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Multiple introduction pathways of non-native *Phoxinus* minnows (Teleostei: Leuciscidae) in Corsica revealed by its hidden diversity and their parasites

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Abstract The introduction of freshwater fish species is a leading cause of aquatic biodiversity erosion and can spread parasites to native populations. Hidden diversity evidenced by recent taxonomic revisions can add further complexity to the issue by rendering biological assessment data incomplete. The Eurasian minnows *Phoxinus* are one such example of cryptic diversity, with several described species being invasive. Current non-native fish populations in the small Mediterranean island of Corsica (France) are the result of successive waves of introductions, including several *Phoxinus* species. This study aims at determining which *Phoxinus* species

were introduced to Corsica using the *cytochrome oxidase subunit I* barcoding marker, reconstructing their introduction routes and examining their parasite communities. The study found four species in Corsica: *Phoxinus phoxinus* and *Phoxinus csikii* mainly in the northernmost studied drainage basin and *Phoxinus dragarum* and *Phoxinus septimaniae* in the Tavignano drainage basin. *P. phoxinus* and *P. csikii* were most likely introduced through a live bait wholesaler while *P. dragarum* and *P. septimaniae* were probably introduced by recreational anglers bringing their bait from continental France. The molecular study of their *Gyrodactylus* (Platyhelminthes: Monogenea) parasites with the ITS marker allowed us to hypothesize inter-drainage basin secondary introduction routes for *P. phoxinus* and *P. dragarum*. In several sampling sites, *Phoxinus* minnows had black spot disease caused by encysted metacercariae of Digenea, likely *Posthodiplostomum cuticola*. These parasites were also found on the brown trout *Salmo trutta* in a locality where this patrimonial species co-occurs with *Phoxinus* minnows. Barcoding should be used in fish communities monitoring to help to accurately identify cryptic species.

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Introduction

The introduction of non-native species, especially fish, is one of the leading causes of the decline of fauna in freshwater ecosystems at global scale, impacting both the abundance and distribution of native species (Cambray 2003; Clavero and Garcia-Berthou 2005; Milardi et al. 2018). Fish introductions have diverse impacts on both invaded ecosystems and native species, ranging from alteration of interactions between fish (e.g. increased predation pressure), increase in prey availability for native predators or competition for trophic resources, alteration of trophic web structure possibly leading to habitat alteration (e.g. eutrophication), and genetic impacts through hybridization and introgression (Ribeiro and Leunda 2012; Witkowski and Grabowska 2012; Ellender and Weyl 2014; Tadese and Wubie 2021). An additional, particularly noteworthy consequence of species introduction is the co-introduction of associated parasites and/or pathogens (Taraschewski 2006; Ribeiro and Leunda 2012; Ellender and Weyl 2014; Goedknecht et al. 2016; Tadese and Wubie 2021). Such organisms are frequently introduced along with their hosts in a new area (Lambert 1997; Prenter et al. 2004; Taraschewski 2006). One reason to focus on these parasite co-introductions is that this phenomenon may have a serious impact as the lack of co-evolution between non-native parasites and native hosts can result in the lack of an adequate immune response to the infection. The scale of the problem may be underestimated when biodiversity assessment data are either lacking or incomplete, which can be the case when dealing with cryptic species. Cryptic species are two or more species usually reported as a single one because of their indistinguishable morphology and a lack of systematic studies (Bickford et al. 2007). One such example of cryptic diversity is the case of minnows *Phoxinus* spp. (Leuciscidae) (Kottelat 2007; Palandačić et al. 2017; Corral-Lou et al. 2019; Denys et al. 2020), small freshwater fish widely distributed across Eurasia, for which reliable diagnosis on the field is impeded by the difficulty of observing the diagnostic characters (Bianco 2014), except the nuptial coloration pattern shown in French species (Denys et al. 2020), *i.e.* only during their spawning period. There are currently 26 valid *Phoxinus* species in Eurasia (Berg 1949; Mitrofanov et al. 1987; Chen 1988; Kottelat 2006, 2007; Bianco and

De Bonis 2015; Zhang and Zhao 2016; Palandačić et al. 2017; Bogutskaya et al. 2020, 2023; Denys et al. 2020; Dyldin et al. 2023; Turan et al. 2023; Artaev et al. 2024; Bayçelebi et al. 2024), 5 more molecular lineages potentially corresponding to distinct species (Palandačić et al. 2017) and the taxonomy of this genus is still under study, owing notably on the emergence of molecular tools. Within the genus, several introduction events have been documented e.g. *Phoxinus csikii* Hankó, 1922 and *Phoxinus septimaniae* Kottelat 2007 were likely introduced in the lower and middle Rhine catchment systems (Netherlands, Belgium and Germany) (Palandačić et al. 2020, 2022); the latter species was also introduced in the western Po river basin (Italy), possibly during the same period with the growing popularity of trout angling (De Santis et al. 2021). Corral-Lou et al. (2019) highlighted the introduction in Catalonia of *P. septimaniae* and of a lineage from the Garonne which may be *Phoxinus dragarum* Denys, Dettai, Persat, Daszkiewicz, Hauteceur and Keith, 2020 which is endemic to the Garonne drainage basin. Similarly, Denys et al. (2020) affirmed the introduction of *P. dragarum* in the Guadalquivir drainage basin on the basis of the nuptial coloration of the specimen illustrated by Sáez-Gómez and Prenda (2019). Garcia-Raventós et al. (2020) noted the introduction of a population from the Charente drainage basin (Western France) in the Sousa river (Portugal). Introductions of *Phoxinus* were due to their use as live bait for angling, or via contamination of Salmonidae used to reinforce stocks to enhance angling (Museth et al. 2007; Miró and Ventura 2015; Garcia-Raventós et al. 2020). These introduction events contributed to the alteration of the distribution of *Phoxinus* minnows and have made their management more complex. Knowledge of this genus' taxonomy has evolved over the last fifteen years (Kottelat 2007; Palandačić et al. 2017, 2020, 2022; Corral-Lou et al. 2019; Denys et al. 2020; Garcia-Raventós et al. 2020; De Santis et al. 2021).

The small Mediterranean island of Corsica (France) displays a unique freshwater fish stock, with a native fish fauna composed of only 4 native fish species: the European eel *Anguilla anguilla* (Linnaeus 1758), the brown trout *Salmo trutta* Linnaeus, 1758, the freshwater blenny *Salariopsis fluviatilis* (Asso 1801) and the three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758; and naturally devoid of several Cypriniformes occurring in the

Ibero-Franco-Italian region (Roule 1933; Changeux 1998); and more than 20 non-native species resulting from successive waves of introductions into the island's rivers and lakes (Roche and Mattei 1997; Roché 2001). These introductions started at the end of the nineteenth century with the addition of the mosquitofish *Gambusia holbrooki* Girard 1859 in an attempt at biological control of malaria vectors (mosquito control). The introductions then continued in the 1970s with the release of the brook trout *Salvelinus fontinalis* (Mitchill 1814) and the domestic brown trout *Salmo trutta* Linnaeus, 1758 in mountain lakes. Several species such as the roach *Rutilus rutilus* (Linnaeus, 1758), the rudd *Scardinius erythrophthalmus* (Linnaeus, 1758), the tench *Tinca tinca* (Linnaeus, 1758), the carp *Cyprinus carpio* Linnaeus, 1758 and the pikeperch *Sander lucioperca* (Linnaeus, 1758) were then introduced into artificial lakes and probably dispersed by anglers to rivers. Several non-controlled introductions occurred afterwards at unknown dates, such as those of *Carassius* sp., the common perch *Perca fluviatilis* Linnaeus, 1758 and gudgeons *Gobio* spp. Being an insular environment, Corsica is especially sensitive to species introductions (Townsend et al. 2006; Donlan and Wilcox 2008). The arrival of *Phoxinus* minnows in Corsica are one such example of fish introductions that may have an impact on native fish populations e.g. the heritage species *Salmo trutta* Linnaeus, 1758. According to Denys et al. (2020), two species of *Phoxinus* are known to occur on the island: *Phoxinus phoxinus* Linnaeus, 1758 in the Golo river and *P. dragarum* in the Tavignano river. However, this data was acquired at only two localities on the island, and owing to the difficulty of discriminating between *Phoxinus* species outside their spawning period, the specific diversity of this genus has yet to be explored in Corsica.

Parasites have been used as a tool for assessment of conservation issues, helping to untangle the introduction history of their hosts, to identify their origin and spreading routes by acting as proxies for their hosts' genealogy and ecology (Whiteman and Parker 2005; Nieberding and Olivieri 2007; Gagne et al. 2022). The shorter generation time and smaller population size of parasites, compared to their hosts, allow the use of parasites as markers to clarify the origin and dispersal of invasive species (Nieberding and Olivieri 2007; Gagne et al. 2022). For this purpose, considerable attention has been paid to viruses (e.g.

Biek et al. 2006; Allen et al. 2010; Wilfert and Jiggins 2014). However, macroparasites have also been used successfully to gain insight into the origin of introduced hosts, their introduction routes and vectors, to highlight contemporary and historical contacts between host populations, to identify the source population of migratory individuals, past migrations and differentiation events (Wickström et al. 2003; Nieberding et al. 2004, 2006; Criscione et al. 2006; Reshetnikov et al. 2011; Huysse et al. 2015; Kmentová et al. 2019; Šimková et al. 2022). A key feature with regard to the ability of a parasite to be a useful marker is its shared history with its host, which is dependent on the strength of the host-parasite interaction and thus on its host specificity, on the absence or presence of intermediate hosts and on the absence or presence of a free-living stage (Page 2003; Clayton and Johnson 2003; Charleston and Perkins 2006; Nieberding and Olivieri 2007). Monogeneans, with their often strong association with their host, their direct life cycle and direct transmission, are good candidates to study their fish host's introductions, dispersion, biogeography and evolutionary history (Pariselle et al. 2011; Lumme et al. 2016; Kmentová et al. 2019; Benovics et al. 2020; Šimková et al. 2022; Rahmouni et al. 2023a).

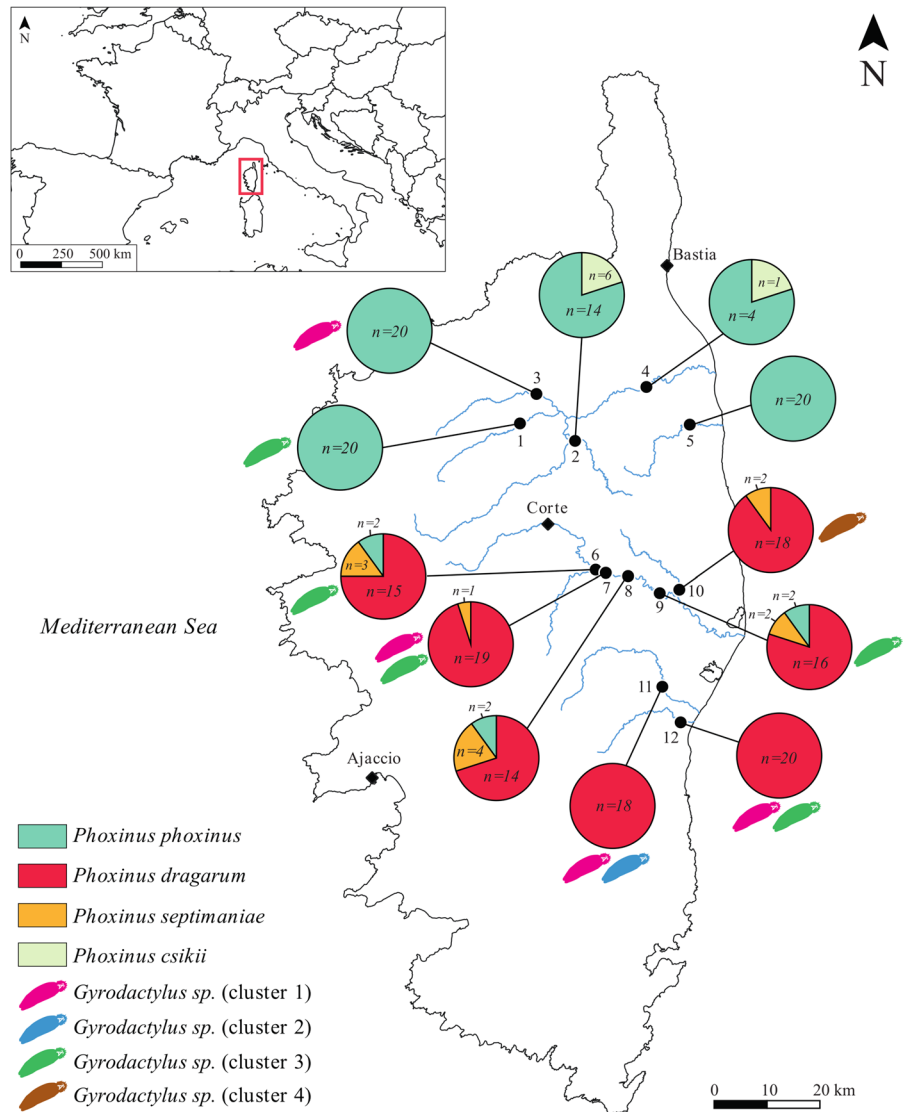
The aims of this study were (1) to inventory the *Phoxinus* species introduced to the small Mediterranean island of Corsica and their intraspecific variability, and to retrace their introduction routes through haplotype networks and parasite fauna, (2) to study their parasite communities for the first time in this region and (3) assess their potential impact on the native fish fauna, especially native *S. trutta*.

Material and methods

Study area and sample collection

A total of 225 *Phoxinus* minnows were taken from 12 different freshwater sampling localities across the Haute-Corse Département (Corsica, French Mediterranean, Fig. 1, Table 1). The sampling localities cover a total of 10 rivers and five drainages basins. The samplings were conducted from June to October 2022 by electrofishing in compliance with French legislation and with the help of the French Agency of Biodiversity (OFB) as well as the Angling Federation

Fig. 1 Sampling localities for *Phoxinus minnows* in Corsica, with the corresponding proportions of *Phoxinus* species and corresponding *Gyrodactylus* clusters. Locality number (river, locality name): 1 (Asco, Moltifao); 2 (Casaluna, Gavignano); 3 (Tartagine, Castifao); 4 (Golo, Barchetta); 5 (Fium'Alto, Taglio-Isolaccio); 6 (Vecchio, Venaco); 7 (Tavignano, Altiani); 8 (Tavignano, Piedicorte-di-Gaggio); 9 (Tavignano, Saint-Georges); 10 (Corsigliese, Pancheraccia); 11 (Fium'Orbo, Ghisonaccia); 12 (Abatesco, Prunelli-di-Fium'Orbo). Diamonds are the major cities in Corsica, *n* is the number of individuals of the corresponding species in the corresponding locality



of Corsica (FDAAPPMA2). Sampled watercourses were selected to include all rivers for which *Phoxinus* minnow abundance was sufficient, on the basis of data obtained through the European Union Water Framework Directive fish monitoring conducted by the OFB.

Dissection and parasitological examination

Phoxinus minnows were euthanized in compliance with French legislation (NOR: AGRG1238753A), transported on ice in individual bags to the laboratory, and kept on ice until examination. Each individual was weighed to the nearest 0.1 g (TW, in g),

and measured to nearest millimeter (TL, in mm). Fin-clips were preserved in 95% ethanol for molecular studies. The stomach, intestine, swim bladder, spleen, and liver were placed in Petri dishes with physiological saline and examined under a stereomicroscope for parasites. The skin, gills, fins, mouth, and abdominal cavity were also checked for parasites. Brains were checked for *Diplostomum phoxini* (Faust, 1959) metacercariae by squashing the brain gently between two microscope slides as described in Müller (1995). All parasites collected were preserved in 70% or 90% ethanol. A subsample of *Gyrodactylus* (Platyhelminthes: Monogenea) were used for further molecular analysis.

Table 1 Specimens analyzed in this study (N, number of specimens; TL, total length in mm (mean ± standard deviation (minimum–maximum)); TW, total weight in g (mean ± standard deviation (minimum–maximum))), and parasitological indices for both main parasites (P, prevalence (%); MA, mean abundance; MI, mean intensity)

Site	N	Species (N)	Site name	Altitude	River	Drainage basin	TL	TW	Gyrodactylus spp.			Black spot disease, metacercariae		
									P	MA	MI	P	MA	MI
1	20	<i>P. phoxinus</i> (20)	Moltifao	310	Asco	Golo	66.2 ± 7.8 (50–79)	2.8 ± 1 (0.9–4.4)	65	3.8 ± 4.4	5.9 ± 4.2	15	1.2 ± 4.7	7.7 ± 11.5
2	20	<i>P. phoxinus</i> (14) <i>P. csikii</i> (6)	Gavignano	254	Casaluna	Golo	73.8 ± 5.2 (66–82)	4.5 ± 0.9 (3.1–6.2)	70	3.9 ± 7.9	5.5 ± 9.0	15	0.3 ± 0.7	1.7 ± 1.2
3	20	<i>P. phoxinus</i> (20)	Castifao	250	Tartagine	Golo	63.4 ± 8.3 (49–83)	2.9 ± 1.3 (1.4–5.9)	90	59.6 ± 94.8	66.2 ± 97.8	55	15.7 ± 54.5	28.5 ± 72.4
4	5	<i>P. phoxinus</i> (4) <i>P. csikii</i> (1)	Barchetta	81	Golo	Golo	67.8 ± 5.4 (62–75)	3.3 ± 0.6 (2.7–4.2)	20	0.2 ± 0.4	1 ± -	0	-	-
5	20	<i>P. phoxinus</i> (20)	Taglio-Isolaccio	60	Fium'Alto	Fium'Alto	35.1 ± 3.1 (28–41)	0.4 ± 0.1 (0.2–0.6)	5	0.1 ± 0.4	2 ± -	0	-	-
6	20	<i>P. phoxinus</i> (2) <i>P. dragarum</i> (15) <i>P. septimaniae</i> (3)	Venaco	199	Vecchio	Tavignano	63.4 ± 5.4 (51–72)	2.9 ± 0.8 (1.4–4.7)	25	0.4 ± 0.7	1.4 ± 0.9	5	0.1 ± 0.2	1 ± -
7	20	<i>P. dragarum</i> (19) <i>P. septimaniae</i> (1)	Altiani	157	Tavignano	Tavignano	52.4 ± 7.8 (39–75)	1.5 ± 0.8 (0.6–4)	45	2 ± 3.3	4.4 ± 3.8	0	-	-
8	20	<i>P. phoxinus</i> (2) <i>P. dragarum</i> (14) <i>P. septimaniae</i> (4)	Piedicorte-di-Gaggio	157	Tavignano	Tavignano	56.3 ± 6.9 (40–72)	2 ± 0.8 (0.7–3.9)	65	3.1 ± 3.5	4.7 ± 3.3	0	-	-
9	20	<i>P. phoxinus</i> (2) <i>P. dragarum</i> (16) <i>P. septimaniae</i> (2)	Saint Georges	57	Tavignano	Tavignano	65.3 ± 7.8 (54–86)	2.9 ± 1.5 (1.4–7.3)	70	2.4 ± 2.1	3.4 ± 1.7	5	0.2 ± 0.9	4 ± -
10	20	<i>P. dragarum</i> (18) <i>P. septimaniae</i> (2)	Pancheraccia	56	Corsigliese	Tavignano	66.6 ± 7.6 (51–85)	3.1 ± 1 (2–5.8)	85	16.8 ± 29.2	19.8 ± 30.9	0	-	-
11	20	<i>P. dragarum</i> (18) Unidentified (2)	Ghisonaccia	22	Fium'Orbo	Fium'Orbo	55.2 ± 10.3 (45–88)	1.9 ± 1.1 (0.9–5.3)	50	2.6 ± 5.0	5.2 ± 6.1	15	1.5 ± 4.3	9.7 ± 7.5
12	20	<i>P. dragarum</i> (20)	Prunelli-di-Fium'Orbo	8	Abatesco	Abatesco	57.8 ± 7.3 (43–69)	1.9 ± 0.7 (0.8–3.4)	25	0.6 ± 1.2	2.2 ± 1.6	30	0.7 ± 1.1	2.2 ± 1.0

Statistical analysis

Parasite indices were calculated following the terminology of Bush et al. (1997): prevalence is the number of hosts infected with at least one individual of a particular parasite species divided by the number of hosts examined and expressed as a percentage; mean abundance is the total number of individuals of a given parasite species in a sample divided by the total number of hosts in that sample; and mean intensity is the total number of individuals of a given parasite species in a sample divided by the number of infected hosts in that sample. As species are considered bio-indicators when their abundance and/or frequency in a particular habitat are significantly higher in this habitat (Mouillot et al. 2002), an analysis of indicator values (IndVal) (Dufrene and Legendre 1997) was used to combine the parasite species' relative abundance (specificity) and relative frequency (fidelity) for a given variable. Specificity is the mean abundance of a parasite species in a given group of *Phoxinus* minnows divided by the same parasite abundance infecting all *Phoxinus* minnows. Fidelity is defined as the percentage of *Phoxinus* minnows in a given group infected by a given parasite species. The IndVal analysis's capacity to include both specificity and fidelity in the same index constitutes an advantage over classical statistical tests (e.g., ANOVA) when looking for indicator species in highly variable communities, such as parasites (Mouillot et al. 2002). Calculations of IndVal and associated *p* values (10,000 permutations) were conducted using the labdsv R package (Roberts 2019).

Molecular analyses

For minnows, a DNA barcoding approach sensu (Hebert et al. 2003) was done with the cytochrome oxidase subunit 1 (COI) marker. DNA extraction, PCR, sequencing and sequences cleaning follow Denys et al. (2020). Concerning parasites, total DNA was extracted following the same protocol as for minnows.

Concerning *Gyrodactylus* parasites, total DNA was extracted with a QIAamp® DNA Micro kit (QIAGEN) following the manufacturer's instructions. The D1+D2 regions of the 28S rDNA gene was amplified using the forward primer C1 5'-ACCCGC TGAATTTAAGCAT-3' and the reverse primer D2

5'-TGGTCCGTGTTTCAAGAC-3' (Wu et al. 2005) and a partial fragment of the Internal Transcribed Spacer (ITS2) region was amplified using the forward primer ITS4.5 5'-CATCGGTCTCTCGAACG-3' and the reverse primer IST2 5'-TCCTCCGCT TAGTGATA-3' (Matejusová et al. 2001). DNA was amplified by PCR in a final 20 µL volume containing 1 µL DMSO, 1 µL of dNTP 6.6 µmol/L, 0.15 µL of Qiagen Taq DNA polymerase, using 2 µL of the buffer provided by the manufacturer and 0.4 µL of each primer at 10 pmol/L; 3 µL of DNA extract was added. After 3 min denaturation at 95 °C (hot start), the PCR was run for 50 cycles (30 s at 94 °C; 1 min at 50 °C for ITS2 and 56 °C for 28S; 1 min 30 s at 72 °C), with a 3 min terminal elongation at 72 °C on a Bio-Rad t100™ thermal cycler. Successful PCRs were selected on ethidium-bromide stained agarose gel. Sanger sequencing was performed in both directions by a commercial company (Eurofins) (<http://www.eurofins.fr>).

Species identification, phylogenetic grouping and haplotype networks

Phoxinus COI sequences were compared with a molecular dataframe from different publications (Geiger et al. 2014; Knebelberger et al. 2015; Behrens-Chapuis et al. 2015, 2021; Thalinger et al. 2016; Schönhuth et al. 2018; Denys and Manne 2019; Denys et al. 2020; De Santis et al. 2021; Zangl et al. 2022; Table S1). Aligning, p-distances and NJ-tree reconstruction based on the DNA barcodes were performed under MEGA X (Kumar et al. 2018) with the Kimura 2 parameter model (K2P; Kimura 1980). Bootstrap values (Felsenstein 1985) with 1000 replicates were also calculated for evaluating the robustness of clusters.

Median-joining networks were built from the COI datasets of each species using Network v.4.6 (Bandelt et al. 1999). We applied a maximum parsimony algorithm and the criterion "frequency > 1" to simplify the complex branching scheme and generate networks representing the most parsimonious relationships. Genetic diversity indices (haplotype diversity (Hd), number of polymorphic site (S) and number of haplotypes (h)) were calculated with DnaSP V6 (Rozas et al. 2017).

Gyrodactylus delineation was performed as follows: data processing and sequence assembling were

done with Geneious Prime ® 2020.2.4 (<http://www.geneious.com>). Sequences were aligned with MAFFT alignment (Kato et al. 2002). PartitionFinder v.2.1.1 (Lanfear et al. 2012) was used to estimate the best evolution model for the Bayesian phylogenetic inference analyses selected under the Bayesian Information Criterion (GTR+G for ITS2 and 28S). The percentage of divergence between sequences was calculated in Geneious Prime. The phylogenetic tree was constructed with MrBayes v.3.2.6 (Ronquist et al. 2012). Two independent analyses were run for 10 million generations, sampling every 200 generations. The convergence of the two analyses was checked and the tree obtained is a consensus with ten percent of the trees discarded as burn-in. Sixteen sequences were obtained for the phylogenetic reconstruction and two sequences of *Benedenia armata* were added from GenBank (LC602801.1 for ITS2 and LC408961.1 for 28S) as out group. A total of 16 sequences were used for the phylogenetic reconstruction based on an alignment of 1390 base pairs (pb).

Results

Diversity of introduced *Phoxinus* in Corsica

The *Phoxinus* minnows sampled in Corsican rivers were molecularly identified at the species level as *P. phoxinus*, *P. dragarum*, *P. septimaniae* and *P. csikii*, as supported by the phylogenetic tree (Fig. 1, S1, Table 1, S2.). The distribution of these recorded species seems to be dependent on the drainage basin, with *P. csikii* recorded exclusively from the Golo drainage basin and *P. septimaniae* from the Tavignano drainage basin. *P. dragarum* was recorded only in the Tavignano drainage basin and those to the south of it. *P. phoxinus* is the majority of the identified individuals in both northernmost sampled drainage basins (Golo and Fium'Alto) (Fig. 1, S1).

Five *P. dragarum* haplotypes were detected in Corsica (Fig. 2). H7 is shared by 19 individuals sampled in the Fium'Orbo and specimens originating from the Garonne drainage basin in the Pyrenees and the Landes (six sequences), H4 is shared by 72 *P. dragarum* from the Tavignano and one specimen from the Garonne drainage basin in the Massif Central (Fig. 2a). Three haplotypes (H5, H6 and H11; respectively 12, 15 and 5 individuals) were new and

did not correspond to any available sequence. Three haplotypes were detected in Corsica for *P. septimaniae* (Fig. 2b): no correspondence was found for H4 and H5 (one and five individuals respectively), which are separated from specimens originating from the Rhone drainage basin by two and three mutational steps, respectively. The haplotype H24 recorded from the Tavignano in Corsica (6 sequences) was shared by minnows sampled in several drainage basins: Mediterranean and Rhone (France, 7 sequences), Middle Rhine (Germany, 12 sequences) and Po (Italy, one sequence). Three *P. phoxinus* haplotypes were recorded in Corsica of which H11 was the major one (69 sequences) and was shared with four specimens originating from the Meuse drainage basin (France), the Middle Rhine (Germany, 178 sequences) and one specimen from the Boyne River in Ireland. No haplotype was shared with specimens from the Seine. The second most represented haplotype in Corsica (H10) was shared with three specimens from the Upper Danube and the Middle Rhine (Germany). Concerning *P. csikii*, only one haplotype (H23) was detected in Corsica (seven sequences) and was shared with specimens from the Middle Rhine (Germany, 132 sequences) and the Po (Italy, two sequences).

Genetic diversity indices are given in Table S2. Comparison of genetic diversity parameters among the four species found in Corsica showed that *P. septimaniae* had the highest haplotype diversity, mean number of pairwise differences and nucleotide diversity while *P. phoxinus* had the lowest. The highest number of haplotypes was reported for *P. csikii* though only one was detected in Corsica. This species also showed the highest number of polymorphic sites. Conversely, the lowest number was reported for *P. dragarum* while this species had the highest number of haplotypes in Corsica and the lowest number of polymorphic sites.

Parasite diversity in *Phoxinus* minnows in Corsica

A total of six distinct parasites were recovered from Corsican *Phoxinus* minnows. *Gyrodactylus* spp. (Monogenea: Gyrodactylidae, recovered from all sampling localities) and a black spot disease-causing metacercariae, most likely *Posthodiplostomum cuticola* (Digenea: Diplostomidae, recovered from half the localities) were the two main parasites recovered from Corsica. Prevalence, mean abundance

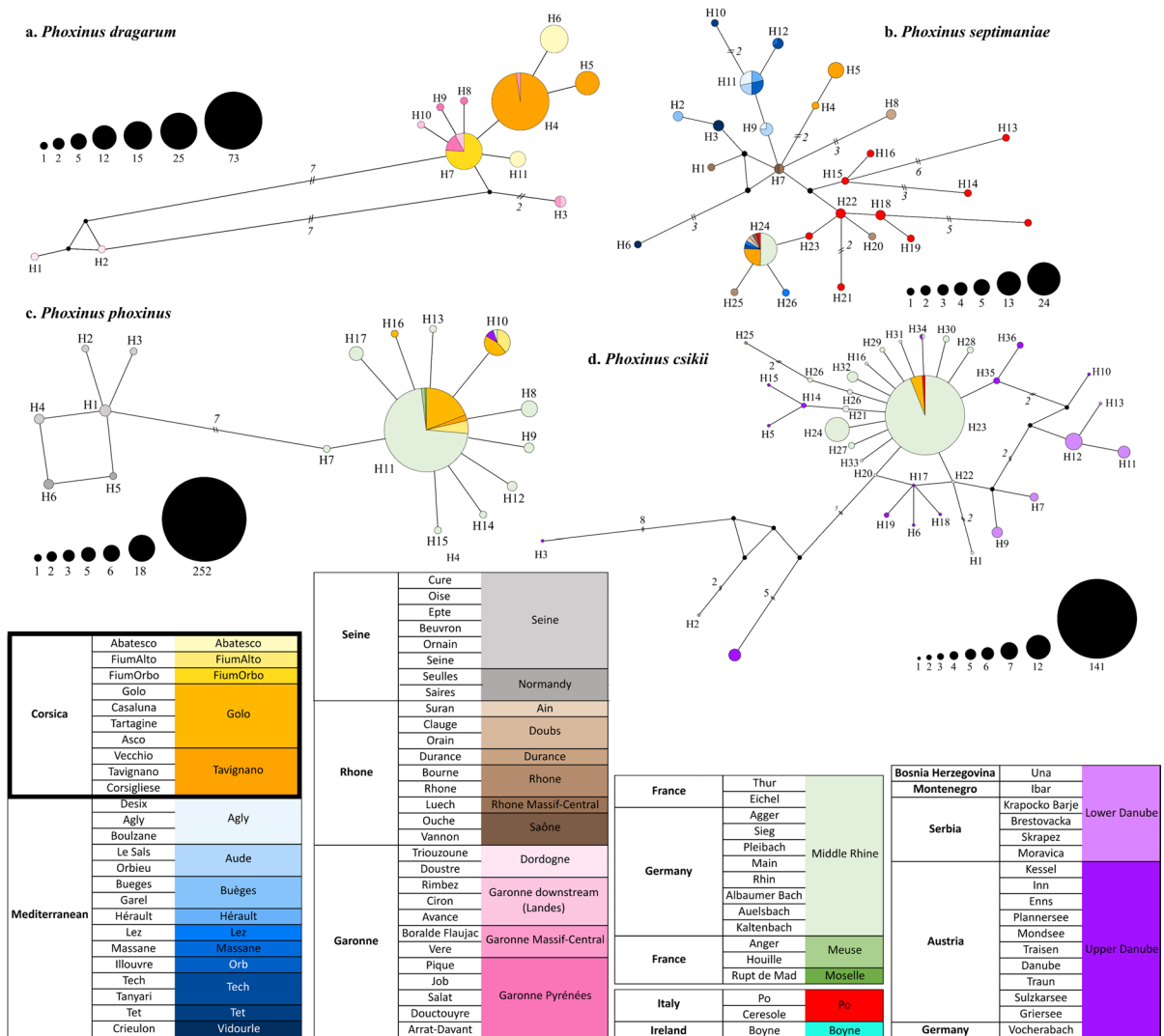


Fig. 2 COI haplotype networks obtained for the four *Phoxinus* species detected in Corsica, on the 745 sequences generated in this study and retrieved from GenBank (Table S1). Circle

size is proportional to the observed haplotype frequencies and black points represent hypothetical haplotypes. Colors high-light drainage basins

and mean intensity for both these two main parasites are reported for each locality and each host species (Tables 1, S3). *P. cuticola* metacercariae were recovered from all *Phoxinus* species except *P. septimaniae* and *Gyrodactylus* from all four species present in Corsica. *Gyrodactylus* species could not be identified at the species level due to the lack of matching sequences in GenBank, but our phylogenetic analysis showed the occurrence of four distinct clusters of *Gyrodactylus* sequences (Figs. 1, 3). The first cluster consists of *Gyrodactylus* recovered

from a *P. phoxinus* from Tartagine river (Golo drainage) and from several *P. dragarum* from Fium’Orbo, Tavignano and Abatesco. Cluster 2 comprises a single sequence from a *P. dragarum* sampled in the Abatesco. The third cluster shows haplotypes being shared between a *P. phoxinus* from Vecchio river (Tavignano drainage basin) and from *P. dragarum* from the Asco (Golo drainage basin), Tavignano and Fium’Orbo rivers. The last cluster (Cluster 4) consists of two sequences originating from a *P. dragarum* from Corsigliese river (Tavignano drainage basin).

Besides *Gyrodactylus* spp. and *P. cuticola*, several parasites could not be identified due to the low number of recovered individuals and development stages lacking diagnostic features. One Trematoda larvae was recorded from the body cavity of *P. dragarum*, Nematoda larvae were observed in the swimbladder of *P. phoxinus*, *P. dragarum* and *P. septimaniae*, two leeches (Hirudinea) were recovered from the skin of a *P. phoxinus* and a *P. dragarum* and a few freshwater mussel glochidia (Bivalvia: Unionidae) were recovered from the gills of three individuals *P. dragarum*, only in locality 9. The examination of brains did not show any *D. phoxini* metacercariae.

Influence of sampling localities on parasite communities

IndVal analysis did not show any preference of *Gyrodactylus* spp. nor black spot disease metacercariae towards a particular *Phoxinus* species and those results are thus not presented here. However, IndVal analysis showed that both black spot disease-causing metacercariae and *Gyrodactylus* spp. are characteristic of locality 3 (Tartagine river) as the IndVal as significantly higher for this locality (Table S4). Fidelity of *Gyrodactylus* spp. was very high whereas the specificity was intermediate as this taxon was recovered from all studied localities. Black spot disease metacercariae showed quite high specificity and intermediate fidelity. This corresponds to the locality showing the highest abundances for both parasites (Fig. 4).

Discussion

Phoxinus minnows in Corsica: underestimated diversity and diverse introduction routes

Only one *Phoxinus* species was reported from Corsica (Roché 2001) until 2020 when Denys et al. (2020) showed not only the presence of *P. phoxinus* in the Golo river, but also of *P. dragarum* in the Tavignano river. The present study reports for the first time the occurrence of two additional species in Corsica: *P. septimaniae* and *P. csikii*. The results of the present study should however be analyzed taking into account the limitation of the molecular marker used. Hybridization events were reported for this genus (Palandačić et al. 2017, 2020, 2022; Corral-Lou et al.

2019). Our study used only a mitochondrial marker (COI) which does not allow the detection of eventual hybrids. However, as numerous Corsican localities present admixed populations, the presence of hybrids is likely. So, using a nuclear marker could be of interest in the future.

All four species of *Phoxinus* species introductions in Corsica are likely to result from their use as live bait by anglers. This mechanism has already been shown to be the cause of *Phoxinus* minnows introductions in other regions in Europe e.g. Norway and Portugal (Museth et al. 2007; Garcia-Raventós et al. 2020). This hypothesis is supported by the isolation of Corsica from the mainland by the Mediterranean Sea and the fact that *Phoxinus* minnows are not part of the native Corsican fish fauna (Changeux 1998; Roché 2001; Keith et al. 2020), indicating a human-mediated introduction and the common use of *Phoxinus* minnows as live bait for recreational fishing e.g. *Salmo trutta* angling (Banha et al. 2016). Two patterns of introduction are supported by the COI haplotype networks (Fig. 5): (1) In both northernmost Corsican drainage basins (Golo and Fium'Alto), *P. phoxinus* was the majority of sampled individuals, and *P. csikii* was only found in the Golo drainage basin, with haplotypes originating from the Middle Rhine, pointing toward an introduction through individuals from a continental Europe wholesaler and used as baits. This pattern resembles the case of *P. csikii* and *P. phoxinus* in Germany and the Netherlands (Palandačić et al. 2022). In France, minnows sold in all angling shops come from a same wholesaler (Amorvif; <http://www.armorvif.com/>) located in Brittany. Costedoat et al. (2014) characterized 50 specimens from this wholesaler and they found that 86% belong to the Meuse lineage of *P. phoxinus*, 6% were identified as *P. csikii*, 6% as *Phoxinus fayollarum* Denys, Dettai, Persat, Daszkiewicz, Haute-cœur and Keith, 2020 and 2% as *P. septimaniae*. The proportions of each species in both northernmost drainage basins (92% *P. phoxinus* and 8% *P. csikii*) add support to the hypothesis of a wholesaler-mediated introduction as the main species is the same in these localities and the wholesaler's stock. The absence of two minor species *P. septimaniae* and *P. fayollarum* and presence of *P. csikii* would result from a random sampling that most likely occurred during the importation process. (2) In the southernmost drainages basins (Tavignano, Fium'Orbo and

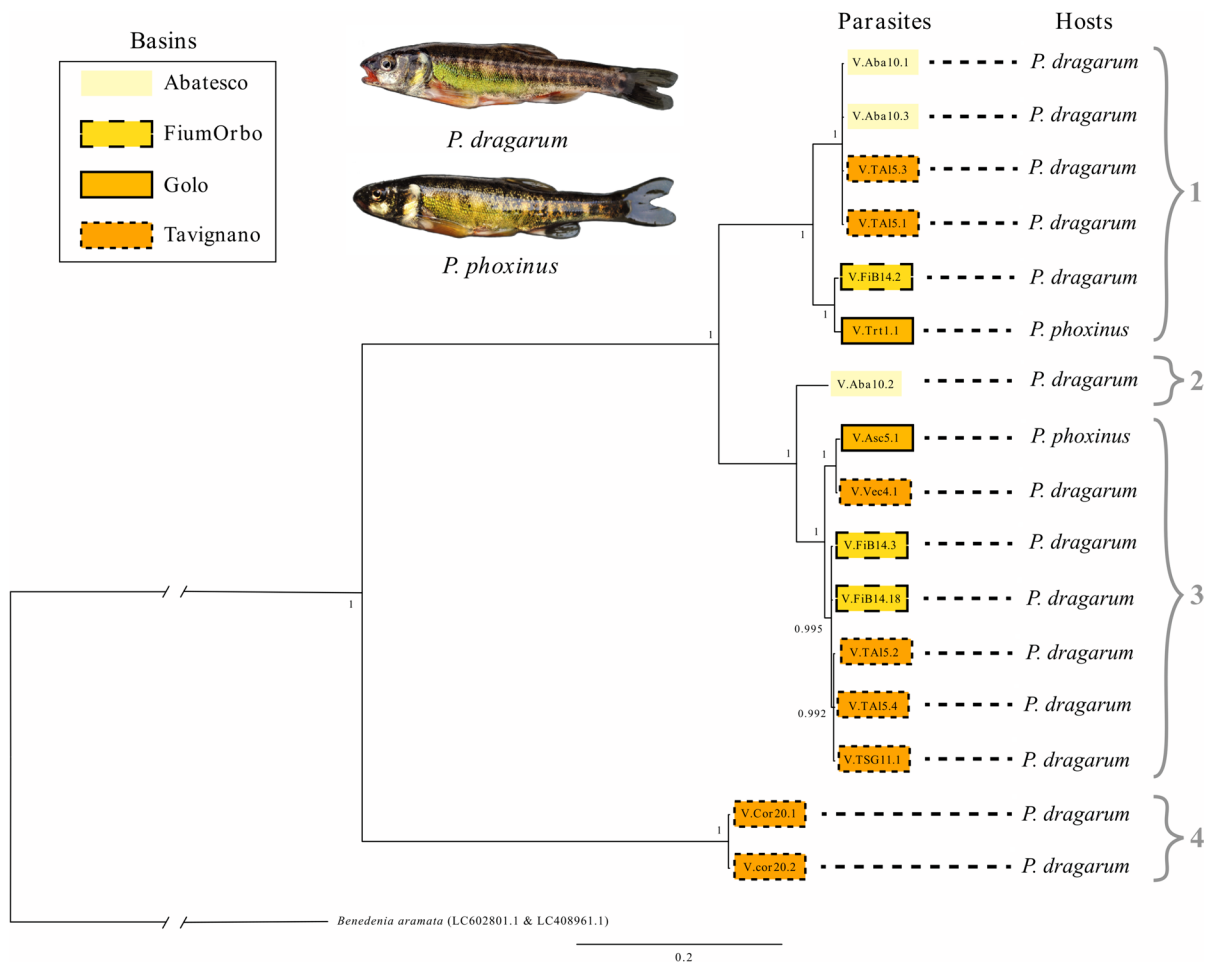


Fig. 3 Phylogenetic tree inferred with MrBayes for the *Gyrodactylus* sampled in Corsica using ITS2 gene, with their corresponding host species. Numbers indicated in grey correspond to the four clusters. Minnows pictures come from Denys et al. (2020)

Abatesco), the occurrence of haplotypes from Southern France (Garonne, Mediterranean and Rhone) for *P. septimaniae* and *P. dragarum* could be indicative of angler-mediated introductions, as has been shown in the case of *P. dragarum* and *Phoxinus bigerri* Kotletat 2007 in the Iberian Peninsula (Corral-Lou et al. 2019). In this region, it is known that anglers travel widely and can use and introduce invasive species (Banha et al. 2016), and it is likely that anglers from continental France bring their live baits with them to be used in Corsican freshwaters.

Six species are recognized from continental France: *P. phoxinus*, *P. csikii*, *P. septimaniae*, *P. dragarum*, *P. fayollarum* and *P. bigerri* (Geiger et al. 2014; Corse et al. 2017; Palandačić et al. 2017; Schönhuth et al. 2018; Denys et al. 2020). To our

knowledge, *Phoxinus* species reported from Italy consist of *Phoxinus lumaireul* (Schinz, 1840), *P. septimaniae* and *P. csikii* (Palandačić et al. 2017; De Santis et al. 2021) and those from Spain are *P. bigerri*, *P. septimaniae* and *P. dragarum* (Geiger et al. 2014; Corral-Lou et al. 2019; Keith et al. 2020). According to the extent of current knowledge concerning the distribution of this genus' species, France is the only country where all species introduced to Corsica were reported. The known distribution of *Phoxinus* species in the countries closest to Corsica is thus an additional argument strengthening the hypothesis of multiple introductions from continental France. The inclusion of more individuals in haplotypes analysis would be of interest to better resolve haplotype networks as those generated in this study showed

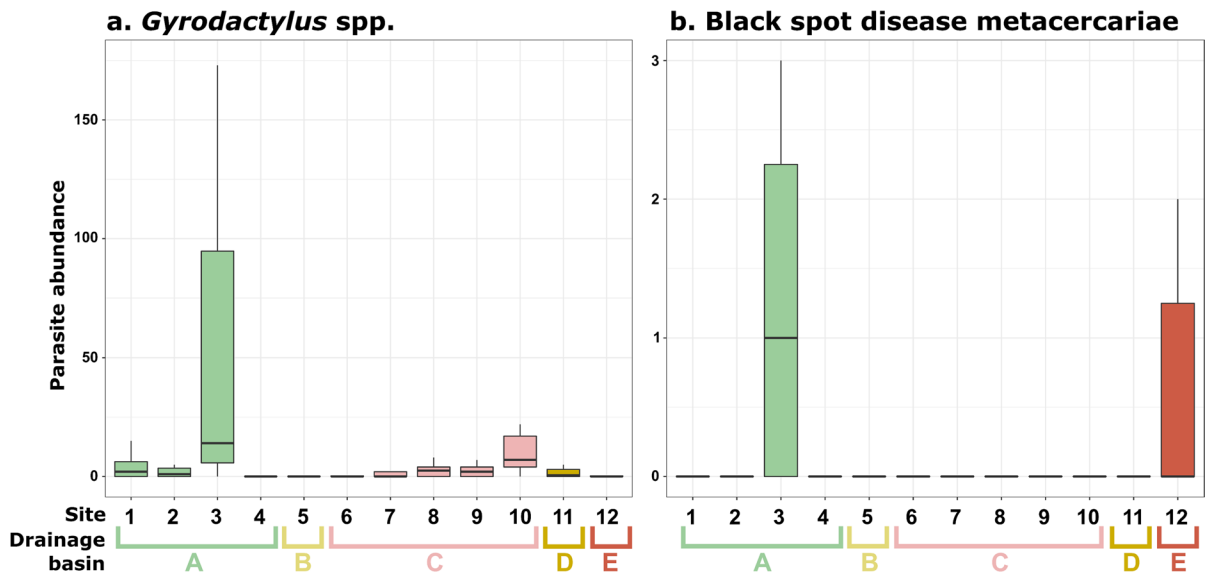


Fig. 4 Abundance of infection for **a** *Gyrodactylus* spp. and **b** Black spot disease (metacercariae) in *Phoxinus* minnows for each locality. Localities: (1) Moltifao, (2) Gavignano, (3) Castifao, (4) Barchetta, (5) Taglio-Isolaccio, (6) Venaco, (7) Altiani, (8) Piedicorte-di-Gaggio, (9) Saint-Georges, (10)

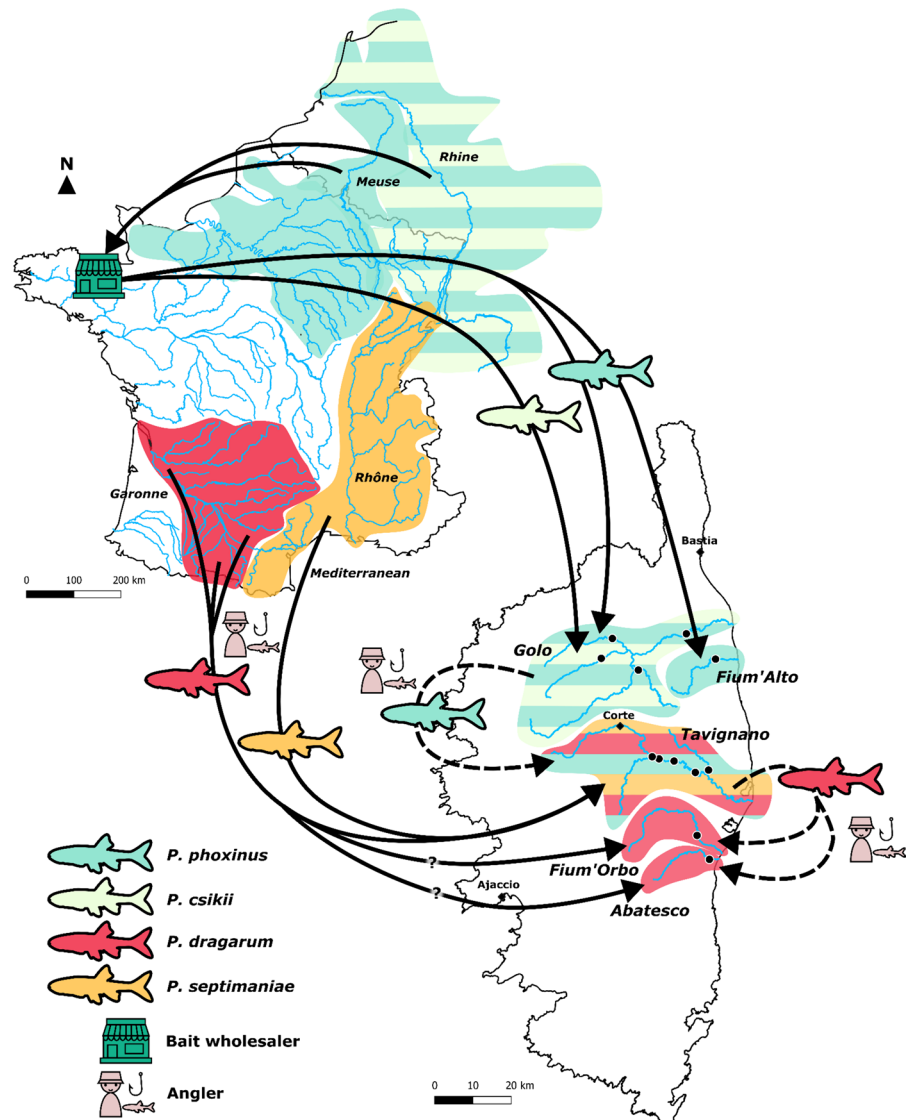
Pancheraccia, (11) Ghisonaccia, (12) Prunelli-di-Fium'Orbo. Drainage basin A: Golo, B: Fium'Alto, C: Tavignano, D: Fium'Orbo, E: Abatesco. Boxplots appearing flat are due to the low prevalence of parasite in the corresponding locality

several hypothetical haplotypes and thus uncertain relationships.

Additionally, the study of these fishes' parasites allows the hypothesis of secondary dispersion routes (Fig. 5). The observation of two shared *Gyrodactylus* clusters (clusters 1 and 3) between *P. phoxinus* from Golo drainage and *P. dragarum* from Tavignano and Fium'Orbo (plus Abatesco for one of them) drainages basins, combined with the absence of observation of *P. dragarum* in the Golo drainage basin, allows the hypothesis of a recreational anglers-mediated secondary dispersion of fish and their parasites from the Golo drainage to the Tavignano, Fium'Orbo and Abatesco drainages basins. As *P. phoxinus* was not observed in the Fium'Orbo and Abatesco rivers, a probable suite of events would be: a first transfer of *P. phoxinus* from the Golo drainage basin to the Tavignano drainage basin, and a second transfer of *P. dragarum* from the Tavignano drainage basin to the Abatesco and Fium'Orbo. The use of parasites as potential markers for introduction routes and historical distribution of hosts has already been discussed e.g. *Kapentagyris* (Monogenea: Dactylogyridae) were used to clarify the origin of the Clupeidae *Limnothrissa miodon* (Boulenger, 1906) in Lake Kariba,

Zimbabwe (Kmentová et al. 2019), the lack of *Gyrodactylus* on the round goby *Neogobius melanostomus* (Pallas, 1814) in Belgium suggested an introduction via ballast water (Huysse et al. 2015), host specific *Dactylogyris* (Monogenea: Dactylogyridae) were used to evidence historical contact between North American and European Leuciscidae as well as contemporary contacts between these fish in North America (Šimková et al. 2022) and the host-specific *Nippotaenia perccotti* (Akhmerov, 1941) (Cestoda: Nippotaeniidae) proved useful to analyze the introduction vectors and dispersion pathways of its host, the Chinese sleeper *Perccottus glenii* Dybowski, 1877 (Reshetnikov et al. 2011). Again with regard to *P. glenii*, the Monogenea *Gyrodactylus perccotti* Ergens & Yuxhimenko, 1973 has been used to suggest distinct introduction events in the Vistula and Danube drainages basins and a migration from the Vistula to the middle Dnieper River (Ondračková et al. 2012; Kvach et al. 2016). Here we show the usefulness of *Gyrodactylus* species as tags to elucidate regional-scale inter-basin transfer of their hosts in the case of *Phoxinus* minnows introduction in a small Mediterranean island.

Fig. 5 Resume of introduction events of *Phoxinus* minnows in Corsica based on results from this study. Solid arrows correspond to primary introduction routes (wholesaler and angler-mediated introductions), dashed arrows to secondary routes (angler-mediated inter-drainage basin translocations)



Low parasite diversity of introduced *Phoxinus* minnows

In Eurasia, wild parasite communities of *Phoxinus* spp. show species richness ranging from four helminths reported from Frongoch lake in the UK (Bibby 1972) to 14 helminths species in the river Pechora, Russia (Dorovskikh and Stepanov 2008, 2009) and 14 parasite species including helminths and Copepoda in the rivers Chulman and Ungra, Russia (Boutorina and Reznik 2015). With five helminth species, the parasite richness of *Phoxinus* minnows in Corsica was thus among the lowest reported for this genus in Eurasia,

along with two reports from the UK and one from a mountain water system in southern Norway (Ashworth and Bannerman 1927; Bibby 1972; Kristoffersen and Teigland 1997). A likely explanation for the low diversity reported from the UK is its insularity whereas the *Phoxinus* examined in southern Norway were qualified as recently spread. Corsica combines both these characteristics, being a small Mediterranean island where *Phoxinus* minnows are part of the non-native fauna, most likely introduced circa 2000 (Roché 2001). Several taxa known to occur in *Phoxinus* in mainland southern Europe were not recorded from Corsica, such as the Acanthocephala, the

Argulidae (Ichthyostraca), the Allocreadiidae (Digenea) and the Cestoda (both Proteocephalidae and Diphyllbothridae) (Cruz et al. 2022). The few parasitological analyses of *Phoxinus* minnows conducted in mainland France showed radically distinct parasite communities, with the presence of *Dactylogyrus* spp., *D. phoxini* and *Diplozoon* spp., taxa not recorded in Corsica (Joyeux and Baer 1953; Euzet and Lambert 1971; Lambert 1977; Le Brun et al. 1988). However, it should be noted that these records originate from localities pertaining to the distribution ranges of *P. fayollarum*, a species not observed in Corsica, and of *P. septimaniae*, which was one of the least abundant *Phoxinus* in our samples and may have been introduced in small numbers. Data concerning the parasite fauna of *Phoxinus* species found in Corsica are currently lacking.

The absence of record for *D. phoxini* in Corsica can appear surprising as this species was reported from *Phoxinus* minnows from Russia in the east to Spain in the west (Dorovskikh et al. 2008; Cruz et al. 2022). However, there are two possible explanations for this: (1) *D. phoxini* “missed the boat” and never reached Corsica i.e., the *Phoxinus* introduced to Corsica were devoid of this parasite, or (2) *D. phoxini* “drowned on arrival”, being unable to complete its life cycle.

As a side observation, there may still be much to unravel concerning *Gyrodactylus* diversity in *Phoxinus*. The four distinct clusters detected in the freshwaters of Corsica and highlighted by the use of ITS2 gene potentially correspond to four different species and two of them were shared between two species of hosts (*P. phoxinus* and *P. dragarum*) while the other two have only be found in *P. dragarum*. Due to technical difficulties, molecular and phylogenetic analysis could not be performed for *Gyrodactylus* specimens sampled on all *Phoxinus* species present in Corsica despite their occurrence on the four species recovered from the island. Numerous species of *Gyrodactylus* are known to occur on *Phoxinus* minnows throughout Eurasia (Harris et al. 2004; Bakke et al. 2007; Lumme et al. 2017), with some species recorded from a restricted number of sampling localities e.g., *Gyrodactylus vimbi* Shulman, 1954 (Finland) (Blazek et al. 2008), *Gyrodactylus prostae* Ergens, 1963, *Gyrodactylus llewellyni* Ergens & Dulmaa 1967 and *Gyrodactylus minimus* Malmberg, 1957 (Mongolia) (Ergens and Dulmaa 1967), and *Gyrodactylus*

konovalovi Ergens 1976 (Russia) (Boutorina and Reznik 2015); and other species known from a wider geographical range e.g. *Gyrodactylus macronychus* Malmberg, 1957 (Czech Republic, Finland, Mongolia, Norway, Russia, the UK and Spain) (Ergens and Dulmaa 1967; Ergens 1976; Matějusová et al. 2000; Ziętara and Lumme 2003; Dorovskikh and Stepanov 2008; Grano-Maldonado et al. 2011; Pettersen et al. 2016; Cruz et al. 2022) or *Gyrodactylus pannonicus* Molnár, 1968 (Czech Republic, Finland, Russia, Slovakia and the UK) (Matějusová et al. 2000; Ziętara and Lumme 2002; Dorovskikh and Stepanov 2008; Blazek et al. 2008; Grano-Maldonado et al. 2011; Lumme et al. 2017). As *Gyrodactylus* is an extremely diversified genus, comprising both generalists and highly specialist species, with an estimated proportion of 30% infecting a single host (Bakke et al. 1992, 2007; Harris et al. 2004), it is likely that the diversity of introduced *Gyrodactylus* in Corsica is still underestimated. As an additional argument, this genus is known for its cryptic diversity resulting from the lack of morphological characters that would enable unambiguous species identification (Hansen et al. 2007; Razo-Mendivil et al. 2016; Ondračková et al. 2020; Rahmouni et al. 2023b, a). Studies of fish Monogenea regularly allow detection and description of new species of *Gyrodactylus* (e.g. Vanhove et al. 2011, 2014; Příkrylová et al. 2012a, b; Ziętara et al. 2012; Lumme et al. 2017; Shigoley et al. 2023; Zhang et al. 2023). More effort focused on the molecular identification of *Gyrodactylus* and their *Phoxinus* minnows hosts across Eurasia would likely reveal a considerable diversity which remains unknown for now. An example of this potential is the description of three new *Gyrodactylus* species from minnows sampled in the Baltic, White Sea and Black Sea basins, and in Mongolia (Lumme et al. 2017).

May introduced minnows transmit parasites to native species?

Black spot disease is caused by encysted metacercariae of Digenea, to which the fish host reacts by forming a fibrous capsule (Wittrock et al. 1991). Fish melanocytes are then attracted by these processes and melanin is deposited around the parasite, creating black spots visible to the naked eye (Tobler and Schlupp 2008). Though black spot disease-causing metacercariae observed in the present study could not

be identified at the species level, there is a restricted number of Digenea known to cause such symptoms. Several species cause black spot disease in marine fish: *Ichthyophaga* sp. (Fecampiida: Piscinquiniliidae) (Justine et al. 2009), *Scaphanocephalus expansus* (Creplin, 1842) and *Scaphanocephalus* sp. (Plagiiorchiida: Heterophyidae) (Kohl et al. 2019; Dennis et al. 2019; Elmer et al. 2019; Cohen-Sánchez et al. 2023), and *Cryptocotyle concava* (Creplin, 1825) and *Cryptocotyle lingua* (Creplin, 1825) and *Cryptocotyle jejuna* (Nicoll, 1907) (Plagiiorchiida: Opisthorchiidae) (Khan 2006; Aalvik et al. 2015; Dufлот et al. 2021, 2023; Kornyychuk et al. 2022). Considering their environmental preferences, it is highly unlikely that the metacercariae found on *Phoxinus* minnows in Corsican freshwaters are any of these species. The genus *Crassiphiala* and *Uvulifer* (Diplostomida: Diplostomidae) are known to occur in American freshwaters (Berra and Au 1978; Quist et al. 2007; Tobler and Schlupp 2008; Wisenden et al. 2012; Achatz et al. 2019; López-Hernández et al. 2023) and *Uvulifer* sp. was detected in Northern Africa (Charo-Karisa et al. 2021). In Eurasia, black spot disease in fish has been attributed to *Apophallus muehlingi* (Jägerskiöld, 1899) and *Apophallus donicus* (Skrjabin and Lindtrop, 1919) (Plagiiorchiida: Opisthorchiidae) (Sándor et al. 2017; Tyutin et al. 2023) but, to the extent of our knowledge, *Posthodiplostomum cuticola* (von Nordmann, 1832) (Diplostomida: Diplostomidae) is the most commonly reported agent of this condition in Europe (Shukhgalter and Chukalova 2002; Ondracková et al. 2004b, a, c; Zrnčić et al. 2009; Kirankaya and Ekmekçi 2011; Maja et al. 2012; Innal et al. 2020; Cech et al. 2021). As *P. cuticola* is also the only black spot disease-causing Digenea to have been reported from *Phoxinus* minnows including in continental France (Nicoll 1924; Kennedy 1974; Prouff 2017; Cruz et al. 2022), assuming that it is the species present in Corsica is a reasonable hypothesis. Unfortunately, parasitological data concerning Corsican fish anterior to this introduction of *Phoxinus* minnows on the island are not available, and it is thus not possible to know whether black spot disease was co-introduced with these fish. Black spot disease-causing Digenea, including *P. cuticola*, typically have an complex life cycle involving a piscivorous bird such as Ardeidae (Dönges 1964). Corsica being a Mediterranean island situated on bird migration routes (Bruderer and Liechti 1999; Jourdain et al. 2007; Maggini

et al. 2020), the parasite could have been transported by migrating birds and have found competent fish hosts among the numerous species introduced in the island waterways, including *Phoxinus* minnows. While black spot disease does not seem to cause mortality in fish hosts, symptoms can include body deformation, muscle fiber necrosis and dysfunction of kidney and liver, which can be particularly harmful to fry (Williams 1994; Marković and Krsmanović 2008; Innal et al. 2020). This pathogenicity could be a point of concern as a preliminary study conducted in 2021 allowed detection of the presence in locality 3 (Tartagine river) of black spot disease-causing metacercariae on brown trout *Salmo trutta* Linnaeus, 1758, a species considered of patrimonial interest in Corsica. This disease had not been detected in *S. trutta* in Corsica before the present study despite extensive surveys of this species' parasitofauna (Quilichini et al. 2007; Quilichini et al. 2010), and, to the extent of our knowledge, *P. cuticola* was only reported once from *S. trutta*, in Poland (Rolbiecki et al. 2009).

Conclusion

Although the extensive range of ecological consequences of *Phoxinus* minnows introduction in Corsica is not yet fully understood, their presence in sustainable populations is of concern as they could compete with native fish. *Phoxinus* minnows are reported to impact native fish e.g. reduced recruitment and growth rates in *S. trutta* in Scandinavia, possibly resulting from competition for trophic resources as there is a dietary overlap between *Phoxinus* minnows and young *S. trutta* (Museth et al. 2007, 2010). It has been suggested that the harsh Mediterranean climatic conditions could limit the establishment of introduced species having evolved under different environmental conditions (Filipe et al. 2010), but *Phoxinus* minnows seem to have broad-enough ecological tolerances to successfully colonize this type of habitat.

The introduction of *Phoxinus* minnows has likely had parasitological impacts on native species, as shown by the case of black spot disease. While it is not possible to know whether these fish co-introduced the parasite responsible for this infection, its occurrence in Corsican freshwaters is most likely favorable to the disease's persistence on the island, and thus its potential transmission to native species. Parasites,

especially *Monogenea* with relatively restricted host spectrum, can be useful markers of small-scale secondary introduction patterns, and allow detection of inter-basin transfer of hosts at the regional scale.

In the context of the European Water Framework Directive, all member states of the European Union have to monitor their fish populations. It has been shown that *Phoxinus* species are reliably identifiable on the basis of the nuptial coloration pattern only during their spawning period (Denys et al. 2020). As the monitoring periods of fish populations are not always compatible with the *Phoxinus* spawning period, the addition of molecular identification (i.e. barcoding) in the context of the Water Framework Directive should be considered. A complementary approach could be the use of eDNA detection methods (Pont et al. 2021).

Another primordial aspect of *Phoxinus* minnows' introductions is the importance of applying management plans able to prevent future introductions. A Europe-wide ban on live bait fishing could be one approach to prevent future fish introductions, especially in other Mediterranean islands that currently seem free of *Phoxinus* minnows, such as Sardinia and Sicily (Orrù et al. 2010; Marrone and Naselli-Flores 2015).

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Data availability The data supporting the findings of this study are available within the article and its supplementary

material. Raw data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Conflict of interest The authors have no financial or proprietary interests in any material discussed in this article.

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