) data from previous research (Chapter 1).

3.2. Laboratory procedures

DNA was extracted from muscle tissue using either the HOTShot technique (Montero‐Pau et al., 2008) or the QIAmp DNeasy Blood and Tissue Kit (QIAGEN, Germany). A total of 22 microsatellites (Grenier, Costedoat, Chappaz, & Dubut, 2013) were amplified in three optimized multiplex reactions (see Figure S2-1). PCR amplifications were performed in a total volume of 11 µl, which contained 10 µl of Qiagen multiplex PCR master mix, distilled water and primer mix following the concentrations described by

Grenier et al. (2013), and 1 µl DNA extract. After the initial test, a positive control was used in each reaction to identify any issues. The PCR touchdown cycle conditions were the same for all multiplexes: denaturation step of 15 min at 95 °C, 30 cycles at 94 °C (30 s), 56 °C (30 s), 72 °C (60 s), and 60 °C (40 min). PCR products were electrophoresed on an ABI 3730 capillary sequencer (Secugen, Madrid, Spain), fragments were sized with LIZ-500 size standard and binned using Geneious (Java Version 11.0.4+11). To ensure data reliability, only samples with an individual amplification success rate of 75% or higher and loci with an amplification rate of 75% or higher were used for analysis. For mitochondrial DNA, we utilized the same individuals that were previously analyzed in Chapter 1, using COI and Cytb datasets.

3.3. Genetic diversity and population analysis

Genetic structure was analyzed hierarchically using the Bayesian algorithm implemented in STRUCTURE 2.3.4 (Falush, Stephens, & Pritchard, 2003; Stephens & Donnelly, 2000) (i.e., starting with an initial assessment of the entire dataset, followed by independent evaluations of the major genetic groups corresponding to each species – see Results for more details). A total of ten independent runs were conducted for various clusters (K) ranging from 1 to 10 using the Bayesian algorithm implemented in STRUCTURE 2.3.4. Each run consisted of a burn-in period of 1×10^5 iterations followed by 5×10^5 iterations, employing the correlated allele frequencies admixture model and lacking prior information about the population of origin. The value of K with the highest ΔK statistic was determined using STRUCTURE HARVESTER 0.6.94 (Earl & VonHoldt, 2012), representing the optimal K that best explained the observed genetic data. For this optimal K, the runs were summarized using Pophelper 2.3.1 (Francis, 2017) and visually displayed in R 2020.0.3 software. In addition, Discriminant Analysis of Principal Components (DAPC; Jombart and Collins (2015)) was run in R using the adegenet package (Jombart, 2008) as an alternative and complementary approach to describe genetic population structure. This analysis primarily aimed at species identification and was based on the four species recognized in previous

studies. The number of retained principal components and discriminant functions were selected following the package's manual guidelines.

Site Number	Country	Site Name	Coordinates	Num. of Microsat	Num. of mtDNA	Num. of species	Num. of lineages
1	Italy	Lake Capezzone	45°56'17.6"N 8°12'33.2"E	3	-	1	1
2	Italy	Lake Gran-Lac	45°38'26.5"N-7°33'24.7"E	5	2	1	1
3	Italy	Lake Balena	45°38'23.8"N-7°32'54.6"E	15	2	1	1
4	Italy	Lake Vernouille	45°38'01.1"N-7°35'34.8"E	19	2	1	1
5	Italy	Lake Leita	45°38'36.5"N-7°33'01.7"E	24	2	1	1
6	Italy	Lake Miserin	45°36'05.6"N-7°31'19.8"E	24	2	1	1
7	Italy	Reservoir Ceresole	45°25'31.9"N-7°14'02.4"E	26	4	3	3
8	France	River Adour (Saint-Paul-lès-Dax)	43°45'16.6"N-1°02'13.9"W	1	1	1	1
9	France	River Adour (Saugnac et Cambran)	43°40'11.4"N-0°59'36.4"W	1	-	1	1
10	France	River Adour (Cheraute)	43°14'03.7"N-0°49'30.3"W	2	1	1	1
11	France	River Adour (Menditte)	43°10'05.4"N-0°53'44.0"W	2	-	1	1
12	France	River Adour (Osse-en-Aspe)	42°59'59.1"N-0°36'16.2"W	1	1	1	1
13	France	River Adour (Assat)	43°14'41.9"N-0°18'28.0"W	2	1	1	1
14	France	River Adour (Juillan)	43°11'54.0"N-0°01'47.6"E	2	2	1	1
15	France	River Adour (Tarbes)	43°14'09.9"N-0°05'07.9"E	1	1	1	1
16	France	River Adour (Estirac)	43°14'03.7"N-0°49'30.3"W	2	1	2	2
17	France	River Garonne (Manent-Montané)	43°20'39.0"N-0°37'29.5"E	2	2	2	2
18	France	River Garonne (Lespiteau)	43°04'05.6"N-0°45'43.2"E	2	2	1	2
19	France	River Garonne (Cierp-gaud)	42°54'59.3"N-0°38'29.8"E	2	2	1	1
20	France	River Garonne (Rivèrenert)	42°57'21.1"N-1°12'48.6"E	1	1	1	1
21	France	River Garonne (Dun)	43°01'15.5"N-1°47'53.9"E	2	1	1	1
22	France	Lake Mort	42°45'53.2"N-1°25'28.5"E	23	7	1	1
23	Spain	River Garonne (Bausen)	42°49'51.1"N-0°43'44.6"E	3	3	1	1
24	Spain	River Garonne (Les)	42°48'41.9"N-0°42'41.0"E	20	2	1	1
25	Spain	River Gállego (Anzánigo)	42°24'15.5"N-0°39'04.3"W	8	1	1	2
26	Spain	River Bellos (Cinca)	42°31'02.1"N-0°06'33.6"E	8	2	1	2
27	Spain	River Noguera Palleresa (Esteri d'Aneu)	42°37'38.9"N-1°07'26.2"E	19	2	1	4
28	Spain	River Noguera Palleresa (Gerri de la sal)	42°18'47.0"N-1°03'39.8"E	5	1	1	2
29	Spain	River Noguera Palleresa (Puigcercós)	42°07'43.8"N-0°54'07.4"E	9	1	1	5
30	Spain	River Segre (Pla de Sant Tirs)	42°18'20.4"N-1°21'56.6"E	10	4	1	1
31	Spain	River Segre (La Seu d'Urgell)	42°21'11.0"N-1°27'41.3"E	17	5	1	1
32	Spain	River Segre (Martinet)	42°22'00.7"N-1°42'44.5"E	10	4	1	2
33	Spain	River Segre (Ribera Salada)	42°01'14.5"N-1°19'11.0"E	3	-	1	1
34	Spain	River Llobregat (Cardener)	41°55'49.1"N-1°38'04.6"E	10	2	1	1
35	Spain	River Ter (Ripoll)	42°11'47.9"N-2°11'32.4"E	5	3	1	1
36	Spain	River Ter (Sant Joan de les Abadeses)	42°14'08.9"N-2°17'07.2"E	3	-	1	1
37	Spain	River Gurri (Llica de Vall)	41°59'01.4"N-2°17'22.2"E	1	1	1	1
38	Spain	River Avenco (Aiguafreda)	41°46'04.5"N-2°14'56.8"E	1	1	1	1
39	Spain	River Tenes (Lliçà de Vall)	41°35'11.6"N-2°14'43.9"E	2	2	1	1
40	Spain	Lake Estanes	42°47'58.1"N-0°35'27.3"W	24	2	1	2
41	Spain	Lake Asnos	42°41'22.2"N-0°16'05.8"W	23	2	1	1
42	Spain	Lake Sabocos	42°41'36.0"N-0°15'27.0"W	24	2	1	1
43	Spain	Lake Lapazosa	42°42'40.7"N-0°04'11.8"W	24	2	1	1
44	Spain	Lake Uridiceto	42°39'54.3"N-0°16'47.7"E	18	2	2	5
45	Spain	Lake El Cao	42°39'11.3"N-0°16'28.2"E	20	5	2	1
46	Spain	Lake Sen	42°37'11.0"N-0°23'30.1"E	26	4	2	3
47	Spain	Lake Anglios	42°35'26.7"N-0°42'39.9"E	8	-	1	3
48	Spain	Lake Xic	42°36'27.1"N-0°52'36.7"E	28	9	1	5
49	Spain	Lake Llebreta	42°32'57.1"N-0°53'18.1"E	8	2	2	4
50	Spain	Lake Llong	42°34'23.2"N-0°56'59.2"E	9	4	2	4
51	Spain	Lake Bassa Nord	42°33'18.2"N-0°56'33.5"E	30	4	1	2
52	Spain	Lake Estanyol Dellui	42°32'51.4"N-0°56'51.8"E	24	2	1	2
53	Spain	Lake Muntanyó d'Àrreu	42°40'21.4"N-1°00'26.0"E	30	5	1	2
54	Spain	Lake Tres estanys del baix	42°40'50.4"N-1°10'53.0"E	33	5	1	2
55	Spain	Lake Tres estanys del mig	42°40'56.7"N-1°10'58.1"E	28	10	1	2
56	Spain	Lake Finestres	42°39'13.9"N-1°11'57.7"E	24	3	1	1
57	Spain	Lake Soliguera	42°39'05.8"N-1°12'04.0"E	21	5	1	1
58	Spain	Downstream Soliguera	42°38'30.0"N-1°12'15.9"E	2	2	1	1
59	Spain	Lake Closell	42°40'58.2"N-1°17'41.7"E	24	11	1	2
60	Spain	Lake Naorte	42°41'23.4"N-1°18'00.1"E	33	6	1	4
61	Spain	Lake Rovinets	42°40'03.3"N-1°20'04.5"E	16	5	1	2
62	Spain	Lake Malniu	42°28'19.2"N-1°47'29.3"E	28	4	1	2

Table 2-1. List of sampling sites with number of samples analysed for each gene, as well as number of species and lineages found.

From the different genetic groups outlined by STRUCTURE, we used GenAlEx 6.503 (Peakall & Smouse, 2006) to estimate observed heterozygosity (H_O), expected heterozygosity (H_E), the mean number of alleles (NA) and population pairwise genetic differentiation (Fst). Additionally, unbiased allelic richness (AR) and private allelic richness (P-AR) were calculated using HP-RARE 1.1 (Kalinowski, 2005).

Also, we used the 'detection of first-generation migrants' option in GeneClass 2.0 to identify individuals born in populations different from the ones where they were sampled. Recent migration data are valuable for analyzing current introduction processes, and GeneClass 2.0 uses a suite of likelihood-based statistics, in combination with resampling methods, to calculate probabilities that individuals are first generation migrants (Bergl & Vigilant, 2007). We chose an alpha level of 0.01 to determine critical values, as simulated data have shown this level to represent an appropriate balance between stringency and power (Paetkau, Slade, Burden, & Estoup, 2004).

4. Result

4.1. Microsatellite genetic data pre-treatment

After cleaning for incomplete samples, a total of 801 remained with a selection of 17 loci. Individual amplification success rates ranged from 72.2% to 94.4%, while locus amplification rates ranged from 75.7% to 100%. Amplification rates of some primers showed differences among species. For instance, locus CypG9 did not amplify at all in *P. septimaniae* individuals from Ceresole (7), as well as in *P. csikii* from Leita (5), Miserin (6), and Vernouille (4). Furthermore, loci CypG30 and Lleb-072 did not amplify in most *P. bigerri* and in some *P. dragarum* individuals, particularly in samples from the Aragonese Pyrenees and the French rivers.

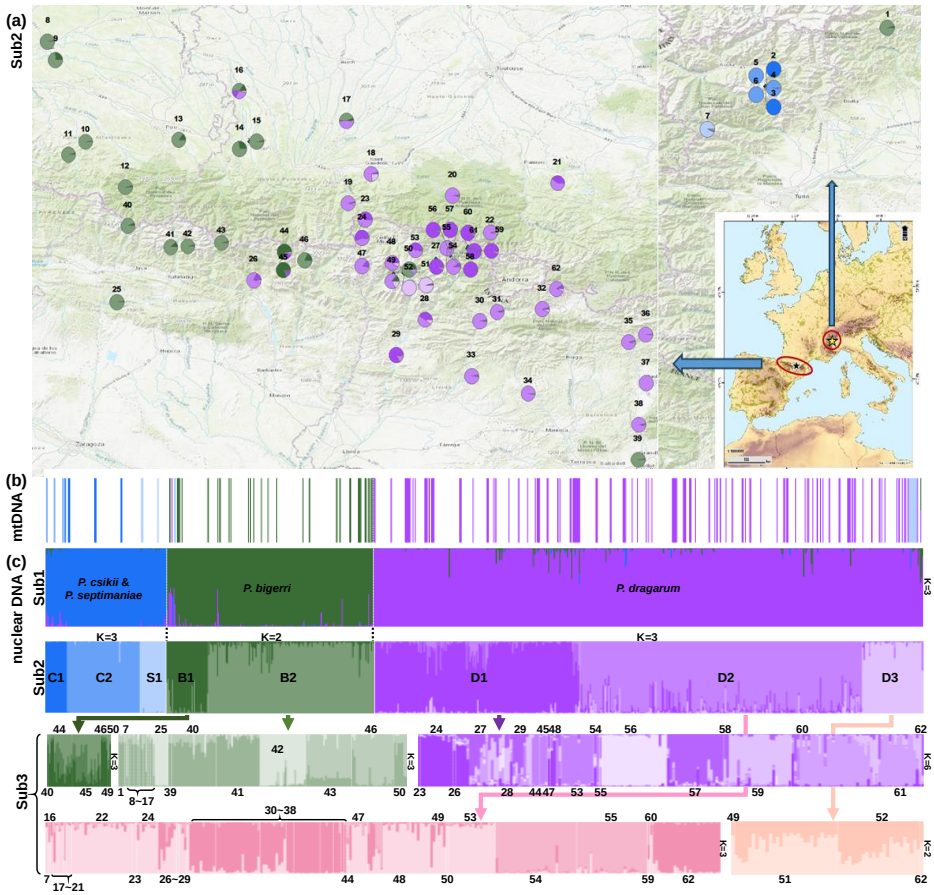


Figure 2-1. Results of *Phoxinus* Bayesian clustering analysis and comparison with mitochondrial DNA results from Chapter 1. Panel (a) shows the geographic distribution of the eight genetic clusters identified by STRUCTURE at the second level of analyses (Sub2). Sampled populations are represented by pie charts highlighting the population cluster membership obtained in STRUCTURE. Panel (b) represents the identification of the different species with mitochondrial DNA (results from Chapter 1) with the same colors as Sub1 but with dark and light blue for *P. csikii* and *P. septimaniae*, respectively. Panel (c) shows STRUCTURE barplot of membership assignment for the three sequential runs (from Sub1 to Sub3). Each individual is represented by a vertical bar corresponding to the sum of assignment probabilities to the K cluster. Numbers in panel (a) and Sub3 from panel (c) are population codes (see Table 2-1).

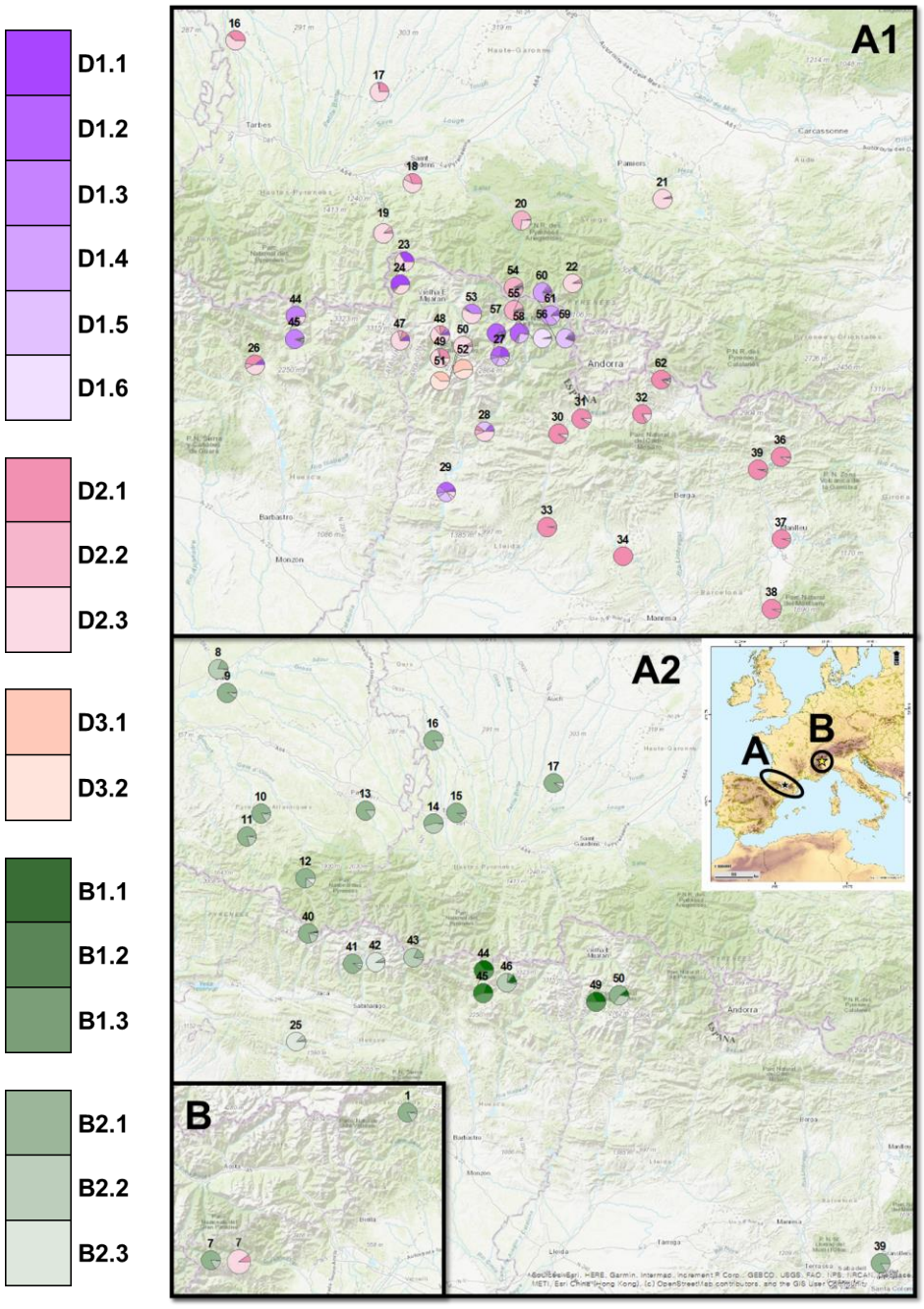


Figure 2-2. Geographic distribution of the eleven of *P. dragarum* (A1 and B) and the six genetic clusters of *P. bigerri* (A2 and B) identified by STRUCTURE at the third level of analyses (Sub3). Numbers at are population codes, see Table 2-1.

4.2. Population analysis using STRUCTURE

A first analysis of the whole data set supported K = 3 as optimal number of STRUCTURE clusters (Sub1 in Figure 2-1). The first (blue) cluster included *P. csikii* and *P. septimaniae* from Italy. The second cluster (green) corresponded exclusively to *P. bigerri*, which comprised individuals from both sides of the western Pyrenees, as well as introduced localities in River Tenes (Catalonia), Lake Llong (Catalan Pyrenees) and the Italian Alps (Lake Capezzone (1) and reservoir Ceresole Reale (7)). The third (purple) cluster comprised only *P. dragarum*, which was present mainly at both sides of the Eastern Pyrenees, but it was also found in Aragon at the River Cinca (26) and Lakes Urdiceto (44) and El Cao (45) and in one site of the Italian Alps (reservoir Ceresole Reale, 7). The distribution ranges of *P. bigerri* and *P. dragarum* were divided diagonally across the Pyrenees.

Structure analysis typically considers the highest-level of genetic subdivisions in a hierarchical manner, so we conducted an additional STRUCTURE analysis (Sub2 in Figure 2-1) for each of the three first clusters separately. Cluster 1 (comprising most Italian Alps samples, belonging to both *P. csikii* and *P. septimaniae*) was further subdivided into three subclusters: one formed by *P. septimaniae* only (S1, comprising most individuals from the lowland reservoir Ceresole Reale (7)), and the other two separating *P. csikii* in two subclusters (C1 with individuals from Lake Gran Lac (2) and Balena (3), and C2 with all the individuals of Lakes Vernouille (4), Leita (5) and Miserin (6)). Cluster 2, the *P. bigerri* group, was divided into two subclsuters (B1 and B2) and cluster 3 (*P. dragarum*) into three subclusters (D1, D2 and D3).

STRUCTURE at the second level of analyses (Sub2). Sampled populations are represented by pie charts highlighting the population cluster membership obtained in STRUCTURE. Panel (b) represents the identification of the different species with mitochondrial DNA (results from Chapter 1) with the same colors as Sub1 but with dark and light blue for *P. csikii* and *P. septimaniae*, respectively. Panel (c) shows STRUCTURE barplot of membership assignment for the three sequential runs (from Sub1 to Sub3). Each individual is represented by a vertical bar corresponding to the sum of assignment

probabilities to the K cluster. Numbers in panel (a) and Sub3 from panel (c) are population codes (see Table 2-1).

Finally, we conducted a final STRUCTURE clustering for each of the eight clusters identified in the second subdivision (see Sub 3 in Figure 2-1). For *P. csikii* and *P. septimaniae* subgroups (C1, C2 and S1; Figure 2-1) there was no further molecular subdivision, with no further subclusters. In the case of the two *P. bigerri* subclusters (B1 and B2; Figure 2-2), three additional subclusters each were detected (B1.1-3, B2.1-3). As for group B1, B1.1 was found only in high mountain lakes of Aragon (Urdiceto (44), El Cao (45), Sen (46), Estanes (40)) and one lake in Catalonia (Lake Llong, 50), while B1.2 and B1.3 showed a clearer population-based differentiation, with some individuals from Urdiceto (44) and El Cao (45) together with Lake Llebreta (49) in the Catalan Pyrenees. On the other hand, B2 was more widespread (Figure 2-2). B2.1 was the lineage present in almost all the French rivers, but it was not so common in high mountain lakes, as it was only present in Lakes Sen, Estanes and Asnos in Aragon, and in Lake Llong in Catalonia. It was also the lineage introduced in River Tenes (39) and in the two Italian Lakes, Lake Capezzone and the reservoir Ceresole Reale. B2.2 was also present in two French sites of the River Adour (8 and 14), in the River Gallego (25) and in two high mountain lakes of Aragon (Lakes Lapazosa (43) and Sabocos (25)) and one from Catalonia (Lake Llong). The third lineage, B2.3, had the smaller distribution, being found only in Lake Sabocos and in the River Gállego.

As for *P. dragarum*, each subcluster (D1, D2 and D3) showed values of K = 6, 3, and 2, respectively. The subcluster D1 was the most diverse, with six different subclusters, and exhibited a complex structure with sites having one single subclusters or others with different subclusters within each site. The subcluster D1.1 was present in two sites of the River Garonne (23 and 24, Aran valley, Spain), in three other sites tributary of the Ebro, the Noguera Pallaresa in Catalonia and Cinca in Aragon (26, 27 and 29, tributary of the Ebro; Spain) and in five lakes, one in Aragon (Lake Angliós, 47) and four in Catalonia, the Lakes Soliguera (57), Naorte (60), Tres estanys de baix (54) and Xic (48) (Figure 2-2). The subcluster D1.2 was mainly present at two sites of the River Noguera Pallaresa (27 and 29) and although it was also present in four lakes

of the Catalan Pyrenees, it was only dominant in Lake Soliguera. In contrast, D1.3 was present in two lakes of Aragon (Lakes Urdiceto (44) and El Cao (45)) and four in Catalonia (Muntanyó d'Àrreu (53), Tres estanys de baix (54), Tres estanys del mig (55) and Xic (48)), and was found in one individual of the rivers Noguera Pallaresa and Garonne. Similar to D1.2, D1.4 was found in one site in the River Noguera Pallaresa and was the dominant subcluster of Lakes Naorte (60) and Rovinets (61). D1.5 was found in three sites of Noguera Pallaresa and in three nearby lakes, Lakes Naorte, Closell (59) and Rovinets, and finally D1.6 was present in Noguera Pallaresa and was the dominant subcluster of Lake Finestres (56). It is interesting to note that this lake outflows to Lake Soliguera, which has minnows of different genetic groups (see above). The cluster D2 was subdivided into three sublineages. Sublineage D2.1 had the clearest spatial pattern, since it was the dominant cluster in most Catalan rivers (see Figure 2-2), but it was also present in some individuals from the River Garonne and the River Bellos (26, Aragon), in one lake from Aragon (Anglios, 47) and in three lakes of Catalonia, where it was the dominant cluster in Lake Malniu (62). Cluster D2.2 was found in one individual from the River Garonne, but was dominant in Lakes Tres estanys de baix (54) and Tres estanys del mig (55) and also present in two more Catalan Lakes. Finally, cluster D2.3 had the highest number of individuals and sites, and was present in the River Bellos (Aragon), the rivers Noguera Pallaresa and Segre in Catalonia, and Adour and seven sites in the Garonne in France. We found it also in the reservoir Ceresole Reale (Italy), in two lakes in Aragon, five lakes in Catalonia, where it was the dominant lineage in Xic and Muntanyó d'Àrreu, and was also the most abundant in Lake Mort (22, France). Cluster D3 was further subdivided into two subclusters, which were both mainly found in samples from two nearby lakes, Lake Bassa Nord (51) and Lake Estanyol Dellui (52), the lake downstream, Lake Llebreta (49) and a lake from the eastern Catalan Pyrenees, Lake Malniu (62).

STRUCTURE Cluster	Species	N	Na	Ho	He	AR	P-AR	Type of water body and location	Site Number
C1	*P. csikii*	20	4.611	0.401	0.448	2.87	0.34	Lakes Italy	2, 3
C2	*P. csikii*	62	5.833	0.376	0.456	3.57	0.5	Lakes Italy	4, 5, 6
S1	*P. Septimaniae*	22	6.333	0.499	0.631	4.6	0.99	Lakes Italy	7
B1.1	*P. bigerri*	10	5.389	0.459	0.584	3.98	0.28	Lakes Aragon	40, 44, 45, 46, 50
B1.2	*P. bigerri*	11	3.833	0.455	0.463	6.67	0.04	Lakes Aragon	44, 45
B1.3	*P. bigerri*	14	3.833	0.385	0.454	6.68	0.1	Lakes Aragon	44, 45, 49
B2.1	*P. bigerri*	64	12.833	0.516	0.707	5.08	0.49	Lakes Aragon, Lakes Italy rivers France	1, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 39, 40, 41, 46, 50
B2.2	*P. bigerri*	46	9.278	0.497	0.643	4.37	0.23	Lakes Aragon, rivers France	8, 14, 25, 43, 46, 50
B2.3	*P. bigerri*	27	7.278	0.490	0.601	4.02	0.45	Lakes and rivers Aragon	25, 42
D1.1	*P. dragarum*	25	7.222	0.534	0.632	4.14	0.23	Rivers Catalonia and Garonne	23, 24, 26, 27, 47, 48, 54, 57, 60
D1.2	*P. dragarum*	32	7.389	0.518	0.607	4.04	0.13	Lakes and rivers Catalonia	27, 29, 57, 58, 60, 61, 62
D1.3	*P. dragarum*	22	5.833	0.411	0.585	3.61	0.05	Lakes Aragon and Catalonia, rivers Catalonia	29, 44, 45, 48, 53, 54, 55, 60
D1.4	*P. dragarum*	40	6.333	0.500	0.598	3.77	0.1	Lakes and rivers Catalonia	27, 60, 61
D1.5	*P. dragarum*	35	7.111	0.508	0.596	3.87	0.11	Lakes and rivers Catalonia	27, 28, 29, 59, 60, 61
D1.6	*P. dragarum*	26	4.667	0.544	0.582	3.41	0.01	Lakes and rivers Catalonia	27, 56
D2.1	*P. dragarum*	90	10.833	0.526	0.653	4.28	0.17	Rivers Catalonia, France and Aragon, Lakes from Aragon and Catalonia	18, 26, 30, 31, 32, 33, 34, 35, 36, 37, 38, 47, 48, 49, 62
D2.2	*P. dragarum*	61	8.333	0.505	0.588	3.79	0.18	Lakes Catalonia, rivers France	20, 48, 54, 55, 59
D2.3	*P. dragarum*	97	12.667	0.533	0.676	4.56	0.17	Lakes and rivers Catalonia, Lakes Aragon, Lakes and rivers France	7, 16, 17, 18, 19, 21, 22, 23, 24, 26, 27, 28, 29, 32, 44, 47, 48, 49, 50, 53, 60
D3.1	*P. dragarum*	22	4.611	0.497	0.584	3.25	0.08	Lakes Catalonia	51, 52
D3.2	*P. dragarum*	34	6.111	0.554	0.608	3.72	0.09	Lakes Catalonia	49, 51, 52, 62

Table 2-2. Diversity parameters for the different STRUCTURE subclusters, including sample size (N), mean number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), allelic richness (AR), private allelic richness (P-AR) and sampling sites included in each group. See Table 2-1 for a complete list of sites and their locations.

4.3. Patterns of genetic diversity

Genetic diversity statistics were calculated from microsatellite markers within the different genetic lineages as determined by the STRUCTURE analysis. Since each group includes individuals from various populations, it doesn't always represent a clear distinction. However, we have presented the characteristics of the major lineages along with the site numbers in Table 2-2. Even within the same species, different levels of diversity were observed among the groups, and no specific trend could be identified. The higher diversity values were found in subclusters B2.1 (Na = 12.8, He = 0.72), B2.2 (Na = 9.3, He = 0.64), D2.1 (Na = 9.3, He = 0.64) and D2.3 (Na = 12.7, He = 0.68), which mostly included high mountain lakes and rivers in Spain and rivers in France.

Site code	Country	Site Name	individuals nDNA	individuals mtDNA	species	mt/nDNA Hybrids	nDNA Hybrids	Sub lineage 1	Sub lineage 2	Species hybridised
1	Italy	Lake Capezzone	3	-	1	0	1	1	1	*P. bigerri & P. dragarum*
4	Italy	Lake Vernouille	19	2	1	0	1	1	1	*P. csikii & P. septimaniae*
7	**Italy**	**Reservoir Ceresole**	**26**	**4**	**3**	**1**	**2**	**3**	**3**	***P. bigerri & P. septimaniae, P. septimaniae & P. dragarum***
13	France	River Adour (Assat)	2	2	1	0	1	1	1	*P. bigerri & P. dragarum*
16	**France**	**River Adour (Estirac)**	**2**	**1**	**2**	**0**	**0**	**2**	**2**	
17	**France**	**River Garonne (Manent-Montané)**	**2**	**2**	**2**	**0**	**1**	**2**	**2**	***P. bigerri & P. dragarum***
23	Spain	River Garonne (Bausen)	3	3	1	0	1	2	2	*P. bigerri & P. dragarum*
30	Spain	River Segre (Pla de Sant Tirs)	10	4	1	2	1	1	1	*P. bigerri & P. dragarum, P. septimaniae & P. dragarum*
31	Spain	River Segre (La Seu d'Urgell)	17	5	1	3	1	1	1	*P. bigerri & P. dragarum, P. septimaniae & P. dragarum*
34	Spain	River Llobregat (Cardener)	10	2	1	0	1	1	1	*P. bigerri & P. dragarum*
35	Spain	River Ter (Ripoll)	5	3	1	2	3	1	1	*P. bigerri & P. dragarum*
36	Spain	River Ter (Sant Joan de les Abadeses)	3	-	1	0	1	1	1	*P. bigerri & P. dragarum*
39	Spain	River Tenes (Lliçà de Vall)	2	2	1	2	2	1	1	*P. septimaniae & P. bigerri & P. dragarum*
42	Spain	Lake Sabocos	24	2	1	0	1	1	1	*P. bigerri & P. dragarum*
44	**Spain**	**Lake Uridiceto**	**18**	**2**	**2**	**0**	**0**	**3**	**5**	
45	**Spain**	**Lake El Cao**	**20**	**2**	**2**	**0**	**0**	**2**	**4**	
49	**Spain**	**Lake Llebreta**	**8**	**2**	**2**	**0**	**1**	**3**	**4**	***P. bigerri & P. dragarum***
50	**Spain**	**Lake Llong**	**9**	**4**	**2**	**1**	**5**	**3**	**4**	***P. bigerri & P. dragarum***
52	Spain	Lake Estanyol Dellui	24	2	1	0	1	1	2	*P. bigerri & P. dragarum*
55	Spain	Lake Tres estanys del mig	28	10	1	0	1	2	2	*P. bigerri & P. dragarum*
		TOTAL	**235**	**54**		**11**	**25**			

Table 2-3. Sites with more than one species. Hybrid individuals either with disagreement between mitochondrial and nuclear DNA (mt/nDNA Hybrids) or within nuclear DNA (nDNA Hybrids). The number of lineages found at each site according to STRUCTURE for the second or third level of clustering (lineages) is also included (see Figure 2-1 or text).

4.4. Hybridization and migration patterns

Summary information on the sites where interspecific hybridization or genetic admixture between species (by comparing mitochondrial and nuclear DNA) was found is presented in Table 2-3. A maximum of three species were found to coexist in one location (Ceresole), while two species were found in some high-altitude lakes in the Pyrenees (Aragon: Urdiceto (44), El Cao (45), and Llong (50), Catalonia: Llebreta (49)) and two rivers in France (Adour (16) and Garonne (17)). In addition, it was relatively frequent to find individuals belonging to different subclusters or lineages in the same site, especially in high mountain lakes or rivers where they are introduced (mean number of lineages per site ± standard deviation of 2 ± 1.3 and 1.7 ± 1.2 for lakes and rivers, respectively) compared to native areas, where the mean number of lineages per site was 1.1 ± 0.3. We found up to 5 lineages per site in high mountain lakes and introduced rivers and never more than two in native areas.

A previous study using mtDNA has provided valuable insights into the colonization history of high mountain lakes of the genus *Phoxinus* (Chapter 1), which included 167 individuals (of which 156 were sequenced for the COI gene fragment and 112 for a fragment of Cytb). Comparing the 167 individuals for which we obtained both mitochondrial and nuclear (microsatellites) information, we found that species identification was consistent. However, there were 11 cases where nDNA and mtDNA species identification did not match, which could be considered as hybrids (6.6 % of the total number of individuals; Table 2-3). Of these, eight individuals were classified as *P. dragarum* based on nDNA but appeared as *P. septimaniae* in mtDNA (Table 2-3), as was the case of most Catalan rivers, and one individual from the reservoir Ceresole Reale. There was also one individual that was identified as *P. bigerri* in nDNA but classified as *P. dragarum* (Lake Llong) and two as *P. septimaniae* (River Tenes) in mtDNA.

We also found individuals that according to STRUCTURE were hybrids or had gone through a process of admixture between two different species (nuclear DNA hybrids; Table 2-3). The proportion of nuclear DNA interspecific admixture was quite low (3% of the individuals), although it was observed at 17 sites, with 7 of them located in high mountain lakes (Italian Alps: 2, Catalan Pyrenees: 4, Aragonese Pyrenees: 1) and 10 in lowland lakes and rivers. The highest number of individuals, 5 in total, was found in Lake Llong (50). Regarding the species that were found to hybridize, two individuals from Italy (Lake Vernouille (4) and Reservoir Ceresole Reale (7)) appeared to be hybrids between *P. csikii* and *P. septimaniae* the former and *P. dragarum* and *P. septimaniae* the latter. Excluding these two cases, the rest of nuclear hybridization was found between *P. bigerri* and *P. dragarum*. The most striking hybrids were those from the River Tenes, where we found that the two nuclear hybrids had the mtDNA from a third species, *P. septimaniae* (see above). Most of the hybrids were found in sites where only one species was present, while in nearly half of the sites with more than one species we found no admixed individuals. In addition, there was no relationship between the

	P. bigerri			*P. dragarum*			*P. csikii*	*P. septimaniae*
	Lakes	***Rivers***		***Lakes***	***Rivers***		***Lakes***	***Rivers***
	Introduced	Introduced	Native	Introduced	Introduced	Native	Introduced	Introduced
individuals	164	12	13	365	105	31	87	24
Sub cluster 2								
lineages	2	1	1	3	2	2	2	1
Mean lineages/ population	1.3	1	1	1.6	1.3	1.3	1	1
admixed (%)	19 (12%)		4 (31%)	38 (10%)	22 (21%)	3 (10%)		
Sub cluster 3								
lineages	6	3	2	11	8	4		
Mean lineages/ population	1.9	1.3	1.1	2.4	1.7	1.4		
admixed (%)	39 (24%)	1 (8%)	1 (8%)	95 (26%)	28 (27%)	3 (10%)		

Table 2-4. Total number of lineages per site per species and class (distinguishing from high mountain lakes and lowland introduced or native rivers), mean number of lineages per population and the number and percentage (in parentheses) of admixed individuals detected in the second and third cluster level (Sub cluster 2 and Sub cluster 3) of STRUCTURE (see Figure 2-1 or text).

number of admixed individuals and the total number of individuals analysed at each site (lack of significant correlation between these two variables).

Finally, the degree of admixture at the intraspecific level was assessed by comparing the genetic admixture of various lineages within species, that were separated in the two hierarchical levels of STRUCTURE subdivision (Sub2 and Sub3). At this level we found no evidence of admixture in *P. csikii* and *P. septimaniae.* For the other two species the average number of lineages of each population and the degree of admixture for each group is indicated in Table 2-4. The proportion of admixed individuals was similar for both species and increased from 13% for both species to 22 for *P. dragarum* and 25% for *P. bigerri* (see also Figure 2-1 for a detailed overview by site).

To identify individuals born in populations different from the ones where they were sampled, we ran GeneClass 2.0. We found that 8% (64 individuals) of the individuals could be attributed to be first generation migrants. Amongst those we looked for cases where individuals migrated from high mountain lakes to other high mountain lakes and found 17 plausible cases. More cases of possible lake to lake migration were detected but were deemed

to be inconsistent with geographical and topographical conditions, since they hypothetically originated from Lake Mort (23; French Pyrenees) and were introduced into Lakes Llebreta (49) and Muntanyo d'Àrreu (53), both in the Catalan Pyrenees. Migration from high mountain lakes to rivers was inferred in 12 cases, and 18 cases of migration from river to river were found. Migration from lowland rivers to high mountain lakes was estimated in 17 cases, with 4 cases inferred to have migrated from French rivers to Lake Estanes (40) in the Aragonese Pyrenees and Lake Ceresole (7) in the Italian Alps. In 12 cases, we detected migration from Catalan inner basin rivers (26, 28, 32, 33, 35, 37, 38) and Garonne basin (24) near the border to lakes in the Pyrenees (40, 45, 47, 48, 50, 53, 59). One case was found to have migrated from River Segre (32) in Catalonia to Lake Mort (22) in France.

5. Discussion

This study, encompassing approximately 800 individuals, covers the distribution range of the genus *Phoxinus* in Spain, France and Italy, with a particular focus on European high mountain lakes of the Pyrenees and the Alps. Previous studies on *Phoxinus* contributed valuable insights into the distribution of various species, the discovery of divergent intraspecific lineages, and even the identification of new species (De Santis et al., 2021; Denys et al., 2020; Garcia-Raventós et al., 2020; Palandačić et al., 2015; Palandačić et al., 2022). However, research on species distribution, introduction history, and naturally fishless lakes has been limited. Our study involved intensive sampling of high mountain lakes and presents results based on nuclear markers (microsatellites), complemented by mitochondrial genes (Cytb and COI), which have been used successfully to scrutinize fine-scale gene flow in other studies (Pöschel et al., 2018; Vamberger et al., 2015) (Chapter 1).

In our previous study (Chapter 1), by using mitochondrial genes we could clearly distinguish amongst the four species present (*P. bigerri*, *P. csikii*, *P. dragarum* and *P. septimaniae*). In this study that utilized nuclear microsatellites, it was also possible to clearly differentiate between the four different species.

Apart from a small proportion of individuals, we found consensus in species identification between the two marker types. However, the inclusion of a higher number of samples allowed us to increase the knowledge on the distribution range of the different species. For example, we have documented, for the first time, the presence of *P. bigerri* and *P. dragarum* in Italy. We have also found *P. bigerri* in high mountain lakes and lowland rivers in Catalonia, and *vice versa*, we have found that *P. dragarum* was also introduced in Aragon. We also documented that both *P. dragarum* and *P. bigerri* could be found out of their native area in the rivers of France (Denys et al., 2020). Another noteworthy observation is the case of *P. csikii* in the Alps, where two lineages were identified, with Gran-Lac (2), and Balena (3) belonging to *P. csikii* lineage 1, and Vernouille (4), Leita (5) and Miserin (5) belonging to *P. csikii* lineage 2. Leita (5), despite the close geographical proximity to *P. csikii* lineage 1, was categorized under *P. csikii* lineage 2. Gene flow between the two *P. csikii* lineages was minimal, suggesting highly restricted movement between lakes in the Alps, with no significant additional inflow after the initial introduction.

5.1. Mito-nuclear discordances and nuclear hybrids

The comparison between mitochondrial and nuclear DNA analyses allowed us to detect discrepancies in the identification for certain individuals. Out of the 167 individuals for which we obtained both mitochondrial and nuclear information, in 11 individuals (7%) the two marker types did not match, which could be attributed to introgressive hybridisation (Toews & Brelsford, 2012). Previous studies have used discrepancies between nuclear and mitochondrial genes to detect cases of introgressive hybridisation, which are relatively common in fishes belonging to relatively close species (Aboim, Mavárez, Bernatchez, & Coelho, 2010; Gante, Collares-Pereira, & Coelho, 2004; Chapter 3). While hybridization is common over evolutionary timescales, some studies have proposed that it may be even more common in contemporary populations where anthropogenic disturbances have modified local environments where fish live and reproduce (Banerjee et al., 2023). One such scenario takes place when allochthonous species are introduced in new environments. On the other hand, in fisheries it is relatively common to

cultivate hybrids with the general belief that they might provide higher production (Bartley, Rana, & Immink, 2000). These hybrids end up in the natural environment (Chapter 3), and in some circumstances might result in generating genotypes that are fertile or even with higher fitness than their parents (e.g. Evolutionary Novelty Model Arnold & Hodges, 1995; Oziolor et al., 2019).

In our study, all but one mitochondrial/nuclear DNA hybrids were from the Catalan rivers, being mostly hybrids between *P. dragarum* and *P. septimaniae*. In fact, all the individuals from Catalan rivers that belonged to *P. septimaniae* in the mtDNA were hybrids. In a previous study on the presence of *Phoxinus* in rivers of northern Iberian Peninsula, Corral-Lou et al. (2019) analysed a relatively large number of minnows from the Catalan rivers, and found that most sites had individuals belonging to both species when analysing mtDNA. By analysing the nDNA we found that all the Catalan River sites and the Eastern high mountain lake (Lake Malniu) belonged to a single lineage (D2), that was also present in the native area of the species (the Garonne River). The fact that hybrids are not frequent, and that all the *P. septimaniae* from Catalonia were hybrids, suggests that they were already introduced as hybrids. The origin of the particular case of the individuals of the river Tenes that were a result of hybridisation between three different species is more difficult to elucidate. However, the fact that we have not found any *P. bigerri* in the rivers of Catalonia suggests that they might have already been introduced as they are. An analysis of more individuals of this river might shed light on this aspect. Similarly, in the rest of the sites with nDNA hybrids of *P. bigerri* and *P. dragarum,* we found that in 75% of the sites there was only one of the two species present. Although it is possible that we simply did not detect the second species, we only detected hybrids in half of the sites where we found two species.

We have found that the mean number of lineages per lake was much higher in the sites where the minnows were introduced than in their native areas in France (River Garonne in France for *D. dragarum* and River Adour and the Cantabric basins for *P. bigerri* (Corral-Lou et al. 2019 ; Denys et al. 2020 ; Chapter 1). The high number of microsatellite lineages found in the present

study suggests that there should be very high levels of spatial segregation within each species in their native areas. We actually found many more lineages in the introduced rivers or lakes than the native areas. Here we did not find any clear spatial pattern in France, which might be expected, since we were not able to sequence many individuals, and we did not cover the whole drainages of the native areas. In addition, the finding of *P. bigerri* in the Garonne and *P. dragrarum* in Adour show that there is a certain flow of translocations within France, which poses a conservation threat for the species in their native areas. A future study of the distribution of the different minnows in their native areas would be of great interest.

5.2. A natural experiment of admixture

The higher number of lineages or species was found in the introduced areas, especially in high mountain lakes. This aligns with other studies that have shown a trend of pure genotypical tendencies in native habitat and increased admixture ratios in contact zones (Antunes, Velo-Antón, Buckley, Pereira, & Martínez-Solano, 2021; Lucati et al., 2020; Pöschel et al., 2018; Vamberger et al., 2015). This can also be an indicator of the minimum number of different introduction events that have taken place in each site. Although one cannot rule out that within a single introduction event a given fisherman might introduce different lineages or species, this seems very unlikely, since fishermen introduce a limited number of individuals each time (only those that remain after the fishing trip), and usually buy them in specialised shops, or catch them directly in local rivers. In addition, one may use these multiple introduction events as a natural experiment to detect cases of inter and intraspecific hybridisation. Although this type of experiment has some clear drawbacks, since one does not have control of the number of introduced individuals and their exact genetic composition, it has also some advantages, since it has taken place simultaneously in many different ecosystems, which are relatively small, with *a priori* unlimited chances of crossings between individuals of different species or lineages. As an example, only in the Catalan and Aragonese Pyrenees, minnows were introduced in 140 lakes up to the year 2000 (Miró & Ventura, 2015). These introductions also date back

from the 80s of the last century, so they have been going on for quite few years. In this study we have, therefore, described the different levels of intra and inter specific admixture or hybridisation.

We have found that the overall frequency of interspecific hybridisation is really small (3%), and that the degree of intraspecific admixture increases with the degree of genetic differentiation from subcluster 2 (13%) to subcluster 3 (24%) (Table 2-4 and Figure 2-1). It seems that despite sharing the same ecosystem, there is relatively low admixture between individuals. This is especially the case since we actually cannot rule out that some of the individuals that are found to be admixed between different lineages were already admixed before being introduced into the lake. Some lakes have a very homogeneous genetic composition, such as Lakes Finestres that flow to Lake Soliguera. These two lakes have different lineages (Lake Finestres: D1.6, Lake soliguera: D1.2), and there is no restriction so that minnows of Finestres may reach Soliguera unharmed. However, we did not find any individual of the Finestres lineage in Soliguera, so it seems that there is no effective gene flow. Despite we only sequenced *ca.* 20 individuals of each population, we took special care of randomly selecting the individuals included in the analysed pool, covering the whole morphological variability (in each lake we sampled several hundreds of individuals). Another case is the one of the individuals found in Lake Llong (50), which was the high mountain lake with the highest interspecific hybrid rate (58%) among all the sites (Table 2-3). Given that the surrounding lakes are dominated by *P. dragarum*, the sole instance of *P. bigerri* dominance in this site raises suspicions of the initial introduction of pre-hybridized individuals.

5.3. The origin of the different lineages

P. bigerri has been described as a native species in the Adour basin of France and the eastern Cantabrian region (Corral‐Lou et al., 2019; Denys et al., 2020). In a previous study (Chapter 1) we showed that most haplotypes were originated in the Adour basin. However, the use of microsatellites has not given light to the history of all high mountain lake introductions. Lineage B2

was found in high mountain lakes in Spain and even in Italy, including the rivers known to be native in France, suggesting an introduction from France to other high mountain lakes. However, lineage B1 had a dominant presence in some lakes from Aragon, but no matching lineages were discovered within Spain or France. Furthermore, our previous study (Chapter 1) confirmed that the haplotype of Cantabria region did not match with those in high mountain lakes (Figure 2-2 (A2), Table 2-2). However, we were unable to analyse any individuals of all the native areas of this species.

P. dragarum also exhibited a similar situation. Lineage D2 was found in high mountain lakes in Spain and France, as well as in rivers in Catalonia and Garonne basin which are known as its native habitat (Denys et al., 2020), suggesting the high possibility of introduction from there. However, D1, which was found in some lakes in Aragon and Catalonia, as well as beyond the Spanish border following the Garonne basin, was not found in France. The possibility of introduction from other regions in Spain, as well as from France, can't be ruled out. For D3, present in only two lakes, Bassa Nord de l'Estanyet Dellui (51) and Estanyol Dellui (52), it was also impossible to determine their area of origin.

5.4. Does downstream migration occur effectively?

GeneClass modelling identified that 8% of the individuals were compatible with being first generation migrants from other populations. Provided that our dispersal system does depend mainly on human-based dispersal, the interpretation of these first-generation migrants must be taken with caution. First, one fisherman might sequentially introduce individuals from one lineage to two lakes, where in one there is already a pre-established lineage of the same species, while there are no minnows in the second lake, where they could establish and fund a new population. This, according to GeneClass, would lead to a scenario of migration from the second lake to the first. However, there are several cases of stream to lake migrations that could be feasible, but the most likely scenario is that they simply point out possible co-introductory routes to different places from a third non-sampled location.

There were also 12 cases where the model found direct migration from lakes to rivers. Most of the cases were highly unlikely, although raise the concern if there are effectively cases where lake populations might colonise the rivers downstream. This is of special concern for those rivers where there are native (and likely endangered) populations of minnows, such as *P. lumarieul* in Italy (De Santis et al., 2021) or the different minnow species in France (Denys et al., 2020). De Santis et al. (2021) did not find any evidence of downstream migration from 16 high mountain lakes. In our case, we tried to obtain minnows from the different streams immediately below the different mountain lakes with negative results. All the minnow populations we sampled came from far away from the nearby lakes, with no direct connection to the streams immediately below the lakes. The case of the Lakes Finestres and Soliguera described above, can be completed with the individuals of minnows found in the stream just below Lake Soliguera. While a few meters below the lake we could find minnows, there were no minnows further down the mountains. Our personal experience is that while downstream migration cannot be ruled out, it is not taking place in most of the cases.

6. Conclusion

Based on previous study that used mitochondrial markers, we utilize nuclear markers to compare these two genetic markers. This enhanced the genetic information available and allowed for a more precise identification and understanding of the distributional range of species. We also aimed to provide information about the distribution of the different intraspecific lineages and their potential introduction pathways through a more detailed analysis of sublineages. Due to limitations in the sampling range and the number of collected individuals, the introduction pathways for some lineages remain uncertain. Nevertheless, we have managed to deduce potential introduction pathways. These results will support the explanation of the origin of various species within the genus *Phoxinus* and contribute to the preservation of high mountain ecosystems, which are colonized by these species.

Part 2: A case study of invasive species (Loaches) in freshwater basins of Catalonia.

Chapter 3

Invaders they are a-changing: a recent, unexpected surge of invasive loaches in Catalonia

1. Abstract

Risk analyses for invasive species often assume that the characteristics of future invaders will resemble those already successful, but these features may change. Here, we use data from more than 3,500 fish sampling events, sequencing of mitochondrial and nuclear genes and analyses of several traits describing non-native fish to describe the irruption and rapid expansion of non-native loaches in Catalonia, north-east Iberian Peninsula, and framing this surge in the knowledge of previous invasions. We report the establishment of at least five (*Cobitis bilineata*, *Misgurnus anguillicaudatus*, *Misgurnus bipartitus*, *Paramisgurnus dabryanus* and a yet undescribed *Barbatula species*) non-native loach species in Catalonia, most of which are currently spreading. Furthermore, one of the two regionally native loach species (*Cobitis paludica*) has been introduced and is spreading through river basins where it is not native. Genetic analyses were fundamental to understand loach invasions, by clarifying specific statuses, identifying the origin of non-native populations and/or detecting independent introduction events. Genetic results also highlighted the unresolved taxonomy of loaches, particularly for European Barbatula. Loaches are recent, diverse, and successful invaders whose traits differ from those of most previously established invasive fish species, signalling potential weaknesses of prevention of biological invasions based on prohibited species lists. We call for the development of more flexible management tools (e.g. based on fish traits and not only on species identities) and prioritising prevention and rapid responses to new introductions.

2. Introduction

Invasive species constitute a critical and growing threat for freshwater ecosystems (Reid et al., 2019), with impacts that spread across trophic networks (Gallardo, Clavero, Sánchez, & Vilà, 2016). Preventing and mitigating the effects of biological invasions are thus management priorities for freshwaters. Eradicating or controlling established invasive species has been shown to benefit aquatic biodiversity (Bosch et al., 2019; Miró et al.,

2020), but these may not be feasible targets for widespread, abundant invaders (Blackburn et al., 2011), as is the case of many of the most impactful invasive species in freshwaters (e.g., Havel, Kovalenko, Thomaz, Amalfitano, and Kats (2015), Toussaint, Beauchard, Oberdorff, Brosse, and Villéger (2016)). Therefore, the prevention of new invasions is generally considered the most effective and efficient management strategy (Keller & Lodge, 2010).

Invasive species profiling has for long been considered a useful tool to prevent future invasions (Kolar & Lodge, 2002). The rationale behind this approach is that characterizing the features of species that have been successful invaders can allow identifying taxa with a high probability of being equally successful and become future invaders. Management efforts should then concentrate on avoiding the introduction of those "high risk" taxa and establishing rapid response protocols in case they are recorded in new areas (Roy et al., 2019). The usefulness of the profiling approach relies on the assumption that future invasions will resemble previous ones (Miguel Clavero, 2011). However, this assumption can be compromised if the characteristics of successful invaders change with time, as has already been described for introduction routes (Cardador et al., 2017) and pathways (Zieritz et al., 2017). Here, we present an example of the irruption and rapid expansion of a group of taxonomically related and ecologically similar invasive species that differ in several characteristics from previously established invaders.

Over 550 freshwater fish species are known to have established self-sustaining populations beyond their native areas globally (Bernery et al., 2022) and the country-level records of new non-native fish have constantly and steeply increased since the start of the 20th century (Seebens et al., 2017). Non-native fish species are one of the main drivers of freshwater fish biodiversity loss worldwide (G. Su et al., 2021), their impacts being often independent of those of other anthopogenic pressures (Light & Marchetti, 2007). The global patterns in fish invasions have a regional reflection in the Iberian Peninsula, with a constant increase of non-native species (Ribeiro, Collares‐Pereira, & Moyle, 2009). Once established, non-native fish species tend to spread across naturally isolated basins (Miguel Clavero & García-Berthou, 2006) and drive

the decline of the highly endemic and threatened native fish fauna (Hermoso, Clavero, Blanco-Garrido, & Prenda, 2011). Fish introductions in the Iberian Peninsula have been primarily related to sport fishing, either involving species targeted by fishermen (large-bodied fish, often predators) or species perceived to favour game fish by providing food (the so-called "forage fish", most often small-bodied cyprinids) (Carpio, De Miguel, Oteros, Hillström, & Tortosa, 2019; Tiberti et al., 2022).

Loaches are small, benthic fish species included in 10 families within the suborder Cobitoidei (sensu Kottelat (2012)). The Iberian Peninsula hosts at least four native loach species, within the genera Cobitis (*C. paludica*, *C. calderoni* and *C. vettonica*) and Barbatula (*B. hispanica*). Loaches have not been traditionally considered relevant invaders in Iberian waters, because there were few known introduced populations, including either Iberian species (e.g. *Cobitis paludica*; Piorno and de la Hoz Regules (2020)) or other European species (e.g. *Barbatula barbatula*; Perea et al. (2011)). However, the dojo loach (*Misgurnus anguillicaudatus*), first recorded in the Ebro River Delta in 2001, has spread over that coastal wetland area (Franch et al., 2008). In recent times, there has been an increasing number of records of non-native loaches in Catalonia, NE Iberian Peninsula, which seem to involve different species.

Here, we report the recent introductions and spread of non-native loaches in Catalonia in order to: 1) clarify the specific identity of these non-native species; 2) update their geographic range and describe spatial patterns of their spread processes; 3) discuss the most plausible mechanisms driving introduction events and secondary spread; and 4) evaluate how the irruption of new profiles among invasive species can hinder the design and implementation of prevention and rapid response management strategies to deal with biological invasions.

3. Materials and Methods

3.1. Study Area

Within some 32,000 km², Catalonia bears a high diversity of ecosystems and environmental conditions, from coastal to high-altitude habitats (up to 3,100 m above sea level) and from humid to semi-arid climates. The river network in Catalonia can be divided in two main units with roughly similar sizes. The western half belongs to the Ebro River basin, and includes the lower part of the Ebro River, its estuary (the Ebro Delta) and most of the basin of the Segre River, the Ebro's main tributary. To the east, there is a series of medium to small-sized coastal basins, draining to the Mediterranean Sea north to the Ebro Delta. The Llobregat and Ter rivers are the most important systems in this sector, with basin areas of approximately 5,000 and 3,000 km², respectively.

As in other Mediterranean areas fish assemblages in Catalonia are characterised by the presence of low native species richness at the local scale and a high number of introduced species (Marr et al., 2010). Currently, the ichthyofauna of inland waters of Catalonia comprises 27 native and 31 exotic species (E Aparicio et al., 2016). Most native species have greatly declined, plausibly due to habitat degradation and expansion of invasive species (Enric Aparicio, Vargas, Olmo, & de Sostoa, 2000; MACEDA‐VEIGA, MONLEON‐GETINO, Caiola, Casals, & de Sostoa, 2010). Catalonia constitutes the main invasion route of freshwater fish into the Iberian Peninsula, since most of introductions originate from France and enter through Catalonia to then spread across other Iberian areas, often in relation to sport fishing activities (Miguel Clavero & García-Berthou, 2006).

3.2. Fish species records

Data on the distribution of loach species were collected from various sources involving different sampling methodologies and periodicities. Most data come from the more than 3,500 sampling events performed by the authors in inland waters of Catalonia from 2007 to 2019, mainly in the framework of

monitoring programs for public agencies. Several records derive from a monitoring network that involves multiple electrofishing surveys of 353 stretches following the European Committee for Standardisation—CEN 14011 standard protocol (CEN 2003). In those areas where the presence of *Misgurnus* loaches was previously known, i.e. tributaries of the lower Ter basin and Ebro River Delta (Franch et al., 2008), spatially and temporally intense monitoring was conducted to determine the spread of populations, involving the use of small-meshed fyke nets and minnow traps, in addition to electrofishing. In the Ter, Fluvià, and Muga rivers and their tributaries, 232 sites were sampled at least twice between 2007 to 2019, but 42 of these sites were sampled with higher frequency, from four to 25 occasions, in the context of different local monitoring programs. In the Ebro Delta, 196 sites were surveyed yearly on average from 2012 to 2019.

Loach species records were summarised using a Universal Transverse Mercator 5 × 5 km grid when the whole Catalonia was presented in maps and using a Universal Transverse Mercator 1 × 1 km grid when maps were focussed on more reduced areas.

3.3. Laboratory procedures

DNA from 71 samples of three fish groups from 21 localities (Table 3-1) was extracted. Overall, 24 samples of Barbartula, 17 samples of Cobitis and 30 of Misgurnus and Paramisgurnus were extracted using QIAGEN DNeasy Blood and Tissue Kit (QiagenTM, Hilden, Germany) according to the manufacturer's protocol, or following the HotSHOT method (Montero - Pau et al., 2008) in a total volume of 100 μl, and subsequently stored at -20 °C.

We sequenced two gene fragments, a 658-nt section of the mitochondrial cytochrome c oxidase gene subunit 1 (COI) and 920-nt section of the nuclear recombination activating gene 1 (RAG-1). Two primer sets were used for COI: LCOI490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) and jgLCO1490 (5′- TITCIACIAAYCAYAARGAYATT-3′) and jgHCO2198 (5′-

TAIACYTCIGGRTGICCRAARAAYCA-3′) (Geller et al., 2013). For RAG-1 we used RAG-1F (5′-AGCTGTAGTCAGTAYCACAARATG-3′) and RAG-9R (5′-GTGTAGAGCCAGTGRTGYTT-3′) (Quenouille, Bermingham, & Planes, 2004) for RAG1. For some taxa, we also carried out a nested PCR using PCR products amplified with the primer pair RAG-1F and RAG-RV1 (5′-TCCTGRAAGATYTTGTAGAA-3′) (Šlechtová, Bohlen, & Tan, 2007) for PCR with the former primer combination.

Species	Basin	Latitude	Longitude	COI	RAG-1	Genbank accession
Barbatula sp4	Fluvià	42°12'54.6"N	2°39'44.8"E	1	2	OP174631 / OP407662, OP407663
Barbatula sp4	Fluvià	42°13'49.6"N	2°36'35.0"E	2	2	OP174631 / OP407662, OP407663
Barbatula sp4	Fluvià	42°15'45.2"N	2°26'34.7"E	6	1	OP174631 / OP407656
Barbatula sp4	Llobregat	41°55'49.1"N	1°38'04.6"E	1	2	OP174631 / OP407662
Barbatula sp4	Segre	42°21'06.7"N	1°27'31.7"E	6	1	OP174631, OP174632, OP174641 / OP407662
Barbatula sp4	Segre	42°22'00.7"N	1°42'44.5"E	1	2	OP174632 / OP407656, OP407662
Barbatula sp4	Ter	42°06'48.6"N	2°12'25.6"E	1	2	OP174631 / OP407656, OP407662
Barbatula sp4	Ter	42°13'34.2"N	2°15'29.6"E	1	2	OP174631 / OP407655
Cobitis bilineata	Ter	42°07'45.2"N	2°46'02.5"E	2	2	OP174637 / OP407664
Cobitis paludica	Besòs	41°32'49.7"N	2°15'57.9"E	2	1	OP174635, OP174636 / OP407659
Cobitis paludica	Ebro	40°42'54.8"N	0°42'19.5"E	2	2	OP174640 / OP407657
Cobitis paludica	Júcar	39°53'03.3"N	0°03'43.4"W	2	2	OP174640 / OP407657
Cobitis paludica	Júcar	39°21'34.1"N	0°27'31.8"W	2	2	OP174633 / OP407658, OP407659
Cobitis paludica	Júcar	39°48'24.3"N	2°08'41.4"W	2	2	OP174634 / OP407660
Cobitis paludica	Ter	41°59'13.1"N	2°36'44.7"E	2	2	OP174638 / OP407659
Cobitis paludica	Girona	38°48'26.5"N	0°03'02.7"W	-	2	- / OP407658
Cobitis paludica	Júcar	39°18'41.8"N	0°34'35.5"W	-	1	- / OP407659
Misgurnus anguillicaudatus	Ebro	40°42'54.8"N	0°42'19.5"E	11	2	OP174639 / OP407665
Misgurnus anguillicaudatus	Ter	42°05'16.6"N	3°07'49.3"E	2	1	OP174643 / OP407667
Misgurnus anguillicaudatus	Ter	41°58'09.2"N	2°50'26.1"E	1	1	OP174643 / OP407667
Misgurnus anguillicaudatus	Júcar	39°24'06.9"N	0°23'08.4"W	1	2	OP174639 / OP407661
Misgurnus bipartitus	Ter	42°05'16.6"N	3°07'49.3"E	3	-	OP174642/ -
Misgurnus bipartitus	Ter	41°58'09.2"N	2°50'26.1"E	5	2	OP174642 / OP407665, OP407666
Paramisgurnus dabryanus	Llobregat	41°24'53.2"N	2°05'51.4"E	6	-	OP174644/ -

Table 3-1. Species, genes amplified (N individuals) and Genbank accession numbers (COI/RAG-1) for the samples collected in the field and analysed for this study.

Total reaction volume (25 μL) consisted of 1× PCR buffer (Silverstar, Eurogentec), 1.5 mm MgCl2, 200 μm of each dNTP, 0.2 μm of each primer, 2 μL of template DNA, 1 U Taq polymerase and UV light-sterilized mQ-H2O for both genes. PCR conditions for the first set of COI primers involved a denaturing step of 5 min at 95°C, five cycles of 1 min at 95°C, 90 s at 45°C and 60 s at 72°C, followed by 35 cycles of 1 min at 95°C, 60 s at 50°C and 60 s at 72°C and a final elongation of 7 min at 72°C. For the second primer set, the amplification conditions were an initial denaturation of 1 min at 94 °C, five cycles of 40 s at 94°C, 40 s at 45°C and 60 s at 72°C, followed by 35 cycles of 40 s at 94°C, 40 s at 51°C and 60 s at 72°C and a final elongation of 5 min at 72°C. For RAG-1, consisted of an initial denaturation of 5 min at 95 °C, touch-down profile of 1 min at 94 °C, 90 s at 60–55 °C (1°C /cycle) and 2 min at 72 °C followed by 30 cycles with annealing temperature held at 54 °C. The reaction was completed by final extension for 7 min at 72 °C. And for the nested reaction we used a simple profile consisting of 2 min at 95 °C, 35 cycles of 45 s at 94 °C, 45 s at 56 °C, 90 s at 72 °C and final elongation step for 5 min at 72 °C. PCR products were purified using NucleoFast 96 PCR Plate (Macherey-Nagel) and sequenced on an ABI 3730 capillary sequencer (Secugen, Madrid, Spain).

3.4. Data treatment and Genetic analyses

The obtained sequences (see Table 3-1 for GenBank accession numbers) were aligned with all available sequences from GenBank (sequence numbers for COI/RAG-1 were: Barbartula: 185/21, Cobitis: 83/72, Misgurnus and/or Paramisgurnus: 380/83). We used the ClustalW algorithm (J. D. Thompson, Higgins, & Gibson, 1994) in MEGA version 7 (Kumar et al., 2016). The alignments were checked by eye and corrected according to the translated amino-acid alignment, and sequence divergences (Kimura 2-parameter model) were calculated using MEGA. Phylogenetic relationships within Barbartula, Cobitis and Misgurnus and/or Paramisgurnus were assessed using a part of each COI gene of 627bp, 565bp and 605bp respectively for each group and using *Lepidocephalichthys hasselti* as outgroup. For the RAG-1 the

fragments finally used were 888bp, 874bp and 893bp long and using *Kottelatlimia pristes* as outgroup.

We used jModeltest (Posada, 2008) to select the best model of nucleotide substitution, and assessed the phylogeny using the Bayesian inference (BI) in MrBayes version 3.2.7 (Ronquist & Huelsenbeck, 2003) and Maximum Likelihood (ML) in PhyML 3.0 (Guindon et al., 2010). For the final phylogenetic trees, jModeltest selected the models for Barbartula, Cobitis and Misgurnus and/or Paramisgurnus data each TIM1+I+G, TVM+I+G, TrN+I+G for COI and TPM2+G, K80+G, SYM+I+G for RAG-1. In BI, two parallel runs of four Monte Carlo Markov chains were run for 3 million generations, trees were sampled every 100 generations, and the first 25% of sampled trees were discarded as a burn-in phase. In PhyML the model was suggested by SMS (Smart Model Selection in PhyML) (Lefort, Longueville, & Gascuel, 2017) and branch support was evaluated by fast likelihood-based methods using aBayes. We performed haplotype network analyses to estimate gene genealogies using HAPLOVIEWER, which turns trees built from traditional phylogenetic methods into haplotype genealogies (Salzburger et al., 2011). We estimated the phylogeny using a maximum-likelihood method with RaxML Blackbox (Stamatakis, 2006), with the model suggested by the program. Input data were COI and RAG-1 sequences from each individual, subsequently collapsed into haplotypes. Sequences with ambiguous bases were not included in the analysis. The best tree (using the log-likelihood criterion) was selected for network construction using HAPLOVIEWER.

3.5. Are invaders a-changing?

We analysed the characteristics of non-native fish species in Catalonia to identify possible temporal changes in their profiles (i.e. average features). We compiled information on traits describing biological characteristics, geographical range, ecological features, and human uses of fish species having non-native populations in Catalonia. Nativeness was considered attending at natural geographic entities (i.e. river basins) and not at administrative units, and thus our non-native fish dataset included some

Variable	Description	Unit	Source
Body size	Maximum body size	cm, Log_{10}-transformed	Fishbase
Average Latitude	Average value of the minimum and maximum latitude limits of range	Decimal degrees	Fishbase
Aquaculture	Use in aquaculture production	1 (yes) – 0 (no)	Fishbase
Gamefish	Sport fishing target	1 (yes) – 0 (no)	Fishbase
Bait	Use as bait in sport fishing	1 (yes) – 0 (no)	Fishbase
Aquarium	Use in domestic aquariums	1 (yes) – 0 (no)	Fishbase
Phytophilic	Spawning associated to plants	1 (yes) – 0 (no)	Aparicio et al. (2016)
Litophillc	Spawning associated to stones	1 (yes) – 0 (no)	Aparicio et al. (2016)
Rheophilic	Spawning associated to runs	1 (yes) – 0 (no)	Aparicio et al. (2016)
Nest guarders	Eggs-offspring guarding	1 (yes) – 0 (no)	Aparicio et al. (2016)
Diet	Maximum feeding level	1 (plants/plakton), 2 (macroinvetebrates), 3 (fish)	Aparicio et al. (2016)
Longevity	Code for maximum age	1 (up to 3 years), 2 (up to 6 years), 3 (>6 years)	Aparicio et al. (2016)
Intolerant	Narrow environmental tolerance	1 (yes) -0 (no)	Aparicio et al. (2016)
Flow velocity	Water current preferences	1 (limnophilic) 2 (generalistic) 3 (rheophilic)	Aparicio et al. (2016)
Benthic	Preference for benthic habitats	1 (yes) -0 (no)	Aparicio et al. (2016)

Table 3-2. Accession codes for the haplotypes collected from Genebank for this study. The table indicates the gene (COI or RAG-1), the loach group as used in this work (*Barbatula*, *Cobitis* or *Misgurnus/Pamisgurnus* loaches), the species identification provided by Genebank and the country of origin of the sample

species that are native to some areas in Catalonia and non-nativein others (e.g. *Luciobarbus graellsii* or *Parachondrostoma miegii*). Overall, we recorded 15 variables (Table 3-2), which we summarised by means of a principal components (PC) analysis in order to extract the main gradients of variation (PCs) from the multivariate dataset. We selected the number of analysed PC axes following the broken-stick Approach (Sergeant, Starkey, Bartz, Wilson, & Mueter, 2016), by which only axes explaining a larger amount of variance than that expected by chance should be interpreted. We also compiled the year in which each species was first recorded in Catalonia, from Manau and Cazorla (2016) and authors' own experience. The possible temporal variation in the characteristics of non-native fish species was analysed by exploring the correlation between the scores along the extracted PCs and the year of first

Species	First record	PC1	PC2	Size	AvLat	Aquacul	Game	Bait	Aquariu m	Phyto ph	Lito ph	Rheo ph	Nest guarders	Diet	Longevity	Intolerant	Flow velo	Benthic
Abramis brama	2004	-1.50	0.81	1.60	57.5	1	1	1	0	1	1	0	0	2	3	0	2	0
Achondrostoma arcasii	1986	1.54	-2.53	1.00	41.5	0	0	0	0	1	1	1	0	2	1	1	3	0
Alburnus alburnus	1992	0.61	0.59	1.26	51.5	1	0	1	0	1	1	0	0	1	2	0	2	0
Barbatula sp	1995	2.70	0.27	1.00	47.6	0	0	1	1	0	1	0	0	2	2	0	3	1
Blicca bjoerkna	2003	0.81	1.11	1.48	52.5	0	0	1	0	1	0	0	0	2	2	0	2	0
Cobitis bilineata	2002	3.49	1.53	1.00	44.7	0	0	1	1	1	0	0	0	2	1	0	3	1
Cobitis paludica	2015	3.62	1.50	1.00	40	0	0	1	1	1	0	0	0	2	1	0	3	1
Esox lucius	1949	-2.54	1.50	2.00	54.5	1	1	0	0	1	0	0	0	3	3	0	2	0
Fundulus heteroclitus	2005	1.92	0.76	1.00	40	0	0	0	1	0	0	0	0	2	1	0	1	0
Gobio sp	1985	2.67	-0.80	1.08	42.5	0	0	1	1	0	1	1	0	2	2	0	3	1
Ictalurus punctatus	1995	-2.06	0.71	1.95	40	1	1	0	1	0	1	0	1	2	3	0	2	0
Leuciscus aspius	2015	-2.68	-0.91	1.95	50.5	1	1	0	0	0	1	1	0	3	3	0	2	0
Luciobarbus graellsii	1965	-0.98	-2.43	1.90	42.5	0	1	0	0	0	1	1	0	1	3	0	3	0
Micropterus salmoides	1955	-2.86	0.87	1.78	35	1	1	0	0	0	1	0	1	3	3	0	1	0
Misgurnus anguillicaudatus	2001	1.66	2.12	1.30	40	1	0	1	1	1	1	0	0	2	2	0	1	1
Parachondrostoma miegii	1975	0.10	-3.70	1.40	42	0	1	0	0	0	1	1	0	1	2	1	3	0
Paramisgurnus dabryanus	2008	1.42	1.48	1.48	33	1	0	0	1	1	1	0	0	1	2	0	1	1
Perca fluviatilis	1975	-1.85	0.80	1.60	56	1	1	0	0	1	1	0	0	3	2	0	2	0
Phoxinus sp	1985	2.37	-2.20	1.00	44.25	0	0	1	0	0	1	0	0	1	1	1	3	0
Pseudorasbora parva	2001	2.06	0.48	1.00	38	0	0	0	1	1	1	0	0	2	1	0	2	0
Rutilus rutilus	1985	-1.03	0.11	1.48	53.5	1	1	0	0	1	1	0	0	1	2	0	2	0
Salvelinus fontinalis	1975	-1.29	-2.84	1.70	48	1	1	0	0	0	1	1	0	2	2	1	3	0
Salvelinus umbla	1985	-1.54	-2.57	1.70	46.5	0	1	0	0	0	1	1	0	2	3	1	1	0
Sander lucioperca	1975	-3.25	1.35	1.95	51.5	1	1	0	0	1	1	0	1	3	3	0	2	0
Silurus glanis	1974	-3.37	1.99	2.30	49	1	1	0	0	1	0	0	1	3	3	0	2	0

Table 3-3. Features of non-native fish species established in Catalonia. The table shows non-native fish species detected for the first time in the wild after 1940, reporting their values for each of the variables listed in Table 2 and the two main gradients extracted from the principal components analysis run to summarize this information (PC1 and PC2)

detection for each species. We concentrated the analyses on the 25 non-native fish species recorded after 1940 (see Table 3-3), to avoid the uncertainties linked to ancient introductions. We used the genus level to characterise those genera in which there were uncertainties in species identification (i.e. *Gobio*, *Phoxinus*, and *Barbatula*).

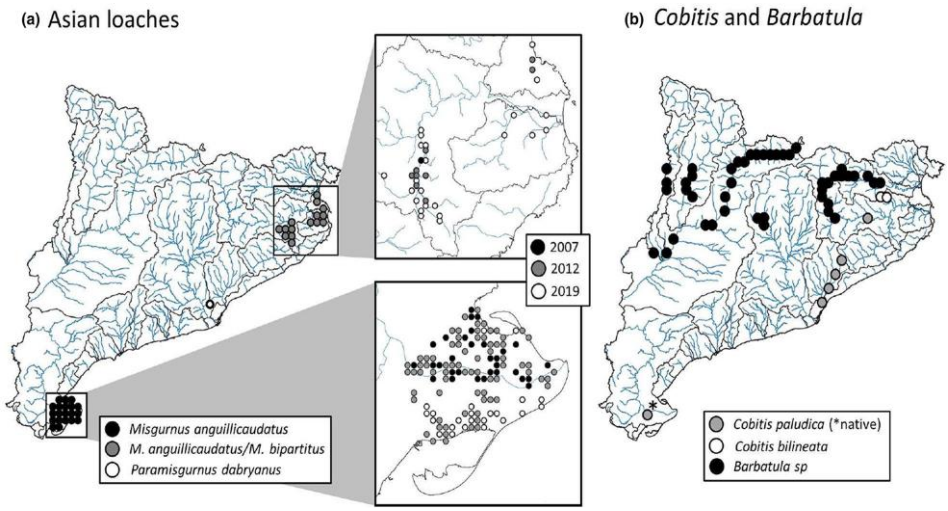


Figure 3-1. Distribution of loach species in Catalonia. (a) Distribution of Asian loaches in genera *Misgurnus and Paramisgurnus* (dot colours represent different species) and range expansion in the Ebro Delta (bottom, right) and north-eastern Catalonia catchments (top, right) between 2007 and 2019 (dot colours represent different years). (b) Distribution of *Cobitis* (native populations are marked with a (*) and *Barbatula* species.

4. Results

4.1. Asian loaches

Asian loaches have been detected in three different areas within Catalonia: The Ebro Delta, the Vallvidriera Reservoir (near the city of Barcelona), and in the Ter basin (Onyar and Cinyana streams; Figure 3-1). The genetic analyses performed depict a complex introduction scenario, and provide the first Iberian records of *Misgurnus bipartitus* and *Paramisgurnus dabryanus* (Figure 3-2).

Paramisgurnus dabryanus was first detected in Vallvidrera Reservoir in 2005, being initially identified as *M. anguillicaudatus*. This population was apparently well-established before being the focus of different control operations between 2015 and 2020, which have not been successful. The presence of *P. dabryanus* is largely restricted to the reservoir, although some specimens have been found in the Vallvidriera Stream, which flows to the Llobregat River (where no specimens have been detected yet). All *P. dabryanus* individuals from Catalonia shared the same COI haplotype.

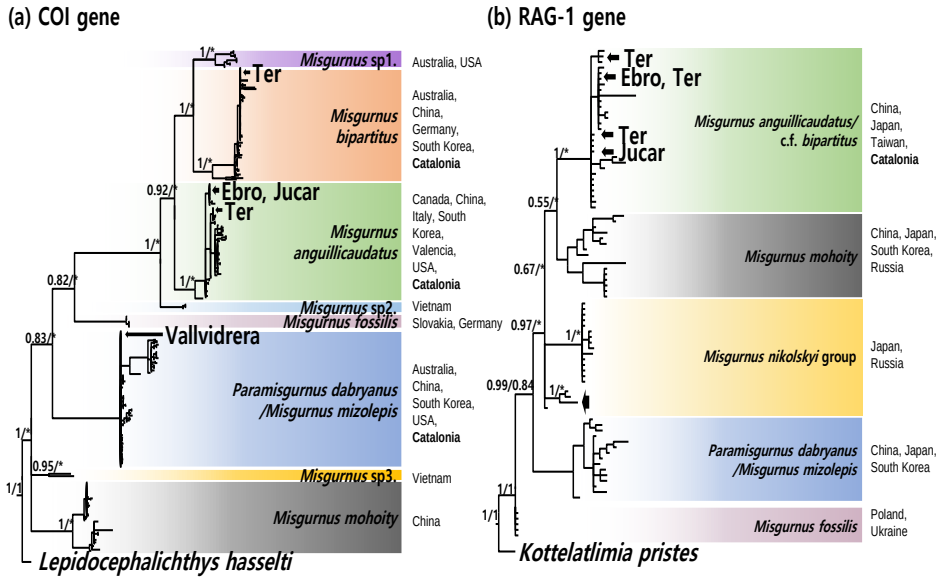


Figure 3-2. Genetic patterns in Asian loaches in the genera *Misgurnus* and *Paramisgurnus*. maximum likelihood phylograms based on a partial sequence of the (a) mitochondrial cytochrome c oxidase gene subunit 1 (COI) and (b) nuclear recombination activating gene 1 (RAG-1) genes. Arrows indicate samples obtained and sequenced in this study and the basin of origin. The territories of origin of samples are indicated to the right of the phylograms, not mentioning unknown locations. See Tables 3-1 and S3-1 for full accounts of accession numbers.

Genetic data confirmed the specific identity of *M. anguillicaudatus* introduced in the Ebro Delta. We found only one COI haplotype among nine individuals from this area (Figure 3-2 (a)), a low genetic diversity that fits well with the assumed origin of the population from an accidental escape (Franch et al., 2008). Despite this low genetic diversity, *M. anguillicaudatus* is currently

widespread in the area (Figure 3-1), occupying all its low-conductivity environments (López, Q., & M., 2012) and being numerically dominant in some of them, such as rice fields (Miguel Clavero, López, Franch, Pou-Rovira, & Queral, 2015).

Misgurnus loaches found in the Ter River basin belong to two different lineages, according to the COI gene. Some of the individuals analysed (three out of 11) were identified as *M. anguillicaudatus*, while most individuals clustered with *M. bipartitus* (Figure 3-2(a)). However, the analysis of the RAG-1 sequences showed that the two mitochondrial lineages are part of a single lineage (Figure 3-2 (b)), suggesting that the *Misgurnus* in the Ter River basin would originate from a single source population that included *M. bipartitus*×*M. anguillicaudatus* hybrids inheriting mitochondrial DNA from M. bipartitus females. These hybrid loaches have successfully established and are still spreading their range (Figure 3-1).

The introduction of *Misgurnus* in the Ter Basin was independent from the *Misgurnus* population in the Ebro Delta, since both areas do not share any COI haplotype. The *M. angullicaudatus* established in the Júcar basin, in the Valencia region, shares the COI haplotype found in the Ebro Delta, but was different according to the RAG-1 gene (Figure 3-2 (b)), suggesting an additional independent introduction event. Overall, our results show that there have been at least three independent introduction events leading to the establishment of wild populations of Asian loaches in Catalonia, and additional independent events in other Iberian regions (i.e., Valencia).

4.2. Cobitis loaches

Cobitis paludica has a very restricted and declining native range in Catalonia, being confined to freshwater springs in the Ebro Delta (M Clavero, Franch, López, Pou-Rovira, & Queral, 2021). However, the range of the species has recently expanded due to introductions. *Cobitis paludica* was first detected in the Besòs basin in 2016, when it was already abundant and widespread in the middle reaches of the Besòs River (Figure 3-1). Surveys performed in those

same reaches in 2008–2009 had not detected the species. In 2017, *C. paludica* was captured in the Ter basin for the first time, being detected in a single reservoir from which the species does not seem to have spread yet. *Cobitis bilineata* was recorded in the karstic Lake Banyoles in the early 1990s (Pou i Rovira, 2004). It successfully established in the area, but has not spread notably, being currently found mainly in the lake outflow channels and some associated stream systems (E Aparicio et al. (2016); Figure 3-1).

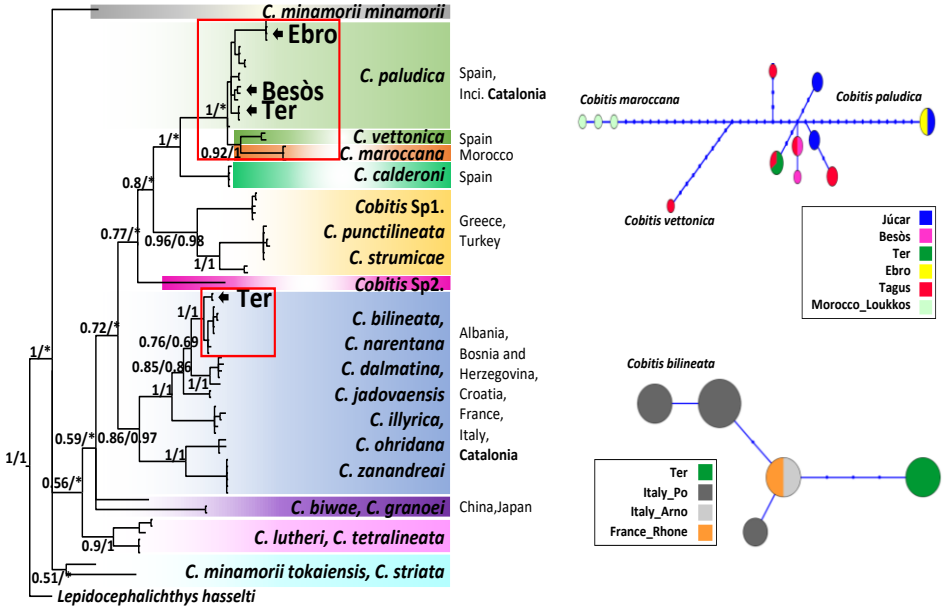


Figure 3-3. Genetic patterns in Afro-European *Cobitis* loaches. Maximum likelihood phylograms based on a partial sequence of the mitochondrial cytochrome c oxidase gene subunit 1 gene (as in Figure 2) and network of haplotypes, in which each circle represents a unique haplotype, and its size is proportional to the number of individuals sharing it. Each branch with more than one mutational step is labelled. The phylogram branches that correspond to the haplotype networks are marked by the brown rectangles. See Tables 3-1 and S3-1 for full accounts of accession numbers.

The analysis of the COI gene shows that the *C. paludica* populations in the Ebro Delta are closely related to populations in other Mediterranean areas (in the Júcar River basin) and highly differentiated from other Iberian populations (Figure 3-3). The origin of the recently introduced *C. paludica* populations in Catalonia is not the native Mediterranean lineage, but populations from central Iberia, probably from the Tagus basin. Genetic

analyses also suggest that *C. paludica* may have been introduced in the Besòs and Ter basins in independent events, since both areas do not share COI haplotypes (Figure 3-3). We confirmed the specific status of *C. bilineata* populations in Lake Banyoles, but results also introduced uncertainty regarding their origin, since the detected COI haplotype is different and relatively distant from those reported to the date from native areas.

4.3. Barbatular loaches

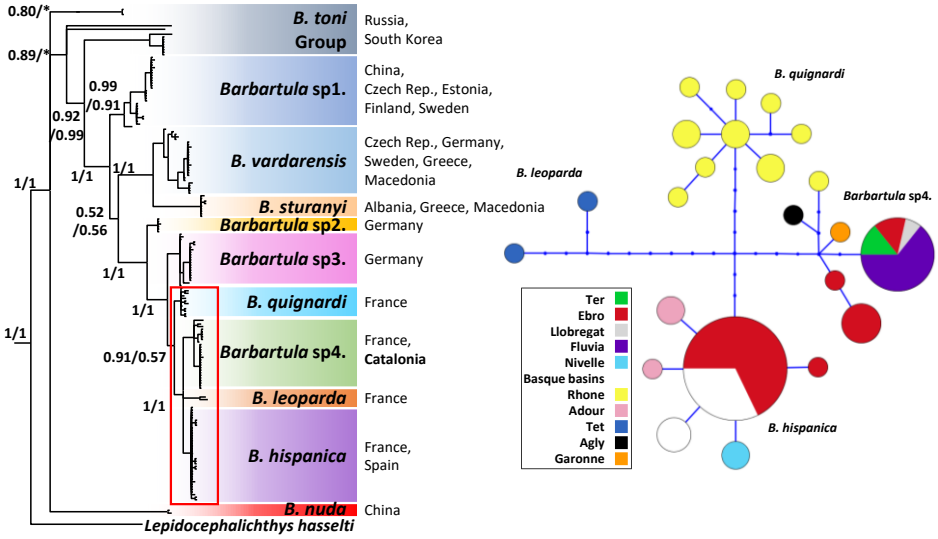


Figure 3-4. Genetic patterns in Eurasian *Barbatula* loaches. Maximum likelihood phylograms based on a partial sequence of the mitochondrial cytochrome c oxidase gene subunit 1 gene (as in Figure 3-2) and network of haplotypes (as in Figure 3-3). The phylogram branch that corresponds to the haplotype network is marked by the brown rectangle. See Tables 3-1 and S3-1 for full accounts of accession numbers.

Barbatula hispanica is native of Spanish and French rivers draining to the south-eastern Gulf of Biscay and to the Ebro basin (Denys et al., 2021). This native area might encompass the Segre River, the largest tributary of the Ebro River, and its tributaries, where *Barbatula* presence is often reported. Our original hypothesis was that the *Barbatula* in the Segre Basin would be native *B. hispanica*, while recently reported populations in other Catalan river basins (Ter, Llobregat, and Fluvià; see Figure 3-1) would be non-native. However,

genetic patterns provided a different picture. All *Barbatula* populations in Catalonia belong to a distinct genetic lineage, plausibly representing a yet undescribed species (*Barbatula* sp4 in Figure 3-4), closely related to *B. hispanica*, *B. quignardi*, and *B. leoparda*. COI haplotypes included in this lineage have been reported from the Rhone, Agly, and Garonne rivers in France, but none of those haplotypes are shared among French basins or between them and Catalan ones (Figure 3-4). The distribution of haplotypes in Catalonia suggests that the original introduction occurred in the Segre basin, which was the source to secondary introductions in other areas across Catalonia.

4.4. Are invaders a-changing?

The PC analysis on the traits of non-native fish species in Catalonia produced two main components (PC1, PC2) that accounted for 53.4% of the variability of the original 15-variable dataset (Table 3-3). Loaches tended to score at extreme positions of both PC1 and PC2. There was a clear relationship between the year of the first detection of a species and its position along PC1 (33.6% of the original variance; Figure 3-5). Older introductions tended to involve large and long-lived fish species, used as gamefish and for aquaculture, while recent ones tended to feature small, benthic fish used as aquarium species and/or as bait in sportfishing. Loaches represented the extreme of this gradient and tended to have been introduced in recent decades (Figure 3-5).

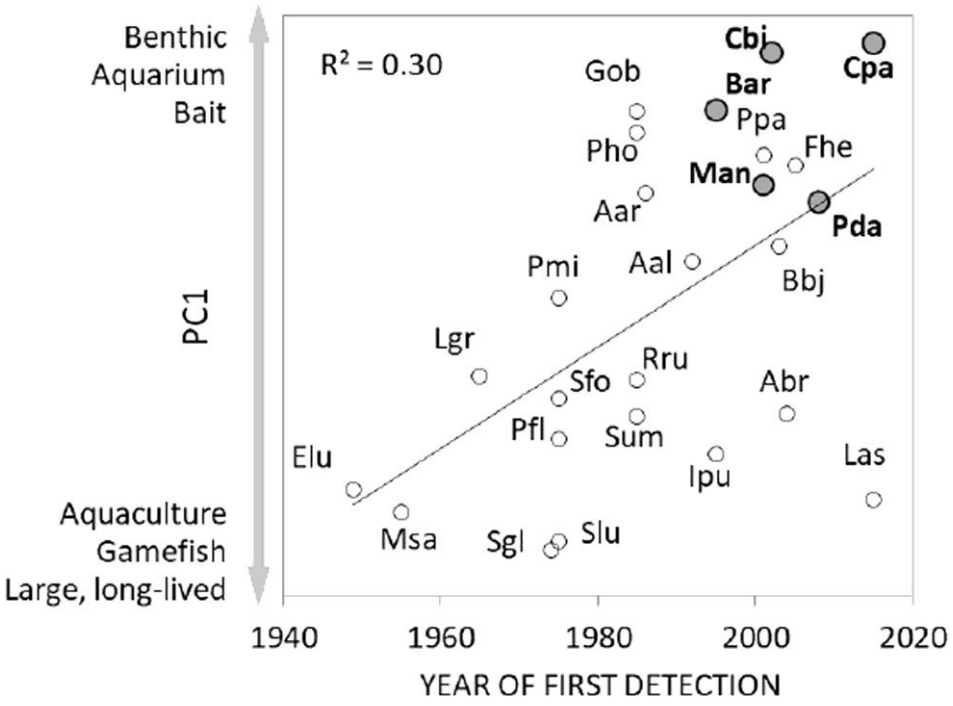


Figure 3-5. Temporal dynamics in the features of non-native fish in Catalonia. Relationship between the main gradient of variation in the traits of non-native fish in Catalan freshwaters and the date in which those species were first detected in Catalonia as non-natives (some of the species have native populations in the region). Larger dots represent loaches. The regression line is statistically significant ($p < 0.005$). Species codes are: Aal (*Alburnus alburnus*); Aar (*Achondrostoma arcasii*); Abr (*Abramis brama*); BAR (*Barbatula* sp., averaging values of *B. quignardi* and *B. barbatula*); Bbj (*Blicca bjoerkna*); Cbi (*Cobitis bilineata*); Cpa (*Cobitis paludica*); Elu (*Esox lucius*); Fhe (*Fundulus heteroclitus*); Gob (*Gobio* sp., averaging values of *G. lozanoi* and *G. occitaniae*); Ipu (Ictalurus punctatus); Las (Leuciscus aspius); Lgr (Luciobarbus graellsii); Msa (Micropterus salmoides); Man (*Misgurnus anguillicaudatus*); Pmi (*Parachondrostoma miegii*); Pda (*Paramisgurnus dabryanus*); Pfl (*Perca fluviatilis*); Pho (*Phoxinus* sp., averaging values of *P. dragarum* and *P. septimaniae*); Ppa (*Pseudorasbora parva*); Rru (*Rutilus rutilus*); Sfo (*Salvelinus fontinalis*); Sum (*Salvelinus umbla*); Slu (*Sander lucioperca*); Sgl (*Silurus glanis*). See Table 3-3 for raw variable values.

5. Discussion

5.1. Loaches in Catalonia

Loaches have been historically rare in Catalan freshwaters, where the only two native species, *C. paludica* and *C. calderoni*, have very restricted ranges. As a result of the introduction of non-native species, the region now hosts at least six loach species, with *C. paludica* having notably expanded its range due to illegal introductions of non-native lineages. Loach introductions in Catalonia are very recent, since *C. bilineata* was introduced in the 1990s and all other species in the 21st century. In fact, it is probable that additional introductions will occur or may have already occurred and that further expansions will continue being reported. This is because of the accumulation of different time-lags may affect the perception of invasion processes. These time-lags include those between the introduction event and either detection (Albano et al., 2018) or notable increases in abundance (Miguel Clavero & Villero, 2014) and those between detection in the field and publication of the non-native records (Zenetos, Gratsia, CARDOSO, & Tsiamis, 2019).

Closely related loach species are often difficult to identify from morphological characters alone (Buj, Šanda, Marčić, Ćaleta, & Mrakovčić, 2014; Kottelat, 2012; Perdices & Coelho, 2020). As in other loach introductions in Europe (e.g. Belle et al. (2017), Zangl, Jung, Gessl, Koblmueller, and Ratschan (2020)), genetic analyses have proven very useful to determine species identities and describe invasion processes in Catalonia. For example, the analyses of the COI gene rejected native *Cobitis* populations as sources of introduced populations in Catalonia and clarified the specific status of Asian loaches found in the region. Previous studies had assumed that all Asian loach nuclei detected in Catalonia would be *M. anguillicaudatus* and would have originated due to a human-mediated spread of individuals from the Ebro Delta population (Franch et al., 2008; Maceda-Veiga, Escribano-Alacid, Martínez-Silvestre, Verdaguer, & Mac Nally, 2019). However, we found that three different Asian loach species have been successfully introduced into Catalonia and that the *M. anguillicaudatus* population of the Ebro Delta has not acted as source for other Iberian populations of this species.

The finding of the *M. bipartitus*×*M. anguillicaudatus* hybrid population in Catalonia highlights the need of integrative taxonomic work on loaches. Although the COI tree clearly discriminated two independent lineages, identified as *M. bipartitus* and *M. anguillicaudatus*, they could not be detected in the RAG-1 tree. This latter result could be due to the lack of truly *M. bipartitus* RAG-1 Sequences in Genebank or to mislabelling issues, but calls for more work on the genetic characterisation of putative *M. bipartitus* populations. Two RAG-1 sequences assigned to *M. bipartitus* from Austria hosted in Genebank (from Zangl et al. (2020)), which we did not include in our analyses due to length disadjustments, seemed to integrate in the *M. mohoity* lineage. Further confusing patterns are provided by sequences labelled as *M. mizolepis*, which formed solid clades with those of *P. dabryanus* for both COI and RAG-1, signalling unsolved taxonomic issues, already signalled for *M. mohoity* (Yi, Zhong, Wang, Huang, & Wang, 2016).

We detected distinct genetic linages that plausibly represent yet undescribed species in all groups analysed. Strikingly, some of these undescribed lineages are known only from areas where they are invasive (eg, *Misgurnus* sp 1). In this line, we found that all *Barbatula* specimens analysed in Catalonia belong to a yet undescribed species with an uncertain native area, plausibly in France. Future work should clarify the specific status of this *Barbatula* lineage, identify its native range and investigate its possible coexistence with *B. hispanica* in the Segre River (Denys et al., 2021). At least four European *Barbatula* lineages probably merit a specific level, following recent description of species within the genus (Gauliard, Dettaï, Persat, Keith, & Denys, 2019).

5.2. Introduction pathways

Identifying the pathways leading to illegal introductions is challenging, because the perceptions and motivations of the releasers have to be deduced or assumed. Loach introductions, and particularly those involving *M. anguillicaudatus*, have been related in some areas with their consumption, which is rooted in the culinary traditions of eastern Asian societies (Gao, Koshio, Nguyen, Wang, & Cao, 2012). However, most loach introductions in

Europe, Australia, and the Americas have been related to aquarists' releases or to their use as live bait in recreational fishing. The environmental tolerance of loaches makes them very suitable organisms as both aquarium fish (e.g. Chan et al. (2019)) and live bait (Norén, Kullander, Nydén, & Johansson, 2018) and several species are widely used as such.

The origin of the *M. anguillicaudatus* population of the Ebro Delta seems clearly linked to the ornamental fish trade, through the escapement of the species from an exotic animal importer (Franch et al., 2008), which has acted as source of introduction for other aquatic species (Joshi & Parera, 2017). Aquarium fish releases could have also been involved in the establishment of other introduced loach populations in Catalonia, particularly in those of Asian loaches. Even though aquarists may be increasingly conscious of the risks of pet releases (Maceda‐Veiga, Domínguez‐Domínguez, Escribano‐Alacid, & Lyons, 2016), specific analyses have revealed that the role of aquarium hobbyists in freshwater species introductions may still be high (Copp, Wesley, & Vilizzi, 2005; Padilla & Williams, 2004). Banha, Diniz, and Anastácio (2019) recently reported that around 8% of Portuguese aquarists admit having released pets into the wild. Even though this proportion could seem low, the figure may be translated to a very high number of freshwater species introductions, because the number of pet owners (i.e. the numerator in that proportion) is constantly increasing (Evers, Pinnegar, & Taylor, 2019). By contrast, that 8% could arguably be an underestimation, since aquarists are being asked to acknowledge having done an illegal action (e.g. Gavin, Solomon, and Blank (2010)). A particular case of aquarium fish releases would involve conservation-minded aquarium hobbyists breeding native and often threatened fish species; such hobbyists are arguably responsible for introducing into the wild threatened Iberian killifishes, such as *Aphanius* toothcarps (Gonzalez et al., 2018; Gonzalez, Pedraza-Lara, & Doadrio, 2014). *Cobitis* loaches have been involved in some of these introductions, such as that of *C. paludica* in the Chícamo River, one of the few habitats of Aphanius iberus in south-eastern Spain (F. J. Oliva-Paterna, personal communication). This kind of illegal conservation-minded release could be involved in the establishment of some Catalan loach populations, particularly in those of *C. paludica*.

Cobitis loaches have been traditionally used as live bait by Iberian fishermen, a use that increased after the introduction of large piscivorous fish (*Micropterus salmoides* and *Esox lucius*) since the mid-20th century (Collares‐Pereira, Cowx, Ribeiro, Rodrigues, & Rogado, 2000). Live bait is generically forbidden, with few exceptions, in both Spain and Portugal, in spite of which fishermen in both countries admit using *Cobitis* loaches as live bait (Banha, Diniz, & Anastácio, 2017; Sánchez-Hernández et al., 2018). In both cases, the percentage of fishermen admitting this use was low, but, as in the case of aquarium releases, these low proportion figures may involve a high number of potential introduction events. Other loach genera, including *Barbatula* and *Misgurnus*, are also used as live bait (Iida, 2002; Norén et al., 2018), and their release from fishermen's buckets cannot be discarded as the origin of some of the Catalan populations. Notably, the detection of *Misgurnus* loaches in the Albufera de València Lake coincided with that of Wels catfish (*Silurus glanis*) and several press releases reported the widespread opinion that loaches had been introduced by catfish fishermen.

The changes in the main pathways of introduction of fish arguably lie in the change in the profiles of non-native fish species introduced into Catalonia. Aquarium releases and live bait discards have been plausibly involved in the irruption of loaches in Catalonia, and both of them have been identified as emerging introduction pathways (see Lapointe, Fuller, Neilson, Murphy, and Angermeier (2016)), which seem to be increasing worldwide (Bernery et al., 2022) and in the Iberian Peninsula (Corral‐Lou et al., 2019; Maceda-Veiga, Escribano-Alacid, de Sostoa, & García-Berthou, 2013). It is thus probable that the dynamics in the relative importance of the different introduction pathways, coupled with changing climate conditions (Vilizzi et al., 2021), will continue driving changes in the profiles of non-native fish introductions, with important implications for environmental management.

5.3. Management implications

Our results show how the characteristics of invasive species found in an area can rapidly change, with changes being two-sided. On the one hand, the

magnitude of the problems associated with loach invasions might have been underestimated due to identification issues. In this sense, this work shows that Catalonia hosts established, non-native populations of at least six loach species. These figures were unexpected and represent an important proportion of the total non-native fish richness found in Catalonia (i.e. around 20%). On the other hand, loaches had not been previously considered among the typical fish types involved in fish invasions in the Iberian Peninsula, which were described to involve mainly game fish and small cyprinid fish used as live bait (e.g. Miró and Ventura (2015)). The outbreak-like irruption of invasive loaches in Catalonia reported here arguably implies a change in the average characteristics of the invasive fish species found in the area, the description of which deserves further research.

The shifting profiles of non-native fish species may hinder effective management of biological invasions, in at least two ways. First, management tools for preventing new invasions largely rely on risk analyses and prohibited species lists (a.k.a. black lists). The frequent procedure involves including a species in a prohibited list, and thus ban its uses, when a risk analysis states that it has similar features to those of species that have already proven to be successful invaders and/or to generate serious impacts. However, the quick irruption of a group of species not fulfilling the previous invasive profiles, as that of loaches in Catalonia, would not have been identified as highly risky in a risk analysis. That same irruption would also change the overall characteristics of successful invaders, what would call for a reassessment of risk analyses. However, legal instruments are most commonly reluctant to change, and their adaptation to rapidly changing situations is challenging (Oficialdegui et al., 2020). Second, knowledge on the environmental impacts of an emerging group of invaders, as loaches in Catalonia are, may not be produced at the same tempo as the invasion progresses, due to lag between research development and in the incidence of invasion impacts. When the profile of a new invader fits that of a previously successful one (e.g. a piscivorous fish), its impacts can be predicted, at least qualitatively, and management decisions can be made attending to those predictions. However, when the new invaders have new profiles, managers face the new environmental issue without enough evidence at hand to make

informed decisions. This is critical, because the efficacy of steeply decline along the invasion process. Therefore, it seems urgent to progress in the knowledge on the life history and population dynamics of these species in the new environments to guide the implementation of effective eradication or population control actions.

The Catalan loach example calls for rethinking the mainstream uses of basing the management of biological invasions solely on prohibited species lists. While these lists are undoubtedly useful when the profiles of new invaders can be predicted, they should be complemented (not substituted) by a combination of permitted species lists (a.k.a. white lists) and more agile and generic instruments. These later instruments should be at least in part independent of the identity of the invaders (i.e., should not rely exclusively on species listings) and should emphasise prevention and the rapid response to new introduction events, aimed at hindering establishment and spread through secondary introductions.

General Discussion

1. High mountain lakes and non-native fish species

High-mountain lakes, maintained by their geographical isolation, retained unique fishless ecosystems. However, the case of the species of the genus *Phoxinus* found in high mountain lakes are all introduced and established as invasive species. They were introduced primarily as live bait through the introduction of salmonids (Miró & Ventura, 2015). Despite their shorter introduction history compared to salmonids, they have exhibited faster invasions (Ventura et al., 2017). Even after the decline or removal of salmonids, their introduction has led to negative impacts on the unique ecosystem of high-mountain lakes, disrupting the native ecology and reducing species diversity as the top predators in these lakes (Miró & Ventura, 2015; Museth et al., 2007; Næstad & Brittain, 2010; Osorio et al., 2022; Werner, Skelly, Relyea, & Yurewicz, 2007). Therefore, research on the introduction pathways of the genus *Phoxinus* in high-mountain lakes is crucial for the restoration and conservation of the high-mountain lake ecosystems.

1.1. Distribution of genus Phoxinus in high mountain lakes and study limitations

In Chapters 1 and 2 of this study, I conducted the identification of the introduced *Phoxinus* species into high-mountain lakes, clearly illustrating the distribution of each species. The results obtained using the mtDNA marker in Chapter 1 and the nDNA marker in Chapter 2 generally coincided. Naturally abundance of organisms is limited by the biological and environmental factors (Andrewartha & Birch, 1954). However, determining the introduction pathways of the genus *Phoxinus* into high-mountain lakes is challenging, as they are primarily associated with human activities, and therefore they do not follow a predefined pattern, lacking a clear spatial pattern.

The haplotype network from Chapter 1 (Chapter 1, Figure 1-3) revealed that *P. bigerri* introduced into high-mountain lakes had its introduction origin in the Adour basin in France, not in the Cantabrian inner or upper Ebro basin in

Spain. This finding was further supported by the analysis using microsatellites in Chapter 2 (Lineage B2). However, the lineage B1 (Chapter 2, Table. 2) predominant in the high-mountain lakes of the Aragon region in Spain, showed a match with the haplotype recorded in the Duero basin individuals (Corral‐Lou et al., 2019). Therefore, indicating that both areas had a similar yet unknown origin. Similarlty the lineage D3, predominant of two high-mountain lakes (51, 52), also presented challenges in tracing its introduction origin. This is despite the utility of genetic approaches for investigating invasive species invasion. It is caused by the indiscriminate introduction of invasive species over an extended period across broad regions, coupled with limitations in research manpower and funding. To overcome this, there is a need for additional microsatellite analysis on samples from other regions in Spain and France (e.g., Cantabria, Garonne River, Adour River and the Basque regions). Furthermore, since Chapters 1 and 2 have revealed that the introduction of fish invasive species occurs across borders, tracking the introduction pathways of invasive species and addressing additional introductions and occurrences would require further research and collaboration on a multinational scale.

1.2. **The impact of introducing non-native fish species into high mountain lakes on downstream ecosystems.**

High-mountain lakes are known to have a low resistance to fish introductions due to the geographical conditions and unique ecosystem (Knapp et al., 2001). Additionally, the introduction of fish into headwater lakes provides fish with access to a larger stream area within a watershed compared to mainstream or low-elevation introductions. This is the most apparent mechanism as limited mobility can restrict fish movement and invasion in steep streams. The rate and spatial extent of stream fish invasions may be influenced by many factors, including dispersal rates, demographic growth in the invasion front, and demographic pressures in potential source populations. The release of fish into headwater lakes can potentially influence each of these factors, resulting in a greater extent of river area invaded from the source than downstream

water bodies (Adams, Frissell, & Rieman, 2001). However, the results from Chapter 2 indicate that downstream migration is does not take place easily. For example, Lake Llebreta did not get any fish from the two the above-mentioned lakes with the lineage D3. Similar results have been found in Italy, where despite different species of minnows are found in high mountain lakes, while the native species *P. lumarieul* was still the only species found in lowland streams (De Santis et al., 2021). This may be a result of the presence of steep slopes that prevent the fish from arriving alive downstream. In fact, we have observed the presence of minnows along the stretches immediately downstream high mountain lakes when there are no steep slopes, but never found them in the lowland reaches below high mountain lake areas. With this scenario, the same slopes that prevent fish from colonizing naturally high mountain lakes, might protect downstream reaches. Additionally, lowland mountain streams with natural presence of trout might be able to eradicate those few minnows reaching alive lowland stream reaches. However, there might be exceptions, for instance, lowland mountain streams with anthropogenic alterations, such as pollution, trout populations alterations for overfishing, or due to the introduction of alien species. therefore, this threat should not be taken lightly.

2. Invasiveness of fish species to lowland rivers and coastal areas.

Invasion of non-native fish to relatively low-elevation habitats has had a negative impact on native aquatic fauna in many large portions of many drainages (Adams et al., 2001). In contrast to the isolated environments of high-mountain lakes discussed in Chapters 1 and 2, the basins in Catalonia described in Chapter 3 (but also in Chapters 1 and 2) have a spatial continuity. While they exhibit relatively high resistance to introduced fish compared to fishless lakes, they conversely, have a higher potential for introductions due to larger accessibility to human activities. This is clearly indicated by the fact that the ichthyofauna of Catalonia currently consists of 27 native species and 31 non-native species (Manau & Cazorla, 2016). While Chapter 1 focused only on one genus, Chapter 3 addressed the introduction of four species groups of

loaches. Therefore, it is considered necessary to explore alternatives tailored to the characteristics of these habitats to prevent and manage the invasion risks of non-native species.

2.1. Distribution of loaches in Catalonia and remaining questions

The results of Chapter 3 demonstrate that, despite the short introduction history, non lative loaches already show a large distribution in Catalonia. Additionally, the difficulty in morphological identification of loaches led to the use of genetic analysis for their idientification (Buj et al., 2014; Kottelat, 2012; Perdices & Coelho, 2020). Through mtDNA analysis, we confirmed the presence of introduced species such as *Cobitis bilineata*, *Misgurnus anguillicaudatus*, *Misgurnus bipartitus*, *Paramisgurnus dabryanus* (first citation of this highly invasive species in Europe), and the discovery of an undescribed *Barbatula* sp.. As well as the habitat expansion of the native species, *Cobitis paludica,* also through anthropogenic interventions. However, despite this, nDNA analysis revealed that the genus *Misgurnus* found in the River Ter, which appeared as two species in mtDNA, showed a single lineage in nDNA, indicating the presence of hybrids. In the case of the genus *Cobitis*, a clear species differentiation was not possible with nDNA. This is attributed to the limited genetic data and the complexity inherent in the species. Hybridization and polyploidy are distinctive characteristics of biodiversity among cypriniform fish (Saitoh, Chen, & Mayden, 2010). Particularly within the loaches, many authors (I. Kim & Lee, 2000) have reported the occurrence of these specific diversification processes in natural habitats (Janko et al., 2007; I.-S. Kim & Lee, 1990; Mezhzherin & Pavlenko, 2007; Vasil'ev, Lebedeva, Vasil'eva, & Ryskov, 2007; Vasil’ev, Vasil’eva, & Osinov, 1989).Therefore, when artificial introductions are added to these natural processes, more complex outcomes are anticipated.

2.2. Difficulty figuring the introduction pathway of invasive fish species in connected aquatic ecosystems

There are also many difficulties in uncovering the origin of introductions in easily accessible lowland areas, where the situation may be somewhat different from the above described case of high mountain lakes. For instance, in the case of the introduction of intercontinental species, such as the case of species originally from Asia (e.g. *Misgurnus* and *Paramisgurnus*), they are usually imported as aquarium fish. When they are released into a connected water system, it becomes difficult to distinguish the specific introduction route even if it took place in Spain or France. While it may be possible to trace the introduction paths if a large number of individuals are released, this scenario is uncommon.

Indeed, the ornamental fish trade is a burgeoning industry, involving over 125 countries and more than 2,500 species, with a market value of several billion dollars. Among these, 60% comprise freshwater fish (Dey, 2016). It is well recognized that the trade in ornamental fish serves as a crucial pathway for the introduction and establishment of fish species (Fuller, 2015; Strecker, Campbell, & Olden, 2011). It includes cases like aquaculture fish, which account for a significant proportion of global establishment events of non-native species, or fish introduced as biological control agents to manage weeds or mosquitoes such as mosquito fish (Gambussia sp.; Beisel & Lévêque, 2010). These have later become established and invasive, causing ecological impacts (Copp, Bianco, et al., 2005). In conclusion, there are many other introduction pathways into lowland aquatic ecosystems (Bernery et al., 2022). As evident from the example of Chapter 3, the severity of invasive species in Catalonia is significant. The impact of invasive species is expected to increase due to various factors mentioned above, emphasizing the urgent need for measures to address this issue.

3. Invasive species and hybridization

One of the most intriguing aspects of this study is the identification of various hybridizations within or between species of the genus *Phoxinus* in Chapter 1 and 2. Additionally, hybrid individuals were also found in the genus *Misgurnus* in Chapter 3. The presence of hybridization and introgressive hybridization, where genetic material from one species is incorporated into the gene pool of another, is relatively common in fish species that are closely related (Aboim et al., 2010; Gante et al., 2004). The introduction of non-native species can induce evolutionary changes in native species, and the interactions between native and non-native species are an important area of research in invasion ecology (Lambrinos, 2004; Schoener, 2011). These interactions have significant conservation implications as they can affect the integrity and evolutionary history of native species.

3.1. Anthropogenic habitat disturbance and hybridization risk.

Anthropogenic habitat disturbance, such as habitat alteration by human activities, can increase the risk of hybridization. Previous studies have shown that habitat disturbance can lead to introgressive hybridization and pose a threat to species with deeper evolutionary histories (Anderson, 1948; Rhymer & Simberloff, 1996; Wiegand, 1935). However, in cases where different species naturally coexist in sympatry, the formation of hybrid swarms resulting from the breakdown of reproductive isolation is not well established (Seehausen, 2006). Theory suggests that under such circumstances, reproductive barriers may be reinforced and resistant to habitat changes (Hasselman et al., 2014).

The results of this study differ from the expectation of a high probability of hybridization or admixture in coexisting species or lineages, as discussed in Chapter 2. In isolated lake environments, different lineages demonstrated better preservation of their genetic integrity, while in rivers, a higher incidence of hybridization or admixture was observed. This suggests that human-induced pressures, such as habitat alteration, play a role in promoting genetic variations and hybridization. In the case of introduced species, there

is a possibility of secondary hybridization when individuals with artificial hybrid forms, as those bred in aquariums, are released into natural ecosystems. Additionally, genetic variation can contribute to the adaptation of invasive species to new environments. Mitochondrial introgression, where mitochondrial DNA from one species is introduced into another through hybridization, has been associated with thermal adaptation in freshwater fish species. This can lead to individuals possessing higher reproductive abilities and potentially having more significant impacts as invasive species.

Additionally, genetic variation can play a role when invasive species adapt to new environments. Mitochondrial introgression is associated with thermal adaptation in different freshwater fish species. For instance, the mtDNA of Arctic char (*Salvelinus alpinus*), a northern species, has become fixed through introgression with northern populations of lake trout (*Salvelinus namaycush*) and brook trout (*Salvelinus fontinalis*), typically found at lower latitudes (Bernatchez & Osinov, 1995; Côté, Perry, Blier, & Bernatchez, 2007; Doiron, Bernatchez, & Blier, 2002; Glemet, Blier, & Bernatchez, 1998). On the other hand, mitochondrial DNA of bull trout (*Salvelinus confluentus*), typically a southern species, has been found in southern populations of dolly varden (*Salvelinus malma*), typically found at higher latitudes (Redenbach & Taylor, 2002). Furthermore, Mee and Taylor (2012) predicted that *Phoxinus neogaeus*, which has long been present in northern regions, would facilitate coexistence in the north through mitochondrial introgression resulting from hybridization with *P. eos-neogaeus*.

Overall, the presence of hybridization and introgressive hybridization in invasive fish species has important implications for understanding the dynamics and impacts of these species on native ecosystems. It highlights the need for further research and monitoring to assess the risks and conservation implications of hybridization in invasion ecology.

4. Ecological risks of invasive fish species

The invasion of alien species has the most immediate and direct impact through resource competition on species occupying the same ecological niche. However, it extends beyond that to potentially influence entire ecosystems. It has often overlooked the impact of the cascading effects of fish introduction, such as trophic cascades, and the ecological connections between invaded lakes and terrestrial habitats. The impact of invasive fish can be exacerbated through interactions with airborne pesticides, infections by molds and viruses, climate changes, increased ultraviolet radiation due to ozone layer thinning, water exploitation, water-level fluctuations, and point sources of organic pollutants (Ventura et al., 2017). While not explicitly addressed in this study, I would like to briefly discuss the latent risks that potentially exist.

4.1. Risk of introduction of new invasive species

Invasive organism profiling has long been considered a useful tool for preventing future invasions (Kolar & Lodge, 2002). Through previous studies, we can better respond to the additional invasion of invasive species. Following similar routes as those of the Genus *Phoxinus* covered in this study, there might be other potential invasive species, such as other species being used as live bait and introduced into lakes. For example, the gudgeon (*Gobio* spp.) has already been found in some lakes of the northern slopes of the Pyrenees (France). This fish is similar in size to minnows and equivalent in flexible ecological traits (Tang et al., 2011), poses a potential threat to ecosystems through a similar introduction process (Ventura et al., 2017). In addition, in the northern Pyrenees, where the use of live bait is permitted, various introduced species such as *Gobio gobio, Scardinius erythrophthalmus, Cyprinideae spp, Rutilus rutilus, Squalius cephalus, Perca fluviatilis, Lepomis gibbosus,* and *Esox Lucius* have already been identified (Didier Galoup Pers. Comm.). Similarly, in the Alps there are introduced populations of *Thymallus thymallus, Cottus gobio, Lota lota, Squalius cephalus,* and *Barbatula barbatula* (Pastorino et al., 2019; Tiberti et al., 2020; Tiberti & Splendiani, 2019). This is a significantly higher number of species discovered compared to the southern

Pyrenees (Spain), where live bait is forbidden, and can be seen as an example of how appropriate legal regulations effectively prevent the possibility of introducing new invasive species. However, the threat due to illegal fishing activities continues.

The situation is not significantly different in lowland rivers and coastal areas. As mentioned in Chapter 3, the scale of an aquarium fish industry is increasing, and the use of live bait, which is prohibited, is also frequent (Banha et al., 2017; Sánchez-Hernández et al., 2018). Many countries (including Portugal and Spain) have special laws on the introduction of alien species but these are not always obeyed (Vinyoles et al., 2007). Vila-Gispert, Alcaraz, and García-Berthou (2005) conducted a study in Catalan streams to compare native and invasive species and identify the characteristics of successful invasive species. According to this study, in the rivers of Catalonia, the highest proportion of native and Iberian exotic species is found in the upper reaches and headwaters (Enric Aparicio et al., 2000; Doadrio, 2001). In contrast, the majority of foreign exotic species are discovered in the middle and lower reaches. Headwater streams and upper reaches in Catalonia experience strong seasonal flow patterns. During the summer, the flow is low, restricting aquatic habitats to small isolated pools, while in winter and spring, high flows occur. Fish native to these seasonal flow conditions, including both native and Iberian exotic species, are adapted to smaller sizes and multiple spawning. This adaptation allows them to survive after floods without losing all offspring, thus enhancing their resilience to the seasonal hydrological dynamics (Vila-Gispert et al., 2005). Loaches, as discussed in Chapter 3, seem to exhibit the latter characteristics. Stone loaches, for instance, are known to seek refuge under banks when rivers flood or in muddy pools. They are also reported to possess strong resistance to organic pollution (Smyly, 1955). Considering these traits, the invasion of stone loaches is expected to be accelerated. Additionally, *G. gobio* has been shown to have resistance to floods (Vila-Gispert et al., 2005). As such, the potential risk of invasive species is always present and may already be present. To respond promptly to the changing invasive species (Chapter 3) based on the characteristics of invasive species, continuous attention and studies are required.

4.2. Potential risks of exotic fish as disease vectors

The potential risks of invasive fish species as disease vectors highlight the importance of monitoring and managing these species (Conn, 2014; Peeler, Oidtmann, Midtlyng, Miossec, & Gozlan, 2011). The introduction of fish can introduce pathogens to native aquatic organisms, posing a threat to both aquatic biodiversity and human health (Hall, Hewitt, Tuffrey, & De Silva, 2008; Serracca et al., 2013). For example, a study conducted on invasive fish species in the high mountain lakes of Italy (Dimon Lake and Balma Lake) sampled *Cottus gobio*, *Phoxinus phoxinus*, and *Salvelinus fontinalis*. The results showed positive reactions for widely distributed bacteria in aquatic environments, such as *Aeromonas sobria* and *Plesiomonas shigelloides*, as well as a major pathogen, *Yersinia ruckeri*, primarily found in facilities that cultivate salmonids. These diseases can disrupt the host-pathogen balance and have implications for ecosystem health(Pastorino et al., 2019).

Furthermore, the presence of invasive fish species in high mountain lakes, which are often tourist destinations, raises concerns about potential interactions between these fish and humans. Activities such as swimming and camping could lead to interactions with invasive fish species, potentially increasing the risk of disease transmission. The absence of adequate health monitoring in these areas may further exacerbate the potential impacts on public health. Another example of fish acting as significant disease vectors is ranavirosis. This is caused by large double-stranded DNA viruses belonging to the Iridoviridae family, infecting amphibians, reptiles, and fish (Duffus et al., 2015). Rosa et al. (2022) used a 40-year dataset to test the hypothesis that the introduction of invasive fish causes disturbance by causing ranavirosis in amphibian hosts. This could impact native communities and disrupt the host-pathogen balance, potentially worsening the threat to ecosystem health. Globally, there has been an increasing occurrence of disease outbreaks in amphibian clusters caused by the genus Ranavirus. It has been confirmed that both the Common midwife toad virus (CMTV) and Frog virus 3 (FV3) have led to mass mortality in the Iberian Peninsula since the late 1980s (Thumsová et al., 2022). However, it remains unclear whether this is associated with the introduction of invasive fish species.

The ecological recovery of high mountain lake ecosystem has been observed following the removal of minnows (T. Buchaca et al., 2019; Miró et al., 2020), but the aspects related to diseases have not been investigated. What is certain is that high mountain lakes hold value as habitats for various amphibian species, including those that are endangered or endemic. Therefore, beyond the existing research and projects focusing on the removal, management, and monitoring of invasive species, there seems to be a need for additional attention to these areas.

5. Way to conserve the native ecosystem and manage the ongoing invasions

Invasion of invasive species has serious negative impacts on ecosystems from alpine lakes to lowland rivers, and is an ongoing but potential threat that will continue to arise in the future due to environmental change and human activities.

5.1. Effective management methods for introduced invasive species

The tradition of using live bait in fishing implied pathways for the introduction of invasive species (DiStefano, Litvan, & Horner, 2009; Kerr, Brousseau, & Muschett, 2005; J. Ward et al., 2011). Improper handling of live bait has resulted in the introduction of at least 14 fish species in the state of Ontario, USA (Kerr et al., 2005). In the English Lake District, a minimum of 12 native and non-native fish species were intentionally moved to Windermere for live bait purposes (Winfield, Fletcher, & James, 2011). In many cases, anglers have been observed releasing unused fish, used as live bait after fishing activities (Kerr et al., 2005; Kilian et al., 2012; Maitland & Campbell, 1992; Winfield et al., 2011), serving as a direct pathway for the introduction of invasive species (Ventura et al., 2017).

In the Pyrenees, the strict prohibition of releasing organisms into certain areas without government approval, such as national parks and conservation areas, is in place (Miró, 2011). However, regulating individual actions on a case-by-case basis is more challenging. Currently, in some research sites of Chapters 1 and 2, there are ongoing or completed high mountain lake restoration plans (LIFE BIOAQUAE, LIFE LIMNO PIRINEUS, LIFE RESQUE ALPYR) are taking place due to their ecological significance. This has led to the long-term restoration of native ecosystems in many lakes. The likelihood of the introduction of non-native fish as live bait in lakes where trout has been removed is low but cannot be completely ruled out. In the lowlands, the presence of many introduced species poses greater challenges for removal and management.

The proposed introduction pathways and species identification presented in this study can contribute to more effective ecosystem conservation efforts. Understanding the pathways of invasive species introduction and alerting local communities can prevent new introductions. Accurate identification of non-native species contributes to conveying information about invasive species to conservation and management projects, facilitating effective control and management. This information, along with comprehensive data on the local environment, including aquatic insects and amphibians, conducted as part of the project, should be shared with fisheries commissions and local communities to enhance preventive measures. Such measures are among the best guiding principles for preventing the spread of invasive species, in conjunction with regulations and legislation (Simberloff et al., 2013).

General conclusions

1. The summary of freshwater species invasion: Identification, distribution and introduction pathways of minnows and loaches.

I. Despite the active research in recent years revealing diverse species diversity within the Genus *Phoxinus*, the study of their distribution has remained predominantly confined to lowland areas. In this study, through various genetic analyses conducted in high mountain lakes in the Pyrenees in Spain, France, and the Italian Alps, we have confirmed the introduction of *Phoxinus bigerri*, *Phoxinus csikii*, *Phoxinus dragarum* and *Phoxinus septimaniae*.

II. *P. bigerri* was primarily found in its native area, the Adour basin, and in high mountain lakes of the west of the Pyrenees, with the regional discontinuity in the haplotype network suggesting its introduction to high mountain lakes from the Adour basin. The higher number of individuals analysed for nuclear DNA analysis allowed to detect for the first time its presence in Catalonia and Italy.

III. *P. csikii* has been introduced in high mountain lakes in the Italian Alps, with haplotype analysis indicating its origin more aligned with haplotypes also present in Rhone or Rhine basins rather than presently detected haplotypes from its native Danube basin. Results from nuclear DNA from high mountain lakes showed that this species had two clearly differentiated lineages.

IV. *P. dragarum* was found in its native Garonne basin, and it was introduced into high mountain lakes to the east of the Pyrenees and

in rivers in Catalonia. The analysis of nuclear DNA allowed to discover its introduction into Italy and Aragon region of Spain.

V. *P. septimaniae* was suspected to have been introduced from its native Rhone basin to high mountain lakes and lowland reservoirs in Italy. It was also found in the rivers of Catalonia.

VI. Contrary to initial expectations that introductions into high mountain lakes or reservoirs could impact downstream ecosystems, this study revealed that downstream migration does not occur easily. Among the 12 cases of downstream migration identified through GenClass analysis, there were virtually no instances where it was deemed feasible considering the local context.

VII. We reported the establishment of at least five non-native loach species in Catalonia (*Cobitis bilineata*, *Misgurnus anguillicaudatus*, *Misgurnus bipartitus*, *Paramisgurnus dabryanus*, and an as-yet-undescribed *Barbatula* species), with the majority currently in the process of spreading. Additionally, one of the region's native loach species, *Cobitis paludica*, has expanded beyond its original habitat and is spreading through river basins, likely through anthropogenic intervention.

VIII. In Catalonia, Asian loach species (genus *Misgurnus* & *Paramisgurnus*) have been discovered in the Ebro Delta, the Vallvidriera Reservoir (near the city of Barcelona), and in the Ter Basin (Onyar and Cinyana streams). Despite the low genetic diversity, their distribution is expanding.

IX. For *Cobitis* loaches, the *C. paludica* population in the Ebro Delta is closely related with populations from other Mediterranean regions (Jucar River basin). In contrast, the recently introduced *C. paludica* in the Besòs and Ter basins originated independently from central Iberian populations, likely from the Tugas River basin. Additionally,

we confirmed the presence of *C. bilineata* in Lake Banyoles, although we could not demonstrate its origin.

X. All the *Barbatula* individuals collected in this study did not belong to the native *B. hispanica,* but were found to belong to a distinct genetic lineage, likely to an undescribed species originating from France.

2. Inter-species hybridization and intra-species admixture: Implications and insights.

I. The coexistence of two or more species within the genus *Phoxinus* in the same site, along with the presence of various haplotypes and lineages, indicates diverse introductions. We found higher number of lineages in high mountain lakes and rivers out of their native range (mean of 2 and 1.7 lineages per site respectively) compared to native rivers (mean of 1.1 per site).

II. In the Catalan rivers, all individuals identified as *P. septimaniae* by mtDNA were identified as *P. dragarum* with nDNA indicating they were mitochondrial/nuclear DNA hybrids (7% of the cases). Our results suggest they were already introduced as hybrids.

III. Contrary to the expectation of a high rate of intraspecific hybridization in isolated lakes, we observed a low proportion of nDNA admixture (3%). Notably, two individuals from River Tenes were identified as a nuclear and mitochondrial hybrids of *P. septemaniae* (mtDNA) and *P. dragarum* x *P. bigerri* (nDNA).

IV. The *Misgurnus* specimens found in the River Ter basin originated from a single source population, including hybrids of *M. bipartitus* × *M. anguillicaudatus*.

3. Other interesting facts…

I. For *P. dragarum*, which had the largest sample size, relatively low mitochondrial genetic diversity was observed in areas where fishing was prohibited. Genetic analysis indicated that fishing regulations reduced the possibility of new introductions.

II. Risk analyses for invasive species often assume that the characteristics of future invaders will resemble those already successful, but these features may change. In the case of Catalonia, the characteristics of introduced fish have evolved over time, from ornamental fish species or targeted game fish to species used in aquariums and live bait for fishing.

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Supplementary Materials

Chapter 1

1.1. TABLE S1-1. Accession codes for the phylogenetic tree collected from Genebank for COI.

The table represents the gene (COI) used for generating phylogenetic tree of genus *Phoxinus*, the species identification provided by Genebank, as well as the country of origin, unique identifier, and source of the sample.

Gene	Species Genbank	Accesion number	Country	Literature
COI	*Phoxinus csikii*	MN816111	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MN816112	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MN816113	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MN816114	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MN816118	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus lumaireul*	MN816091	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus lumaireul*	MN816097	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816126	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816127	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816128	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816131	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818242	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816132	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816133	Austria	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MF407985	Austria	Schonhuth,S. et al. 2018
COI	*Phoxinus marsilii*	MF407988	Austria	Schonhuth,S. et al. 2018
COI	*Phoxinus marsilii*	MF407986	Austria	Schonhuth,S. et al. 2018
COI	*Phoxinus marsilii*	MF407987	Austria	Schonhuth,S. et al. 2018
COI	*Phoxinus* sp.	KX673427	Austria: Grundl	Ramler,D., Palandacic,A. et al. 2016
COI	*Phoxinus* sp.	KX673425	Austria: Grundl	Ramler,D., Palandacic,A. et al. 2016
COI	*Phoxinus* sp.	KX673432	Austria: Lunzer	Ramler,D., Palandacic,A. et al. 2016
COI	*Phoxinus* sp.	MF407701	Bosnia and Herzegovina	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407679	Bosnia and Herzegovina	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407697	Bosnia and Herzegovina	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407699	Bosnia and Herzegovina	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407708	Bosnia and Herzegovina	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407709	Bosnia and Herzegovina	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407710	Bosnia and Herzegovina	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407711	Bosnia and Herzegovina	Palandacic,A. et al. 2018
COI	*Phoxinus strandjae*	MN816080	Bulgaria	Palandacic,A. et al. 2020

COI	*Phoxinus strandjae*	MN816074	Bulgaria	Palandacic,A. et al. 2020
COI	*Phoxinus strandjae*	MN816075	Bulgaria	Palandacic,A. et al. 2020
COI	*Phoxinus strandjae*	MN816076	Bulgaria	Palandacic,A. et al. 2020
COI	*Phoxinus strandjae*	MN816077	Bulgaria	Palandacic,A. et al. 2020
COI	*Phoxinus strandjae*	MN816078	Bulgaria	Palandacic,A. et al. 2020
COI	*Phoxinus strandjae*	MN816079	Bulgaria	Palandacic,A. et al. 2020
COI	*Phoxinus krkae*	MN816034	Croatia	Palandacic,A. et al. 2020
COI	*Phoxinus krkae*	MN745549	Croatia	BOGUTSKAYA, Nina G., et al 2020
COI	*Phoxinus lumaireul*	MN816089	Croatia	Palandacic,A. et al. 2020
COI	*Phoxinus lumaireul*	MN816090	Croatia	Palandacic,A. et al. 2020
COI	*Phoxinus lumaireul*	MN816087	Croatia	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MF407741	Croatia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407742	Croatia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407739	Croatia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407740	Croatia	Palandacic,A. et al. 2017
COI	*Phoxinus bigerri*	MN816045	France	Palandacic,A. et al. 2020
COI	*Phoxinus bigerri*	MN816046	France	Palandacic,A. et al. 2020
COI	*Phoxinus bigerri*	MN816049	France	Palandacic,A. et al. 2020
COI	*Phoxinus bigerri*	MN816050	France	Palandacic,A. et al. 2020
COI	*Phoxinus bigerri*	MN816051	France	Palandacic,A. et al. 2020
COI	*Phoxinus bigerri*	MN816052	France	Palandacic,A. et al. 2020
COI	*Phoxinus bigerri*	MN816053	France	Palandacic,A. et al. 2020
COI	*Phoxinus dragarum*	MT975793	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975788	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975787	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975762	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975760	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975757	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975753	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975746	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975745	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975732	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975720	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975716	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975715	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975711	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975710	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975709	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975708	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus dragarum*	MT975707	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975769	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975768	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975766	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975752	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975736	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975735	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975734	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975733	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975725	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975724	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975704	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975703	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus fayollarum*	MT975702	France	Denys,G.P.J. et al. 2020
COI	*Phoxinus septimaniae*	KJ554499	France	Geiger,M.F. et al. 2014

COI	*Phoxinus septimaniae*	KJ554474	France	Geiger,M.F. et al. 2014
COI	*Phoxinus septimaniae*	MF407785	France	Palandacic,A. et al. 2020
COI	*Phoxinus septimaniae*	MF407786	France	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MF407729	France	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	KJ554392	France	Palandacic,A. et al. 2020
COI	*Phoxinus colchicus*	MN816067	Georgia	Palandacic,A. et al. 2020
COI	*Phoxinus colchicus*	MN816068	Georgia	Palandacic,A. et al. 2020
COI	*Phoxinus colchicus*	KU729254	Georgia	Palandacic,A. et al. 2020
COI	*Phoxinus colchicus*	KU729255	Georgia	Palandacic,A. et al. 2020
COI	*Phoxinus phoxinus*	KU729257	Germany	Behrens-Chapuis,S. et al. 2016
COI	*Phoxinus phoxinus*	KU729258	Germany	Behrens-Chapuis,S. et al. 2016
COI	*Phoxinus phoxinus*	KM287014	Germany	Knebelsberger,T. et al. 2015
COI	*Phoxinus phoxinus*	KM286877	Germany	Knebelsberger,T. et al. 2015
COI	*Phoxinus phoxinus*	KM286879	Germany	Knebelsberger,T. et al. 2015
COI	*Phoxinus phoxinus*	KM286880	Germany	Knebelsberger,T. et al. 2015
COI	*Phoxinus phoxinus*	KM286881	Germany	Knebelsberger,T. et al. 2015
COI	*Phoxinus* sp.	MN816042	Germany	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816043	Germany	Palandacic,A. et al. 2020
COI	*Phoxinus lumaireul*	MG806860	Greece	Schonhuth,S. et al. 2018
COI	*Phoxinus strymonicus*	KJ554101	Greece	Geiger,M.F. et al. 2014
COI	*Phoxinus strymonicus*	KJ554135	Greece	Geiger,M.F. et al. 2014
COI	*Phoxinus strymonicus*	KJ554301	Greece	Geiger,M.F. et al. 2014
COI	*Phoxinus strymonicus*	KJ554359	Greece	Geiger,M.F. et al. 2014
COI	*Phoxinus phoxinus*	KU729256	Ireland	Behrens-Chapuis,S. et al. 2016
COI	*Phoxinus lumaireul*	MT385939	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MT385940	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984796	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984797	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984798	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984799	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984800	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984801	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984802	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984803	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984804	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984805	Italy	De Santis,V. et al 2021
COI	*Phoxinus lumaireul*	MK984806	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984810	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984811	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984812	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984813	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984814	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984815	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984816	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984817	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984818	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984819	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984820	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984821	Italy	De Santis,V. et al 2021
COI	*Phoxinus septimaniae*	MK984822	Italy	De Santis,V. et al 2021
COI	*Phoxinus karsticus*	MF407746	Montenegro	Palandacic,A. et al. 2017
COI	*Phoxinus karsticus*	MF407761	Montenegro	Palandacic,A. et al. 2017
COI	*Phoxinus karsticus*	MF407762	Montenegro	Palandacic,A. et al. 2017
COI	*Phoxinus karsticus*	MG806859	Montenegro	Schonhuth et al. 2018

COI	*Phoxinus* sp.	MF407703	Montenegro	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407705	Montenegro	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407706	Montenegro	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407704	Montenegro	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407767	North Macedonia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407766	North Macedonia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407765	North Macedonia	Palandacic,A. et al. 2017
COI	*Phoxinus marsilii*	MN816129	Poland	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816130	Poland	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816139	Romania	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816138	Romania	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816140	Romania	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816141	Romania	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816142	Romania	Palandacic,A. et al. 2020
COI	*Phoxinus phoxinus*	MG806858	Russia	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816062	Russia	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816063	Russia	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816065	Russia	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816066	Russia	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816084	Russia	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MF407722	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus csikii*	MF407738	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus csikii*	MF407724	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus csikii*	MF407728	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus csikii*	MN816109	Serbia	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MN816110	Serbia	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MF407942	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407943	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407945	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407947	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407950	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407952	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407953	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407954	Serbia	Palandacic,A. et al. 2017
COI	*Phoxinus lumaireul*	MF407788	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus lumaireul*	MF407787	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus lumaireul*	MF407789	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus lumaireul*	MF407790	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus lumaireul*	MN816085	Slovenia	Palandacic,A. et al. 2020
COI	*Phoxinus lumaireul*	MN816086	Slovenia	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MF407886	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407887	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407888	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407889	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407908	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407909	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus* sp.	MF407910	Slovenia	Palandacic,A. et al. 2017
COI	*Phoxinus bigerri*	MN816044	Spain	Palandacic,A. et al. 2020
COI	*Phoxinus bigerri*	MN816047	Spain	Palandacic,A. et al. 2020
COI	*Phoxinus bigerri*	MN816048	Spain	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MN816115	Switzerland	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MN816116	Switzerland	Palandacic,A. et al. 2020
COI	*Phoxinus csikii*	MN816117	Switzerland	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816136	Ukraine	Palandacic,A. et al. 2020

COI	*Phoxinus marsilii*	MN816134	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816135	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818259	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818260	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818261	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818262	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818263	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818264	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818265	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN818266	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus marsilii*	MN816137	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816073	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816071	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816072	Ukraine	Palandacic,A. et al. 2020
COI	*Phoxinus* sp.	MN816070	Ukraine	Palandacic,A. et al. 2020

1.2. **TABLE S1-2. Accession codes for the phylogenetic tree collected from Genebank for Cytb.**

The table represents the gene (Cytb) used for generating phylogenetic tree of genus *Phoxinus*, the species identification provided by Genebank, as well as the country of origin, unique identifier, and source of the sample.

Gene	*Species Genbank*	Accesion number	Country	Literature
Cytb	*Phoxinus csikii*	MN820801	Austria	Palandacic,A., Kruckenhauser,L. et al. 2020
Cytb	*Phoxinus csikii*	MN820805	Austria	Palandacic,A., Kruckenhauser,L. et al. 2020
Cytb	*Phoxinus csikii*	MN820814	Austria	Palandacic,A., Kruckenhauser,L. et al. 2020
Cytb	*Phoxinus csikii*	MN820815	Austria	Palandacic,A., Kruckenhauser,L. et al. 2020
Cytb	*Phoxinus csikii*	MN820817	Austria	Palandacic,A., Kruckenhauser,L. et al. 2020
Cytb	*Phoxinus csikii*	MN820818	Austria	Palandacic,A., Kruckenhauser,L. et al. 2020
Cytb	*Phoxinus csikii*	MN820819	Austria	Palandacic,A., Kruckenhauser,L. et al. 2020
Cytb	*Phoxinus* sp.	KX673469	Austria	Ramler,D., Palandacic,A. et al. 2016
Cytb	*Phoxinus* sp.	KX673470	Austria	Ramler,D., Palandacic,A. et al. 2016
Cytb	*Phoxinus* sp.	KX673471	Austria	Ramler,D., Palandacic,A. et al. 2016
Cytb	*Phoxinus lumaireul*	HM560123	Bosnia and Herzegovina	Perea,S., Bohme,M. et al. 2010
Cytb	*Phoxinus* sp.	KT166574	Bosnia and Herzegovina	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166575	Bosnia and Herzegovina	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166576	Bosnia and Herzegovina	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166591	Bosnia and Herzegovina	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus lumaireul*	MG681338	Croatia	Vucic,M., Jelic,D. et al. 2018
Cytb	*Phoxinus lumaireul*	MG681360	Croatia	Vucic,M., Jelic,D. et al. 2018
Cytb	*Phoxinus lumaireul*	MG681381	Croatia	Vucic,M., Jelic,D. et al. 2018
Cytb	*Phoxinus lumaireul*	MG681401	Croatia	Vucic,M., Jelic,D. et al. 2018
Cytb	*Phoxinus lumaireul*	MG681402	Croatia	Vucic,M., Jelic,D. et al. 2018
Cytb	*Phoxinus lumaireul*	MG681487	Croatia	Vucic,M., Jelic,D. et al. 2018
Cytb	*Phoxinus lumaireul*	MG681503	Croatia	Vucic,M., Jelic,D. et al. 2018
Cytb	*Phoxinus* sp.	KT166543	Croatia	Palandacic,A., Bravnicar,J. et al. 2015

Cytb	*Phoxinus* sp.	KT166544	Croatia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166545	Croatia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166546	Croatia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166547	Croatia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166548	Croatia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166722	Croatia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166723	Croatia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166724	Croatia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus .phoxinus*	Y10448	France	Briolay,J., Galtier,N. et al. 1998
Cytb	*Phoxinus septimaniae*	MK787817	France	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787818	France	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MG806684	France	Schonhuth,S., Vukic,J. et al. 2018
Cytb	*Phoxinus* sp.	MK787813	France	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787814	France	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787815	France	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787816	France	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MF408211	Germany	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus* sp.	MF408215	Germany	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus* sp.	MF408216	Germany	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus* sp.	MF408217	Germany	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus marsilii*	MF408191	Hungary	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus marsilii*	MF408192	Hungary	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus septimaniae*	MF408210	Italy	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus septimaniae*	MF408213	Italy	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus septimaniae*	MF408214	Italy	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus* sp.	KT166616	Kosovo	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166617	Kosovo	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166678	Macedonia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166679	Macedonia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166680	Macedonia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166691	Montenegro	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166692	Montenegro	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166693	Montenegro	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166808	Montenegro	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166809	Montenegro	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166810	Montenegro	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus marsilii*	MF408190	Poland	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus* sp.	KT166613	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166762	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166763	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166764	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166767	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166768	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166769	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166790	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166791	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166792	Serbia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166595	Slovenia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166596	Slovenia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166597	Slovenia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166627	Slovenia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166628	Slovenia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus* sp.	KT166629	Slovenia	Palandacic,A., Bravnicar,J. et al. 2015
Cytb	*Phoxinus bigerri*	MK787469	Spain	Corral-Lou,A., Perea,S. et al. 2019

Cytb	*Phoxinus bigerri*	MK787479	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787480	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787484	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787486	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787490	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787491	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787496	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787500	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787501	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787506	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787508	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787509	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787511	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787513	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787516	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787518	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787519	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787521	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787523	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787524	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787525	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787526	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787531	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787538	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787540	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787542	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787543	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787545	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787547	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787548	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787549	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787550	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787551	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787552	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787553	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787554	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787555	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787556	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787557	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787558	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787559	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787560	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787561	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787562	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787563	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787564	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787565	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787566	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787567	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787568	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787569	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787573	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787574	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787585	Spain	Corral-Lou,A., Perea,S. et al. 2019

Cytb	*Phoxinus bigerri*	MK787588	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787619	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787622	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787627	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787632	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787639	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787641	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787651	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	MK787667	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus bigerri*	HM560122	Spain	Perea,S., Bohme,M. et al. 2010
Cytb	*Phoxinus septimaniae*	MK787595	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787680	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787684	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787689	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787703	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787705	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787708	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787709	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787710	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787714	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787715	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787720	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787722	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787727	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787728	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787730	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787731	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787732	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787735	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787736	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787737	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787738	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787741	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787742	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787744	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787753	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787754	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787755	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787756	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787757	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787758	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787759	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787760	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787761	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787762	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787765	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787766	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787767	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787769	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787770	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787772	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787773	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787774	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787775	Spain	Corral-Lou,A., Perea,S. et al. 2019

Cytb	*Phoxinus septimaniae*	MK787776	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787777	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787778	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787779	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787781	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787782	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787783	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787784	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787785	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787787	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787796	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787797	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787798	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787799	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787800	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787801	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787802	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787803	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787804	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787805	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787806	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787807	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787808	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787809	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787810	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787811	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787812	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787826	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787827	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787828	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787829	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787830	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787831	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787832	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787833	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787834	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787835	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787836	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MK787837	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787668	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787669	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787670	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787671	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787672	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787673	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787674	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787675	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787676	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787677	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787678	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787679	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787681	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787682	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787683	Spain	Corral-Lou,A., Perea,S. et al. 2019

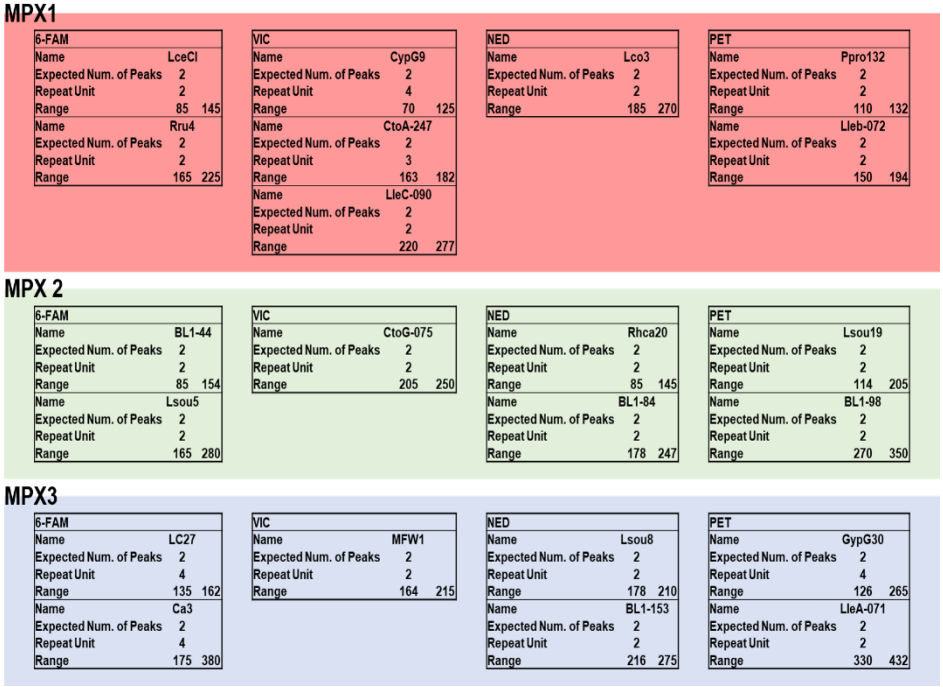
Cytb	*Phoxinus* sp.	MK787685	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787686	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787687	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787688	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787690	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787691	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787692	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787693	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787694	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787695	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787696	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787697	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787698	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787699	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787700	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787701	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787702	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787704	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787706	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787707	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787711	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787712	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787713	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787716	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787717	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787718	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787719	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787721	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787723	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787724	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787725	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787726	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787729	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787733	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787734	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787739	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787740	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787743	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787745	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787746	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787747	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787748	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787749	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787750	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787751	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787752	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787763	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787764	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787768	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787771	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787780	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787786	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787788	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787789	Spain	Corral-Lou,A., Perea,S. et al. 2019

Cytb	*Phoxinus* sp.	MK787790	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787791	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787792	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787793	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787794	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787795	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787819	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787820	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787821	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787822	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787823	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787824	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus* sp.	MK787825	Spain	Corral-Lou,A., Perea,S. et al. 2019
Cytb	*Phoxinus septimaniae*	MF408209	Switzerland	Palandacic,A., Naseka,A. et al. 2017
Cytb	*Phoxinus septimaniae*	MF408212	Switzerland	Palandacic,A., Naseka,A. et al. 2017

Chapter 2

1.3. **Figure S2-1. Information about multiplex of microsatellite loci.**

The figure of three optimized multiplex including 22 microsatellites (Grenier et al., 2013).



Chapter 3

1.4. TABLE S3-1. Accession codes for the haplotypes collected from Genebank for COI and RAG-1.

The table indicates the gene (COI or RAG-1), the loach group as used in this work (*Barbatula*, *Cobitis* or *Misgurnus*/*Pamisgurnus* loaches), the species identification provided by Genebank and the country of origin of the sample

Gene	Group	Species Genbank	Accesion number	Country
COI	Barbatula	*Barbatula barbatula*	KT716378	China
COI	Barbatula	*Barbatula barbatula*	HQ960701	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960702	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960703	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960704	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960856	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960857	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960858	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960859	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960860	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960923	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960924	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960927	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960936	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960954	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960955	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ960968	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ961012	Czech Republic
COI	Barbatula	*Barbatula barbatula*	HQ961013	Czech Republic
COI	Barbatula	*Barbatula barbatula*	MF172067	Estonia
COI	Barbatula	*Barbatula barbatula*	MF172068	Estonia
COI	Barbatula	*Barbatula barbatula*	MF172062	Finland
COI	Barbatula	*Barbatula barbatula*	MF172070	Finland
COI	Barbatula	*Barbatula barbatula*	KP715096	France
COI	Barbatula	*Barbatula barbatula*	MF458555	France
COI	Barbatula	*Barbatula barbatula*	MF458556	France
COI	Barbatula	*Barbatula barbatula*	MF458557	France
COI	Barbatula	*Barbatula barbatula*	MF458558	France
COI	Barbatula	*Barbatula barbatula*	NC_027192	France
COI	Barbatula	*Barbatula barbatula*	HM392023	Germany
COI	Barbatula	*Barbatula barbatula*	KM286463	Germany
COI	Barbatula	*Barbatula barbatula*	KM286464	Germany
COI	Barbatula	*Barbatula barbatula*	KM286465	Germany
COI	Barbatula	*Barbatula barbatula*	KM286466	Germany
COI	Barbatula	*Barbatula barbatula*	KM286467	Germany
COI	Barbatula	*Barbatula barbatula*	KM286468	Germany
COI	Barbatula	*Barbatula barbatula*	KM286469	Germany
COI	Barbatula	*Barbatula barbatula*	KM286470	Germany
COI	Barbatula	*Barbatula barbatula*	KM286471	Germany

COI	Barbatula	*Barbatula barbatula*	KM286472	Germany
COI	Barbatula	*Barbatula barbatula*	KM286473	Germany
COI	Barbatula	*Barbatula barbatula*	KM286474	Germany
COI	Barbatula	*Barbatula barbatula*	KM286475	Germany
COI	Barbatula	*Barbatula barbatula*	KM286476	Germany
COI	Barbatula	*Barbatula barbatula*	KM286477	Germany
COI	Barbatula	*Barbatula barbatula*	KM286478	Germany
COI	Barbatula	*Barbatula barbatula*	KM286479	Germany
COI	Barbatula	*Barbatula barbatula*	KM286480	Germany
COI	Barbatula	*Barbatula barbatula*	KM286481	Germany
COI	Barbatula	*Barbatula barbatula*	KM286482	Germany
COI	Barbatula	*Barbatula barbatula*	KM286483	Germany
COI	Barbatula	*Barbatula barbatula*	KM286484	Germany
COI	Barbatula	*Barbatula barbatula*	KM286485	Germany
COI	Barbatula	*Barbatula barbatula*	KM286486	Germany
COI	Barbatula	*Barbatula barbatula*	KM286487	Germany
COI	Barbatula	*Barbatula barbatula*	KM373639	Germany
COI	Barbatula	*Barbatula barbatula*	KM373640	Germany
COI	Barbatula	*Barbatula barbatula*	KM373656	Germany
COI	Barbatula	*Barbatula barbatula*	KM373665	Germany
COI	Barbatula	*Barbatula barbatula*	KM373672	Germany
COI	Barbatula	*Barbatula barbatula*	KM373673	Germany
COI	Barbatula	*Barbatula barbatula*	KM373684	Germany
COI	Barbatula	*Barbatula barbatula*	KJ128426	Sweden
COI	Barbatula	*Barbatula barbatula*	KJ128427	Sweden
COI	Barbatula	*Barbatula barbatula*	MF158258	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172053	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172054	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172055	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172056	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172057	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172058	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172059	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172060	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172061	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172063	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172064	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172065	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172069	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172071	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172072	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172073	Sweden
COI	Barbatula	*Barbatula barbatula*	MF172074	Sweden
COI	Barbatula	*Barbatula hispanica*	MZ087707	France
COI	Barbatula	*Barbatula hispanica*	MZ087708	France
COI	Barbatula	*Barbatula hispanica*	MZ087709	France
COI	Barbatula	*Barbatula hispanica*	MZ087710	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087711	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087712	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087713	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087714	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087715	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087716	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087717	Spain

COI	Barbatula	*Barbatula hispanica*	MZ087718	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087719	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087720	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087721	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087722	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087723	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087724	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087725	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087726	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087727	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087728	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087729	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087730	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087731	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087732	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087733	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087734	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087735	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087736	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087737	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087738	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087739	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087740	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087741	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087742	Spain
COI	Barbatula	*Barbatula hispanica*	MZ087743	Spain
COI	Barbatula	*Barbatula leoparda*	MK518369	France
COI	Barbatula	*Barbatula leoparda*	MZ189976	France
COI	Barbatula	*Barbatula nuda*	KT716379	China
COI	Barbatula	*Barbatula nuda*	KT716380	China
COI	Barbatula	*Barbatula quignardi*	KJ552895	France
COI	Barbatula	*Barbatula quignardi*	KJ552969	France
COI	Barbatula	*Barbatula quignardi*	KJ553021	France
COI	Barbatula	*Barbatula quignardi*	KJ553139	France
COI	Barbatula	*Barbatula quignardi*	KJ553150	France
COI	Barbatula	*Barbatula quignardi*	KJ553233	France
COI	Barbatula	*Barbatula quignardi*	KJ553263	France
COI	Barbatula	*Barbatula quignardi*	KJ553271	France
COI	Barbatula	*Barbatula quignardi*	MK518367	France
COI	Barbatula	*Barbatula quignardi*	MK518368	France
COI	Barbatula	*Barbatula sturanyi*	KJ552812	Albania
COI	Barbatula	*Barbatula sturanyi*	KJ552885	Albania
COI	Barbatula	*Barbatula sturanyi*	KJ553273	Greece
COI	Barbatula	*Barbatula sturanyi*	KJ552772	Macedonia
COI	Barbatula	*Barbatula sturanyi*	KJ552811	Macedonia
COI	Barbatula	*Barbatula sturanyi*	KJ552861	Macedonia
COI	Barbatula	*Barbatula sturanyi*	KJ552947	Macedonia
COI	Barbatula	*Barbatula sturanyi*	KJ553097	Macedonia
COI	Barbatula	*Barbatula sturanyi*	KJ553120	Macedonia
COI	Barbatula	*Barbatula toni*	HQ536283	Korea
COI	Barbatula	*Barbatula toni*	HQ536284	Korea
COI	Barbatula	*Barbatula toni*	HQ536285	Korea
COI	Barbatula	*Barbatula toni*	HQ536286	Korea
COI	Barbatula	*Barbatula toni*	HQ536287	Korea

COI	Barbatula	*Barbatula toni*	HQ536288	Korea
COI	Barbatula	*Barbatula toni*	HQ536289	Korea
COI	Barbatula	*Barbatula toni*	KM261769	North Korea
COI	Barbatula	*Barbatula toni*	KT247665	Russia
COI	Barbatula	*Barbatula toni*	KX039652	Russia
COI	Barbatula	*Barbatula toni*	KX039657	Russia
COI	Barbatula	*Barbatula toni*	KX039660	Russia
COI	Barbatula	*Barbatula toni*	MN605509	Russia
COI	Barbatula	*Barbatula toni*	MK560751	South Korea
COI	Barbatula	*Barbatula vardarensis*	KJ552857	Greece
COI	Barbatula	*Barbatula vardarensis*	KJ553163	Greece
COI	Barbatula	*Barbatula vardarensis*	KJ553241	Greece
COI	Barbatula	*Barbatula vardarensis*	KJ552836	Macedonia
COI	Barbatula	*Barbatula vardarensis*	KJ553068	Macedonia
COI	Cobitis	*Cobitis bilineata*	KJ553018	France
COI	Cobitis	*Cobitis bilineata*	KJ552762	Italy
COI	Cobitis	*Cobitis bilineata*	KJ552804	Italy
COI	Cobitis	*Cobitis bilineata*	KJ552893	Italy
COI	Cobitis	*Cobitis bilineata*	KJ553166	Italy
COI	Cobitis	*Cobitis bilineata*	KJ553176	Italy
COI	Cobitis	*Cobitis bilineata*	KJ553211	Italy
COI	Cobitis	*Cobitis bilineata*	KJ553227	Italy
COI	Cobitis	*Cobitis biwae*	AP011344	Japan
COI	Cobitis	*Cobitis calderoni*	KJ553101	Spain
COI	Cobitis	*Cobitis calderoni*	KJ553130	Spain
COI	Cobitis	*Cobitis calderoni*	KJ553149	Spain
COI	Cobitis	*Cobitis calderoni*	KJ553275	Spain
COI	Cobitis	*Cobitis dalmatina*	KJ553131	Croatia
COI	Cobitis	*Cobitis granoei*	KF908768	China
COI	Cobitis	*Cobitis granoei*	NC_023473	China
COI	Cobitis	*Cobitis illyrica*	KJ552766	Bosnia and Herzegovina
COI	Cobitis	*Cobitis illyrica*	KJ552890	Bosnia and Herzegovina
COI	Cobitis	*Cobitis illyrica*	KJ552942	Bosnia and Herzegovina
COI	Cobitis	*Cobitis illyrica*	KJ552992	Bosnia and Herzegovina
COI	Cobitis	*Cobitis illyrica*	KJ553256	Bosnia and Herzegovina
COI	Cobitis	*Cobitis jadovaensis*	KJ552968	Croatia
COI	Cobitis	*Cobitis jadovaensis*	KJ553096	Croatia
COI	Cobitis	*Cobitis lutheri*	HQ536324	
COI	Cobitis	*Cobitis lutheri*	HQ536325	
COI	Cobitis	*Cobitis lutheri*	HQ536326	
COI	Cobitis	*Cobitis maroccana*	KJ553105	Morocco
COI	Cobitis	*Cobitis maroccana*	KJ553110	Morocco
COI	Cobitis	*Cobitis maroccana*	KJ553155	Morocco
COI	Cobitis	*Cobitis minamorii minamorii*	AP013309	
COI	Cobitis	*Cobitis minamorii tokaiensis*	AP013305	
COI	Cobitis	*Cobitis narentana*	KJ552840	Bosnia and Herzegovina
COI	Cobitis	*Cobitis narentana*	KJ552915	Bosnia and Herzegovina
COI	Cobitis	*Cobitis narentana*	KJ553012	Bosnia and Herzegovina
COI	Cobitis	*Cobitis narentana*	KJ553072	Bosnia and Herzegovina
COI	Cobitis	*Cobitis ohridana*	KJ552794	Albania
COI	Cobitis	*Cobitis ohridana*	KJ552816	Albania
COI	Cobitis	*Cobitis ohridana*	KJ553005	Albania
COI	Cobitis	*Cobitis paludica*	KJ552855	Spain

COI	Cobitis	*Cobitis paludica*	KJ553196	Spain
COI	Cobitis	*Cobitis paludica*	KJ553225	Spain
COI	Cobitis	*Cobitis punctilineata*	KJ552769	Greece
COI	Cobitis	*Cobitis punctilineata*	KJ552981	Greece
COI	Cobitis	*Cobitis punctilineata*	KJ552998	Greece
COI	Cobitis	*Cobitis punctilineata*	KJ553028	Greece
COI	Cobitis	*Cobitis punctilineata*	KJ553153	Greece
COI	Cobitis	*Cobitis punctilineata*	KJ553247	Greece
COI	Cobitis	*Cobitis* sp.	KJ552870	Turkey
COI	Cobitis	*Cobitis* sp.	KJ552978	Turkey
COI	Cobitis	*Cobitis* sp.	KJ553029	Turkey
COI	Cobitis	*Cobitis* sp.	KJ553126	Turkey
COI	Cobitis	*Cobitis* sp.	KJ553164	Turkey
COI	Cobitis	*Cobitis* sp.	AP013307	
COI	Cobitis	*Cobitis striata striata*	AP013311	
COI	Cobitis	*Cobitis strumicae*	KJ553048	Greece
COI	Cobitis	*Cobitis strumicae*	KJ553143	Greece
COI	Cobitis	*Cobitis tetralineata*	MN206506	
COI	Cobitis	*Cobitis tetralineata*	MN206507	
COI	Cobitis	*Cobitis tetralineata*	MN206508	
COI	Cobitis	*Cobitis vettonica*	KJ553016	Spain
COI	Cobitis	*Cobitis vettonica*	KJ553226	Spain
COI	Cobitis	*Cobitis vettonica*	KJ553242	Spain
COI	Cobitis	*Cobitis vettonica*	KJ553300	Spain
COI	Cobitis	*Cobitis zanandreai*	KJ552927	Italy
COI	Cobitis	*Cobitis zanandreai*	KJ553001	Italy
COI	Cobitis	*Cobitis zanandreai*	KJ553015	Italy
COI	Cobitis	*Cobitis zanandreai*	KJ553180	Italy
COI	Cobitis	*Cobitis zanandreai*	KJ553193	Italy
COI	Cobitis	*Cobitis zanandreai*	KJ553270	Italy
COI	Misgurnus & Paramisgurnus	*M. anguillicaudatus x M. bipartitus*	NC_043847	China
COI	Misgurnus & Paramisgurnus	*M. anguillicaudatus x P. dabryanus*	MG735453	China
COI	Misgurnus & Paramisgurnus	*M. anguillicaudatus x P. dabryanus*	MG938589	China
COI	Misgurnus & Paramisgurnus	*M. anguillicaudatus x P. dabryanus*	MK714038	China
COI	Misgurnus & Paramisgurnus	*M. anguillicaudatus x P. dabryanus*	KJ939363	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KJ669523	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KJ669524	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KX224162	Canada
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KX224170	Canada
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KX224173	Canada
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	HM446336	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	HM446337	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	HM446338	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	JN177217	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KC509900	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KC734881	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KC762740	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KC823274	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KC881110	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KC884745	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610758	China

COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610759	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610760	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610761	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610762	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610763	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610764	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610765	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610766	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610767	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610768	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610769	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610770	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610771	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610772	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610773	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM610774	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KP112318	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KP112319	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KP112320	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KP112321	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KP112322	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MF122497	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MF122498	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MF122499	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MF122500	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MF122501	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MF122502	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MG938590	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MK093946	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN116750	China
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KX865082	Germany
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KX865083	Germany
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KX865084	Germany
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KJ553659	Italy
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KJ553755	Italy
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KJ553886	Italy
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MF579257	South Korea
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	JN027250	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KY780064	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KY780065	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KY780066	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KY780067	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KY780068	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KY780069	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MW856898	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	AP011291	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	DQ026434	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	EU670785	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KF732665	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM186181	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	LC532166	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	LC532167	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	LC532168	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN127923	

COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN127924	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN127925	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN127926	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN127927	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN709578	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN709579	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN709580	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN709581	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN709582	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN913478	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN913479	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MN913480	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MT413318	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MT413319	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MT413320	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MT413321	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MT667249	
COI	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	MT896815	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KF562047	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505218	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505219	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505220	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505221	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505222	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505223	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505224	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505225	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505226	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505227	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505228	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505229	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505230	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505231	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505232	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505233	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505234	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505235	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505236	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505237	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505238	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505239	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505240	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505241	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505242	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505243	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505244	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505245	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505246	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505247	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505248	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505249	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505250	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505251	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505252	China

COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505253	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505254	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505255	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505256	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505257	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505258	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505259	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505260	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505261	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505262	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505263	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505264	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505265	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505266	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505267	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505268	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505269	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505270	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505271	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505272	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505273	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	KX505274	China
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127929	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127930	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127931	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127932	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127933	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127934	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127935	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127936	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN127937	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN709583	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN709584	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN709585	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN709586	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MN709587	
COI	Misgurnus & Paramisgurnus	*Misgurnus bipartitus*	MT413323	
COI	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	KM286762	Germany
COI	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	KM286763	Germany
COI	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	KM286764	Germany
COI	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	KM286765	Germany
COI	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	JQ011427	Slovakia
COI	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	JQ011436	Slovakia
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	AP017654	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	FJ197700	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	KF732664	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709513	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709514	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709517	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709519	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709520	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709521	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709523	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709524	

COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709527	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709528	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709533	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709535	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709536	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709541	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709542	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709545	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709546	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709547	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709548	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709549	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709551	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709552	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709553	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709554	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709555	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709556	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709557	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709558	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709560	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709561	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709562	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709563	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709564	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709565	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	MN709566	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	NC_038151	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	OL674449	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	OL674450	
COI	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	OL674451	
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KF386025	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505167	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505168	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505169	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505170	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505171	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505172	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505173	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505174	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505175	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505176	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505177	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505178	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505179	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505180	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505181	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505182	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505183	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505184	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505185	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505186	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505187	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505188	China

COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505189	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505190	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505191	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505192	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505193	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505194	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505195	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505196	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505197	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505198	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505199	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505200	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505201	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505202	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505203	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505204	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505205	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505206	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505207	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505208	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505209	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505210	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505211	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505212	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505213	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505214	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505215	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505216	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	KX505217	China
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	MN127944	
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	MN127945	
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	MN127946	
COI	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	MN127947	
COI	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	AB242171	China
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011416	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011417	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011419	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011422	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011426	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011428	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011434	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011435	Australia
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011423	China
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011424	China
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011425	China
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011418	South Korea
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011420	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011421	USA
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011432	Viet Nam
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011433	Viet Nam
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011437	Viet Nam
COI	Misgurnus & Paramisgurnus	*Misgurnus* sp.	JQ011438	Viet Nam
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	JQ011429	Australia
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	HM446339	China

COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	HM446340	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	HM446341	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	HM446342	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	HM446343	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	JN177218	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KF771003	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KJ027397	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KJ699181	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KM610787	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KM610788	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KM610789	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KM610790	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KM610791	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KM610792	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112350	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112351	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112352	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112353	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112354	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112355	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112356	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112357	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP112358	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KR349175	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122611	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122612	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122613	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122614	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122615	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122616	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122617	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122618	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MF122619	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MG725379	China
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	JQ011430	South Korea
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	JQ011431	South Korea
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	AP012124	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KM186182	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KM186183	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN127938	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN127939	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN127940	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN127941	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN127942	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN127943	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709567	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709568	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709569	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709570	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709571	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709572	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709573	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709574	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709575	

COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709576	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MN709577	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MT413333	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	MT413334	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	OL494211	
COI	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	OL494290	
RAG-1	Barbatula	*Barbatula barbatula*	KP738564	Czech Republic
RAG-1	Barbatula	*Barbatula barbatula*	KP738565	Czech Republic
RAG-1	Barbatula	*Barbatula barbatula*	EU711107	
RAG-1	Barbatula	*Barbatula nuda*	EU930366	Korea
RAG-1	Barbatula	*Barbatula toni*	EU930367	Korea
RAG-1	Barbatula	*Barbatula toni*	EU711133	
RAG-1	Barbatula	*Barbatula toni*	KM818291	
RAG-1	Cobitis	*Cobitis bilineata*	EF672415	Italy
RAG-1	Cobitis	*Cobitis bilineata*	EF672416	Italy
RAG-1	Cobitis	*Cobitis bilineata*	EF672417	Italy
RAG-1	Cobitis	*Cobitis bilineata*	EF672418	Italy
RAG-1	Cobitis	*Cobitis bilineata*	KP161139	Italy
RAG-1	Cobitis	*Cobitis bilineata*	EF056382	Italy
RAG-1	Cobitis	*Cobitis calderoni*	KP161143	Spain
RAG-1	Cobitis	*Cobitis calderoni*	KP161144	Spain
RAG-1	Cobitis	*Cobitis dalmatina*	KP161145	Bosnia and Herzegovina
RAG-1	Cobitis	*Cobitis dalmatina*	EF672419	Croatia
RAG-1	Cobitis	*Cobitis illyrica*	KP161150	Bosnia and Herzegovina
RAG-1	Cobitis	*Cobitis jadovaensis*	KJ487504	Croatia
RAG-1	Cobitis	*Cobitis jadovaensis*	KJ487505	Croatia
RAG-1	Cobitis	*Cobitis lutheri*	EF508613	Republic of Korea
RAG-1	Cobitis	*Cobitis lutheri*	EF508614	Republic of Korea
RAG-1	Cobitis	*Cobitis lutheri*	JN858832	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858834	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858835	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858836	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858837	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858838	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858839	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858840	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858841	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858843	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858844	Russia
RAG-1	Cobitis	*Cobitis lutheri*	JN858845	Russia
RAG-1	Cobitis	*Cobitis maroccana*	KP161154	Morocco
RAG-1	Cobitis	*Cobitis maroccana*	KP161155	Morocco
RAG-1	Cobitis	*Cobitis narentana*	KP161158	Bosnia and Herzegovina
RAG-1	Cobitis	*Cobitis narentana*	KP161159	Bosnia and Herzegovina
RAG-1	Cobitis	*Cobitis narentana*	KP161160	Bosnia and Herzegovina
RAG-1	Cobitis	*Cobitis ohridana*	EF672431	Albania
RAG-1	Cobitis	*Cobitis ohridana*	EF672432	Albania
RAG-1	Cobitis	*Cobitis ohridana*	KP161161	Albania
RAG-1	Cobitis	*Cobitis ohridana*	KT717937	Albania
RAG-1	Cobitis	*Cobitis ohridana*	EF672433	Greece
RAG-1	Cobitis	*Cobitis paludica*	KP161162	Spain
RAG-1	Cobitis	*Cobitis paludica*	KP161163	Spain
RAG-1	Cobitis	*Cobitis punctilineata*	KP161164	Greece
RAG-1	Cobitis	*Cobitis punctilineata*	KP161165	Greece

RAG-1	Cobitis	*Cobitis punctilineata*	KP161166	Greece
RAG-1	Cobitis	*Cobitis strumicae*	MH843034	Turkey
RAG-1	Cobitis	*Cobitis tetralineata*	KP161183	Republic of Korea
RAG-1	Cobitis	*Cobitis tetralineata*	KP161184	Republic of Korea
RAG-1	Cobitis	*Cobitis tetralineata*	KP161185	Republic of Korea
RAG-1	Cobitis	*Cobitis vettonica*	KP161188	Spain
RAG-1	Cobitis	*Cobitis vettonica*	KP161189	Spain
RAG-1	Cobitis	*Cobitis vettonica*	EF056330	Spain
RAG-1	Cobitis	*Cobitis zanandreai*	EF672444	Italy
RAG-1	Cobitis	*Cobitis zanandreai*	EF672445	Italy
RAG-1	Cobitis	*Cobitis zanandreai*	KP161190	Italy
RAG-1	Cobitis	*Cobitis zanandreai*	KP161191	Italy
RAG-1	Cobitis	*Cobitis zanandreai*	KP161192	Italy
RAG-1	Cobitis	*Cobitis zanandreai*	KP161193	Italy
RAG-1	Cobitis	*Cobitis zanandreai*	KP161194	Italy
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	HQ454322	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	HQ454327	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	JN177189	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	AB531306	Japan
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	EF056344	Japan
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	EF508651	Japan
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM818293	Korea
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	KM818303	Korea
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	EU670842	Republic of Korea
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	EF508652	Taiwan
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus anguillicaudatus*	EU711122	
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	EF056339	Poland
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	EF508653	Ukraine
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	EF508656	Ukraine
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	EF508654	
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus fossilis*	EF508655	
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	EU670843	Republic of Korea
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	KM818286	
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mizolepis*	KM818294	
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	HQ454343	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	HQ454344	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	HQ454345	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	HQ454346	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	EF056392	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	JN858807	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	JN858808	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	JN858809	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	JN858810	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus mohoity*	EF508657	
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	AB531312	Japan
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858811	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858812	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858813	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858814	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858815	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858816	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858817	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858818	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858819	Russia

RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858820	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858821	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858822	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858823	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858824	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	JN858825	Russia
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus nikolskyi*	EU711140	
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454328	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454329	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454330	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454331	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454332	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454333	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454334	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454335	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454337	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454338	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454340	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454341	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	HQ454342	China
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	LC530852	Japan
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	EF508659	Republic of Korea
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	EF508660	Republic of Korea
RAG-1	Misgurnus & Paramisgurnus	*Misgurnus* sp.	EF508658	Russia
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	EF508675	China
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	EF508676	China
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	HQ454347	China
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	JN177188	China
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	KP695644	China
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	LC530846	Japan
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	LC530847	Japan
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	LC530848	Japan
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	LC530849	Japan
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	LC530850	Japan
RAG-1	Misgurnus & Paramisgurnus	*Paramisgurnus dabryanus*	LC530851	Japan