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INVASION NOTE

Origin and history of *Phoxinus* (Cyprinidae) introductions in the Douro Basin (Iberian Peninsula): an update inferred from genetic data

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Abstract The number of non-native freshwater fishes in the Iberian Peninsula has been greatly increasing. In this study, individuals of the genus *Phoxinus* were detected in 18 out of 138 stream sites sampled across the Douro Basin in 2017 and 2018. A total of 26 individuals were barcoded using partial cytochrome c oxidase subunit I (COI) and cytochrome b (cytb) genes for species identification and determination of geographical origin. Molecular data provided the first record of a second *Phoxinus* species in western Douro (Portugal, Iberian Peninsula), with haplotypes closely matching those found in the Charente River (southern France). This species is suspected to be a recent introduction associated with the use of minnows as live bait by freshwater anglers, which was facilitated by human movements between France and Portugal. Individuals from watercourses in eastern Douro (Spain) were genetically assigned to *Phoxinus bigerri*, an introduced species previously known for that region, which confirms reports of introduction events from Ebro to Douro Basin probably also related to freshwater angling and facilitated by geographic proximity. The potential ecological

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impacts of this genus in the region are unknown and need further investigation.

Keywords Biological invasions · Cryptic diversity · Freshwater fish · DNA barcoding · Streams

Introduction

Biological invasions have rapidly increased over the past century, constituting a major threat to biodiversity and ecosystem services (Simberloff et al. 2013). Freshwater ecosystems are extensively altered and especially vulnerable to biological invasions (Carpenter et al. 2011). In the Iberian Peninsula, the main river basins currently harbour more non-native than native fish species, with ecological impacts ranging from the individual to ecosystem levels (Clavero 2011). Nevertheless, the number of non-native fishes detected continues to increase, and established species are expanding their ranges (Clavero 2011; Filipe et al. 2013; Anastácio et al. 2019). The early detection of a non-native species followed by the identification of its region of origin, pathways and vectors of dispersal, are crucial for a quick risk assessment and a proper anticipation of the species invasiveness and consequent mitigation of potential impacts (Simberloff et al. 2013). However, such knowledge is often absent, delayed or incomplete (Simberloff et al. 2013). To offset at least some of these problems, molecular methods are increasingly being applied (Kamenova et al. 2017).

The *Phoxinus* genus (minnows) comprise small freshwater fishes of the family Cyprinidae and is an example of cryptic diversity (Palandačić et al. 2017; Corral-Lou et al. 2019; Palandačić et al. 2020). The European minnow *Phoxinus phoxinus* (Linnaeus 1758) is a species complex widely distributed across Eurasia (Kottelat 2007), with occurring introduction events (e.g. in Norway; Museth et al. 2007). Within *Phoxinus* species, phenotypic plasticity is high and dependent upon habitat features, which influences some characters proposed for species delimitation and, consequently, species identification using exclusively morphology remains limited (Ramler et al. 2016). In the Iberian Peninsula, native minnow populations are restricted to Ebro Basin and small Atlantic coastal basins (NE Spain and Andorra). These populations were considered *P. phoxinus* until a systematic revision based on morphological characters differentiated them, together with those located in the Adour Basin (SW France), from the ones occurring in Europe (Kottelat 2007). After this revision, these populations were renamed as the Pyrenean minnow *P. bigerri*. In the beginning of twentieth century, *P. bigerri* was supposedly introduced to the Pedroso River (Douro Basin) and to some northern Iberian basins as prey to trout populations (Doadrio and Garzón 1986). Another introduction event of *P. bigerri* was registered in Galicia (NW Spain; Sánchez-Hernández et al. 2012), although, to our knowledge, the accurate taxonomy and geographical origin of this population remains unknown. Recently, Corral-Lou et al. (2019) explored the genetic structure of *Phoxinus* genus in the Iberian Peninsula, supporting the occurrence of two new species, one likely to be *P. septimaniae* resulting from an introduction from France, and other apparently native and not yet described (lineage 21; Palandačić et al. 2020).

Overall, this study explores the use of molecular tools to identify non-native *Phoxinus* at the species level and detect their geographic origin. Herein, we (1) record the occurrence of a second *Phoxinus* species in the Douro Basin so far unknown, (2) examine the geographic origin(s) of minnow's introduction in the Douro Basin, and (3) infer possible introduction pathway(s) and vector(s).

Methods

Study area and sample collection

The study was conducted in early summer of 2017 and 2018, as part of a survey to characterise freshwater fish communities in the Douro Basin (north Iberian Peninsula; Portugal and Spain). Sampling sites were selected with the aim of covering habitat heterogeneity within the basin, ranging from Mediterranean-type to temperate-type regime streams, and from headwaters to large rivers. From the 150 selected sites, 12 were

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found dry at the time of sampling (Fig. 1). At each site, the freshwater fish community was characterized using a single anode electrofishing gear, and all specimens were morphologically identified to the lowest taxonomic level based on taxonomy and known distributions, measured, weighed, and immediately released in the watercourse. In the case of *Phoxinus,* specimens were identified to the species level based on known species distribution ranges. For individuals which could not be confidently classified according to their geographic origin e.g. due to being out of known distribution ranges, a tissue sample was extracted from a pelvic fin and preserved in 96% ethanol for molecular analyses of mtDNA.

Laboratory processing and sequencing

We analysed tissue samples from 26 *Phoxinus* individuals (up to five samples per site) from six and two sites located in eastern and western Douro, respectively (Fig. 1. See Table 1 and S1 for codes). The DNA was extracted using the Easy Spin® Nucleic Acid Extraction kit (Citomed, Lisbon; Portugal). To identify samples at the species level, we targeted the 5′ region of the cytochrome c oxidase subunit I gene (COI; 652 bp) and the complete cytochrome b gene (cytb; 1140 bp) using the universal primer sets HCO-2198 and LCO-1490 for COI (Folmer et al. 1994), and Gludg-L (Palumbi 1996) and H16460 (Perdices and Doadrio 2001) for cytb. Each 10.5 µL PCR reaction was carried out using 5 µL of QIAGEN Multiplex PCR kit (Qiagen), 0.4 µL of each primer at 10 mM, 3.2 µL ultrapure H_2O, and 1.5 µL template DNA. After an initial preheat cycle at 95 °C for 10 min, 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 53 °C and 45 s extension at 72 °C for COI and 60 s denaturation at 95 °C, 60 s annealing at 54 °C and 90 s extension at 72 °C for cytb were performed,

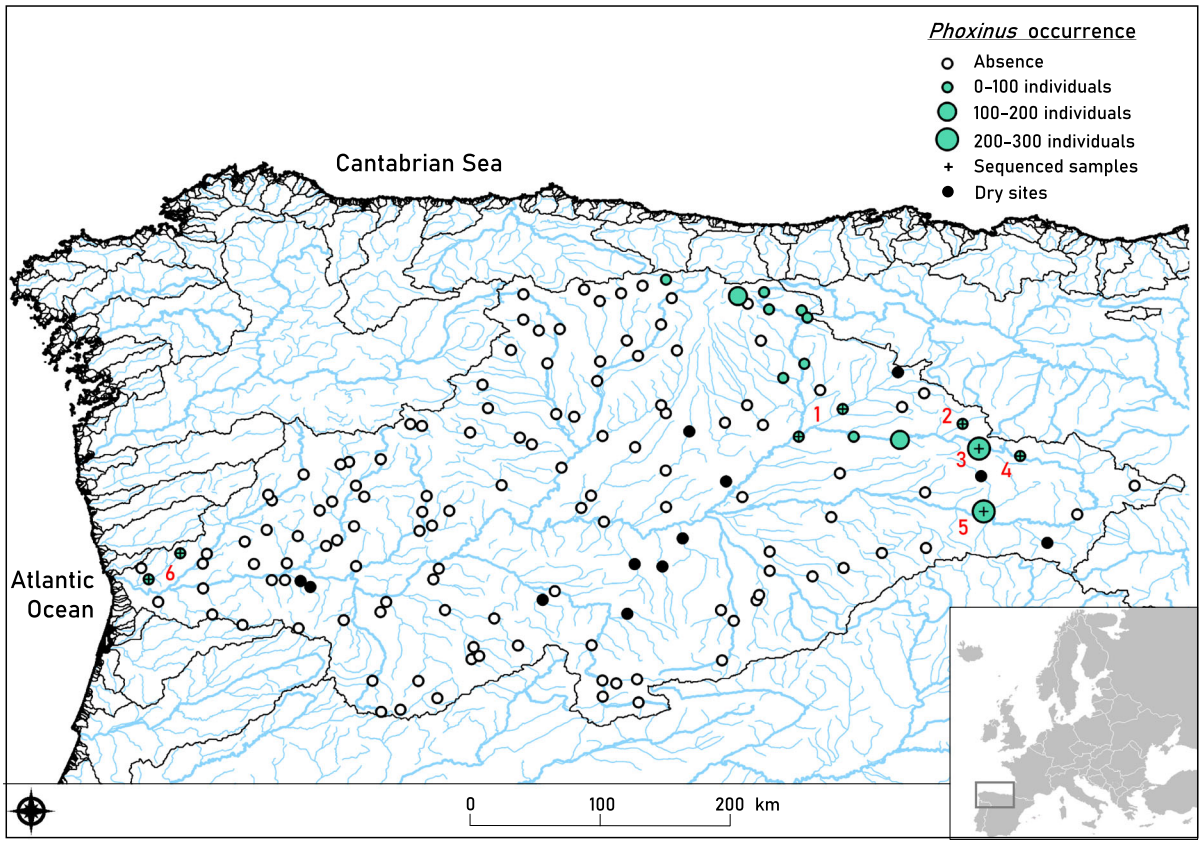

Fig. 1 Location of the 138 sampled sites using electrofishing and the 12 dried sites in the Douro Basin (black circles). Sites where the genus *Phoxinus* was present (green-filled circles) or absent (open circles) are represented. The inner cross indicates sampling sites with molecular data

Table 1 Species identification of the 26 samples of *Phoxinus* sp. collected in the Douro Basin (Portugal and Spain) using the BOLD-IDS (5′-COI region) or NCBI-BLAST (cytb) engines, including percentage of similarity and location of the 'best match' (river and country: SP, Spain; FR, France; PT, Portugal)

Query		COI				cytb			
Sample	Location	Accession no.	Species ID	Location	%	Accession no.	Species ID	Location	%
FIS0056	1, Arlanzon, SP	EUFWF4684-18	*P. bigerri*	Zadorra, SP	99.84	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0096	1, Arlanzon, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0100	1, Arlanzon, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0112	1, Arlanzon, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0113	1, Arlanzon, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0175	2, Pedroso, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0189	2, Pedroso, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0194	2, Pedroso, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.63
FIS0198	2, Pedroso, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.63
FIS0363	3, Arlanza, SP	EUFWF4684-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.53
FIS0365	3, Arlanza, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0370	3, Arlanza, SP	EUFWF4684-18	*P. bigerri*	Zadorra, SP	99.84	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0376	3, Arlanza, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0425	4, Douro, SP	EUFWF4684-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.53
FIS0426	4, Douro, SP	EUFWF4684-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.53
FIS0427	4, Douro, SP	EUFWF4684-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.53
FIS0435	4, Douro, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0556	4, Douro, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0557	5, Ucero, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.63
FIS0569	5, Ucero, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS0578	5, Ucero, SP	EUFWF4685-18	*P. bigerri*	Zadorra, SP	100	MG681515	*P. bigerri*	Adour, FR	98.62
FIS1709	6, Sousa, PT	GBMIN133349-17	*P. phoxinus*	Charente, FR	99.84	EF094550	*P. phoxinus*	n.a.	92.46
FIS1731	6, Sousa, PT	GBMIN133349-17	*P. phoxinus*	Charente, FR	99.84	EF094550	*P. phoxinus*	n.a.	92.46
FIS1851	6, Sousa, PT	GBMIN133349-17	*P. phoxinus*	Charente, FR	99.84	EF094550	*P. phoxinus*	n.a.	92.46
FIS1863	6, Sousa, PT	GBMIN133349-17	*P. phoxinus*	Charente, FR	100	EF094550	*P. phoxinus*	n.a.	92.46
FIS1880	6, Sousa, PT	GBMIN133349-17	*P. phoxinus*	Charente, FR	100	EF094550	*P. phoxinus*	n.a.	92.46

Numbers in query sample correspond to locations in Fig. 1

followed by a final extension step at 72 °C for 10 min in both genes. A negative control containing no template DNA was also included in each PCR reaction. The PCR products were visualized on a 2% agarose gel under UV light, and purified using ExoSAP-IT® PCR clean-up Kit (GE Healthcare, Piscataway, NJ, USA). Samples were bidirectionally sequenced with the same primers, to verify sequence consistency and quality, using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3130 × l Genetic Analyser (Applied Biosystems). Both forward and reverse sequences were assembled against a reference, edited and aligned using Geneious R8 (Biomatters Ltd., Auckland, New Zealand), and translated to detect the existence of stop codons. Sequences were further deposited in BOLD (Barcode of Life Database) and NCBI (National Center for Biotechnology Information) databases.

Species identification, phylogenetic grouping and haplotype networks

For species identification, the sequences obtained were matched with published sequences from reference libraries. We used BOLD-IDS (Tree Based Identification; Ratnasingham and Hebert 2007) and NCBI BLAST (Johnson et al. 2008) to search for the

most similar sequences to identify the species and the possible geographical origin of the individuals.

Due to the phylogenetic complexity of this genus, we used phylogenetic tree inference (FastTree; Price et al. 2009) and network estimation (TCS; Clement et al. 2000) to assess the distinct genetic groups and retrieve existing sequences similar to our samples. The FastTree software infers approximately-maximum-likelihood phylogenetic trees, and was applied using the generalized time-reversible (GTR) model of evolution, with *Luciobarbus bocagei* (NCBI accession no. KJ553688 for COI and AY004727 for cytb) as outgroup. TCS was also used to (1) determine the number of genetic groups (at 95% connection limit) from all *Phoxinus* sequences, and (2) to retrieve parsimony-based haplotype networks for the genetic groups in which our samples assembled (at 95% connection limit) and define subgroups (at 99% connection limit), in order to illustrate the haplotype structure and distribution within the study area. Prior to analyses, we created two sequence alignments in Geneious (Drummond et al. 2010), one for each gene (652-bp COI and 1140-bp cytb), combining the sequences produced in this study and others retrieved from BOLD and NCBI searches (Table S1). The 115 COI sequences included in the phylogenetic tree analyses were selected based on BOLD-IDS tree results and including sequences from Iberian Peninsula retrieved from GenBank not available in the BOLD database (Table S1a). For TCS analysis, new sequences published by Palandačić et al. (2020) were also included, totalizing 131 sequences (Table S1b). For cytb, the first 100 blast matches with higher similarity scores to each of our sequences in NCBI database were selected to build the phylogenetic tree (Table S1c). Network estimation combined those with the 371 sequences from Corral-Lou et al. (2019), totalizing 549 sequences (Table S1d). Given that sequences from Corral-Lou et al. (2019) were shorter, cytb alignment was trimmed to 1089 bp prior to network analysis for this gene (see Table S1d). The possible deficit of molecular studies and the lack of morphological identification leads to incongruences in species nomenclature between public databases (namely from NCBI or BOLD engines) and recent literature (i.e. Palandačić et al. 2017, 2020; Corral-Lou et al. 2019). Thus, recognizing that public databases can be outdated, we followed the nomenclature from the recent literature. Anyway, we present both in Table S1 for clarification. Given this situation, the potential unconformity of species identification is addressed in the discussion.

Results

During fieldwork, 966 individuals identified as *Phoxinus* were captured at two sampling sites in Portugal ($N = 17$; Sousa River, west Douro Basin) and at sixteen sites in Spain ($N = 949$; Arlanzon, Arlanza, Pedroso, Ucero and Douro Rivers, north and eastern Douro Basin) (Fig. 1). *Phoxinus* from Spain were identified in the field as *Phoxinus bigerri* based on its known distribution. However, the individuals from the western part of the basin were registered as *Phoxinus* sp. (Fig. S1) since they were located outside the currently known distribution of the former species.

From the 26 samples collected for molecular analyses of mtDNA, COI gave the highest percentage of identity (Table 1). The minnows found in Spain were molecularly confirmed at the species level as *P. bigerri.* The unidentified *Phoxinus* individuals collected in the Sousa River showed high similarity to sequences entered as *P. phoxinus* in the public databases, but they are henceforth referred to as *Phoxinus* sp. (BOLD:ABV9938) since they were found to be distinct from the haplotypes described for that species (see recent revision in Palandačić et al. 2020). Both phylogenetic trees (Fig. 2, Fig. S2, Fig. S3) and TCS clustering (Table S1b and S1d) supported these results. More specifically, regarding COI, samples collected in eastern Douro belonged to a significantly distinct haplotype group represented by *P. bigerri* (PH4, support value = 1; Fig. 2, see also Table S1a), with high similarity to sequences from Zadorra River in Ebro Basin (99.84–100%; Table 1). Regarding cytb, our sequences from eastern Douro grouped together with *Phoxinus bigerri* sequences from upper Ebro and eastern Cantabria in PH18a (Fig. S3, Table S1c). The COI haplotypes found in Sousa River clustered with *Phoxinus* sp. haplotypes from Charente River in France (99.84–100%; Table 1) into the group PH7 (support value = 1; Fig. 2, Table S1a). No other cytb references clustered with our Portuguese haplotypes in PH12 (Fig. S3, Table S1c).

From the eleven COI haplotypes within the 21 *P. bigerri* samples, we found three haplotypes in Douro


0.79
PH1: Phoxinus morella
Czech Republic, Germany [BOLD:AAC8037]
1
PH2: Phoxinus septimaniae
France [BOLD:AAY8765]
0.97
PH3: Phoxinus phoxinus
Germany [BOLD:ACE5740]
1
PH4: Phoxinus bigerri
Spain, France [BOLD:AAY8807]
0.89
0.72
PH5: Phoxinus sp. Spain [BOLD:ACE6570]
PH6: Phoxinus sp.6 France [BOLD:ACE6570]
1
PH7: Phoxinus sp.
Portugal, France [BOLD:ABV9938]
0.99
PH8: Phoxinus lumaireul,
Phoxinus csikii,
Phoxinus strymonicus,
Phoxinus sp.2,
Phoxinus sp.3
Bosnia and Herzegovina,
Croatia, France, Serbia,
Slovenia [BOLD:AAC8034]
Austria [BOLD:AAC8036]
Greece [BOLD:ACL6823]
Montenegro [BOLD:ADJ6834]
Bosnia and Herzegovina
[BOLD:ADL3298]
Luciobarbus bocagei
0.03

◀**Fig. 2** Phylogenetic tree inferred with FastTree for the genus *Phoxinus* using 5′-COI gene, showing the eight groups supported by TCS analysis (PH1–PH8) and their location. Species designation is presented according to Palandačić et al. (2017, 2020) nomenclature. BOLD Barcode Index Numbers (BINs) are indicated in each sequence cluster. The two species detected in this study are highlighted in blue

(PH4a, PH4c and PH4d), two of which were shared with the Zadorra River population in Ebro (Fig. 3, see also Table S1b). These new haplotypes found in Douro appear as the basal haplotype group for this species (i.e. centred in PH4a). They differed by at least two mutational steps from those found in northern basins (Ugarana River and Estuary of Bilbao; PH4e and PH4f), one from those in southern France (Adour Basin; PH4g, PH4h, PH4j and PH4k), and seven from a distinct haplotype found in Ebro (PH4i). Results found for COI are also supported by cytb network (Fig. 4). Specifically, two cytb haplotypes were shared between *P. bigerri* populations found in eastern Douro and Ebro (PH18a1 and PH18a2). Our samples differed by at least one mutational step from PH18a3 in Cantabria and Ebro, two from PH18a4 and five from PH18a9 both found only in Ebro, and four from PH18a5-8 haplotypes located only in Cantabria (Fig. 4, see also Table S1d).

Regarding the five individuals detected in western Douro (Portugal), we found two distinct COI haplotypes co-occurring in Sousa River that clustered with haplotypes from Charente River in France (Fig. 3, Table S1b). Also, a new haplotype was found in Sousa River (PH7b) that differed by one mutation from one of the haplotypes found in Charente (PH7c). The two

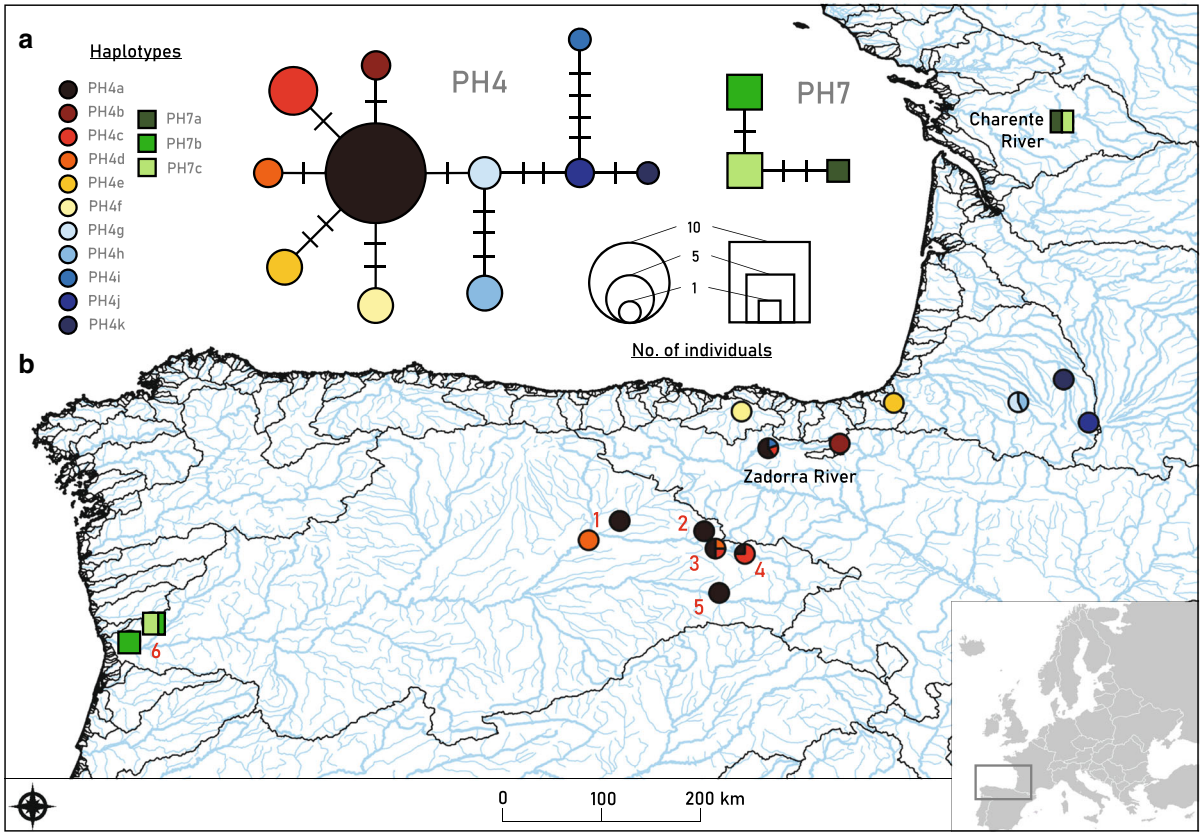

Fig. 3 Phylogeographic analyses for the *Phoxinus bigerri* (PH4) and *Phoxinus* sp. (PH7) using the 5′-COI region (652 bp) (see groups in Table S1b). **a** Haplotype networks. Black lines represent nucleotide mutations and coloured circles denote unique haplotypes. Circle size is proportional to haplotype frequency. **b** Geographical distribution of the populations and their haplotype composition. Populations are coloured according to haplotype networks in (**a**), and pie size is proportional to haplotype frequency

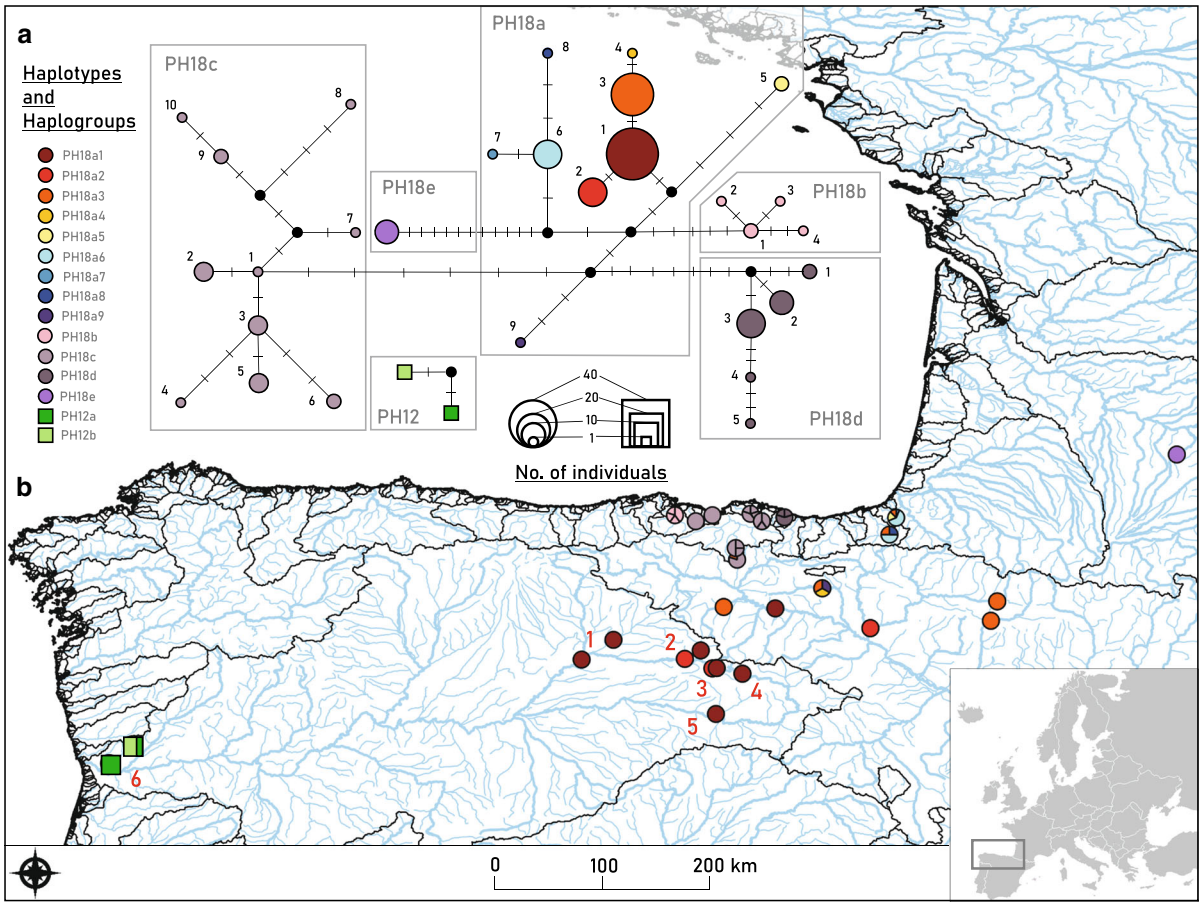

Fig. 4 Phylogeographic analyses for the *Phoxinus bigerri* (PH12) and *Phoxinus* sp. (PH18) using cytb (1089 bp) (see groups in Table S1d). **a** Haplotype networks. Black lines represent nucleotide mutations, coloured circles denote observed unique haplotypes, and black points represent unobserved haplotypes and potential intermediates. Circle size is proportional to haplotype frequency. Network of *P. bigerri* is divided in subgroups as given by TCS analysis (PH18a-e). **b** Geographical distribution of the populations and their haplotype composition. Populations are coloured according to haplotype networks in (**a**), and pie size is proportional to haplotype frequency

observed cytb haplotypes (PH12a-b), differing by two mutational steps (Fig. 4, Table S1d), were the first described for the species and, therefore, the population of origin could not be confirmed using this gene.

Discussion

Our study (1) records for the first time the presence of a second *Phoxinus* species in the Douro Basin detected in a tributary located in northwest Iberian Peninsula (Sousa River, Portugal); (2) in the case of this *Phoxinus* sp., the introduction was possibly originated from a population in Charente Basin (France), whereas the high genetic similarity between western Douro and upper Ebro populations suggests the latter region is likely to be the geographic origin of the *P. bigerri* introduction, supported with previous reports (Doadrio and Garzón 1986; Corral-Lou et al. 2019); and (3) we inferred that both introductions may be related to its use as freshwater live bait. In fact, in the Iberian Peninsula anglers travel widely and sporadically use invasive species as live bait, and therefore releases of unused live bait in the water may occur (Banha et al. 2017). Because of this, the use of *Phoxinus* as live bait by anglers, which is allowed in some countries (e.g. France), has been reported as a likely dispersal mechanism outside native ranges (Museth et al. 2007).

The distribution of *Phoxinus* sp. appear to be an example of such mechanism. To our knowledge this is

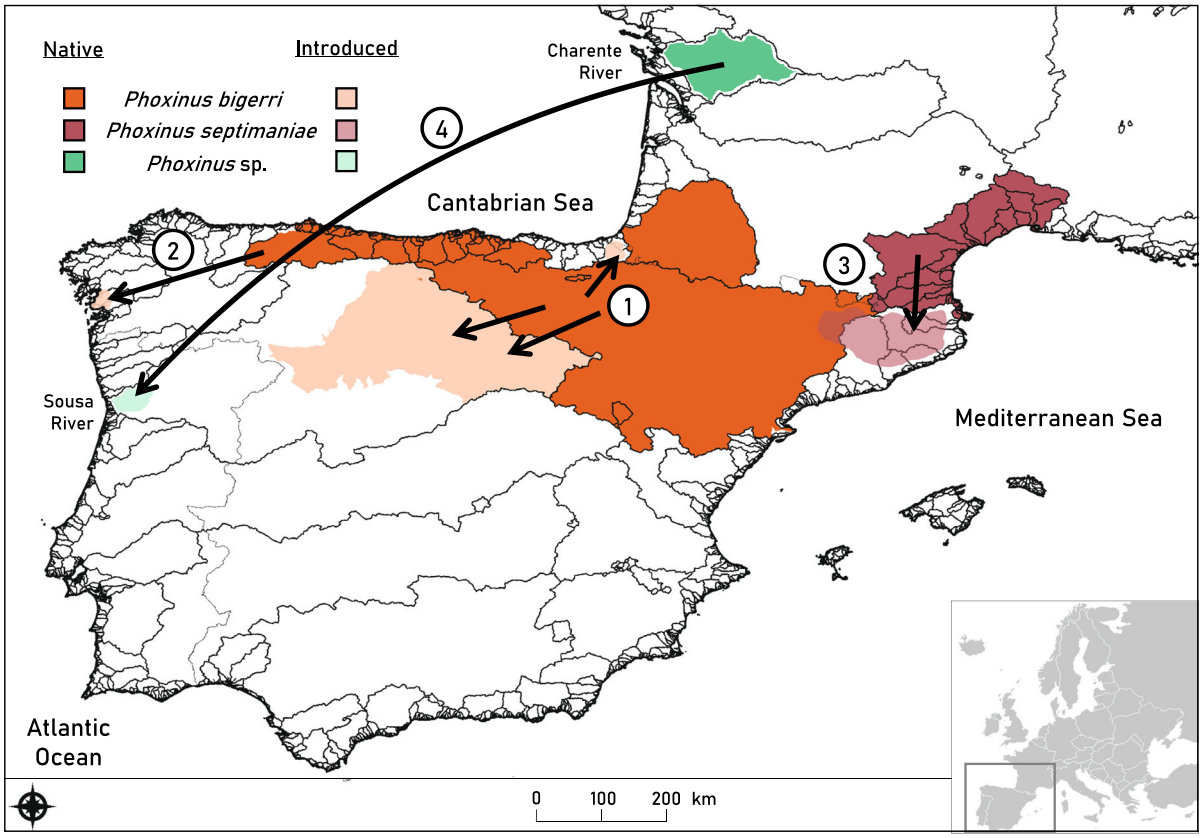

Fig. 5 Resume of known introduction events of *Phoxinus* genus in the Iberian Peninsula based on previous records and results from this study. (1) *Phoxinus bigerri* was introduced to the Douro Basin and northern basins as a consequence of its use as live bait since early 20th (Doadrio and Garzón 1986; this study). (2) Translocation of *Phoxinus bigerri* in a small coastal basin in Galicia, northwest Spain (Sánchez-Hernández et al. 2012). (3) *P. septimaniae* in lower Ebro and Catalonia Basins introduced from southern France (Corral-Lou et al. 2019). (4) First record of *Phoxinus* sp. from the Charente Basin region, southern France, in the Sousa River, Portugal (this study). The distribution of *Phoxinus* sp. described in Corral-Lou et al. (2019) (lineage 21 in Palandačić et al. 2020; PH19-20 in this study, Table S1d) is not shown since there are still no evidences confirming either its introduction or its native range in the Iberian Peninsula

the first record of this taxa in the Iberian Peninsula, and our study suggests that the putative geographic origin of the Sousa population is the Charente River (France). This is supported by the hypothesis of a human-mediated introduction from south France to Portugal given (1) the long geographic distance between localities (origin and place of introduction distanced $>$ 1000 km) and the lack of streams connectivity which would definitely represent an obstacle to its natural dispersal, (2) the common use of species from this genus as a live bait for freshwater angling as we mentioned before (Doadrio and Garzón 1986; Banha et al. 2017), and (3) the frequent human movements by car between northern Portugal and France (Ribeiro and Veríssimo 2014). Indeed, during the period between the 1960s and the 1980s, close to 1 million of Portuguese emigrated to France (Baganha 1994; INSEE, Recensement 2010) and, since then, human movements are recurrent.

Integrating all reported cases, it is now possible to resume the introduction history of minnows in the Iberian Peninsula in four main events (Fig. 5): (1) *P. bigerri* has been introduced into the eastern part of Douro Basin from nearby watercourses of the Ebro Basin; (2) probably this species was also introduced to a coastal Atlantic basin in Galicia (Sánchez-Hernández et al. 2012), hence genetic studies are needed to confirm taxonomy and geographical origin since no data is available; (3) *P. septimaniae* introduced in lower Ebro Basin and Catalonia basins from southern

France rivers (Corral-Lou et al. 2019); and (4) another *Phoxinus* species was introduced in the Sousa River (Douro Basin, Portugal) from southern France (Charente River). The chronological order of such events, however, remains unclear and needs further investigation.

The findings of this study should be interpreted in the light of some constraints: (1) Although BLAST results identified Sousa population as *P. phoxinus*, according to the most recent revisions of the genus (Palandačić et al. 2017, 2020), this species belongs to a genetically distinct lineage (lineage 10) occurring in Germany. Since the haplotypes from western France were not included in the referred revisions (including Charente River), the individuals in Sousa River could not be assigned to any described *Phoxinus* species. Taking this limitation into account, and to avoid possible misunderstandings due to the use of an outdated nomenclature, we decided to designate the population found in Portugal as *Phoxinus* sp. until new revisions disentangle the complexity of the genus. (2) The ability of DNA barcoding to identify species is also compromised if identical mitochondrial sequences are present in related species due to introgression (Palandačić et al. 2017), which is a concern in freshwater fishes and specially *Phoxinus* where numerous hybridization events and mitochondrial captures have been reported (e.g. Palandačić et al. 2020). Therefore, the results reported in this study still need to be further evaluated using nuclear markers to control for such events. (3) Despite we were able to assign individuals to putative regions, this study applies genetic analysis on individual mitochondrial genes and differences may be found regarding tree topologies between this and other studies that use combined genes and more comprehensive analyses to properly study *Phoxinus* phylogeny (Palandačić et al. 2017, 2020; Corral-Lou et al. 2019). For this reason, there is a need for additional genetic analysis (e.g. restriction site-associated DNA markers) in order to infer the whole history of introduction events of these species in the Iberian Peninsula (e.g. number of founders and propagules, number of events, time of the introduction).

To conclude, it is likely that *Phoxinus* sp. but also *P. bigerri* will continue to spread throughout the Douro Basin. Although the ecological consequences of such introductions are still uncertain, their spread is of significant conservation concern because of the potential competition with the native fishes (Clavero 2011). *Phoxinus* species have a broad physiological tolerance and ecological impacts of its introduction have been reported (e.g. Museth et al. 2007). However, the Mediterranean climate and its harsh environmental conditions may restrict the establishment of both species (Filipe et al. 2010). Overall, we highlight the importance of continuing to monitor both introductions in Douro Basin over the next years to assess the potential ecological impacts on native fish communities. We also emphasize the importance of designing and applying management plans to prevent or at least detect future introduction events at the earliest stage, with special focus on fisherman's habits and activities.

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