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Distribution of Eurasian minnows (*Phoxinus*: Cypriniformes) in the Western Balkans

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Abstract – The Eurasian minnows of the genus *Phoxinus* are small cyprinid species, widely distributed across Europe and Asia. Currently, there are at least 15 species in *Phoxinus*, with preliminary data suggesting more to be described. Despite the discernible increase in research integrating molecular phylogenetic approaches with traditional taxonomy and systematics, inter- and intraspecific relationships in the genus *Phoxinus* are still poorly known. The aims of this research were to: (i) compile data on the distribution of *Phoxinus* species in Croatia and Bosnia and Herzegovina from the sampling conducted by authors from 2006 to 2016 and from literature sources, and (ii) provide a detailed insight on distribution ranges of genetic clades and species of *Phoxinus* in the western Balkans. Additional 118 localities in Croatia and 8 locations in Bosnia and Herzegovina were added to already known 160 localities from the literature data. Molecular analyses of mitochondrial DNA indicate: (i) the presence of *Phoxinus marsilii* in Croatia (Drava drainage, the Papuk Mountain), around 260 km south of its known distribution range in Hungary, and (ii) an exceptional genetic variability of *P. lumaireul* in the Western Balkans.

Keywords: *Phoxinus marsilii* / *Phoxinus lumaireul* / cryptic species / distribution range / cytochrome b

Résumé – Répartition des vairons d'Eurasie (*Phoxinus*: Cypriniformes) dans les Balkans occidentaux. Les vairons eurasiens du genre *Phoxinus* sont de petites espèces de cyprinidés, largement répandues en Europe et en Asie. À l'heure actuelle, il y a au moins 15 espèces de *Phoxinus*, et des données préliminaires suggèrent d'autres espèces à décrire. Malgré l'augmentation perceptible de la recherche intégrant les approches phylogénétiques moléculaires avec la taxonomie et la systématique traditionnelles, les relations interspécifiques et intraspécifiques dans le genre *Phoxinus* sont encore mal connues. Les objectifs de cette recherche étaient les suivants: i) compiler des données sur la répartition des espèces de *Phoxinus* en Croatie et en Bosnie-Herzégovine à partir de l'échantillonnage effectué par les auteurs de 2006 à 2016 et de sources bibliographiques, et ii) fournir un aperçu détaillé des aires de répartition des clades génétiques et des espèces de *Phoxinus* dans les Balkans occidentaux. 118 localités supplémentaires en Croatie et 8 localités en Bosnie-Herzégovine ont été ajoutées aux 160 localités déjà connues d'après les données de la littérature. Les analyses moléculaires de l'ADN mitochondrial indiquent: i) la présence de *Phoxinus marsilii* en Croatie (bassin de la Drava, montagne Papuk), à environ 260 km au sud de son aire de répartition connue en Hongrie, et ii) une variabilité génétique exceptionnelle de *P. lumaireul* dans les Balkans occidentaux.

Mots-clés : *Phoxinus marsilii* / *Phoxinus lumaireul* / espèces cryptiques / aire de répartition / cytochrome b

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1 Introduction

The genus *Phoxinus* has a wide distribution range throughout the Palaearctic region (basins of Atlantic, North and Baltic Seas, the Arctic and the northern Pacific Ocean), from the Ebro drainage in Spain eastward to Anadyr and Amur drainages in Russia and China. Since the beginning of the 20th century, minnows have expanded outside their native distribution range, especially in the mountain areas of Scandinavia where they were used as live bait (Museth *et al.*, 2007) and Pyrenees as a by-product of trout stocking (Miró and Ventura, 2015). Presently, the Eurasian minnows of the genus *Phoxinus* include at least 15 species (Eschmeyer *et al.*, 2017) adapted to cold and well-oxygenated waters of fast-flowing mountain streams, large lowland rivers and lakes (Kottelat and Freyhof, 2007).

Besides type species *Phoxinus phoxinus* (Linnaeus, 1758), following species of *Phoxinus* in Europe were described based on morphological characters: *Phoxinus lumaireul* (Schinz, 1840), *Phoxinus colchicus* Berg, 1910, *Phoxinus strandjae* Drensky, 1926, *Phoxinus biggeri* Kottelat, 2007, *Phoxinus septimaniae* Kottelat, 2007, and *Phoxinus strymonicus* Kottelat, 2007 (Kottelat and Freyhof, 2007). According to Kottelat and Freyhof (2007), two *Phoxinus* species inhabit the Western Balkans, with Croatia and Bosnia and Herzegovina as its parts: (i) *P. phoxinus* in the Danube drainage, and (ii) *P. lumaireul* in the Adriatic basin. However, genetic evidence from Palandačić *et al.* (2015, 2017) suggested several new species in the Western Balkans. Also, morphological examination of specimens collected in Italy, Croatia, Bosnia and Herzegovina, Montenegro and Albania led to description of *Phoxinus ketmaieri* Bianco and De Bonis, 2015, *Phoxinus likai* Bianco and De Bonis, 2015, *Phoxinus apollonicus* Bianco and De Bonis, 2015, and *Phoxinus karsticus* Bianco and De Bonis, 2015.

Although Croatian and Bosnian karstic area is known for its high diversity and endemism of freshwater ichthyofauna (Jelić *et al.*, 2016), three of four species described by Bianco and De Bonis (2015) cannot be validated using molecular species delimitation analyses (Palandačić *et al.*, 2017). Two of them (*P. ketmaieri* and *P. likai*) were synonymised with *P. lumaireul*, whereas *P. apollonicus* was synonymised with *P. karsticus* (Palandačić *et al.*, 2017). Also, the 8% genetic divergence between *P. lumaireul* and *P. phoxinus* on cytochrome c oxidase subunit 1 (COI) supports revalidation of *P. lumaireul* by Kottelat (2007) and rejects synonymization of *P. lumaireul* and *P. phoxinus* made by Bianco and De Bonis (2015).

The morphological examination of Eurasian minnows is a challenging task due to phenotypic plasticity of the group (Ramler *et al.*, 2017), the lack of holotype specimens and the existence of cryptic species. Cryptic species within the genus *Phoxinus* are morphologically indistinguishable and molecular taxonomy studies could provide efficient means for uncovering cryptic species diversity (Palandačić *et al.*, 2017). Within the European *Phoxinus*, morphological characters which are traditionally used in taxonomy, cannot provide clear phylogenetic information, and sometimes disagree in validity of some *Phoxinus* species (e.g. *P. lumaireul*) (Palandačić *et al.*, 2017). Morphology of *Phoxinus* is very affected by habitat type and

intraspecific differences in body shape that can be large as those observed on interspecific level. Intraspecific morphological differences can be linked to different swimming modes and foraging in distinct habitats such as lakes and streams (Ramler *et al.*, 2017; Collin and Fumagalli, 2011).

The lack of holotype specimen of type species *P. phoxinus* was resolved by creating its neotype from the Agger River (Rhine drainage) in Germany (Kottelat, 2007). However, recent barcoding of fishes in rivers Rhine and Weser indicated the existence of two divergent *Phoxinus* lineages (Knebelberger *et al.*, 2014). Moreover, the mixing zone of four lineages was detected in the same area in Germany (Palandačić *et al.*, 2017). The designations of many *Phoxinus* species as synonyms of *P. phoxinus* (e.g. the list of synonyms provided by Kottelat, 2007) become questionable because analysed material originated from hybrid zone. However, there is a good example of research (Palandačić *et al.*, 2017) which combines analyses on molecular data and morphology to resolve taxonomic problems in *Phoxinus*; the DNA analyses of museum material enabled re-establishing two species: *Phoxinus csikii* Hankó, 1922 from the Bijelo polje in Montenegro, and *Phoxinus marsilii* Heckel, 1836 from the Danube River near Vienna.

The high level of diversity and complex phylogeography of species which co-exist with the Eurasian minnows in the Balkans (Klobučar *et al.*, 2013; Buj *et al.*, 2008; Mamos *et al.*, 2016; Vitecek *et al.*, 2017; Simonović *et al.*, 2017), together with dynamic geology (Karamata, 2006), suggest that the “Balkan minnow” represents heterogeneous complex (Palandačić *et al.*, 2015, 2017). In line with this, the aims of this research were to: (i) collect all available literature data on distribution of *Phoxinus* in Croatia and Bosnia and Herzegovina, and conduct targeted sampling for new and missing data, (ii) compare the compiled data in the light of recent molecular research (Palandačić *et al.*, 2015, 2017) and (iii) provide an insights of the present situation within the genus *Phoxinus* in Western Balkans, using new sequences of mitochondrial DNA obtained in the present study.

2 Materials and methods

2.1 Data collection

The present study comprises data collected by the authors from 2006 to 2016 (Tab. S1), and all available literature data on species of *Phoxinus* in Croatia and Bosnia and Herzegovina (Tabs. S1 and S2).

The sampling of *Phoxinus* was conducted by hand net sampling and electrofishing method. Electrofishing was performed with Hans Grassl EL63 IIGI (300/600 V, 5000 W) and SAMUS 725 MP (max. 1000 V, 650 W; Samus Special Electronics, Poland). Sampling was performed for 20 min on a 100 m transect upstream from the previously chosen starting point. All types of habitats were covered on every site. The collected specimens were determined as members of the genus *Phoxinus* using morphological characters described in Kottelat and Freyhof (2007). Further identification of species was based on sequences of mitochondrial gene for cytochrome b (cyt-b).

Literature data were collected from the following references: Heckel and Knerr (1858), Seeley (1886),

Steindachner (1866), Glowacki (1885, 1896), Brusina 1892, Hirc (1896), Plančić (1956), Sabioncello (1967), Pažur (1969), Vuković and Ivanović (1971), Sofradžija (1981, 2009), Leiner (1985, 1998), Tvrtković (1985), Bianco (1986), Lelek (1987), Mrakovčić and Mišetić (1988), Meštrov *et al.* (1989a, b), Fašaić *et al.* (1990), Delić (1993), Mrakovčić *et al.* (1996, 2007, 2010, 2014), Trgovčić (2003), Kottelat (2007), Sallai and Mrakovčić (2007), Primc Habdija *et al.* (2008), Habdija *et al.* (2008), Delić *et al.* (2009), Buj (2010), Jalžić *et al.* (2010), Miočić-Stošić *et al.* (2012), Valić *et al.* (2013), Palandačić *et al.* (2015) and Simonović *et al.* (2015) (Tabs. S1 and S2).

2.2 Molecular analyses

Total genomic DNA was isolated from pectoral fin tissue using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) and following the manufacturer protocol. DNA was stored on -20°C until the polymerase chain reaction (PCR) was conducted. Fragments of the *cyt-b* gene were amplified using primer pair GluF and ThrR (Machordom and Doadrio, 2001), following cycling conditions as described in Perea *et al.* (2010). PCR reactions were performed using HotStarTaq Master Mix Kit (Qiagen), in a total volume of $50\ \mu\text{L}$ comprised of 2.5 U HotStarTaq DNA Polymerase, 1.5 mM MgCl_2 , 200 μM each dNTP, 0.2 μM of each primer and 20 ng of DNA template. Purification and sequencing of the PCR products were prepared by Macrogen Inc. (Seoul, South Korea) using the same primers used for gene amplification. Purified PCR products were sequenced on ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, USA). Sequence trace files were analysed using SEQUENCHER (version 5.3; Gene Codes Corp., Ann Arbor, USA).

Phylogenetic analyses were conducted using the *cyt-b* sequences obtained in the present study and the available sequences of *Phoxinus* (1091 BP) from Croatia, Bosnia and Herzegovina and neighbouring countries which were downloaded from the GenBank (Tab. S1; Palandačić *et al.*, 2015). New sequences were deposited in the GenBank under accession numbers shown in Table S1. Phylogenetic relationships were inferred using two methods (Maximum Likelihood – ML and Bayesian Inference – BI). The Tamura-Nei (TrN) model (Tamura and Nei, 1993) with a gamma-distributed rate variation among sites (+G) was selected as the best-fit evolutionary model using the Bayesian information criterion (BIC) in jModelTest2 (version 2.1.6; Darriba *et al.*, 2012). The ML was run in RAxML-HPC2 Workflow on XSEDE (version 8.2.8; Stamatakis, 2014) on the Cipres Science Gateway (version 3.1; <http://www.phylo.org>; Miller *et al.*, 2010) with optimised parameters. ML analysis was conducted with 200 search replicates to find the ML tree and 1000 nonparametric bootstrap replicates, using MrBayes 3.2 (Ronquist *et al.*, 2012) on the Cipres Science Gateway. Two independent runs with four MCMC chains were run for 50 million generations and sampled every 5000 generations. Temperature parameter was set to 0.2 and the first 12.5 million generations were discarded as burn-in. The effective sample sizes of parameters were screened using TRACER 1.5 (Drummond and Rambaut, 2007) whereas the convergence of runs was checked using AWTY

(Nylander *et al.*, 2008). Nodes in the phylogenetic tree which have bootstrap values $P \geq 70$ in ML, and posterior probabilities (pp) values ≥ 0.95 in BI were considered supported.

In cases of uncertain determinations of genetic clades in phylogram, Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to obtain their systematic position.

The Median-Joining (MJ) haplotype networks were used to infer the intraspecific relations (Bandelt *et al.*, 1999). The MJ networks were constructed using PopART (Population Analysis with Reticulate Trees) v1.7 (Leigh and Bryant, 2015).

The number of base substitutions per site from estimation of net average between sequence groups in *P. lumaireul* was calculated using the TrN + G model in MEGA6 (Tamura *et al.*, 2013). Standard error estimates were obtained by a bootstrap procedure (1000 replicates).

All of the data were analysed with ESRI ArcInfo GIS v10.2.2 software (ESRI, 2014) to plot distribution ranges of *Phoxinus* species in Croatia and Bosnia and Herzegovina.

3 Results

3.1 General overview on the distribution of *Phoxinus* in Croatia and Bosnia and Herzegovina

In this paper, we present the distribution of *Phoxinus* in Croatia and Bosnia and Herzegovina, in rivers which are part of the Black Sea basin (rivers Sava, Drava and Danube) and Adriatic basin. Altogether 126 new localities were sampled, with 118 in Croatia and 8 in Bosnia and Herzegovina (Fig. 1, Tab. S1). From 160 localities cited in the literature, 133 are from Croatia and 27 are from Bosnia and Herzegovina (Fig. 1, Tabs. S1 and S2). From a total of 286 localities, 73 sites were positioned in the Adriatic basin and 213 sites were in the Black Sea basin (Fig. 1).

The Eurasian minnows were recorded in small streams on the slopes of continental mountain systems in Croatia with peaks up to 953 m above sea level (Papuk, Bilogora, Krndija, etc.; Drava and Sava drainages). Minnows were also recorded in the fast flowing, well-oxygenated lowland streams, tributaries of the largest Croatian rivers Drava and Sava (near Varaždin in northern Croatia and the Banovina region in central Croatia, respectively). Specimens morphologically determined as *Phoxinus* were also found in the upper courses of Istrian rivers in the Adriatic basin (Mirna, Raša), as well in the rivers Zrmanja, Krka and Neretva and their tributaries (Dalmatia region, southern Croatia, Adriatic basin). Analysis of literature and sampling data showed that the Eurasian minnows are distributed in the upper and middle courses of large rivers in Bosnia and Herzegovina, such as Una, Drina, Bosna and Vrbas, as well in their smaller tributaries (Sava drainage). Species of *Phoxinus* were absent from the lower courses of Sava, Drava and Danube. There is only one literature record of *Phoxinus* sp. in the lower course of Sava River, the largest tributary of the Danube River in Croatia and Bosnia and Herzegovina (Brusina, 1892). Specimens of *Phoxinus* were not recorded in lower courses of rivers Mirna and Raša (Adriatic basin, Istra region, Croatia), the Cetina River in the middle part of Dalmatia in Croatia (Adriatic basin), as well as in several large karst fields (poljes) in the

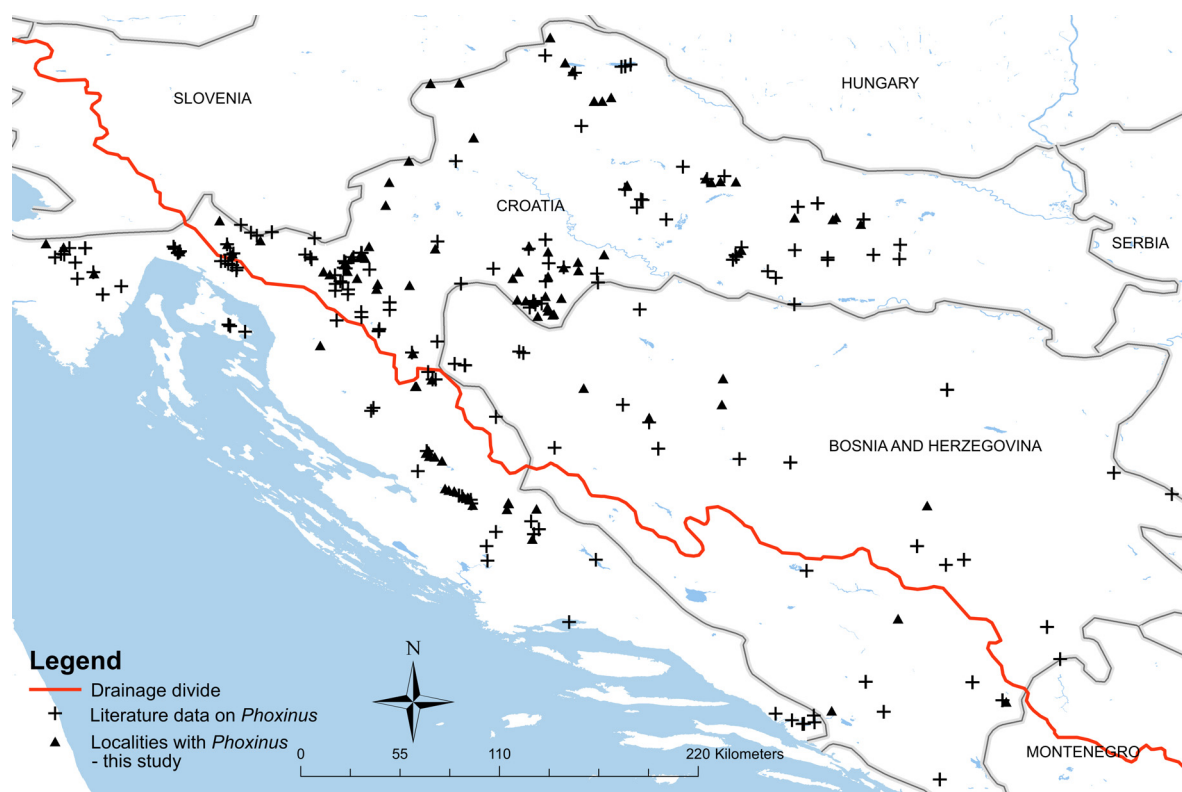


Fig. 1. Distribution of *Phoxinus* (Cypriniformes: Cyprinidae) in Croatia and Bosnia and Herzegovina. New localities from the sampling conducted by authors of the present study are presented by triangles, whereas localities reported in the literature are marked by crosses. The drainage divide between the Black Sea basin and the Adriatic basin is indicated by the thick red line.

Dinaric Alps, such as Duvanjsko, Livanjsko and Grahoračko polje (Adriatic basin, Bosnia and Herzegovina). The species of *Phoxinus* were also not found in other small and isolated karst poljes, such as Mostarsko blato, Nevesinjsko polje, Dabarsko polje, Fatničko polje (Neretva drainage, Adriatic basin, Bosnia and Herzegovina), Drniško polje (Čikola River system, Krka drainage, Adriatic basin, Croatia), Stajničko polje, Drežničko polje, and Crnac polje (Dobra River system, Sava drainage, Croatia).

3.2 Distribution of genetic clades/species of *Phoxinus* in Croatia and Bosnia and Herzegovina

Phylogenetic reconstruction (Fig. 2) combined with the BLAST search resulted in detection of *P. marsilii*, *P. lumaireul* (clades 1a, 1b, 1c and 1d), *P. karsticus*, Clade 2: *Phoxinus* sp. 1 *sensu* Palandačić *et al.* (2017), and Clade 6: *Phoxinus* sp. 4 *sensu* Palandačić *et al.* (2017).

P. marsilii was recorded in Croatia for the first time (Drava drainage). In total eight new haplotypes of *P. marsilii* were detected in three populations on the northern slopes of Papuk Mountain (Fig. 3, Tab. S1). MJ networks using all available sequences of *P. marsilii* (Fig. 4) indicated that the examined specimens from Croatia belong to Clade 9a *sensu* Palandačić *et al.* (2017). The central haplotype in the part of MJ network with Croatian haplotypes (H187, Fig. 4a) differs by one

mutational step from haplotype H194 which is distributed in Austria and Poland.

In the present study, 83 haplotypes of *P. lumaireul* are recorded, 72 of which are new (Fig. 2, Tab. S1). Intraspecific relations in *P. lumaireul* were additionally analysed in the MJ haplotype network (Fig. 5) using 185 available haplotypes (Tab. S1). *P. lumaireul* are represented by clades 1a, 1b, 1c and 1d, all of which were recorded in Croatia, whereas only Clade 1b was found in Bosnia and Herzegovina. Two clades of *P. lumaireul* were recorded for the first time in Croatia: (i) Clade 1c in the westernmost river systems of Sava drainage (Sutla River, Bregana River, and Jamno Stream in the Medvednica Mountain), and (ii) Clade 1d in the westernmost river systems of Drava drainage in Croatia (Zbelava Stream in the Plitvica River system, and a channel near Varaždin; Figs. 3 and 5). The highest number of haplotypes (75; Fig. 5) was detected in Clade 1b.

Additionally, we introduced nine lineages within *P. lumaireul* clades 1a, 1b, 1c and 1d to describe fine-scale geographic distribution of genetic groups in *P. lumaireul*, which differ by at least 8 mutational steps in MJ network (Clade 1a Lineage HR, Clade 1a Lineage SI/HR, Clade 1a Lineage IT1, Clade 1a Lineage IT2, Clade 1a Lineage IT3, Clade 1b Lineage HR/SI/BA, Clade 1b Lineage HR/BA, Clade 1c Lineage SI/HR, and Clade 1c Lineage SI; Fig. 5). Moreover, the TrN + G genetic distances among genetic groups in *P. lumaireul* were calculated (Tab. 1), showing a minimum of

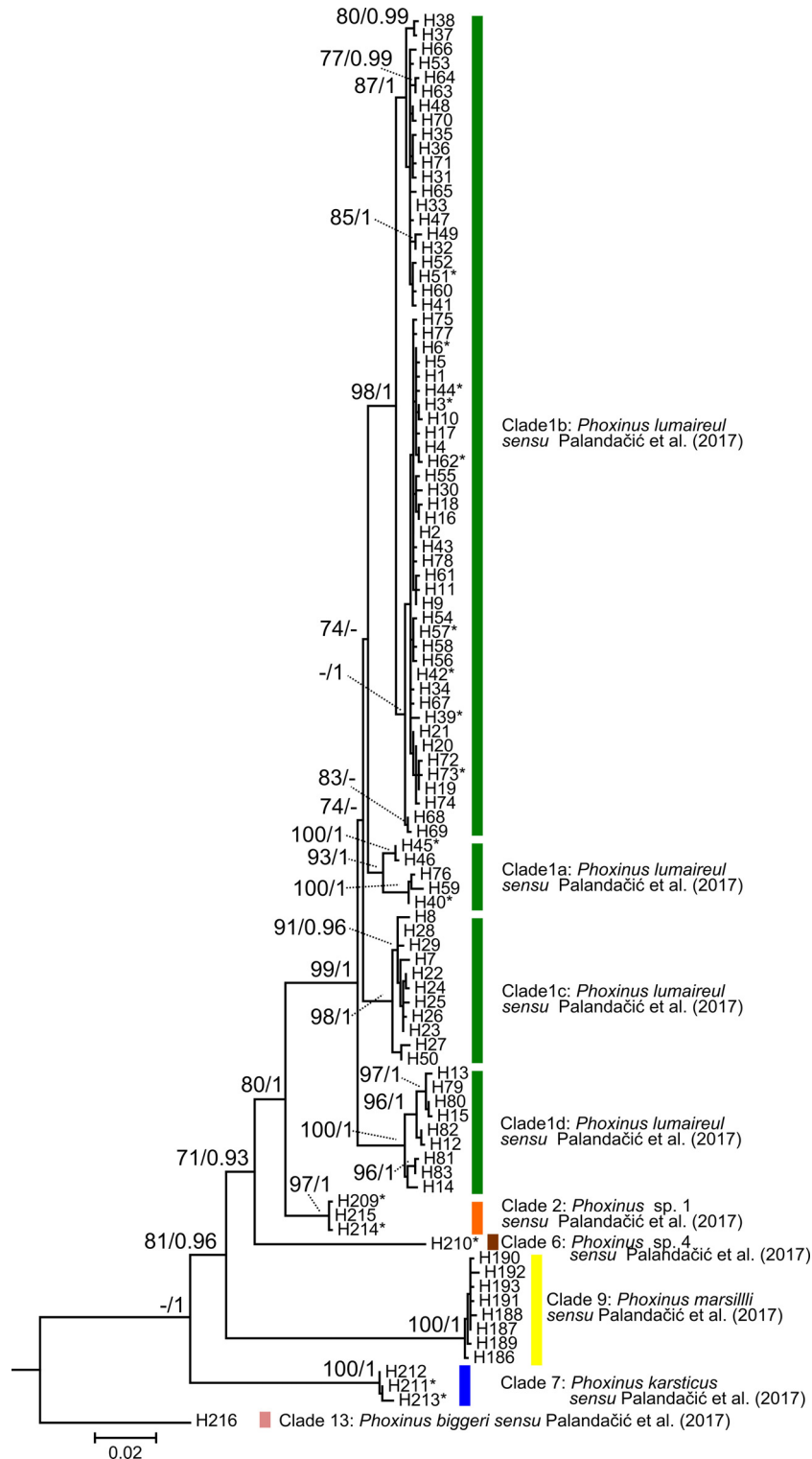


Fig. 2. Phylogram inferred by Maximum Likelihood (ML) analysis using cytochrome b (cyt-b) sequences obtained in the present study. Node supports are given as bootstrap values (P) in ML analysis (showing values ≥ 70) and posterior probabilities (pp) in Bayesian Inference (BI; showing values ≥ 0.9). *Phoxinus biggeri* from France was used as outgroup.

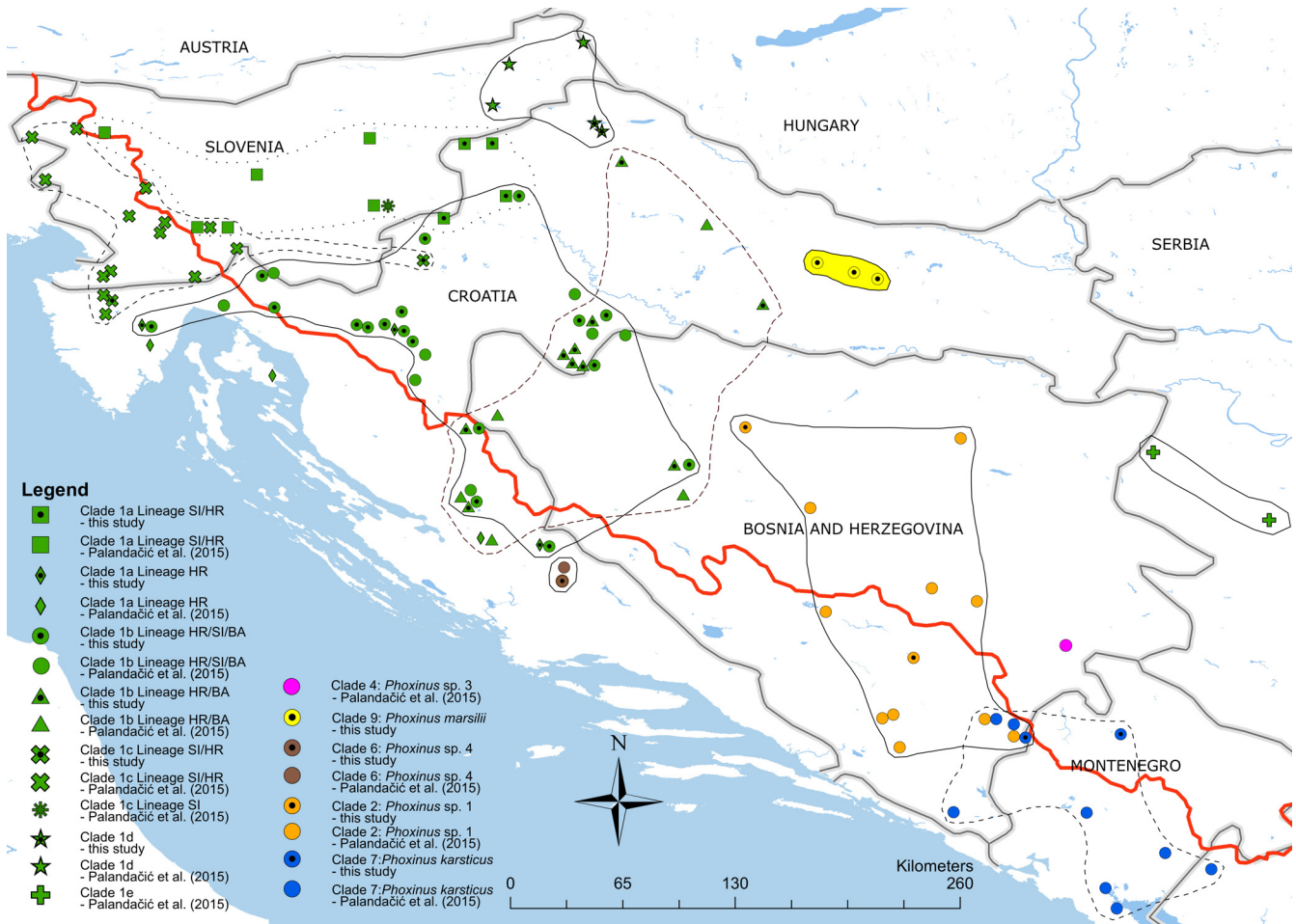


Fig. 3. Distribution ranges of genetic clades, lineages and species. Names of clades and species follow Palandačić *et al.* (2017). The genus *Phoxinus* in the Western Balkans is presented with data on localities from the sampling conducted in present study compiled with localities from Palandačić *et al.* (2015). The drainage divide between the Black Sea basin and the Adriatic basin is indicated by the thick red line.

0.6% [standard error (S.E.) 0.2%] base substitutions per site from estimation of net average between sequence groups which correspond to Clade 1a Lineage SI/HR and Clade 1a IT3, and a maximum of 4.1% (S.E. 0.7%) between Clade 1a Lineage IT2 and Clade 1f *sensu* Palandačić *et al.* (2017). Geographic distribution of clades and lineages of *P. lumaireul* is presented in Figure 3.

4 Discussion

4.1 Collection of literature and sampling data of the genus *Phoxinus* in Croatia and Bosnia and Herzegovina – general remarks on the distribution range

Literature and sampling data indicate that species of *Phoxinus* are common and widely distributed in Croatia and Bosnia and Herzegovina (Fig. 1). Despite its wide distribution range in both countries, the European minnow can be found mostly in cold and well-oxygenated brooks and streams of uplands of Danube drainage and Adriatic basin, a common type

of *Phoxinus* habitat as described in Kottelat and Freyhof (2007). Species of *Phoxinus* seem to be absent from the lower courses of large lowland rivers of the Black Sea basin (rivers Sava, Drava and Danube), lower courses of rivers in Istria and Ravni Kotari (Adriatic basin) and the Cetina drainage, as the only large drainage in the Adriatic basin where *Phoxinus* was not detected. The largest lowland rivers in Croatia and Bosnia and Herzegovina (Sava, Drava and Danube) presumably do not contain favourable slope with fast flowing water and enough turbidity and oxygenation to support populations of *Phoxinus*. Species of *Phoxinus* were recorded in the upper courses of large Bosnian rivers (Una, Drina, Bosna and Vrbas; Sava drainage) as well as in their small tributaries. However, they were not recorded in their lower courses (Fig. 1; Simonović *et al.*, 2015).

4.2 Updates of molecular data for the genus *Phoxinus* in Western Balkans

4.2.1 The first record of *Phoxinus marsilii* in Croatia

Detection of *P. marsilii* in the Drava drainage in Croatia supports the placement of this drainage in the Upper Danube

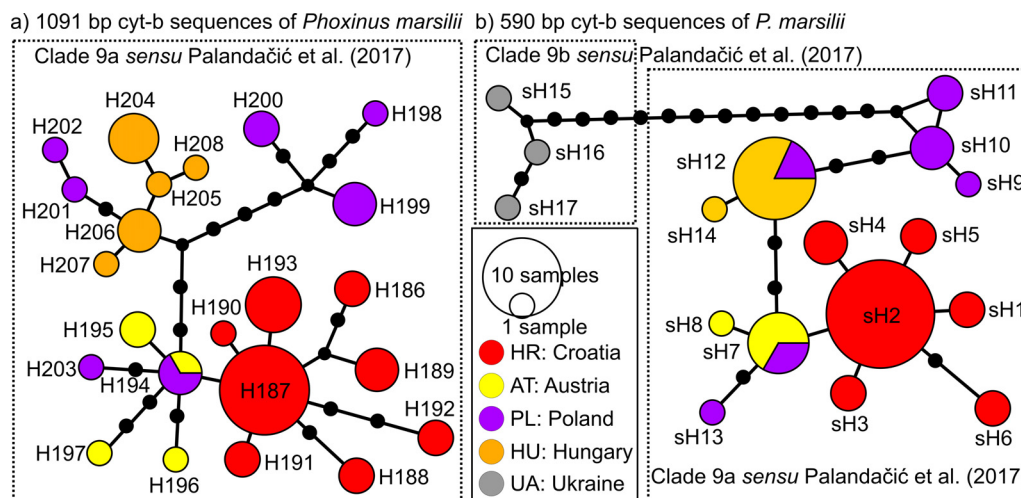


Fig. 4. Median-Joining (MJ) haplotype networks of *Phoxinus marsilii*. Haplotypes were shown with codes ranging: (a) from H186 to H208 in 1091 BP cytb sequences, and (b) from sH1 to sH14 in 590 BP cytb sequences. Mutational steps and median vectors were represented by black circles.

freshwater ecoregion *sensu* Abell *et al.* (2008), which was also confirmed by the presence of Clade 1d of *P. lumaireul* in Drava drainage in Croatia and Slovenia. The distribution range of *P. marsilii* in Croatia overlaps with the distribution range of the Clade VI of *Sabanejewia balcanica* (Karaman, 1922) detected in the river systems on the northern slopes of Papuk Mountain (Buj *et al.*, 2008) and the Mur River in Austria (Perdices *et al.*, 2003).

The only one mutational step between a haplotype obtained from Croatian specimens of *P. marsilii* (H187, Fig. 4) and a haplotype detected in specimens of *P. marsilii* from Austria and Poland (H194, Fig. 4) indicate that the dispersal of *P. marsilii* over a large area in Central Europe can be connected either with the last Pleistocene glacial-interglacial periods or human-mediated translocation.

4.2.2 New insights into the genetic variability of *Phoxinus lumaireul*

Results from molecular data confirmed that *P. lumaireul* is a dominant species of minnow in Croatia. This species is also reported in the westernmost river systems of Sava drainage in Bosnia and Herzegovina (Fig. 3). A remarkable genetic variability of *P. lumaireul* across the entire known distribution range (Italy, Slovenia, Austria, Croatia, Bosnia and Herzegovina, and Serbia) indicates a complex evolutionary history driven by geological features of karstic freshwater environment (Bosák, 2008; Prelovšek, 2010) and climate oscillations during Pleistocene (Schmitt, 2007). It can be presumed that the distribution of clades and lineages of *P. lumaireul* is connected with the existence of several glacial refugia, point out to the phylogeographic scenario of “refugia within refugia” (Gómez and Lunt, 2006). Moreover, evolutionary histories of many freshwater taxa with the overlapping distribution ranges with *P. lumaireul* can be explained by this scenario *e.g.* the brown trout (Simonović *et al.*, 2017), caddisflies (Previšić *et al.*, 2009), *Asellus aquaticus* (Verovnik *et al.*, 2005), the stone crayfish (Klobučar *et al.*, 2013) and the white-clawed crayfish (Jelić *et al.*, 2016).

High dispersal potential of *P. lumaireul* enabled secondary contacts and mixture of clades and lineages (Figs. 3 and 5).

Moreover, *P. lumaireul* crossed through the drainage divide between the Black Sea and Adriatic basins in Slovenia and Croatia (Fig. 3), making it hard to interpret whether by natural dispersal or human-mediated translocation. Particularly, the central haplotype H45 in Clade 1a Lineage SI/HR (Fig. 5) was detected in Kupa (Kolpa) River system (Sava drainage, Croatia and Slovenia), several isolated river systems in Slovenia (Sava drainage and Adriatic basin) and Istra peninsula (Adriatic basin, Croatia).

The sympatry of minnows and salmonids (Museth *et al.*, 2007; Kottelat, 2007; Kottelat and Freyhof, 2007) supports the hypothesis of introductions of *Phoxinus* species throughout the Europe through fish trade and recreational fishing practice. Caution is needed in biogeographic interpretation because human-mediated translocations of *Phoxinus* species could mask their natural distribution ranges (Kottelat, 2007). For example, the mixture of clades 1a (H140, H170, and H171) and 1b (H51 and H167) of *P. lumaireul* in the Zrmanja drainage (Figs. 3 and 5; Palandačić *et al.*, 2015) could be a result of water exchange between Razovac Lake (Zrmanja drainage, Dalmatia region, Adriatic basin, Croatia) and Štikada Lake (Ričica and Otuča river system, Lika region, Adriatic basin, Croatia) by the reversible power plant “Veletbit” rather than a natural dispersal.

4.2.3 Remarks on other species in Croatia and Bosnia and Herzegovina

According to Palandačić *et al.* (2015, 2017), the population from the Krka drainage (Adriatic basin, Croatia) represents a very distinct clade (Clade 6: *Phoxinus* sp. 4) and could be considered a new species. The examination of Krka specimens confirmed two haplotypes reported in Palandačić *et al.* (2015) and detected one new haplotype (Fig. 2). Even though the distribution range of this species remained unchanged, extensive research on the size and genetic parameters of the population and its distribution is required.

The Clade 4: *Phoxinus* sp. 3 described from the Drina River system by Palandačić *et al.* (2017) was not detected in

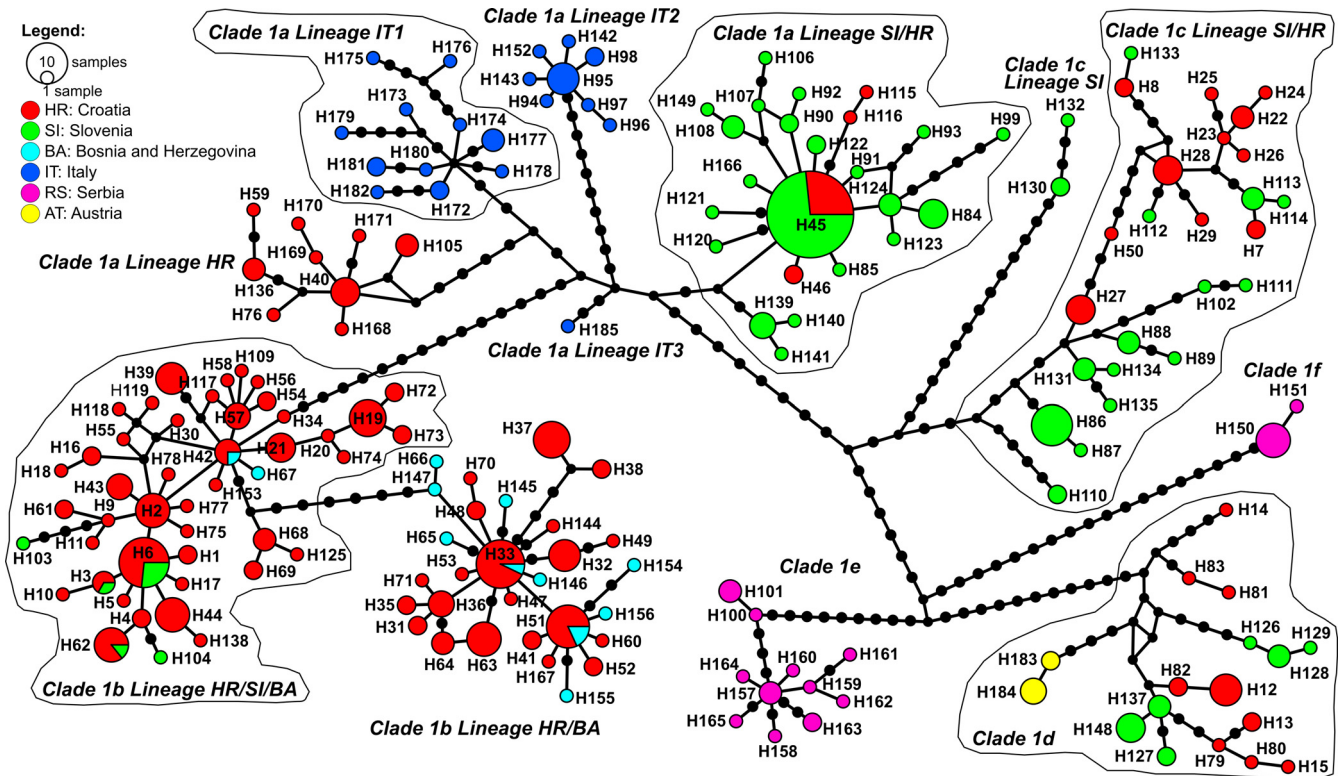


Fig. 5. Median-Joining (MJ) haplotype network of *Phoxinus lumaireul*. Haplotypes were shown with codes ranging from H1 to H185. Mutational steps and median vectors were represented by black circles.

Table 1. The number of base substitutions per site from estimation of net average between sequence groups using the Tamura-Nei model with a gamma-distributed rate variation among sites (Tamura and Nei, 1993). Values are shown as percentage. Sequence groups correspond to clades and lineages in *Phoxinus lumaireul*. Standard error estimates are shown above the diagonal.

Clades and lineages	1	2	3	4	5	6	7	8	9	10	11	12
1. Clade 1a Lineage HR	–	0.3	0.3	0.4	0.3	0.5	0.4	0.6	0.5	0.6	0.6	0.6
2. Clade 1a Lineage SI/HR	1.2	–	0.3	0.4	0.2	0.5	0.4	0.5	0.4	0.5	0.5	0.6
3. Clade 1a Lineage IT1	0.9	1	–	0.4	0.3	0.5	0.5	0.6	0.5	0.6	0.5	0.6
4. Clade 1a Lineage IT2	1.4	1.4	1.6	–	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.7
5. Clade 1a Lineage IT3	1.1	0.6	0.9	1.3	–	0.4	0.4	0.5	0.4	0.5	0.5	0.6
6. Clade 1b Lineage HR/BA	2.5	2.2	2.4	2.5	2.1	–	0.3	0.5	0.4	0.6	0.5	0.6
7. Clade 1b Lineage HR/SI/BA	2.1	2	2.1	2.5	1.9	0.9	–	0.5	0.4	0.6	0.5	0.6
8. Clade 1c Lineage SI	2.8	2.3	2.8	2.7	2.5	2.2	2.6	–	0.4	0.6	0.5	0.6
9. Clade 1c Lineage SI/HR	2.3	1.8	2.3	2.3	1.9	2	2	1.9	–	0.5	0.4	0.6
10. Clade 1d	3.1	2.8	3.2	3.1	2.9	3.1	3.1	3.1	2.6	–	0.4	0.4
11. Clade 1e	3.5	3	3.4	3.6	3.1	3.2	3	3.7	2.6	2.4	–	0.5
12. Clade 1f	3.5	3.3	3.5	4.1	3.3	3.9	3.5	3.4	3.2	2.4	2.6	–

sampling in the present study. Changes of distribution ranges (Fig. 3) were reported for Clade 2: *Phoxinus* sp. 1 *sensu* Palandačić *et al.* (2017) and Clade 7: *P. karsticus*. The Clade 2: *Phoxinus* sp. 1 was reported in the Vrbas River in Banja Luka (Sava drainage, Bosnia and Herzegovina) what presumably represents the western boundary of its distribution

range. The Clade 7: *P. karsticus* was found in the Tara River (Drina River system in Sava drainage, Montenegro), indicating there could be its hybridization zone with Clade 4: *Phoxinus* sp. 3 (Fig. 3). Denser sampling is needed to study distribution ranges of four *Phoxinus* species in Bosnia and Herzegovina.

5 Conclusions

The distribution range of *Phoxinus* species in Europe considerably expanded throughout the 20th century, especially in the mountain areas of Norway and Pyrenees, due to the usage of minnows as live bait for angling (Museth *et al.*, 2007; Miró and Ventura, 2015). Translocation of minnows without previous species determinations could have led to the introduction of non-native *Phoxinus* species in drainages occupied by autochthonous species of *Phoxinus* (Kottelat, 2007). Moreover, introductions of minnows can represent a serious threat to trout fisheries (Museth *et al.*, 2007).

Species descriptions and synonymization in the genus *Phoxinus* cannot be done solely on morphology and should be supported by molecular analyses. Since the habitat-induced body shape changes (*e.g.* head length, eye diameter, gape and body width) may blur morphological differences between species or genetic clades (Walker, 1997; Langerhans *et al.*, 2007; Langerhans and Reznick, 2010; Lucek *et al.*, 2016), they should be regarded in all future morphological species delimitations in the genus *Phoxinus* (Ramler *et al.*, 2017).

Additional taxonomic studies are needed, specifically molecular taxonomy and barcoding projects (Palandačić *et al.*, 2017). However, despite providing a better insight into the systematics of the genus *Phoxinus*, both molecular taxonomy and barcoding methods have setbacks. Interpretation of results obtained by these methods, especially of COI barcoding as a single-gene approach, should be taken with caution (Palandačić *et al.*, 2017). Besides taking additional samples, possible solutions in resolving the specific and complex phylogenetic relationships in the genus *Phoxinus* include the use of several unlinked genes (mitochondrial and nuclear), morphology, data on geographic distribution and ecological parameters.

Supplementary Material

Tables S1 and S2.

The Supplementary Material is available at <https://www.kmae-journal.org/10.1051/kmae/2017051/olm>.

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