ARTICLE

European minnows through time: museum collections aid genetic assessment of species introductions in freshwater fishes (Cyprinidae: *Phoxinus* species complex)

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Abstract

Massive fish introductions have taken place throughout much of the world, mostly over the last 70 years, and present a major threat to the genetic diversity of native fishes. Introductions have been reported for European *Phoxinus*, a ubiquitous small cyprinid that populates a wide variety of habitats. Species delineation in European *Phoxinus* has proven difficult with one reason being ranges of distribution that often traverse drainage boundaries. The present study combines recent samples with museum samples to better understand the current distribution of *Phoxinus* species and their distributions prior to the massive introductions of fishes in Europe, and to evaluate the use of museum specimens for species distribution studies. For these purposes, genetic lineages from sites collected prior to 1900 ($n = 14$), and between 1900 and 1950 ($n = 8$), were analysed using two mitochondrial and nuclear markers. Although possible fish introductions were detected, our results show that the distribution of genetic lineages of museum samples is comparable to that of the extant lineages of European *Phoxinus* present in those areas. These observations suggest that in the studied ranges the distribution of *Phoxinus* lineages has been driven by natural processes.

Introduction

Fish introductions present, along with habitat loss, one of the main threats to genetic diversity of native fishes (Bruton 1995; Harrison and Stiassny 1999; Jug et al. 2005; Scott and Helfman 2001). It is known that the Romans undertook such introductions (Balon 2004), but, according to Crivelli (1995), 60% of introduced fish species have been stocked during the last 70 years. Reasons for introductions include sport and aquaculture (De Silva et al. 2006; Stanković et al.

2015), but also stocking of ornamental ponds (Padilla and Williams 2004; Ahnelt 2016) and biological control (Pyke 2008). Nevertheless, many introductions have occurred unintentionally, such as with ballast waters (Johansson et al. 2018), as by-products of stocking of commercial species, or from angling with live baits (Gozlan et al. 2010).

Minnows of the genus *Phoxinus* Rafinesque 1820 are ubiquitous small cyprinids that populate a wide variety of habitats throughout northern Eurasia. They are found in both still and running waters, including mountain streams, lowland rivers and lakes, spanning a range of altitudes and climatic zones (Banarescu 1992; Frost 1943; Kottelat and Freyhof 2007; Tack 1940). According to the latest revision of European *Phoxinus* (Palandačić et al. 2017), the genus contains ten valid species and at least eight additional well-resolved genetic lineages. Although for some lineages a species name is available (e.g., *Phoxinus morella*), not all lineages have been taxonomically formalised due to a lack of morphological data. Recently, a new species *Phoxinus krkae* was described based on a previously detected genetic lineage (Bogutskaya et al. 2019). In general, species delineation in European *Phoxinus* has proven difficult because of the phenotypic diversity present within the different genetic lineages (Collin and Fumagalli 2015;

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Ramler et al. 2017; Bogutskaya et al. 2019). Compounding the problem are the diverse distribution ranges of the *Phoxinus* species and lineages. While for the Leuciscinae subfamily (at present, at the family level Leuciscidae) distributions are usually limited to particular river drainages (e.g., Hrbek et al. 2004; Doadrio and Carmona 2004, Perea et al. 2010), in European *Phoxinus* the species and lineage distributions do not follow such zoogeographical patterns, often traversing drainages or even basin boundaries (Palandačić et al. 2015, 2017). Moreover, at some sampling sites, hybrids between different genetic lineages have been detected (Palandačić et al. 2015, 2017; Corral-Lou et al. 2019). The origin of the different ranges of *Phoxinus* species can be attributed to anthropogenic translocations, as several species introductions have been reported in the literature. For example, Miro and Ventura (2015) report the spread of *Phoxinus* contemporary with trout introductions in Pyrenean lakes, while Museth et al. (2007) suggest their spread has been due to angling with live baits in Norway. In addition, Knebelsberger et al. (2015) conclude that minnows from the River Danube have been introduced into the River Rhine. Nevertheless, some of the observed species ranges might be natural (Palandačić et al. 2015).

Museum specimens have been used to address questions of taxonomy (Krajewski et al. 1997), phylogenetics (Cooper et al. 1992; Su et al. 1999), population genetics (Thomas et al. 1990), and biogeography (Alfaro et al. 2012), and to assess the impact of stocking of commercially important fishes (Nielsen et al. 1999, Hansen et al. 2009). However, working with museum samples is challenging, because of the degradation and fragmentation of the DNA (Wandeler et al. 2007; Zimmermann et al. 2008). In *Phoxinus*, museum specimens have been used for taxonomic revision of the genus in Europe (Palandačić et al. 2017) and for geometric morphometric analysis of habitat influence on body shape (Ramler et al. 2017). They have also been helpful in determining the ancestor among the four different genetic lineages currently present in the River Rhine (Knebelsberger et al. 2015). However, in those and most other studies using museum material (see Burrell et al. 2014 and the references within), only one locus (the barcoding section of *cytochrome oxidase I, COI*) was used.

The primary aim of the present study was to combine recent and museum samples (for definitions see below) to better understand the current distribution of *Phoxinus* species and lineages, and to compare their ranges prior to the massive introductions of fishes in Europe. A second aim was to evaluate the use of museum samples for such studies, i.e. to map historical species distributions. For these purposes, a combination of two mitochondrial sections (*COI*; and *cytochrome b, cytb*) were used to determine the genetic lineages of both museum and recent samples. Because hybridisation and introgression have been recorded (as

reported above) in European *Phoxinus*, the nuclear *recombination activating gene 1 (RAG1)* was added to the dataset. However, as difficulties have previously been reported for amplification of *RAG1* in museum samples, the *internal transcribed spacer 1 (ITS1)* was also included in the analysis.

Materials and methods

Mitochondrial DNA (mtDNA)

In the present study, the term 'museum' is applied to samples deposited in the various museums that were accessed (Museum of Natural History Berlin, Germany; Natural History Museum of Geneva, Switzerland; Natural History Museum Vienna, Austria) but which were collected prior to the year 2000, while the term 'recent' is applied to samples collected in contemporary *Phoxinus* studies, mostly after the year 2000. Altogether, 186 museum samples from 18 countries, and 48 recent samples from eight countries, were combined with the available sequences deposited in Genbank (Geiger et al. 2014; Knebelsberger et al. 2015; Palandačić et al. 2015, 2017; Ramler et al. 2017; Schönhuth et al. 2018; Denys and Manne 2019). DNA extraction and amplification followed protocols described in Palandačić et al. (2017), adhering to all requirements for working with museum material, including using UV-irradiated utensils, a clean room, negative extraction controls, among others. Following polymerase chain reaction (PCR), *COI* and *cytb* fragments were visually inspected, aligned with MEGA 6.0 (Tamura et al. 2011) and combined together to form single sequences. During this process, overlapping fragments were checked for congruity. The barcoding region of *COI* was fully recovered (650 bp), while for *cytb* fragments of length 473 or 590 bp were obtained, depending on the DNA quality. The program MEGA 6.0 (Tamura et al. 2011) was used for constructing simple neighbour-joining trees from the *COI* and *cytb* sequences with which the genetic lineages of museum samples were determined.

Because *cytb* was not available for representatives of all putative species and clades in European *Phoxinus*, a phylogenetic tree was calculated using *COI* sequences only. The tree was constructed from complete *COI* sequences using Bayesian inference implemented in BEAST 1.8.0 (Drummond et al. 2012) and the Maximum-Likelihood method in PhyML (Guindon et al. 2010) following protocols (including model selection, program settings and burn in) described in detail in Palandačić et al. (2017). In contrast to that last study, phylogenetic analysis was performed with an outgroup chosen based upon the phylogenetic study of Imoto et al. (2013). The species chosen for the outgroup were *Rhynchocypris lagowskii* (AP009147), *Tribolodon*

hakonensis (AB626855, now in the genus *Pseudaspius* according to Imoto et al. 2013) and *Oreoleuciscus potanini* (AB626851).

Nuclear DNA (nDNA)

To assist determination of possible hybridisation and introgression events, two nuclear genes were added to the dataset. *RAG1* has been used previously in *Phoxinus* phylogenetic and taxonomic studies (Palandačić et al. 2015), but without successful amplification from museum samples (Palandačić et al. 2017). Therefore, a second nuclear gene—*ITS1*—was added to the dataset. Because *ITS1* is short (350 bp in *Phoxinus*) and exist in cells in many copies, it is a promising candidate for such amplification. *RAG1* was amplified using the protocol of Palandačić et al. (2017), while *ITS1* was amplified using primers ITS1F (Wyatt et al. 2006) and ITS3R (Palandačić et al. 2010, following the protocol therein). Sequencing was performed in both directions at LGC Genomics (Berlin, Germany) and Microsynth (Vienna, Austria).

RAG1 sequences displayed heterozygous positions, and their gametic phase was determined using Phase 2.1 (Stephens et al. 2001; Stephens and Scheet 2005), implemented in DnaSP 5.10 (Librado and Rozas 2009). In *ITS1*, many sequences had a clean start, but exhibited double or even triple peaks at one or another point in the sequence. Even though studies have shown (e.g., Bos et al. 2007; Harrigan et al. 2008) that the coalescent-based Bayesian algorithm implemented in Phase is a reliable alternative to cloning, *ITS1* sequences exhibited a complicated structure, pointing to insertions or deletions (indels) and to the possibility of more than two haplotype variants per individual. Thus, 2−5 individuals from each species and each genetic lineage were cloned to resolve their gametic phase.

For cloning, PCR was repeated with the same *ITS1* primers but with high fidelity Platinum™ *Taq* DNA polymerase (Invitrogen). Initial denaturation, denaturation and extension followed Platinum *Taq* instructions, while for annealing, two temperatures were used: two cycles at 58 °C and 35 cycles at 52 °C. The PCR products were cleaned with enzymes Exo and Sap (Affymetrix), following the manufacturer's protocol, and cloned with the TOPO-TA© cloning kit (Invitrogen). Six clones were chosen from each sample, purified with the Qiagen PCR purification kit and sequenced with M13 universal primers in both directions by Microsynth Austria.

After cloning, the redundant clones (with identical haplotypes) were removed from the alignment. However, as suspected, more than two different haplotypes were detected within a single sample (Table S1, supplementary material). According to Buckler et al. (1997) and Bailey et al. (2003), there are several ways to distinguish between pseudogenes and functional copies in *ITS* sequences. Here, pseudogenes were identified by examining the sequences of the flanking regions (30 bp long at the 5′ end and 82 bp long at the 3′ end), which are the highly conservative *18S* and *5.8S* genes, where no single nucleotide polymorphisms (SNPs) are expected. Thus, the sequences that exhibited SNPs in the flanking regions were excluded as possible pseudogenes. Subsequently, cloned sequences, homozygotes and simple heterozygotes (with one-step indels or wobbles, which could be resolved by Phase; see above) were aligned with MAFFT V 7.305 on XSEDE (Katoh and Toh 2008) the CIPRES Science Gateway (version 3.3; 197; Miller et al. 2010). The alignment was adjusted manually, and is reported in the supplementary material. Following the coding protocol described in FastGap (Borchsenius 2009), gaps were coded as nucleotides. However, FastGap only codes gaps as present or absent, regardless of the nucleotide in that state. If the nucleotide in a certain state is present, it codes the nucleotide as A and a gap as C. Nevertheless, in the alignment, there might be present a third state, which Fastgap ignores. Thus, the gaps were inspected visually and missing signs (−) changed to a nucleotide not present in that state. If, for example, the three possibilities in a chosen state were −, A or G, the missing sign would be changed to one of the remaining possible nucleotides (C or T), hence gathering maximum information from the dataset.

Finally, unrooted minimum-spanning networks were constructed from *RAG1* and *ITS1* sequences with the median-joining algorithm (Bandelt et al. 1999) implemented in Network 5.1 (www.fluxus-engineering.com) with default settings.

Results

mtDNA

All museum and recent samples for which a genetic lineage was successfully determined (MEGA 6.0; Tamura et al. 2011) are reported in Table S1, including samples deposited in Genbank for which at least one mitochondrial gene and sample locality are available. For all the samples for which both genes (*COI* and *cytb*) are available (regardless of whether museum or recent), there were no incongruences in the genetic lineage detected. The main phylogenetic tree based on *COI* sequences showed good support for the clades, though it was unable to resolve the relationships among the lineages. Nevertheless, it confirmed the inclusion of museum samples in their respective clades and subclades (Fig. 1). The total number of samples for each lineage and the number of different haplotypes are reported in Table S2 (supplementary material). Based on these two analyses (lineage determination based on *cytb* and *COI* and

Fig. 1 Phylogenetic reconstruction of European *Phoxinus* based on *cytochrome oxidase I*. Phylogenetic tree constructed from the barcoding region of *COI* using Bayesian inference (BI) with BEAST 1.8.0 (Drummond et al. 2012). Branches carry posterior probabilities and bootstrap supports (BS) from the tree constructed with the Maximum-Likelihood (ML) method (PhyML; Guindon et al. 2010). The tree is shaded according to the value of posterior probabilities: the lighter the shade the weaker its support. Only posterior probabilities above 0.9 are shown. A lack of bootstraps originating from the difference between the BI and ML trees is denoted with −. Genetic lineages are presented in the diagram in the upper left corner. The genetic lineages, which are valid species, are written in black and the lineages, for which the species name is available but not valid, are in grey. For the genetic lineages, which were collected at a single sampling site, their locality is given. The outgroup consist of *Rhynchocypris lagowskii* (AP009147), *Tribolodon hakonensis* (AB626855) and *Oreoleuciscus potanini* (AB626851).
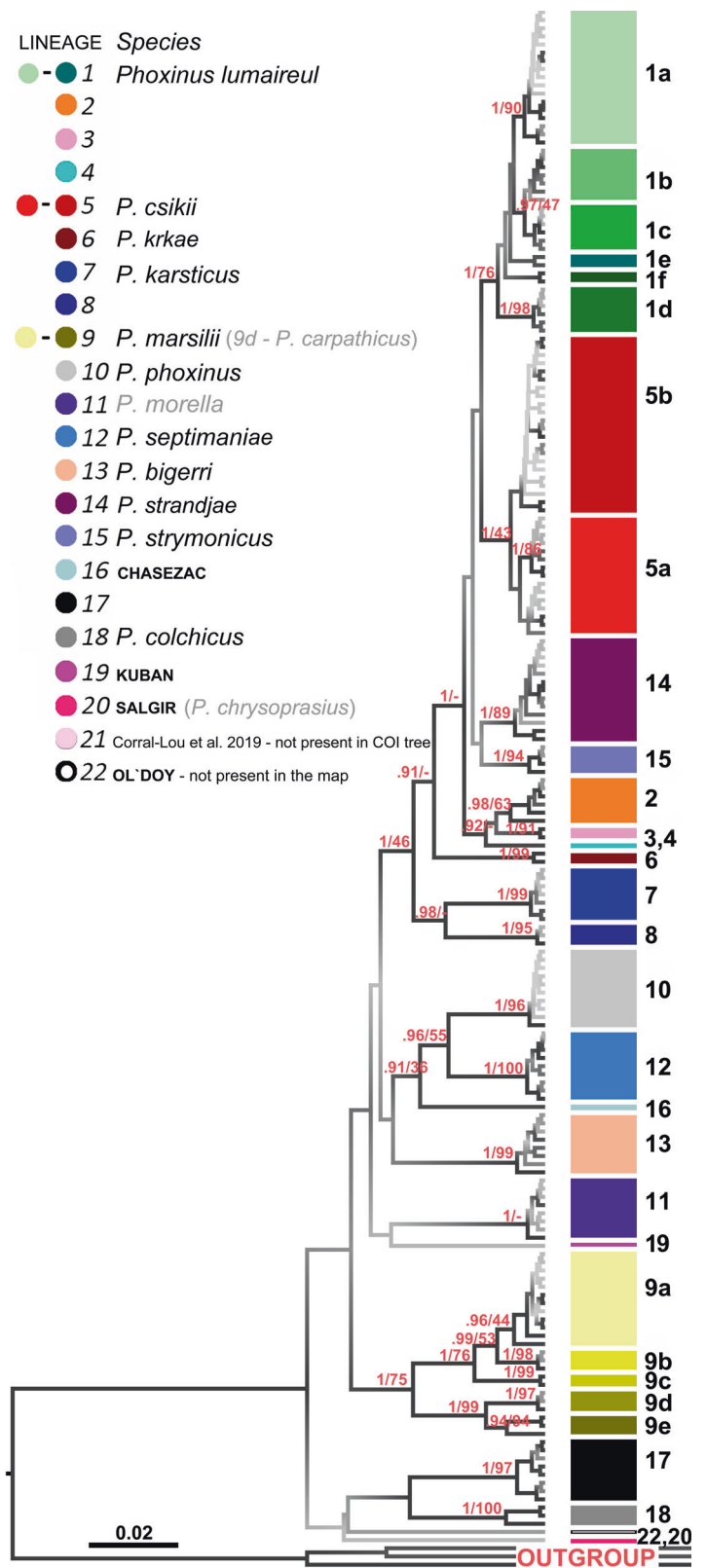
phylogenetic analysis based on *COI*), 1190 samples from 268 localities were used to create a map showing the current distribution of *Phoxinus* lineages (Fig. 2). Besides the 18 clades reported previously in Palandačić et al. (2017), four new ones were also identified: two from the samples newly analysed in the present study (19, Kuban, Russia; and 20,
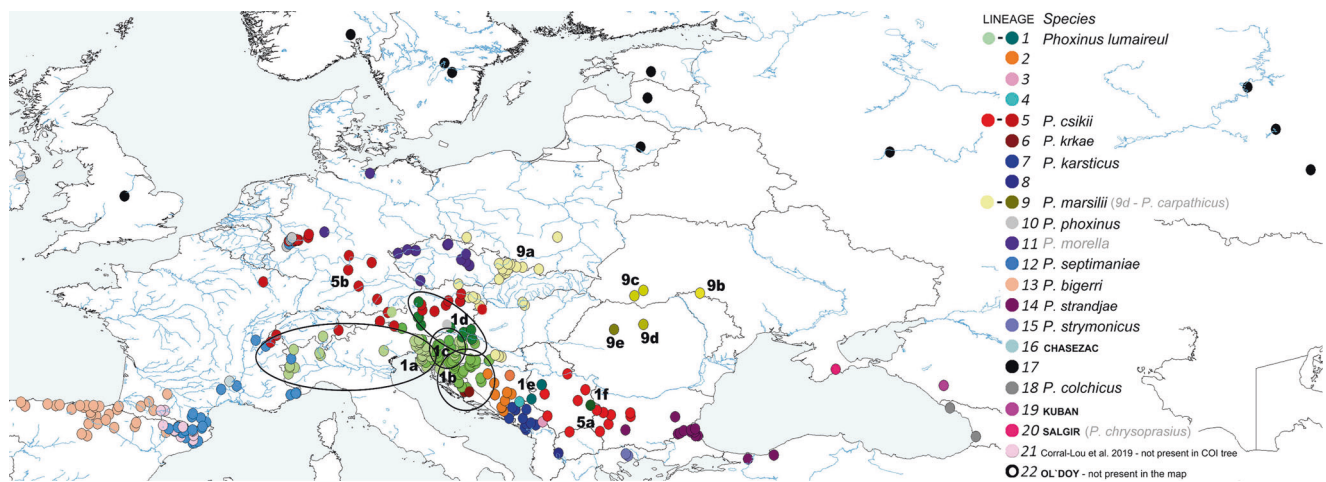
Fig. 2 Current distribution of European *Phoxinus* genetic lineages based on *COI* and *cytb*. All studies are included. For clarity, sub-clades are annotated with letters, 1a–f, 5a–b, and 9a–e and black circles represent the approximate currently known distribution of subclades 1a–d. Major river drainages are shown.
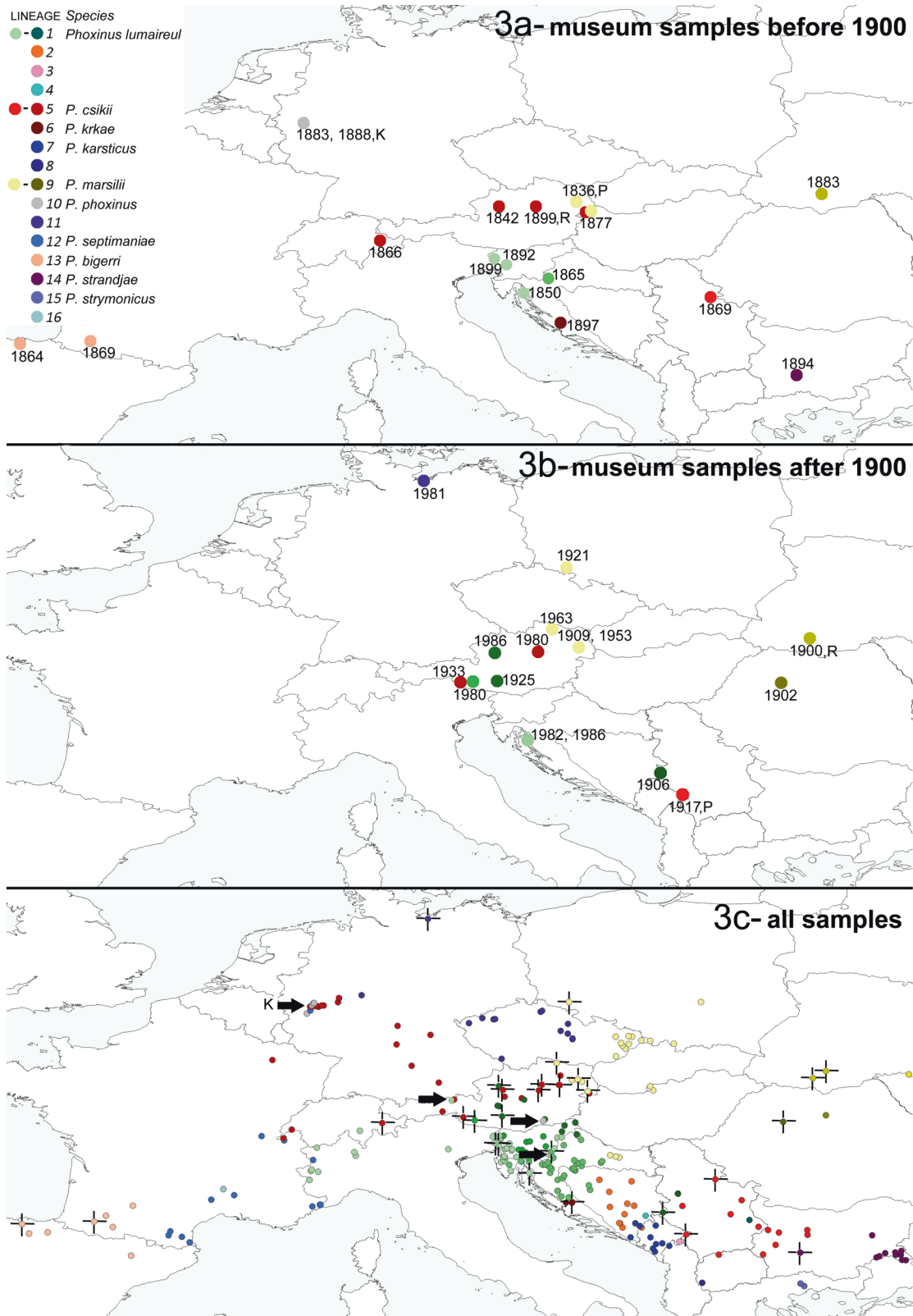
Salgir, Ukraine), and one from a recently published study (21, East Pyrenees mountain range, Corral-Lou et al. 2019). That last study was based upon variation at *cytb* and could not be included in the main *COI* phylogenetic tree. The fourth lineage (22, Ol'doy, Russia, Schönhuth et al. 2018) is also presented on the *COI* phylogenetic tree, though, because of its remoteness (eastern Russia) and because it is only one sample, it is absent from the map. In addition to new clades, three new subclades were also detected in clade 9 (9c, museum samples from Kolomyja, Ukraine; 9d, recent samples from Mures, Romania; and 9e, museum samples from Cluj, Romania). As seen from the previous studies, some clades and subclades represent species and potential species that are not taxonomically formalised. Thus, for a clearer understanding, the term genetic lineage will be used hereafter instead of the terms species, clade or subclade. Nevertheless, the species names are clearly depicted in all of the figures.

Of 186 museum samples, the genetic lineage of 64 (34%) was successfully determined. Because the congruence of overlapping parts of adjacent fragments is an important quality control check, only samples with two or more successfully amplified overlapping fragments were used for further analysis. Amplification of all four *COI* fragments was successful in 53 museum samples, while in another four, the first three adjacent fragments were sequenced. In one sample, the last three fragments were successfully sequenced. Partial amplification of *cytb* was successful in 19 of 83 museum samples, of which six were not represented by *COI* (Table S1). Table S1 also includes details on the sampling sites, the GPS coordinates, and Genbank number and references where applicable.

For clarity, the museum samples are presented in Fig. 3 in two groups: those collected before 1900 and those

collected subsequently. All museum samples are shown and those originating from previous studies are denoted with their respective reference. The oldest sample for which the genetic lineage was successfully determined originates from the year 1836 (Austria, Palandačić et al. 2017). In addition, lineages of 14 samples collected before 1900, from Spain, France, Switzerland, Austria, Slovenia, Croatia, Serbia, Bulgaria, Ukraine and Germany (Knebelsberger et al. 2015), were successfully determined (Fig. 3a). The detected genetic lineages generally correspond to those that are currently present in the same areas (Fig. 3a, c). Moreover, some of the localities of the museum samples collected before 1900 and of the recent samples are (almost) the same, as are those of the lineages detected at the following sites: (i) Spain in 1864 and 1869; (ii) Austria in 1836 and 1889 (Ramler et al. 2017); (iii) Slovenia in 1892 and 1899; (iv) and Croatia in 1850 and 1897. However, when comparing the recent samples with the lineages detected in the samples collected in Germany in 1883 and 1888 (Knebelsberger et al. 2015), in Austria in 1842, and in Croatia in 1865, additional lineages were detected in the recent samples (marked with black arrows in Fig. 3c). In the three samples collected in Austria in 1877, two were assigned to genetic lineage 9a (*P. marsilii*) and one to lineage 5b. For other museum sampling sites (Switzerland, 1866; Serbia, 1869; Ukraine, 1883; and Bulgaria, 1894), comparative recent material from those sites or in their vicinity was not available.

Figure 3b shows the locations of samples collected between 1900 and 2000. Those collected in Germany in 1981, Austria in 1953, 1963, 1980 (two samples) and 1986, and Croatia in 1982 and 1986 were subsequent to the time of massive fish introductions and are, from this respect, equivalent to recent lineages. Samples from Croatia and

LINEAGE *Species*
- 1 *Phoxinus lumaireul*
- 2
- 3
- 4
- 5 *P. csikii*
- 6 *P. krkae*
- 7 *P. karsticus*
- 8
- 9 *P. marsilii*
- 10 *P. phoxinus*
- 11
- 12 *P. septimaniae*
- 13 *P. bigerri*
- 14 *P. strandjae*
- 15 *P. strymonicus*
- 16

3a- museum samples before 1900

3b- museum samples after 1900

3c- all samples

Austria also exhibit the same genetic lineages as the recent samples, except for those collected in Austria in 1980, which belong to the lineage otherwise present in central

Slovenia (1c, light green), and samples collected in 1986, which belong to lineage 1d. This lineage is distributed in Austria also in the recent samples and in the samples

Phoxinus. This presents a closer view of Fig. 2, showing only the part of Europe for which the museum material is available. Genetic lineages were determined based on the mitochondrial genes *COI* and *cytb*. Lineages and attributed (currently valid) species are presented in the legend. Year of collection is noted beside each sampling site. Previous studies: K, Knebelsberger et al. 2015; P, Palandačić et al. 2017; R, Ramler et al. 2017. **a** Museum samples collected prior to 1900. **b** Museum samples collected after 1900. **c** All samples; museum samples denoted with a crosshair; arrows denote sample sites where fish introductions are identified.

collected in 1925, but further south. The samples collected in Germany in 1981 were classified to genetic lineage 11. However, no recent samples are available for comparison. There are also no recent samples for comparison with the one collected in Poland in 1921 (lineage 9a), the sample collected in Ukraine in 1900 (9c; Ramler et al. 2017), and the one in Romania in 1902 (9e). Samples collected in Montenegro in 1917 (Palandačić et al. 2017) were classified as lineage 5a together with the recent samples from that area, though no recent data from the proximity are available. Meanwhile, the samples collected in Bosnia-Herzegovina in 1906 (lineage 1e) correspond to the recent ones from Serbia (Fig. 3c), where both clades 1e and 5a are present.

At two sites, sampling has been undertaken several times over the last 200 years. In Vienna, samples were collected in 1836, 1909, 1953 and 2014, and all exhibited the same haplotype (lineage 9a). Krk Island, Croatia, was sampled in 1850, 1982, 1986 and 2000, the lineage detected from which was 1a, and has not changed, though the haplotypes were not the same. In three samples from central Germany (Durkheim, collected in 1838), the complete *COI* was amplified successfully. However, the samples clustered into a clade together with Lake Ohrid (North Macedonia) samples. The same result was observed when amplifying *cytb* and *ITS1*, indicating a mistake in the labelling; these samples were thus excluded from the analysis.

nDNA

For the gametic phase determination, 97 1258-bp-long *RAG1* sequences were used, 55 of which were new, and 107 downloaded from Genbank. However, some of the Genbank sequences (Schönhuth et al. 2018) included unknown nucleotide bases (N), and were thus omitted from further analysis. In addition, some of the calculated haplotypes exhibited low probabilities for several heterozygous positions and were also excluded. Finally, only the haplotypes that had no ambiguous sites, and no more than one heterozygous position determined with a probability of less than 0.9, were included in the network construction. Altogether, the median-joining network was constructed from 140 sequences (280 resolved haplotypes) representing 27 of 33 genetic lineages

known in *Phoxinus*. The *RAG1* network (Fig. 4a) presented an unclear structure, with the lineages based upon mtDNA forming few identifiable groups (lineages 3, 4, 6, 7 + 8, 14, 15 and 18). The haplotypes of other well-resolved mtDNA lineages are scattered across the network, with the haplotypes from samples as distant from each other as lineages 13 (Iberian Peninsula) and 19 (Kuban River, Russia) clustering together. The newly detected lineages, first reported in the present paper, are marked with arrows.

The *ITS1* dataset included 290 cloned sequences, homozygotes and simple heterozygotes, representing 13 of the *Phoxinus* genetic lineages. Of those, 23 samples of lineages 1a, 2, 3, 5a, 6, 7, 8 and 9a were successfully cloned (Table S1), while clades 4, 11, 12, 13 and 14 were represented by homozygotes and simple heterozygotes (with cloning unsuccessful). Cloning of museum samples failed, though homozygotes and simple heterozygotes were successfully sequenced. In the *ITS1* network, the lineage grouping is familiar and confirms the lineages detected by mtDNA. However, due to technical problems (unsuccessful cloning, insufficient pseudogene designation, gap coding), the results for that network are not very reliable.

Discussion

The distribution of species in the genus *Phoxinus* does not correspond to river drainages or even sea basins, an unusual pattern for leusciscins (Gómez and Lunt 2007; Perea et al. 2010; but see Levin et al. 2017). Thus, the question arises of whether this distribution is natural or anthropogenic in origin. According to Museth et al. (2007), human-assisted introduction of *Phoxinus* species in Norway had already taken place in the late nineteenth century, mostly in the southern part of the country, with translocations becoming more frequent after 1950. Miro and Ventura (2015) report that the first introductions of *Phoxinus* in the Pyrenees took place after 1970. In the present study, the genetic lineage of 14 samples collected prior to 1900 and eight samples collected between 1900 and 1950 (Fig. 3a, b) were analysed, and the distribution of the detected lineages is comparable to those present in the same areas today. Moreover, in the samples collected in Austria in 1877, two bordering lineages (5b and 9a) were detected, pointing to a natural co-occurrence of two lineages. Nevertheless, as detected in Germany (Knebelsberger et al. 2015), the results point to fish introductions also in Austria and, possibly, Croatia. For some of the museum samples, comparison with recent samples was not possible. However, their inclusion helps expand the knowledge on some of the species ranges. Thus, combining museum and recent material proves to be a good strategy for better understanding species ranges, especially in genera where fish introductions are common.

a: RAG1

b: ITS1
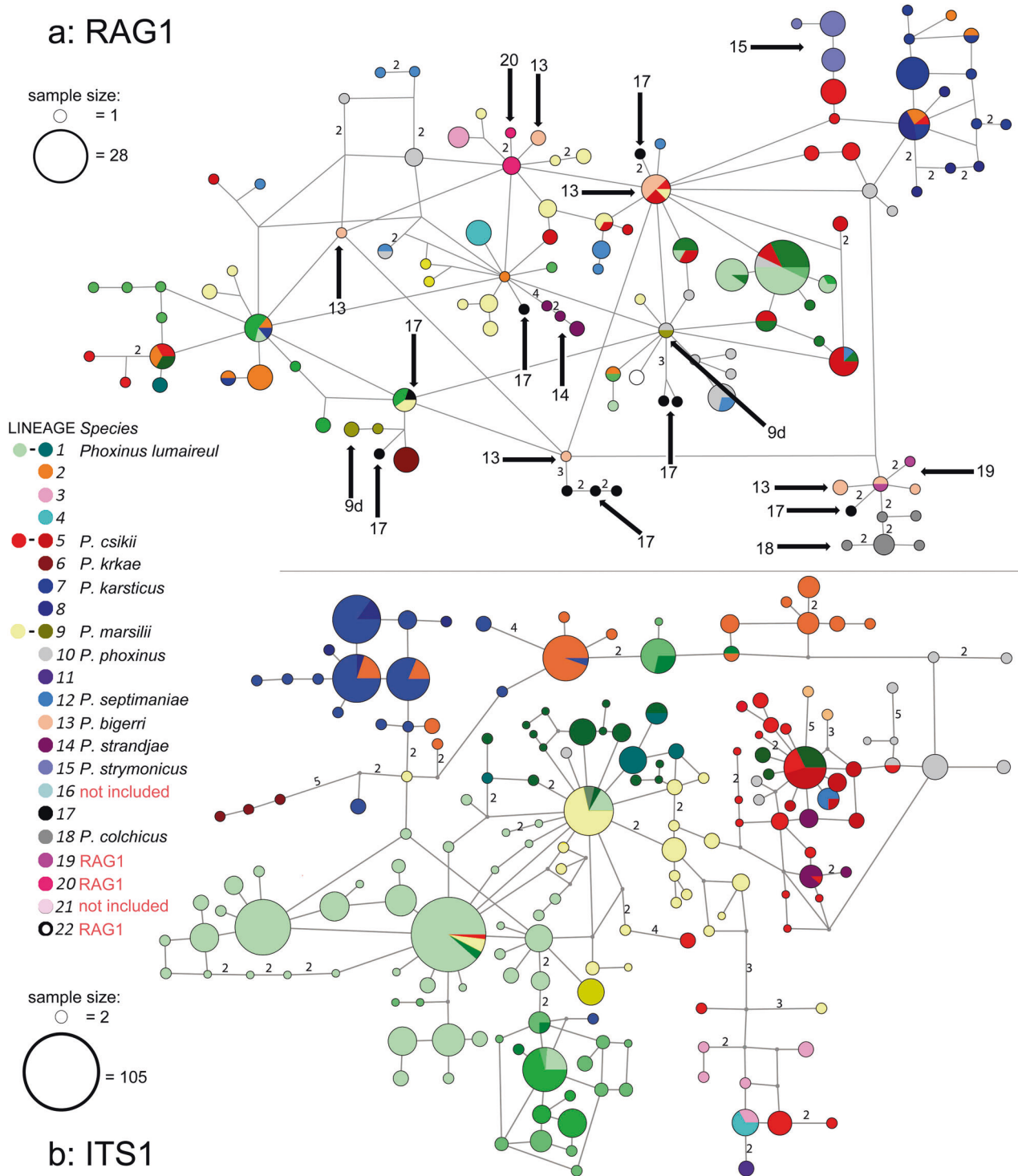
Understanding the distribution of European *Phoxinus* using a combination of museum and recent material

In the last two decades, considerable efforts have been made to inventorise the number of genetic lineages comprising the European *P. phoxinus* species complex (Kottelat and Freyhof 2007; Bianco 2014; Palandačić et al. 2015, 2017; Corral-Lou et al. 2019, Bogutskaya et al. 2019). Even so, the exact number remains unknown and their taxonomic formalisation incomplete. In the present study, museum material has helped to confirm the distribution areas of some *Phoxinus* genetic lineages that are probably a consequence of natural

processes, and highlight the areas where fish introduc-
tions have taken place.

In the Balkan Peninsula, an unusual distribution of
Phoxinus lineages has been reported previously (Palandačić
et al. 2015, 2017), attributed to migration of fishes through
underground water connections characteristic of karst
landscapes, reported also in other studies (Borowsky and
Mertz 2001; Dillman et al. 2010; Palandačić et al. 2012).
Yet, the pattern being a consequence of stocking could not
be excluded. Here, museum material collected in Serbia
(1869), Bosnia-Hercegovina (1906) and Montenegro (1917)
seems to confirm the patchy distribution of lineages 1e
(dark green) and 5a (red) in the central Balkans area even
before most of the massive introductions took place. The
results appear to support the hypothesis that the unique
species distributions of *Phoxinus* in the karst areas are a
result of natural processes (Palandačić et al. 2015).

On a larger scale, the museum samples used in the pre-
sent study confirm the distribution of seven genetic lineages
(1d, 1e, 5a, 5b, 9a, 9c, 9d) in the Danube drainage, where at
least 17 lineages (Table S1: 1a–f, 2, 3, 4, 5a–b, 9a–e and 11)
have been detected previously. These lineages are dis-
tributed discretely in the upper, the middle, and the lower
Danube, and in left and right tributaries (Figs. 2 and 3).
Museum samples confirm the distribution of lineage 13
from north-west Spain to south-west France, in congruence
with earlier studies (Kottelat 2007; Geiger et al. 2014).
Moreover, the samples collected in Pau, France, in 1869 are
in close proximity to (about 50 km from) the type locality of
P. bigerri (lineage 13; Adour River in Tarbes), supporting
its historical distribution in this area. In addition to museum
material, recent samples provide further information on the
ranges of *Phoxinus*, mostly for lineage 9, which is dis-
tributed in the left tributaries of the middle and lower

Danube, and lineage 17, which seems to be spread across
northern Europe all the way to the Ural Mountains. Besides
adding new data on the distribution of previously recog-
nised lineages, two new lineages (19 and 20) were detected
among the recent samples included in the present study.
Lineage 19 is located in Kuban, Russia, while lineage 20 is
from Salgir, Crimea. For the latter, a species name is
available, *P. chrysoprasius* (Pallas 1814). However, the
lineage is not well supported by the *RAG1* network. In
contrast, lineage 9d, for which a name is also available
(*P. carpathicus* Popescu Gorji and Dimitriu 1950; type
locality, Lake Rosu (Red Lake), Romania), is slightly
supported in the *RAG1* network (Fig. 4a).

While the threat of fish introductions is more pronounced
with commercially interesting fish species (Sušnik et al.
2004; Jug et al. 2005), the co-occurrence of minnows with
salmonids caused the introduction of the former across
much of Europe (Kottelat 2007; Kottelat and Freyhof 2007;
Museth et al. 2007). Furthermore, the Rhine–Main–Danube
canal and the Rhone–Rhine canal connect together most of
the biggest river drainages on the continent. In congruence
with this, Knebelsberger et al. (2015) found four different
Phoxinus species or clades in the River Rhine, with some
introduced from the Danube. Meanwhile, Corral-Lou et al.
(2019; see also the references therein) report the introduc-
tion of *P. septimaniae* in north-east Spain, while, in Croatia,
mixed haplotypes in one Adriatic river have been attributed
to water exchange serving a reversible power plant (Vučić
et al. 2018). In general, introductions of *Phoxinus* are well
recorded (Aparicio et al. 2000; Kottelat 2007; Kottelat and
Freyhof 2007; Museth et al. 2007; Schreiber and Sosat
2007; Benejam et al. 2010; Maceda-Veiga et al. 2010;
Knebelsberger et al. 2015; Miro and Ventura 2015), but, in
contrast, only two cases of fish introduction are suggested
here (lineages 1a and 10 in Austria, and lineage 1a in
Croatia), pointing to a lesser effect of fish introductions on
European *Phoxinus* than reported in the previous studies.
Nevertheless, these results could be influenced by the lim-
itations of museum material (e.g., number of samples ana-
lysed, see section 'Usefulness of museum samples in
species distribution studies' and Table S1). Thus, con-
servation of *Phoxinus* autochthonous populations should
be taken seriously, especially because detailed studies of
the genus throughout Europe are still missing. As seen in
the present and previous studies, every time new data are
reported, new genetic lineages of European *Phoxinus*
emerge. In addition, the use and interpretation of nuclear
markers has been challenging, with most studies (including
the present one, see the section 'Some comments on nuclear
markers used') relying on mitochondrial data. Thus, more
studies, mostly concerning northern European discharges,
are needed to finalise species delimitation and form con-
servation guidelines. Meanwhile, fish management should

be more environmentally protective. Supplemental stocking (if needed at all) should be conducted with care, and introduction of non-target (or by-product) species should be prevented.

Usefulness of museum samples in species distribution studies

The present study confirms the usefulness of museum material (some of which is more than 180 years old) to gain a better understanding of species distributions, especially in cases where fish introductions have been common. Nevertheless, using museum material does present challenges. In large, old museum collections, such as at the Natural History Museum Vienna, mislabelling of specimens can occur (previously reported for this collection by Bogutskaya and Zupančič 1999) and was also detected in the present study. The population that, according to the museum label, originated from central Germany clustered with Lake Ohrid samples based upon analysis of three different genes, indicating that the label information needs to be considered with caution (see also Boessenkool et al. 2010; Rawlence et al. 2014; Paterson et al. 2016). Further, successful amplification of fragments depends upon the preservation methods used for museum samples (Hall et al. 1997), while the exact method is usually unknown. In fishes, conservation methods can be distinguished, as alcohol-fixed specimens possess white eyes, while formalin-fixed fishes have clear black eyes (De Bruyn et al. 2011). Here, most (175 out of 186) of the specimens analysed were alcohol-fixed, yet, the genetic lineage was successfully determined in only one-third of individuals. Finally, degradation and fragmentation of DNA —resulting in a low success rate despite a large amount of work—is the reason that most studies use only a few loci when analysing museum samples (Burrell et al. 2014). This is especially true for nuclear loci, as they are typically less variable than mtDNA and longer stretches of DNA are needed for the analysis. Correspondingly, analysis of nuclear loci was the most challenging in the present study.

Some comments on nuclear markers used

Because of several cases of hybridisation or introgression, informative nuclear markers are crucial for studying the phylogeny of the *P. phoxinus* species complex. As found previously (Palandačić et al. 2017), *RAG1* could not be amplified in museum samples. Moreover, it showed only limited resolution in species delimitation investigations (Palandačić et al. 2015; Corral-Lou et al. 2019). In the present study, *RAG1* analysis was challenging (see Results), and the network that was constructed was weakly resolved, with many cross-connections among the haplotypes (Fig. 4a). Most notably, despite inclusion of more samples,

representing almost all the genetic lineages detected with mtDNA analysis (27 of 33), no more information became available, with haplotypes from otherwise distant and well-resolved lineages scattered across the network (see, e.g., lineages 13 and 17 in Fig. 4). Due to the problems with *RAG1* analysis, it is hard to say whether these incongruities are a consequence of artefacts of the sequencing or gametic phase determination, or of the lack of signal, or both. The second nuclear marker—*ITS1*—seems more promising as it is short (350 bp) and sequencing from museum material was successful. In addition, it has previously been used for barcoding in fungi and plants (Schoch et al. 2012; Cheng et al. 2016) and for testing for hybridisation in fishes (Wyatt et al. 2006; Hamilton and Tyler 2007). Indeed, in comparison to the *RAG1* network, the *ITS1* network presented better resolution (support) for the genetic lineages detected with mtDNA (Fig. 4b), especially when large numbers of samples were sequenced (e.g., lineage 1a). However, several outliers (possible pseudogenes in, e.g., lineage 13) pointed to unsuccessful amplification or detection, or both, of functional *ITS1* copies. Thus, the weaknesses with the use of *ITS1* as a marker, resulting from concerted evolution, unequal crossing-over, and existence of pseudogenes and multiple paralogues (Arnheim 1983; Bayly and Ladiges 2007) are apparent. It was not an aim of the present study to evaluate *RAG1* and *ITS1* as markers, but it can be deduced that neither is appropriate for species delimitation and both have limited power for detecting hybrids in European *Phoxinus*.

With new techniques on the rise, the limitations of using PCR-based methods on degraded museum material might be overcome by using DNA capture hybridisation and whole genome sequencing methods (Bailey et al. 2016; Souza et al. 2017), thus enabling the use of many nuclear markers and whole mitochondrial genomes (Mason et al. 2011). As museum specimens are unique biological material and their existence represents a finite resource, sampling is better justified with material being analysed with the latest (and thus most promising) techniques. Nevertheless, using those methods for extracting molecular data in a cost-effective way may still represents an obstacle (Knyshov et al. 2019). Thus, this study shows that even with Sanger sequencing and a limited number of markers, a combination of museum and recent material can provide valuable insights for the conservation of fishes.

Data archiving

All sequences are available under Genbank accession numbers MN820726−MN820819, MN816034−MN816147, MN818456−MN818521, MN818001−MN818455. In addition, the datasets supporting this article have been uploaded as supplementary material (Table S1, *ITS1* aligned fasta file).

Compliance with ethical standards

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

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