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Molecular and osteological verification of the taxonomic status of *Phoxinus sedelnikowi* (Berg, 1908) (Teleostei: Leuciscidae)

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Abstract

Phoxinus sedelnikowi is a Central Asian representative of the genus *Phoxinus*, occurring in the tributaries of Lake Zaisan at the southern foot of the Altai Mountains. Its systematic position has so far been unclear. It was considered a subspecies of *Rhynchocypris czekanowskii*, *R. lagowskii* and *P. phoxinus*. Here, we sequenced the cytochrome b gene and ATP-ase subunits 6 and 8 of representatives of *P. sedelnikowi* and two populations of *P. phoxinus* (including the topotypical population) and *R. czekanowskii*, *R. lagowskii*, and *R. percnurus*. We also performed an analysis of the shapes of the seven bones of the skull of all six forms examined and then performed a phylogenetic interpretation of the obtained results. Both *P. sedelnikowi* and *Phoxinus* sp. from the Solonovka population appeared to be separate from *P. phoxinus* from the topotypical population by Cytb: 0.11 and ATP: 0.13 distance. Therefore, they may be considered as independent species within the genus *Phoxinus*. We found the generic level genetic distance between *Phoxinus* and *Rhynchocypris* (Cytb: 0.24 and ATP: 0.68), which thus confirmed their independence. Genetic analyses revealed that one of the studied individuals of *R. lagowskii* shares the ATP haplotype with *R. czekanowskii*. The Cytb haplotype of this individual is also closest to that of *R. czekanowskii*, indicating an introgression between these forms and questioning their species status. The UPGMA and MP analysis of the selected osteological features confirmed the differentiation between *Phoxinus* and *Rhynchocypris* on the generic level. The results at lower taxonomic levels are not consistent with the identified genetic diversity, suggesting that osteological features may not be sufficient to distinguish forms at the intra-generic level.

Keywords: *Phoxinus sedelnikowi*, *Phoxinus phoxinus*, *Rhynchocypris*, molecular phylogenetics, systematics

Introduction

Minnows of the genus *Phoxinus* are small cyprinid fish inhabiting rivers and lakes across a vast area of Europe and northern Eurasia (Kottelat & Freyhof 2007). Their hidden diversity and unclear systematics are the objects of numerous studies and discussions (Berg 1949; Bănărescu 1964; Gąsowska 1979; Howes 1985; Ito et al. 2002; Sakai et al. 2006; Kottelat 2006, 2007; Sasaki et al. 2007; Bogutskaya et al. 2008; Paśko et al. 2014; Palandačić et al. 2015, 2017; Schönhuth et al. 2018). Two species of the genus *Phoxinus* are known from the Kazakhstan part of the upper Irtysh basin – the Eurasian minnow *P. phoxinus*

and *P. sedelnikowi* (Berg 1949) –, but their systematic relationships are ambiguous.

The Eurasian minnow *Phoxinus phoxinus* is type species of the genus *Phoxinus*. Its distribution range extends from the Pyrenees and the British Isles to the Chukchi Peninsula, northern Sakhalin, Sikhote-Alin, the Korean Peninsula and northern China (Berg 1949; Shedko 2001; Nikitin 2010). However, some recent studies suggest that it is most probably not a monotypic species but rather a complex of undescribed species (Kottelat 2006, 2007; Bogutskaya et al. 2008; Paśko et al. 2014; Palandačić et al. 2015, 2017). The neotype of *P. phoxinus* was designated, and a number of new

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species have been identified in Europe (Kottelat 2007; Palandačić et al. 2017).

A recent morphometric study of scattered populations of *P. phoxinus* across a wide range of distribution revealed significant morphological distinctiveness in the central Asian populations from the upper Irtysh basin and the basin of Balkhash Lake (Paško et al. 2014). This indicates a possible separate phylogenetic lineage of *Phoxinus* in that region.

Berg (1908) described another form of *Phoxinus* from the Zaisan Lake different to that from the upper Irtysh basin – a new form – and treated it as a subspecies of Czekanowski's minnow *P. czekanowskii sedelnikowi*. Berg (1912, 1916) considered this form to be a variety of the Eurasian minnow *P. phoxinus* var. *sedelnikowi*, but soon, Dybowski (1916) assigned it to a subspecies of Amur minnow, *P. lagowskii sedelnikowi*. Berg (1932, 1949) designated it as a separate species – *P. sedelnikowi* – without additional comments.

According to Berg (1908, 1912, 1916, 1932, 1949), *P. sedelnikowi* is similar to *P. phoxinus* but differs mainly by the higher and shorter caudal peduncle and by the presence of the dark midline stripe. Relatively shortened and higher caudal peduncle in forms from Kazakhstan were highlighted as a feature of the majority of Kazakhstan populations by Paško et al. (2014). This corresponds well with the Berg statement. It is worth noting that Berg (1908), in his first description, used only two adult specimens from Lake Zaisan, and freshly collected and preserved material from that locality was not used in any other study. Moreover, due to the allometric type of fish growth, the proportions of the caudal peduncle are a rather doubtful diagnostic feature.

Mitrofanov and Mitrofanov (1987) concluded that the species status of *P. sedelnikowi* is not justified and considered it to be a subspecies of *P. phoxinus*. According to Kottelat (2006), minnows from the Chinese part of the upper Irtysh basin may belong to a separate species. Along with *P. sedelnikowi*, he included them in the synonymy of *P. uymonensis*. However, no specimens from the type localities of these two forms were studied by Mitrofanov and Mitrofanov (1987) or Kottelat (2006).

None of the previous studies (Berg 1908, 1912, 1932, 1949; Mitrofanov & Mitrofanov 1987; Kottelat 2006) provide a convincing basis for a clear verification of the systematic status of *P. sedelnikowi*. The situation is complicated by the fact that *P. czekanowskii* and *P. lagowskii*, which *P. sedelnikowi* was referred to, are now considered representatives of another genus – *Rhynchocypris* (Ito et al. 2002; Sakai et al. 2006). Thus, both the generic and species status of *P. sedelnikowi* remain unclear.

Until now, the genetic diversity of *P. phoxinus* sensu lato from the area of Northern and Central Asia has not been the subject of comprehensive research, which does not allow for the verification of the diversity of this taxon, and species designations based on incomplete morphological premises remain questionable. Some information on the systematic position of this species within Leuciscinae can be found in the work of Imoto et al. (2013). In the work of Kartavtsev et al. (2017) there are also limited conclusions on the systematic relationships of *P. phoxinus* from Europe and *P. uymonensis* from Central Asia. Data on phylogenetic relationships of the Far East populations of *P. phoxinus* and representatives of local populations of species previously included in the genus *Phoxinus*, and now belonging to the genus *Rhynchocypris* (*R. czekanowskii*, *R. kumgangensis*, *R. lagowskii*, *R. oxycephalus*, *R. percnurus*, *R. semotilus*), are included in the work of Sakai et al. (2006).

Our goal was to verify the taxonomic status of *P. sedelnikowi* and other taxa to which it has historically been included, based on (1) the sequences of two mtDNA fragments (Cytochrome b and ATPase 6 and 8 subunits) and (2) selected osteological features.

To establish the relationship between genetic and osteological variability, we conducted analyses of seven flat jaws and opercular bones, taking into account their descriptive features.

Material

Used fish were caught by electrofishing or by net traps. The fin clips for the genetic study were preserved in 70% ethanol, and voucher specimens were preserved in 4% formaldehyde.

The osteological material consisted of specimens of *P. sedelnikowi* from the Kenderlik River (a tributary of the eastern part of Lake Zaisan – the *terra typica* of this form, according to Berg 1908), a sample of *P. phoxinus* from the type locality of this species – the Agger River in Germany, and *Phoxinus* sp. from the Solonovka River belonging to the system of the Bukhtarma Reservoir, as well as of specimens of *R. lagowskii* from the Bira River (Amur River basin), *R. czekanowskii* from the Bolshoy Pit River (Yenisey River basin) and *R. percnurus* from Yakutsk (Lena River basin) (Table I). The taxonomic status of the topotypic Agger specimens was confirmed on the basis of osteological features and pattern of breast scale patches across the breast indicated by Kottelat (2007), Palandačić et al. (2017) and Tagayev (2013). The location of the sampling sites is shown in Figure 1.

Table I. Sample locations and numbers of specimens used. The numbering in the first column corresponds to the markings on the map (Figure 1).

No.	Species	Locality and coordinates(N/E)	River basin	N(osteology)	N(Cytb)	N(ATP)
1	<i>P. phoxinus</i>	Sieburg, Agger River 50.8122°/7.1849°	Rhine River	3	3	3
2	<i>P. sedelnikowi</i>	Zaisan, Kenderlik River 47.5374/84.7815	upper Irtysh River	6	2	2
3	<i>Phoxinus</i> sp.	Solonovka, Solonovka River 49.3058°/84.7389°	upper Irtysh River	8	5	5
4	<i>R. lagowskii</i>	Birobidzhan, Bira River 48.7849°/132.9333°	Amur River	4	-	-
5	<i>R. lagowskii</i> (GenBank AP009147)	Ulan Bator ~47.89°/106.91°	Yenisey River	-	1	1
6	<i>R. lagowskii</i> (GenBank KJ641843)	north China	Amur River	-	1	1
7	<i>R. czekanowskii</i>	Bolshoy Pit River 60.1103°/94.9514°	Yenisey River	2	-	-
8	<i>R. czekanowskii</i>	Tynda, Tynda River 55.1457°/124.7514	Amur River	-	3	5
9	<i>R. percnurus</i>	Yakutsk 61.9131°/129.5750°	Lena River	4	5	8
10	<i>G. gobio</i> (GenBank AB239596)	Plana ~49.87°/12.74°	Elbe River	-	1	1

Specimens of the *P. sedelnikowi* population had a well-expressed diagnostic character – a dark midline stripe extending from the operculum to the caudal fin base (Berg 1949; Mitrofanov & Mitrofanov 1987) (Figure 2). This feature was not present in the studied specimens of *P. phoxinus*.

The genetic material consisted of samples taken from specimens of *P. sedelnikowi* and *P. phoxinus* as well as from *R. czekanowskii* Tynda (Amur River basin) and *R. percnurus* from Yakutsk (*terra typica*). In the case of *R. lagowskii*, the AP009147 and

KJ641843 sequences from GenBank were used. The outgroup was *Gobio gobio*; its mitochondrial DNA sequence, accession number AB239596, was also obtained from GenBank (Table I).

Methods

Osteology

Osteological material was prepared according to a modified method of Hanken and Wasserug (1981).

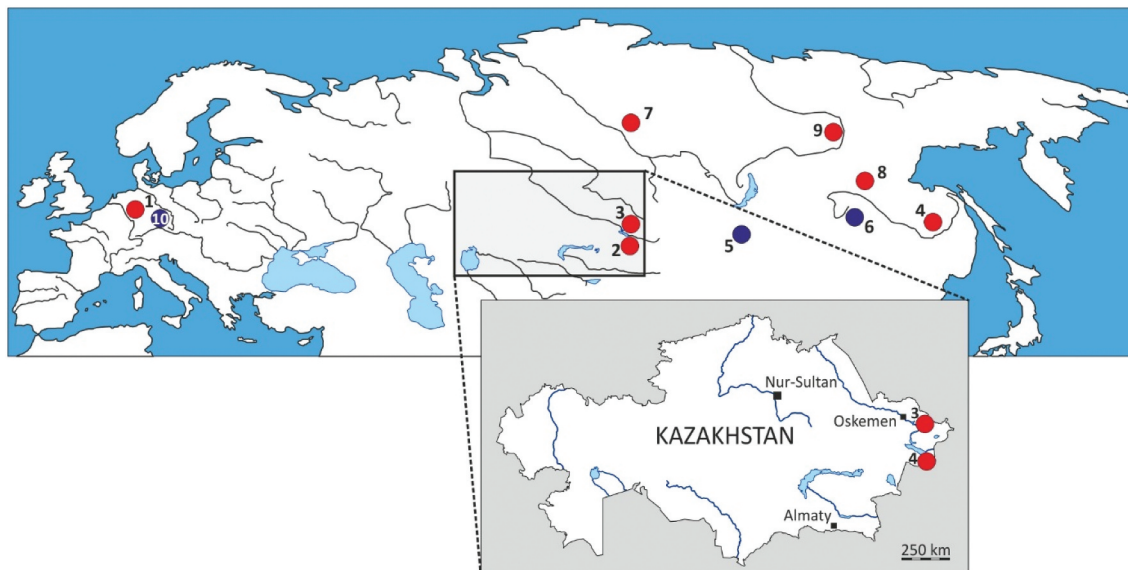


Figure 1. The location of the sampling sites. The numbering corresponds to Table I. Red dots – individuals used for osteological and genetic analyzes, purple dots – samples from GenBank.



Figure 2. Live colouration of the *Phoxinus sedelnikowi*.

Selected specimens were cleared in a solution of KOH (0.7–1%) with the addition of glycerol for 1.5–2 months. The bones were stained by alizarin.

Only the flat bones of the jaws (premaxillary, maxillary, dentary) and the operculum (interopercle, opercle, preopercle, subopercle) were used in the osteological analysis. We believe that they are particularly useful for such a study due to their simple, almost two-dimensional form, which facilitates comparative osteological analysis with the use of drawings. Only the bones of the left side of the fish bodies were used.

All osteological analyses and drawings were made using a NIKON SMZ 800 stereoscopic microscope with a drawing attachment (Nikon, Japan).

The following osteological features were taken into account: shape of the ascending process of the premaxillary (narrow/broad), shape of the posterior ascending process of the maxillary (narrow/broad), shape of the concavity on the ventral margin of the dentary (long and deep/short and shallow), shape of the posterior margin of the opercle (concave/almost straight), shape of the posteroventral margin of the opercle (slightly extended/strongly extended), shape of the anterodorsal process of the opercle (narrow/broad), presence of the posterodorsal process of the interopercle (present/absent), position of the preopercular sensory canal termination (at about the middle of the preopercle/near the dorsal end of the preopercle), shape of the subopercle (short/elongated), shape of the anterodorsal process of the subopercle (broad/narrow). The osteological characters were binary coded, and simple matching coefficient (SM) was calculated between groups. This highly simple value reflects the number of variables that are the same for the two comparing objects (NTSys 2.2, Rohlf 2010). From the matrix of coded binary characters, the maximum parsimony tree (50% consensus from SPR search in Mesquite - Maddison & Maddison 2019) was generated. The SM matrix was summarized by UPGMA graph.

Molecular phylogenetics

The two fragments of mitochondrial cytochrome b gene and ATP-ase subunits 6 and 8 (abbreviated

as Cytb and ATP later in the text) were sequenced to analyse the phylogenetic relationships among the studied populations. The molecular analysis was conducted according to the following procedures: Total DNA was extracted from fin clips using modifications of the phenol-chloroform method or the Qiagen DNeasy Tissue Kit following the manufacturer's protocols. The target DNA fragments were mostly amplified using the ABI GeneAmp 9600 thermocycler and the Perpetual Hot Start OptiTaq DNA Polymerase Kit. The PCR primer sequences and detailed amplification profiles are available upon request. The purified PCR products were cycle-sequenced in both directions using the same primers and the ABI PRISM BigDye Terminator v. 3.1 Cycle Sequencing Kit on ABI 3100Avant. All DNA sequences were edited manually and assembled into contigs using Aligner 3.5.7 (CodonCode Corporation). The sequences were pre-aligned in Clustal W with default settings and subsequently trimmed and aligned in the SOAP1.1b4 program for multiple alignments (Löytynoja & Milinkovitch 2001). No unstable nucleotide blocs (ambiguously aligned positions) were detected, and the obtained alignment matrix was submitted to subsequent analyses as Fasta files.

We decided to conduct separate phylogenetic analyzes of both matrices of gene fragments. Samples from Central Asia used in the research are very difficult to collect. Not all of them provided good quality sequences for both analyzed markers from the same individual. Our priority was the high quality of the sequences. Taking into account only a part of the existing samples in the analysis would result in an excessive reduction of the sample. On the other hand, combining sequences from different individuals, while technically possible, would affect the result in an unpredictable manner. Taking these premises into account, we decided to conduct separate analyzes for all sequences, but the conclusions only took into account the clearly supported consensus.

To avert long branch attraction, we based our phylogenetic analysis primarily on Bayesian inference (Huelsenbeck et al. 2001, 2002). The models of nucleotide substitutions estimated with the help of JModelTest2 (Darriba et al. 2012) for Bayesian Analysis were GTR+I for Cytb and GTR+G for ATP. The analyses were performed in the MrBayes 3.2.1 (Ronquist et al. 2012) parallel version that was run on a WCSS supercomputer SuperNova. To reduce the chance of reaching the apparent stationarity on local optima, two separate runs, consisting of four Markov chains for each analysis, were performed. Each chain was performed by 20×10^6

generations and was sampled every 500 generations. Stationarity and convergence of analyses was estimated by default MrBayes statistics: Average Standard Deviation of Split Frequencies (ASDSF) and Potential Scale Reduction Factor (PSRF). The analysis was terminated when value of ASDSF for Cytb obtained 0.000805 and for ATP = 0.001038. This is in accordance with accepted standards (as recommended, when ASDSF \leq 0.001). The PSRF values obtained in both cases, as expected, 1.00. Burn-in trees and parameters were discarded (50% samples or 10×10^6 generations in every case). The maximum parsimony (MP) tree (50% consensus from 500 bootstrap pseudoreplications) was generated using MEGA7 (Kumar et al. 2016) and the maximum parsimony haplotype network with the help of TCS 1.21 (Clement et al. 2000). Parsimony haplotype network method implemented in TCS creates connections between haplotypes only if they have at least 95% probability. All connections drawn in Figure 4 meet this criterion. This is especially useful in practical groups delimitation.

The delimitations of the taxonomic level for reconstructed clades presumed as possible taxonomic units were done. For this purpose, the genetic distance matrices between the main clades were calculated. We used Tamura - Nei (TN Cytb) and Tamura Nei + G (TN+G ATP) models, with the help of MEGA X (Kumar et al. 2018). These models of nucleotide substitution were the closest to ones estimated in jModelTest2 from those available in MEGA. To determine whether the distances between the main clades indicated by the phylogeny reconstructions for Cytb and ATP were of the same meaning, the correlation of the distance matrix (Pearson's r) was calculated, as well as the Mantel test of the significance of the relationship between the matrices (NTSyst 2.2, Rohlf 2010).

Terminology remarks

The term "population" used in figure captions and throughout the text indicates the geographic origin of an individual or group of individuals. The term "clade" is used in the text and captions to mean "monophyletic group" regardless of whether the reconstructed branches are scaled for length or not.

Results and discussion

Genetics

The studied populations form two main monophyletic groups in all Bayesian and MP trees (Clade 1 and Clade 2) (Figure 3(a–d)). The support for both

clades is extremely high and amounts to 1. One of these groups consists of all *Rhynchocypris* representatives used in the analyses (Clade 1), while the second one contains all three analysed forms of the genus *Phoxinus* (Clade 2). Haplotype networks (Figure 4(a), (b)) show five separate groups and do not support main basal dichotomy (Clade 1–Clade 2). However, there is no connection between the haplotypes that falsifies the possibility of this dichotomy.

Within the *Phoxinus* clade, there are also two distinct clades with maximal support (Figure 5). These clades consist of: (1) the topotypical *P. phoxinus* population (Clade 2A) and (2) *P. sedelnikowi*, as well as *Phoxinus* sp. from the Solonovka population (Clade 2B). Within Clade 2B, there is no constant pattern of divergence for all trees. It likely covers forms belonging to the same formal taxon, both haplotype networks are consistent with this pattern in showing Clade 2A and Clade 2B but these are not connected.

R. lagowskii and *R. czechanowskii* do not create two monophyletic groups on any tree (Figure 3(a–d)). They are a paraphyletic group in which gene flow is likely to exist (shared haplotype: ATP_1). *R. percunurus* is a distinct monophyletic group (Clade 1A) on all trees. In the case of the Cytb network, (Figure 4(a)) it is linked to two haplotypes of *R. czechanowskii*. In both networks (Figure 4(a), (b)), *R. lagowskii* (only one of the two tested individuals) is associated with the *R. czechanowskii* group. One of the *R. lagowskii* individuals, having the Cytb_7 and ATP_6 haplotypes, may constitute a separate clade (Clade 1B). Both on Bayesian trees and on both networks, it remains very distinct from other *R. lagowskii* and *R. czechanowskii* individuals.

Almost all haplotypes examined are exclusive. This may indicate a relatively low gene flow between forms, although samples from individual populations are sparse, which does not allow for a more precise analysis of within-group variability.

In addition to the phylogeny pattern, the number of changes that occurred between clades is also helpful in estimating the taxonomic level of divergence between them. The values of the TN distance for the Cytb fragment, calculated for the clades presented above (Figures 3 and 5, Table S1) and compared with the values of distances typical for intraspecific, intra-generic, and inter-generic differentiation for vertebrate species (Kartavtsev 2011) allows us to estimate the level of systematic differentiation of the identified clades. We did not find similar comparative data for the ATP fragment. Page and Hughes (2010) report that this fragment

diverges faster than Cytb. The correlation between the genetic distance matrices for Cytb and ATP is high ($r = 0.94$), the result of the Mantel test confirms a strong relationship between them ($p < 0.01$). Thus, it can be concluded that the differentiation of the ATP fragment supports the conclusions obtained

from the Cytb fragment. The variation between each examined clade for ATP is greater than it appears from the analysis of the Cytb fragment (Figures 3 and 5, Table S1 and S2). The average distance between all haplotypes for Cytb is 0.17 (S.E. = 0.01), and for ATP is 0.46 and 0.07, respectively. According to our

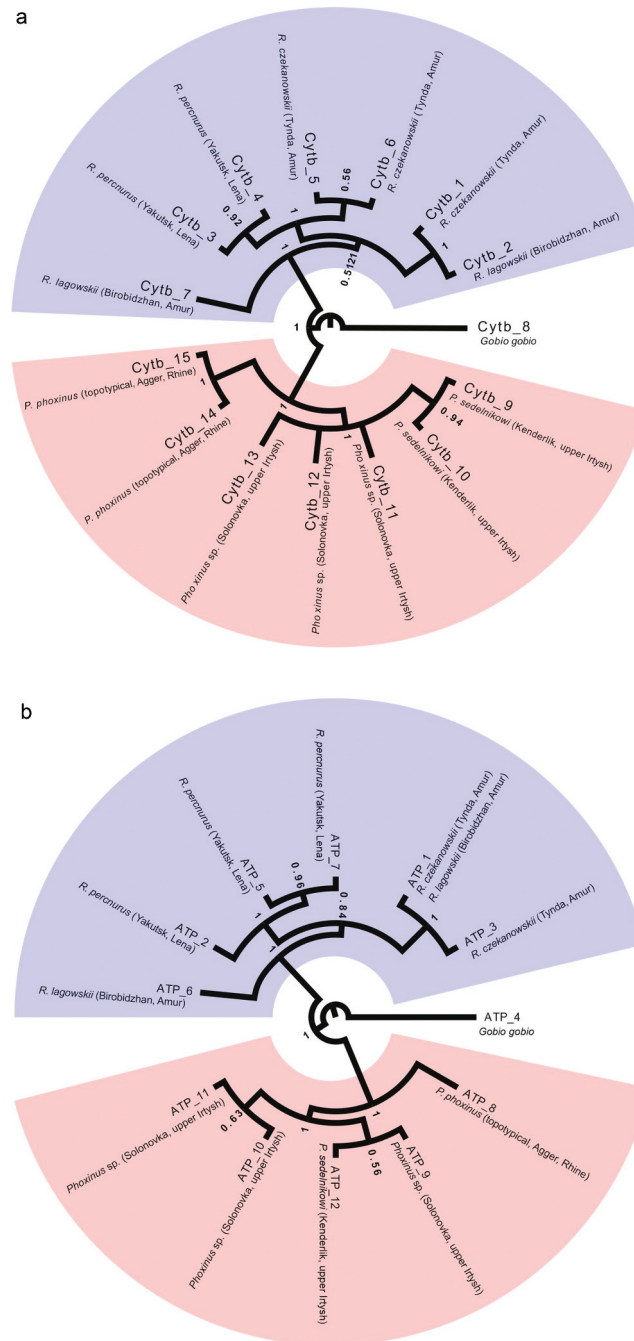


Figure 3. (a) Results of phylogenetic analyses – Bayesian consensus tree from cytochrome b. Colors indicate basal dichotomy reconstructed by every tree. (b) Results of phylogenetic analyses – Bayesian consensus tree from ATP-ase. Colors indicate basal dichotomy reconstructed by every tree. (c) Results of phylogenetic analyses – Maximum parsimony tree from cytochrome b. Colors indicate basal dichotomy reconstructed by every tree. (d) Results of phylogenetic analyses – Maximum parsimony tree from ATP-ase. Colors indicate basal dichotomy reconstructed by every tree.

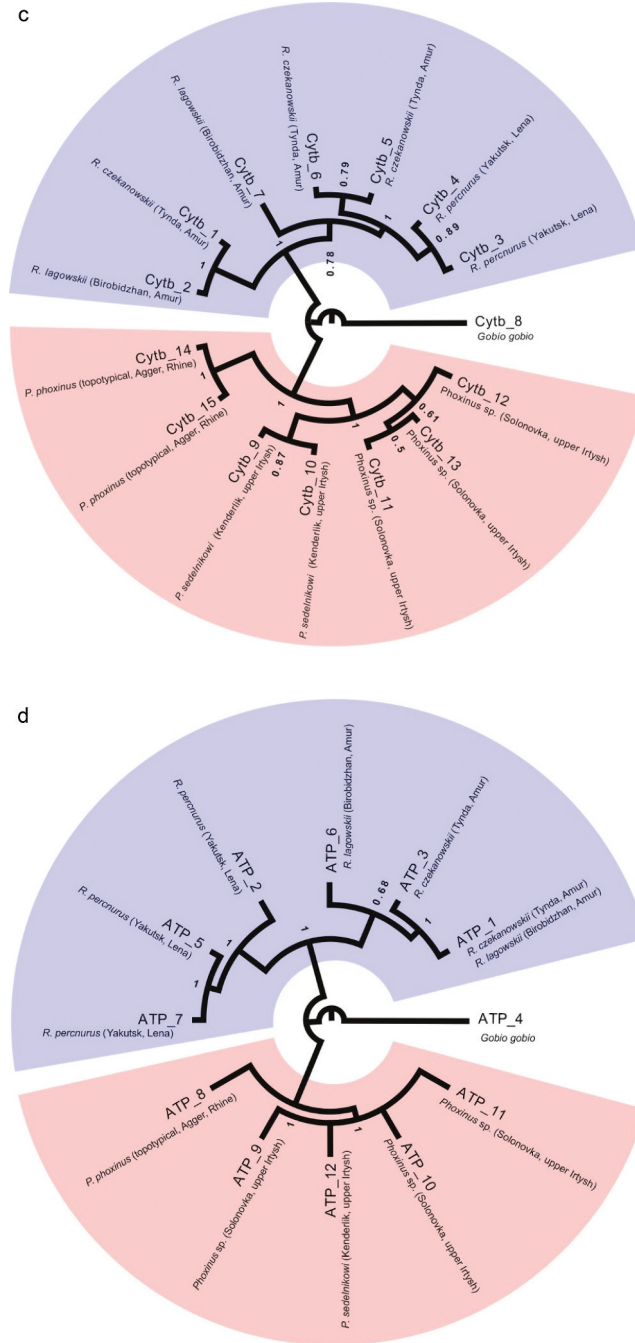


Figure 3. (Continued).

data, the ATP fragment divergence rate is on average 2.7 times faster for the Cytb fragment. Considering the phylogenetic patterns of trees, networks (Figures 3–5), and the distances between clades (Table S1 and S2), we make the following taxonomic conclusions:

1. The main dichotomy separating Clade 1 and Clade 2, and the distance between them (Cytb: 0.24, ATP: 0.68), confirm the level of divergence

corresponding at least to the formal genus (Cytb mean: 0.19 from Kartavtsev 2011). This confirms the validity of the genera *Rhynchocypris* and *Phoxinus*. Given this, the inner clades within Clade 1 and Clade 2 may even have species status. This was also confirmed by the minimum number of shared haplotypes between groups (only ATP_1 in Clade 1). Similar results confirming the independence

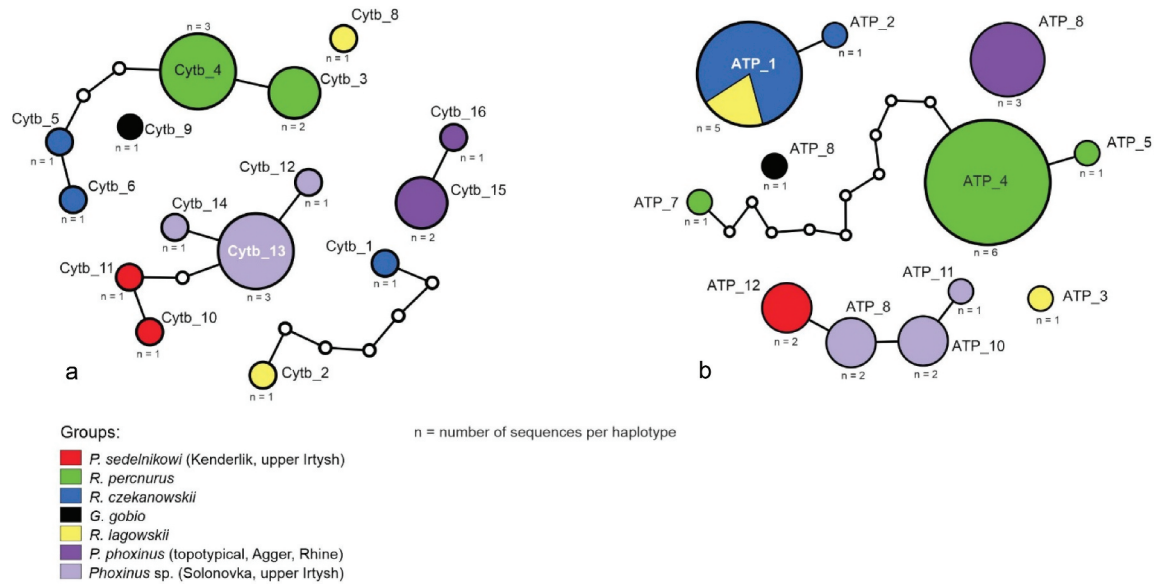


Figure 4. Results of the analysis of haplotypic relations. (a) – Maximum parsimony network from cytochrome b. (b) – Maximum parsimony network from ATP-ase.

of the genera *Rhynchocypris* and *Phoxinus* were obtained by Sakai et al. (2006) as a result of a study of the Far East populations of *P. phoxinus* and local species of the genus *Rhynchocypris*. According to these authors, *Rhynchocypris* and *Phoxinus* are supported as

monophyletic taxa by both allozyme and mtDNA analyzes. Our conclusions are also confirmed by the results of phylogenetic studies by Imoto et al. (2013) based on complete mitochondrial genome sequences of 31 species of Leuciscinae from the entire range of

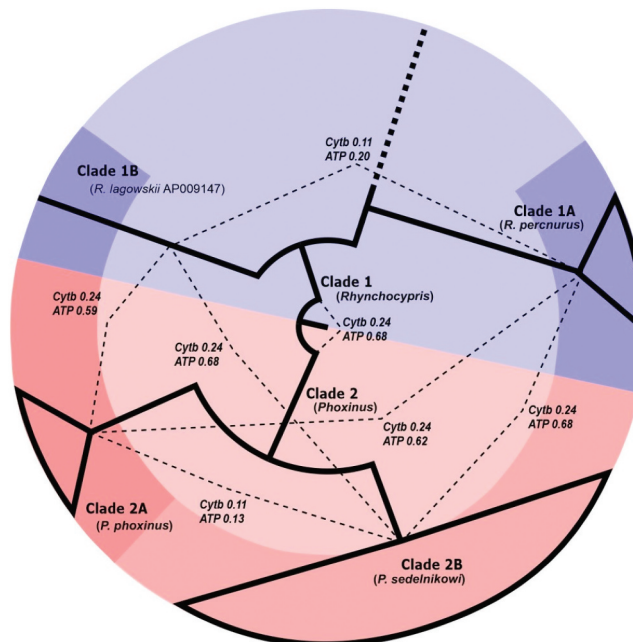


Figure 5. Systematic conclusions. Colors indicate basal dichotomy reconstructed by every tree from Figure 3.

Cytb - TN distance value for pair of clades (dotted line)

ATP - TN+G distance value for pair of clades (dotted line)

Collapsed clade - not significant for the taxonomical problem (thick dotted line)

this subfamily. According to these authors, *Rhynchocypris* and *Phoxinus* are independent taxa. Their populations of *P. phoxinus* (including the studied population from Europe) formed a monophyletic clade which evolved as an independent lineage within the long evolutionary time in the Leuciscinae species.

2. Representatives of *Phoxinus* from the topotypical population (Clade 2A) are separated by the distance Cytb 0.11 and ATP 0.13 from *P. sedelnikowi* and the Solonovka population (Clade 2B). Distance Cytb 0.10 indicates morphologically distinct species within genera (Kartavtsev 2011). Variability within Clade 2A and Clade 2B is minimal; net distances for Cytb and ATP between both clades equal also 0.11 and 0.13, respectively. Therefore, it can be concluded that Clade 2A and Clade 2B are two good species within the genus *Phoxinus*: *P. phoxinus* (Clade 2A) and *Phoxinus sedelnikowi* (Clade 2B). The systematic independence of European populations of *P. phoxinus* and another Central Asian species of the genus *Phoxinus* - *P. uymonensis* is suggested by the results of Kartavtsev et al. (2017). In this situation, it would be interesting to investigate the phylogenetic relationships between the Central Asian phoxinins.

3. Specimen of *R. lagowskii* with the number KJ641843 (Clade 1B, having haplotypes Cytb_7 and ATP_6) may have species status within the genus *Rhynchocypris*. Distances between Clade 1B and Clade 1A equal 0.11 (Cytb) and 0.20 (ATP) and correspond to morphologically distinct species within the genus (Kartavtsev 2011). The distance between KJ641843 and the second specimen *R. lagowskii* included in the analyses is also relatively high and equals 0.12 (Cytb) and 0.17 (ATP), indicating divergence on good species level. The distances between working group constructed from all *R. czekanowskii* specimens and specimen LKJ64 are, respectively, 0.11 (Cytb) and 0.17 (ATP), being practically the same as between two *R. lagowskii* specimens (Supplement Table S1 and S2).

The second analysed specimen of *R. lagowskii* (AP009147) shares the ATP haplotype with one *R. czekanowskii* specimen and the Cytb haplotype closest to *R. czekanowskii*. Concluding, KJ641843 could belong to typical *R. lagowskii* and AP009147 could be hybrid *R. lagowskii* - *R. czekanowskii*. Therefore, *R. czekanowskii* and *R. lagowskii* are probably not good biological species, and introgression between them is possible. Of course, studies on larger sample sizes are needed.

4. *R. percnurus* seems to be a separate species included within the *R. czekanowskii* and *R. lagowskii* diversity. However, because it is

distributed throughout a vast area, a large sample containing local populations from the entire range is needed to verify this view.

5. Our results support the hypothesis presented by other authors (Kottelat 2006; Bogutskaya et al. 2008), according to which the range of *P. phoxinus* in the east does not extend to Central Asia, and the populations found there, still included in this taxon, in fact form a complex of species.

Osteology

The shapes of seven studied bones showed a clear pattern for all the investigated populations, which facilitated the comparison and partly allowed the systematic inference.

The premaxillary is an elongated and curved bone; a notable ascending process is present in its anterior portion. It is narrow in specimens of *P. sedelnikowi* and *P. phoxinus* from the topotypical population, as well as in *R. percnurus* (Figure S1A, C, F), and broad in *Phoxinus* sp. specimens from the Solonovka population, *R. lagowskii* and *R. czekanowskii* (Figure S1B, D, E).

The maxillary is an elongated and curved bone bearing five distinguished processes (Ramaswami 1955; Chen 1996). Only the posterior ascending process of the maxillary shows some variation among the studied populations. It is broad in *P. sedelnikowi*, *P. phoxinus* and *R. lagowskii* (Figure S2A-D) and narrow in *R. czekanowskii* and *R. percnurus* (Figure S2E, F).

The dentary is the largest bone of the lower jaw. It is curved and has a large coronoid process at its dorsal margin. The anterior part of the bone is curved ventrally and medially, thus forming the arcuate concavity on its ventral margin. The concavity is long and deep in *P. sedelnikowi* and *P. phoxinus* (Figure S3A-C) and short and shallow in *R. lagowskii*, *R. czekanowskii* and *R. percnurus* (Figure S3D-F).

The opercle is the largest element of the operculum. It is square-shaped, and its posterior margin is noticeably concave in specimens of *P. sedelnikowi* and both studied populations of *P. phoxinus* (Figure S4A-C), whereas in *R. lagowskii*, *R. czekanowskii* and *R. percnurus*, it is almost straight (Figure S4D-F). In *P. sedelnikowi*, *P. phoxinus* and *R. percnurus*, the posteroventral angle of the opercle extends slightly posteriorly (Figure S4A-C, F), whereas in *R. lagowskii* and *R. czekanowskii*, it extends strongly (Figure S4D, E). A short process articulating with the hyomandibula is present at the anterodorsal angle of the opercle (anterodorsal process). It is narrow in specimens of *P. sedelnikowi* and both populations of *P. phoxinus* (Figure S4A-C) and broad in *R. lagowskii*, *R. czekanowskii* and *R. percnurus* (Figure S4E-F).

The interopercle is an elongated bone broadened posteriorly. All the studied species of the genus *Rhynchocypris* have a well-expressed posterodorsal process in the broad posterior part of the interopercle (Figure S5D–F). In *P. sedelnikowi* and *P. phoxinus*, this process is absent (Figure S5A–C).

The preopercle is an elongated crescent-shaped bone, tapering at both ends and bearing a preopercular sensory canal. The general shape of this bone showed no interspecific differences, whereas the preopercular sensory canal differentiated the genera *Phoxinus* and *Rhynchocypris*. In *P. sedelnikowi* and *P. phoxinus*, this canal terminates in about the middle of the bone (Figure S6A–C), whereas in *R. lagowskii*, *R. czeakanowskii*, and *R. percnurus*, it almost reaches its dorsal tip (Figure S6D–F).

The subopercle is an elongated bone tapered posteriorly. It is rather short in *P. sedelnikowi* and *P. phoxinus* (Figure S7A–C) and elongated in *R. lagowskii*, *R. czeakanowskii*, and *R. percnurus* (Figure S7D–F). In *P. sedelnikowi* and *P. phoxinus*, there is a small broad process at the anterodorsal angle of the bone (Figure

S7A–C), whereas in *R. lagowskii*, *R. czeakanowskii* and *R. percnurus*, it is narrow (Figure S7D–F).

We noticed a significant variability in 10 osteological features among the studied populations. We revealed no features common for all six studied groups. Nine of the features were common for the studied populations of *P. sedelnikowi*, *Phoxinus* sp. (Solonovka) and *P. phoxinus* (topotypical). No differences were revealed between *P. sedelnikowi* and *P. phoxinus* (topotypical), whereas only one feature differentiated from the *Phoxinus* sp. population of the Solonovka River. All of the *Phoxinus* sp. populations differed from *R. lagowskii*, *R. czeakanowskii*, or *R. percnurus* by 8 to 10 features. The species of *R. lagowskii*, *R. czeakanowskii*, and *R. percnurus* shared seven studied features (Table II and S3).

The species *R. lagowskii* and *R. czeakanowskii* showed minor differences in the shape of the maxillary, whereas *R. percnurus* differed from these species in two features, namely the shape of the premaxillary and the opercle. The

Table II. Variability of the osteological features of investigated forms. Feature states characteristic of the topotypical population of the *P. phoxinus* were italicized.

Feature	<i>P. sedelnikowi</i>	<i>P. phoxinus</i> (topotypical)	<i>Phoxinus</i> sp. (Solonovka River)	<i>R. lagowskii</i>	<i>R. czeakanowskii</i>	<i>R. percnurus</i>
Ascending process of the premaxillary	<i>narrow</i>	<i>narrow</i>	broad	broad	broad	<i>narrow</i>
Posterior ascending process of the maxillary	<i>broad</i>	<i>broad</i>	<i>broad</i>	<i>broad</i>	narrow	narrow
Concavity on the ventral margin of the dentary	<i>long and deep</i>	<i>long and deep</i>	<i>long and deep</i>	short and shallow	short and shallow	short and shallow
Posterior margin of the opercle	<i>concave</i>	<i>concave</i>	<i>concave</i>	almost straight	almost straight	almost straight
Posteroventral margin of the opercle	<i>slightly extended</i>	<i>slightly extended</i>	<i>slightly extended</i>	strongly extended	strongly extended	<i>slightly extended</i>
Anterodorsal process of the opercle	<i>narrow</i>	<i>narrow</i>	<i>narrow</i>	broad	broad	broad
Posterodorsal process of the interopercle	<i>absent</i>	<i>absent</i>	<i>absent</i>	present	present	present
Preopercular sensory canal	<i>terminates at about the middle of the preopercle</i>	<i>terminates at about the middle of the preopercle</i>	<i>terminates at about the middle of the preopercle</i>	terminates near to the dorsal tip of the preopercle	terminates near to the dorsal tip of the preopercle	terminates near to the dorsal tip of the preopercle
Subopercle	<i>short</i>	<i>short</i>	<i>short</i>	elongated	elongated	elongated
Anterodorsal process of the subopercle	<i>broad</i>	<i>broad</i>	<i>broad</i>	narrow	narrow	narrow

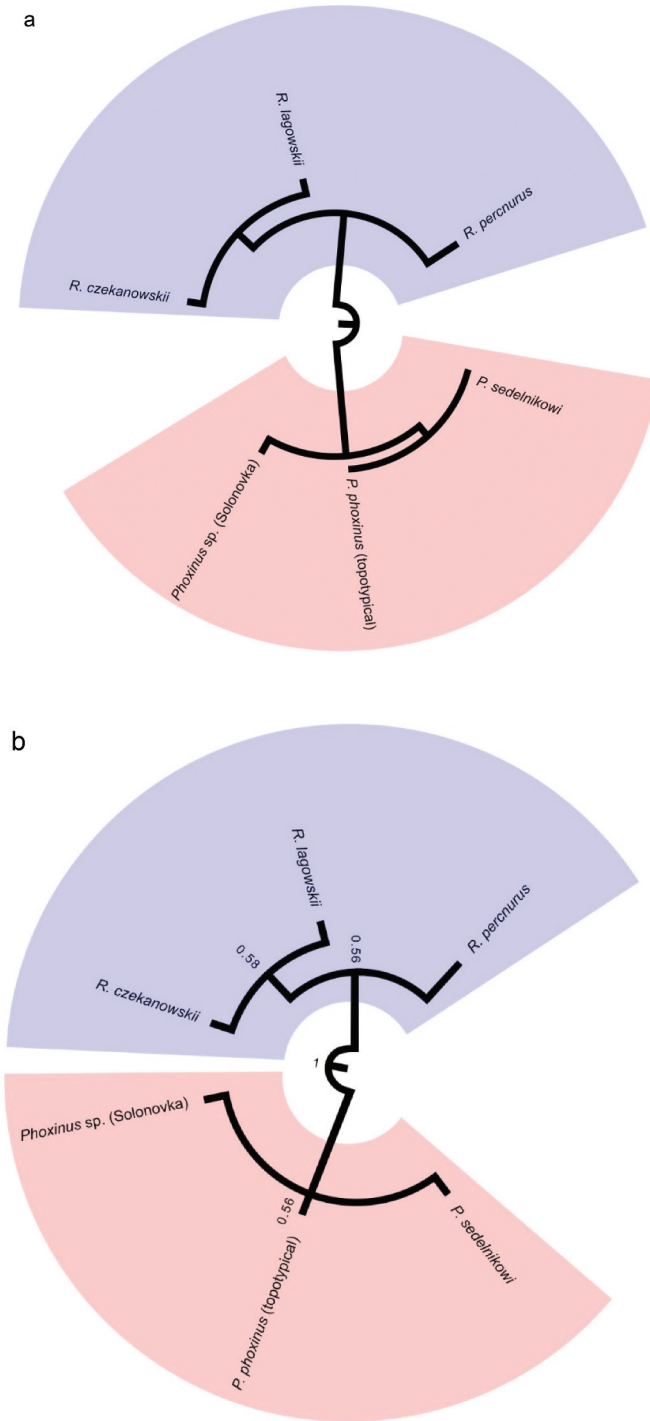


Figure 6. (a) The UPGMA tree calculated from the osteological SM similarity matrix. Colors indicate basal dichotomy. (b) The Maximum Parsimony tree (50% consensus from SPR search) from the osteological data. Colours indicate basal dichotomy.

osteological distinctiveness of *R. percnurus* was also revealed in previous studies (Gąsowska 1979; Howes 1985).

The results of osteological comparisons confirmed some characteristic osteological features of *Phoxinus*

from the topotypical population, the population of Solonovka and *P. sedelnikowi* illustrated in previous studies (Gąsowska 1979; Chen 1996), such as a broad posterior ascending process of the maxillary, a long and deep concavity on the ventral margin of

the dentary, a concave posterior margin of the opercle, a slightly extended posteroventral margin of the opercle, a narrow anterodorsal process of the opercle and the preopercular sensory canal terminating at about the middle of the preopercle. The lack of variation between *P. sedelnikowi* and both *P. phoxinus* populations in all but one of the studied osteological features does not reflect the phylogenetic patterns revealed in our study for interpopulation relationships inside the *Phoxinus* clade (Clade 2). In a recent study of the morphological diversity of *P. phoxinus* (Paško et al. 2014), the authors showed that populations from the upper Irtysh basin (which also includes the Kenderlik and Solonovka rivers samples) form a group that is morphologically distinct from the typical populations of *P. phoxinus*. The differences revealed by these authors were not large, but this morphometric pattern slightly supports our genetic results.

In addition, the phylogenetic relationships as recovered by our genetic analysis in *Rhynchocypris* clade (Clade 1) concerning the paraphylicity of *R. lagowskii* and *R. czekanowskii* group were not confirmed by osteological analysis. Osteology is congruent with classical views on these forms as separate species.

The MP tree and UPGMA tree calculated from the osteological data (Figure 6(a), (b)) clearly indicate the existence of two clades at the genus level: Clade 1 (*Rhynchocypris*) and Clade 2 (*Phoxinus*). At the lower taxonomic levels, the divergence patterns from osteological characters are not congruent with the genetic divergences.

Our both trees (Figure 6(a), (b)) suggest that the shape of the selected bones can be useful in the separation of representatives of the two genera. We believe that in the case of the genus *Phoxinus*; however, the osteological features may not be sufficient to distinguish forms at the species level.

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Disclosure statement

No potential conflict of interest was reported by the authors.



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Supplementary material

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