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Citation	ZOOLOGICAL SCIENCE, 23(4), 323-331 https://doi.org/10.2108/zsj.23.323
Issue Date	2006-05
Doc URL	http://hdl.handle.net/2115/14529
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Type	article (author version)
File Information	ZS_23_323.pdf



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**Phylogenetic and taxonomic relationships of northern Far Eastern
phoxinin minnows, Phoxinus and Rhynchocypris (Pisces,
Cyprinidae), as inferred from allozyme and mitochondrial 16S rRNA
sequence analyses**

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Abbreviated title: Far Eastern Minnow Relationships

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Section: Animal diversity and Evolution

ABSTRACT - Analyses of allozyme (18 loci) and partial mitochondrial DNA (mtDNA) sequences (1295 bp, 16S rRNA) support the classification of phoxinin minnows from the northern Far East into 2 genera of 8 species: Phoxinus phoxinus, Rhynchocypris oxycephalus, R. perenurus, R. czekanowskii, R. kumgangensis, R. semotilus, R. lagowskii and R. sp. (bergi ?). Although R. lagowskii from Japan and the Amur basin and R. sp. from Vladivostok region to Korea have been classified into a single species by many authors as R. lagowskii, they form separate clusters in both analyses, suggesting different specific status. Some R. oxycephalus and R. perenurus had the mtDNA haplotypes of R. lagowskii and R. czekanowskii, respectively, which probably indicates that local introgression of mtDNA occurred through inter-specific hybridization. Rhynchocypris forms a monophyletic cluster with dace genera Tribolodon and Pseudaspius, not with Phoxinus. Eurasian and American Phoxinus are suggested to be paraphyletic.

Key words: biodiversity, biogeography, Cyprinidae, introgression, Sea of Japan, allozyme, 16S rRNA

INTRODUCTION

The northern Far East territory, especially the area abutting the Sea of Japan, exhibits a rich biodiversity. It has been thought to have formed a speciation center for animals, including freshwater fishes, during the Tertiary to Quaternary Period (e.g. Lindberg, 1972; Nishimura, 1974).

Many phoxinin minnows are distributed around the Sea of Japan and they have been variously classified, resulting in their becoming the subject of much confusion (Table 1): e.g., three species of the genus Moroco (Nakamura, 1969) or Phoxinus (Hosoya, 2002) from Japan, one species of Phoxinus and five species of Moroco (Uchida, 1939) or Rhynchocypris (Kim, 1997) from Korea, and four species of Phoxinus from Russia (Berg, 1949; Chereshev, 1998). In China, only one species, Phoxinus lagowskii oxycephalus sensu Berg (1949), is known except species in the Amur River basin (Berg, 1949). On the other hand, Howes (1985) divided the Far East Asian species into five genera, Eupallasella, Lagowskiella, Phoxinus, Rhynchocypris, and Tribolodon, in his revision of phoxinin minnows.

Recently, Ito et al. (2002) analyzed allozyme allelic composition and tentatively proposed seven species groups of Far Eastern minnows: oxycephalus, lagowskii, perenurus, czekanowskii, kumgangensis, semotilus and phoxinus groups. They also suggested that the former six and the last species might be classified into different genera, Rhynchocypris and Phoxinus, respectively. However, their results unsatisfactorily address minnow taxonomy and relationships because of restricted localities examined (21 populations).

In order to reconfirm and develop the results of Ito et al. (2002), the authors analyzed partial mitochondrial DNA (mtDNA) sequences as well as the allozymes of many more populations from nearly the whole range of the northern Far East region.

Cytoplasmic and maternally inherited mtDNA offer phylogenetic information different from that of allozymes, most of which are inherited from both sexes. When the same or similar mtDNA haplotypes are recognized in different species, it can also indicate inter-specific mtDNA introgression through hybridization in the past (Avice, 1994). The results of the present study basically accept the classification proposed by Ito et al. (2002), and imply some possibilities of mtDNA introgression. An additional phylogenetic analysis among some related groups suggests the necessity of future taxonomic revision of the Far Eastern phoxinin genera.

MATERIALS AND METHODS

Allozyme analysis

Forty population samples of minnows were collected from the northern Far East region (Table 2, Fig. 1) and tentatively classified into species beforehand based on morphological differences shown by Uchida (1939), Berg (1949) and Ito et al. (2002): Rhynchocypris oxycephalus (Sauvage and Dably, 1874), R. lagowskii (Dybowski, 1869), R. perenurus (Pallas, 1814) (species name followed Eschmeyer, 1998), R. czekanowskii (Dybowski, 1869), R. kumgangensis (Kim, 1980), R. semotilus (Jordan and Starks, 1905), and Phoxinus phoxinus (Linnaeus, 1758). The genus name also followed Ito et al. (2002) tentatively.

The gene products of the same 18 allozyme loci as those analyzed by Ito et al. (2002) were investigated by standard horizontal starch gel electrophoresis and zymogram methods (Hillis and Moritz, 1990): aspartate aminotransferase (E. C. 2.6.1.1, AAT-1*, AAT-2*), alcohol dehydrogenase (E. C. 1.1.1.1, ADH*), glycerol-3-phosphate dehydrogenase (E. C. 1.1.1.8, G3PDH*), glucose-6-phosphate isomerase (E. C. 5.3.1.9, GPI-1*, GPI-2*), isocitrate dehydrogenase (E. C. 1.1.1.42, IDHP*),

L-lactate dehydrogenase (E. C. 1.1.1.27, LDH-1*, LDH-2*, LDH-3*), malate dehydrogenase (E. C. 1.1.1.37, MDH-1*, MDH-2*, MDH-3*), phosphoglucomutase (E. C. 5.4.2.2, PGM*), L-idoitol dehydrogenase (E. C. 1.1.1.14, IDDH*), superoxide dismutase (E. C. 1.15.1.1, SOD*) and general protein (PROT-1*, PROT-2*).

Mitochondrial DNA analysis

Forty-eight individuals, each one individual from all population samples except for Locality No. 9. Sukhodol (3 individuals), 10. Manoma (3 individuals) and 29. Anadyr (5 individuals) in which mtDNA introgression was presumed, were used for mtDNA analysis. Total DNA was isolated from a piece of fin or muscle by standard methods: proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation (Hillis and Moritz, 1990). PCR amplification (Saiki et al., 1988) was carried out to amplify partial mtDNA 16S rRNA gene using two primer pairs, L1854 (5'-AAACCTCGTACCTTTTGCAT-3') - H2582 (5'-ATTGCGCTACCTTTGCACGGT-3') and L2503 (5'-CACAAGCCTCGCCTGTTTACCA-3') - H3058 (5'-TCCGGTCTGAACTCAGATCACGTA-3') (Watanabe et al., 2000) with a thermal cyclor (Gene Amp PCR System 2400, Perkin Elmer). Amplified and purified DNA was sequenced directly on an automated DNA sequencer (ABI PRISM 310, Applied Biosystems). The nucleotide sequences determined were deposited in DDBJ / EMBL / GenBank (accession numbers AB100697 – AB100736).

The DNA sequences (about 1290 base pairs) were edited and aligned manually with the multiple-sequence editor DNASIS ver. 3.4 (Hitachi Co. Ltd.). Nucleotide substitutions, separated into transitions (Ts) and transversions (Tv), were counted and plotted versus Kimura's (1980) two-parameter model distance (K2P) in order to

judge the saturation of substitutions.

Phylogenetic analysis

Neighbor-joining (NJ) dendrograms (Saitou and Nei, 1987) based on Nei's (1972) genetic distance (D) for the allozyme data and on K2P distance for the mtDNA data were constructed with 1000 bootstrap (Efron, 1979) replications using the computer package PHYLIP v. 3.57c (Felsenstein, 1995). Weighting schemes $Ts/Tv = 2, 4, \text{ and } 8$ were tried but only the result of $Ts/Tv = 4$ is presented because all the results did not differ so much, the mean observed Ts/Tv ratio being 4.2. Gaps were excluded from the phylogenetic analysis as missing data. For the populations 9. Sakhodol, 10. Manoma, and 29. Anadyr, mtDNA data of each one individual was used for phylogenetic analysis because individuals in the same populations exhibited the same sequence.

Selecting one individual from each recognized species, further phylogenetic analyses were conducted adding the data for American Phoxinus erythrogaster (DDBJ/EMBL/GenBank accession number AF038490), P. neogaeus (AF038493) (Simons and Mayden, 1998), Japanese Tribolodon hakuensis (AB162634) (species name followed Eschmeyer, 1998, as hakonensis usually used in Japan is an unjustified emendation), T. sachalinensis (AB162632) (species name followed Shedko, 2005, as ezoe usually used in Japan is a junior synonym), T. brandtii (AB162630) (brandti often used in Japan is not the original spelling), T. nakamurai (AB162637), Siberian Pseudaspius leptocephalus (AB162638) and Leuciscus waleckii (AB162640) (data for Tribolodon, Pseudaspius and Leuciscus by K. Watanabe et al., unpublished data) with Cyprinus carpio (X61010, Chang et al., 1994) as an out group, in order to survey the relationships among Far Eastern phoxinins. Cavender and Coburn (1992) divided the subfamily Leuciscinae into two

phyletic groups based on detailed morphological comparison, the leuciscins including Eurasian forms such as Leuciscus and the phoxinins including Phoxinus, Tribolodon, Rhynchocypris, Pseudaspius, Eupallasella (R. perenurus in the present study, see Table 1), and Lagowskiella (R. lagowskii in the present study, see Table 1). Therefore the design of comparative genera in the present study is thought to be reasonable.

Heuristic maximum-likelihood (ML), heuristic maximum-parsimony (MP) and NJ analyses were conducted with 1000 bootstrap replications using PAUP*4.0b10 (Swofford, 2002) for phylogenetic analyses including the related genera. The HKY model (Hasegawa et al., 1985) with some sites assumed to be invariable and with variable sites assumed to follow a discrete gamma distribution (HKY + I + G) was selected as the best-fit model of nucleotide substitution by ModelTest version 3.06 (Posada and Crandall, 1998) for ML analysis. Gaps were considered as missing data. The HKY85 distance was employed for NJ analysis.

RESULTS

Phylogeny based on allozyme analysis

Phoxinus phoxinus was the most genetically distant from the others (mean \underline{D} = 0.846-2.487, 11-16 loci being displaced), with Rhynchocypris kumgangensis and R. semotilus being next distant from the remaining species (\underline{D} = 0.699-1.143, 8-13 loci being displaced) (Table 3). Rhynchocypris lagowskii was split into two genetically different groups, lagowskii 1 and lagowskii 2 (see also Fig. 2), and these two and R. oxycephalus were closely related to each other (\underline{D} = 0.365-0.439, 4-7 loci being displaced), but were somewhat far from the remaining species, R. perenurus and R. czekanowskii (\underline{D} = 0.630-0.725, 6-11 loci displaced). Rhynchocypris oxycephalus, R.

perenurus and R. czekanowskii were relatively close to one another ($\underline{D} = 0.280-0.579$, 2-9 loci being displaced).

In the NJ dendrogram based on the allozyme data (Fig. 2A), Phoxinus phoxinus was distantly related to the Rhynchocypris species, which were clustered at a high bootstrap probability ($p = 91\%$). In the latter genus, R. kumgangensis and R. semotilus were connected as the outermost branches. Rhynchocypris oxycephalus, R. czekanowskii and R. perenurus each formed three monophyletic clusters, although the bootstrap probabilities were not so high ($p = 44, 32$ and 26% , respectively), with the latter two species forming a cluster ($p = 71\%$) and then connecting with the former ($p = 33\%$). On the other hand, R. lagowskii populations were divided into two clusters, lagowskii 1 from Japan and the Amur basin ($p = 81\%$) and lagowskii 2 from Vladivostok to Korea ($p = 24\%$).

Mitochondrial phylogeny based on 16S rRNA sequence

Sequences of 1295 bp were aligned unambiguously. Excluding indels, 1277 bp were utilized for phylogenetic analysis. Nucleotide substitutions (Ts and Tv) among haplotypes were not so saturated (Fig. 3), with K2P distances becoming larger linearly according to the number of substitutions. However, the plots could be roughly divided into four zones (Fig. 3, see also Table 4) corresponding to intra-specific data (zone 1; mean K2P = 0.003-0.017, Ts/Tv = 0-25/0-8), inter-specific data for Rhynchocypris species other than R. semotilus and R. kumgangensis (zone 2; K2P = 0.030-0.040, Ts/Tv = 22-46/5-13), those between R. semotilus or R. kumgangensis and the other Rhynchocypris species (zone 3; K2P = 0.043-0.064, Ts/Tv = 43-58/6-24), and inter-generic data between Rhynchocypris and Phoxinus (zone 4; K2P = 0.089-0.107, Ts/Tv = 78-96/18-32).

In the NJ dendrogram based on mtDNA data (Fig. 2B), Phoxinus phoxinus was

very distant from Rhynchocypris species, the latter forming a cluster with a high bootstrap value ($p = 100\%$). Rhynchocypris kumgangensis and R. semotilus were connected as the monophyletic outermost branch ($p = 54\%$) in the genus.

Rhynchocypris lagowskii populations were divided into two clusters, lagowskii 1 from Japan and the Amur basin and lagowskii 2 from Vladivostok to Korea, as in the case of the allozyme analysis. Except for some haplotypes of R. oxycephalus and R. perenurus included in different clusters (described below), the other species each formed a cluster with a high bootstrap value: R. oxycephalus ($p = 95\%$), R. perenurus ($p = 100\%$), R. czekanowskii ($p = 100\%$), R. lagowskii 1 ($p = 94\%$) and R. lagowskii 2 ($p = 98\%$).

Rhynchocypris oxycephalus from around Vladivostok (9. Sukhodol, 3 individuals) and the Amur (10. Manoma, 3 individuals) were included in the R. lagowskii 1 cluster, and R. perenurus from Anadyr (29. Anadyr, 5 individuals) was in the R. czekanowskii cluster.

Generic relationships

Figure 4 represents the ML dendrogram with bootstrap probabilities in percentage on major nodes (for ML/MP/NJ analyses), indicating phylogenetic relationships among related genera. The MP dendrogram showed the same topology as the ML dendrogram, whereas the NJ dendrogram differed slightly in that Rhynchocypris semotilus was also connected to the Tribolodon-Pseudaspius cluster with R. kumgangensis paraphyletically by very low probability. In all analyses, Tribolodon and Pseudaspius fitted inside Rhynchocypris, forming a monophyletic cluster with relatively high probability ($p = 52/92/97\%$). Phoxinus phoxinus was connected next to this cluster ($p = 80/46/36\%$) but was paraphyletic with the American Phoxinus (P. erythrogaster and P. neogaeus). Leuciscus waleckii joined

after them.

DISCUSSION

Recognized species

Phoxinus phoxinus, Rhynchocypris oxycephalus, R. perenurus, R. czekanowskii, R. kumgangensis and R. semotilus are supported as monophyletic taxa by both allozyme and mtDNA analyses as shown by Ito et al. (2002), except for some mtDNA haplotypes of R. oxycephalus and R. perenurus, which are included in different species clusters, as discussed in the following section in terms of introgression. Rhynchocypris lagowskii is divided into two clusters, lagowskii 1 and 2, as also suggested by Ito et al. (2002). The taxonomy of lagowskii 1 and 2 is touched on in the section after the next. The generic taxonomy is discussed in the last section.

Russian scientists have treated oxycephalus and lagowskii as two subspecies of Phoxinus lagowskii (e.g. Berg, 1949; Chereshev, 1998) (see Table 1), but they clearly comprise three taxonomic entities or species (Rhynchocypris oxycephalus, R. lagowskii 1 and 2) in our genetic analyses.

Howes (1985) included a nominal species jouyi (Jordan and Snyder, 1901), a subspecies of oxycephalus according to Hosoya (2002), in a dace genus Tribolodon (Table 1). However, fish from Tsushima Island (6. Tsushima), the type locality of jouyi, are members of the oxycephalus cluster in both analyses, and, therefore, jouyi should be a junior synonym of or a subspecies of Rhynchocypris oxycephalus.

Hosoya (2002) treated the nominal species Phoxinus steindachneri Sauvage (1883) originally described from Lake Biwa, Japan, as a subspecies of lagowskii, described from the Amur River (21. Chita in the present study). Kim (1997) adopted the former species name and Uchida (1939) used the latter name for the Korean

species. On the other hand, Howes (1985) recognized them as two species in two genera (Table 1). Our analyses, however, indicate they are included in the lagowskii 1 cluster, suggesting steindachneri is a junior synonym of or a subspecies of Rhynchocypris lagowskii.

Local mitochondrial introgression between species

Rhynchocypris oxycephalus from the Amur and Sukhodol rivers and R. perenurus from the Anadyr River have mtDNA haplotypes quite similar to those of R. lagowskii 1 and R. czekanowskii, respectively (Fig. 2B). They would have experienced mitochondrial introgression through past hybridization events. However, R. lagowskii 2, not lagowskii 1, is distributed around Vladivostok (present study), and R. czekanowskii is not distributed in the Anadyr River (Chereshnev, 1998).

Rhynchocypris oxycephalus around Vladivostok might have come from the Amur basin, presumably through a tributary change event between the Ussuri and Vladivostok sides in the past, as suggested by Lindberg (1972). In a similar way, R. perenurus, presumably already having undergone mtDNA introgression, might have settled in the Anadyr River from neighboring waters such as the Kolyma River, which is inhabited by R. czekanowskii (Chereshnev, 1998). An alternative possibility is that R. lagowskii 1 and R. czekanowskii had hybridized with R. oxycephalus and R. perenurus at Vladivostok and Anadyr, respectively, and then disappeared. Further clarification must follow investigations on populations from a wider range.

The species pairs presumed to experience mtDNA introgression are apparently distinct species according to the allozyme data and the presumed introgressed mtDNAs are closely related to those of the donor species. Therefore, the possibility of an alternative explanation that the similarity in mtDNA sequence between species is due to the incomplete mitochondrial lineage sorting would be small.

Subdivision of species and its biogeographical implication

Rhynchocypris lagowskii 1 and 2 consistently formed separate clusters in both allozyme and mtDNA analyses with the exception of introgressed R. oxycephalus (discussed above). They are genetically different entities from each other and should be classified into two species. The nominal species lagowskii, originally described from the Amur, has usually been designated a continental fish, and a nominal species steindachneri, described from Japan, has mainly been designated a Japanese fish. However, both are included in the lagowskii 1 cluster in the present study as noted above. Therefore, the prior name lagowskii should be adopted as the species name for lagowskii 1. As for lagowskii 2, a nominal species Pseudaspius bergi Jordan and Metz (1913) from North Korea may be a candidate. The taxonomy of R. lagowskii 1 and 2 will be treated elsewhere by some of the authors. The biogeographical reason that the R. lagowskii 1 range is divided into two regions, Amur and eastern Japan, interrupted by R. lagowskii 2 and also by R. oxycephalus on the Korean Peninsula, is unknown and a very challenging question.

There remain other possible incidences of subspecific differentiation, such as between Japanese-Korean and Russian populations of R. oxycephalus or between Japanese and Russian populations of R. perenurus.

Russian populations of R. oxycephalus appear to have the mtDNA haplotypes of R. lagowskii 1, as discussed in the previous section. They also differ slightly from Japanese-Korean populations of R. oxycephalus in allozyme allelic composition, probably indicating a difference in biogeographical history. The Amurian freshwater fish fauna is considered to include the Chinese element that came from China through a tributary change event between the Ryao and Amur rivers (Nishimura, 1967). Rhynchocypris oxycephalus in the Amur basin would have been a member of

the Chinese element, underwent mitochondrial introgression from Amurian R. lagowskii 1, and then some of them would have invaded the Vladivostok region. Japanese and Korean scientists have often applied the species or subspecies name jouyi, originally described from Tsushima Island, to the Japanese fish and oxycephalus, originally described from Beijing, China, to the Korean fish (Table 1). However, the Japanese and Korean populations are more closely related than are the Korean and Russian populations. If different subspecific status should be given to Russian and Japanese-Korean populations, it would be necessary to clarify to which populations the Beijing population is closer before deciding their subspecies names.

Two Hokkaido populations of Rhynchocypris perenurus form a monophyletic line in the species cluster in both allozyme and mtDNA analyses (Fig. 2). Especially in mitochondrial analysis, the species cluster is divided into Japanese and Russian subclusters, except for the Anadyr population that is thought to have undergone mtDNA introgression. This species has usually been divided into two subspecies, sachalinensis (Berg, 1907) from the Hokkaido and Sakhalin Islands, and mantschuricus (Berg, 1907) from Siberia (e.g. Nakamura, 1969; Chereshev, 1998). However, the Sakhalin population (24. Tym) was included in the Siberian subcluster in the present study. The nominal species sachalinensis was originally described from the southernmost part of Sakhalin Island. If the type locality population is a member of the Hokkaido group genetically, two historically different groups are to inhabit the Sakhalin Island. The clarification of the formation of the biogeographical pattern of R. perenurus may throw light on the enigma of how the freshwater fish fauna of Hokkaido and Sakhalin Islands was formed (Maekawa and Goto, 1982).

For any such clarification and classification, however, further genetic and morphological research on more populations from a wider range is necessary because the distribution patterns of such probable species or subspecies pairs are different

from those described up until now in the literature.

Generic taxonomy

Both sets of genetic data strongly support two major divisions among the northern Far East minnows (Figs. 2 and 3), the genera Phoxinus and Rhynchocypris, verifying the suggestion of Ito et al. (2002). For the species included in Rhynchocypris, Howes (1985) applied several genus names including Rhynchocypris, Eupallasella, Lagowskiella, and so on (Table 1). However, his L. lagowskii and L. czekanowskii did not form a monophyletic cluster in either analysis, and his Phoxinus semotilus was more closely related to Rhynchocypris species than to P. phoxinus (Fig. 2). Of them, the genus Rhynchocypris has priority, first described as R. variegatus from China in 1889 and later synonymized with P. lagowskii (after Berg, 1949; Howes, 1985).

In the generic relationship analysis (Fig. 4), Rhynchocypris species formed a monophyletic group with the dace genera Tribolodon and Pseudaspius rather than with the minnow genus Phoxinus. This result supports the division between Rhynchocypris and Phoxinus. Fujita and Hosoya (2003) argued that there is no evidence indicating the monophyly of Rhynchocypris as well as of Phoxinus, and that, therefore, the former name should be used for both groups. However, it is at least evident that the sister group of Rhynchocypris is not Phoxinus but Tribolodon and Pseudaspius. This result produces a problem about relationships among Rhynchocypris, Tribolodon and Pseudaspius. Sakai et al. (2002) have already reported that Pseudaspius is most closely related to Tribolodon in terms of allozyme allelic composition. Cavender and Coburn (1992) have also documented that Rhynchocypris and Tribolodon share several morphological characteristics, suggesting a closer relationship between the two than with any other genera. The

dace genera Tribolodon and Pseudaspius may have been a specialized clade in or from a minnow clade that includes Rhynchocypris, adapting to a large body of water such as the sea. The clarification of their relationships must follow more genetic and morphological comparative studies. At any rate, the Far Eastern minnows are apparently paraphyletic and should tentatively be divided into two genera, Rhynchocypris and Phoxinus.

Eurasian Phoxinus phoxinus and American Phoxinus species are also suggested to be paraphyletic (Fig. 4). Howes (1985) recognized several species of Phoxinus in both Eurasia and North America. Chen (1996) also considered Phoxinus as only one cyprinid genus distributed on the two continents, with three species in Eurasia and six in North America. There remains a possibility that the American Phoxinus should be classified into a different genus after a worldwide study on genetic comparison among species of the genus Phoxinus.

ACKNOWLEDGMENTS

We appreciate the help of the following persons: Valentina G. Sideleva, Sergei F. Zolotukhin, Hwa-Kun Byeon, Mutsumi Nishida, Katsutoshi Watanabe, Keisuke Takata, Hitoshi Ida, Keiichiro Iguchi, Kazuhiko Katsura, Yuji Yamazaki, Hiroshi Takahashi. Thanks also go to Dr. Margaret D. Ohto for English correction. This study was supported in part by Grants-in-Aid for Overseas Scientific Survey Nos. 06041004, 09041138 and 15405008 from the Ministry of Education, Science, Sports and Culture, Japan.

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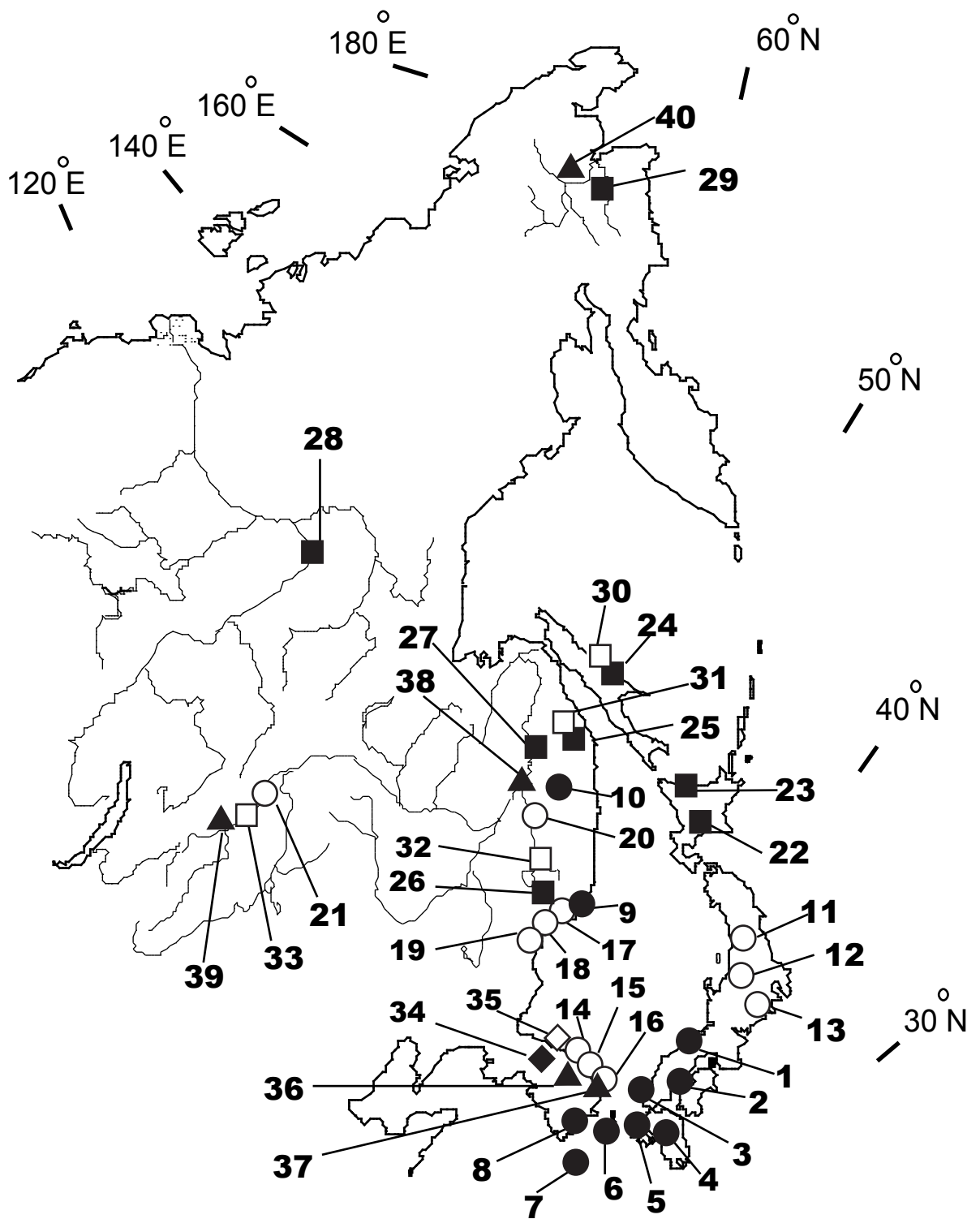
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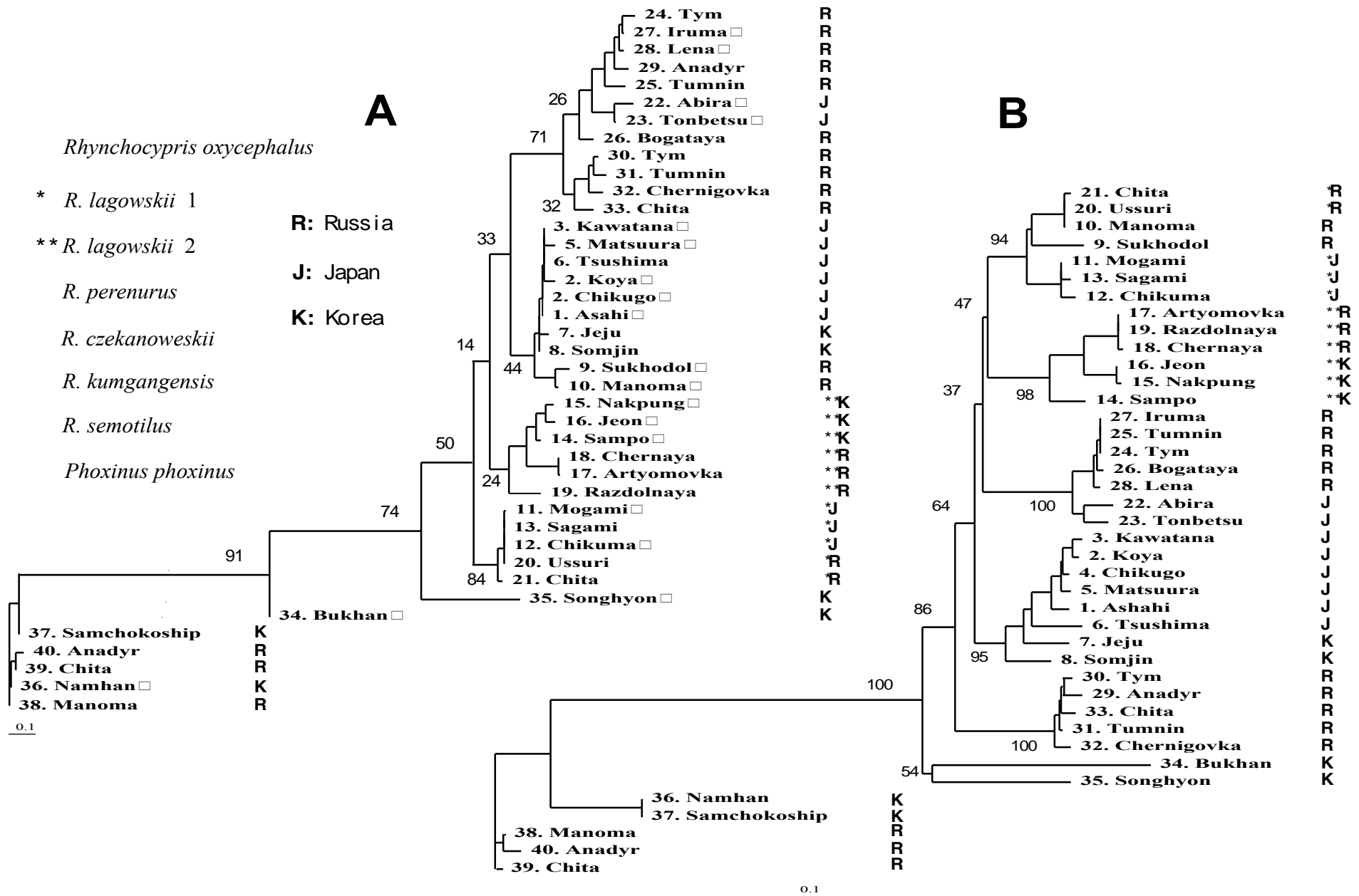
Fig. 1. Sampling localities of phoxinin minnows from the northern Far East region: Rhynchocypris oxycephalus (closed circles), R. lagowskii group (open circles; populations 11-13, 20 and 21 are R. lagowskii 1, and populations 14-19 are R. lagowskii 2), R. perenurus (closed squares), R. czekanowskii (open squares), R. kumgangensis (closed diamond), R. semotilus (open diamond), and Phoxinus phoxinus (closed triangles). Locality numbers correspond to those in Table 1.

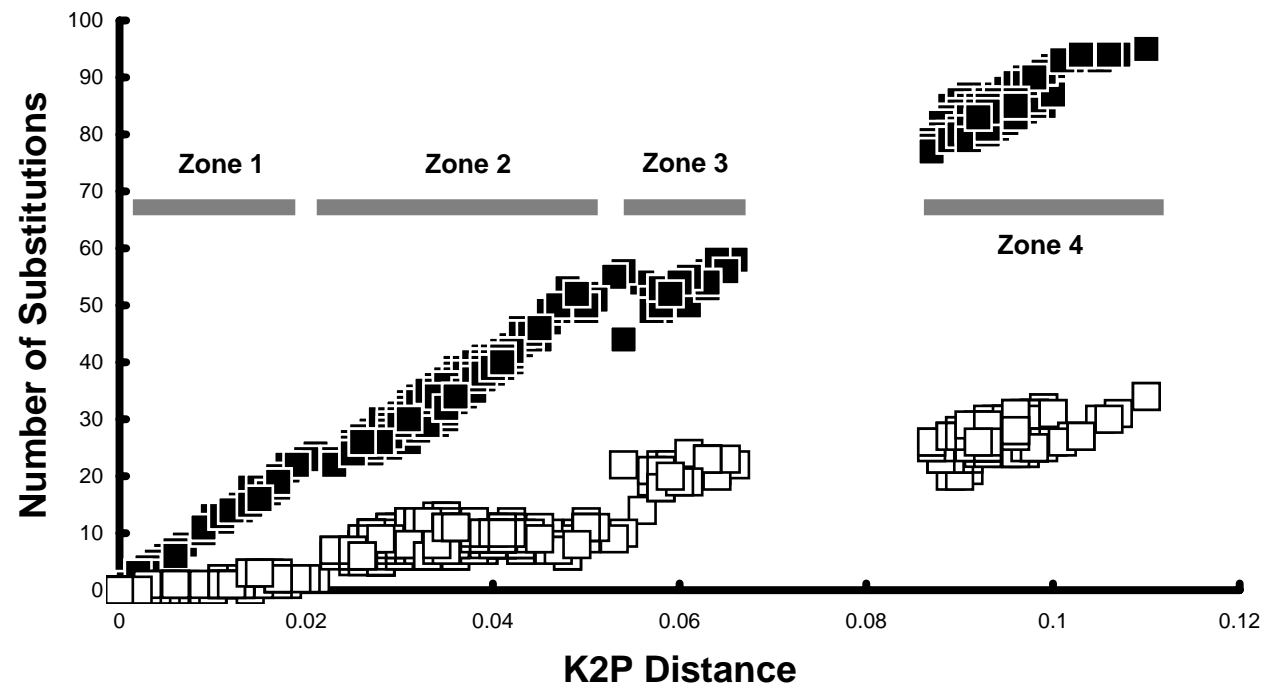
Fig. 2. Neighbor-joining tree of 40 populations of phoxinin minnows from the northern Far East region based on Nei's (1972) genetic distance for allozyme data (A), and that of 40 individuals representative of the 40 populations based on Kimura's (1985) two parameter distance for mtDNA 16S rRNA gene data (B). Locality numbers correspond to those in Table 1. Bootstrap probabilities in % for 1000 replications are indicated at nodes.

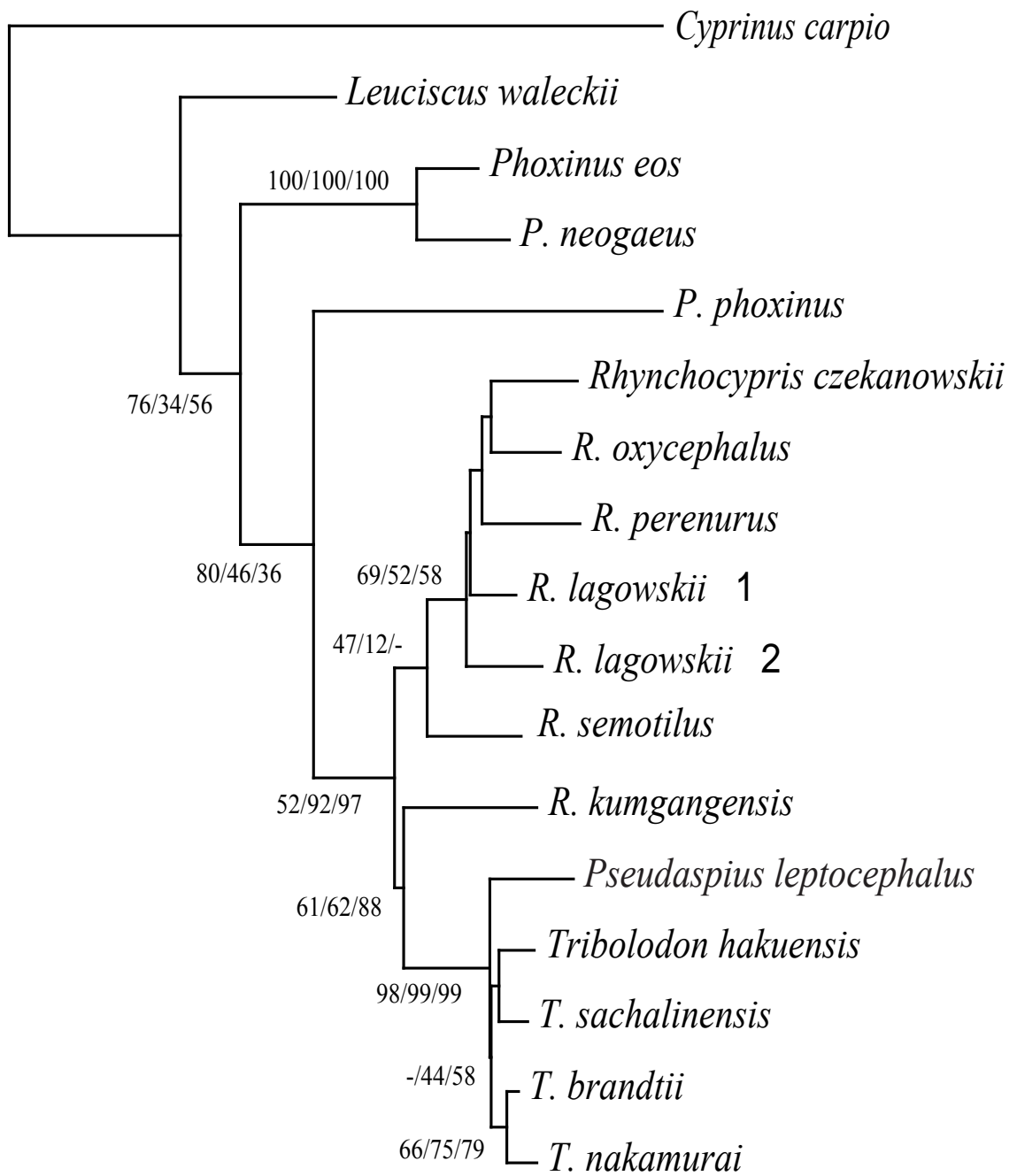
Fig. 3. Absolute number of substitutions in transitions (closed squares) and transversions (open squares) against Kimura's (1985) two parameter distance for all pairwise comparisons between 40 individuals of northern Far East phoxinin minnow. Zones 1, 2, 3, and 4 show intra-specific data, inter-specific data for Rhynchocypris species other than R. semotilus and R. kumgangensis, data between R. semotilus - R. kumgangensis and other Rhynchocypris species, and inter-generic data between Rhynchocypris and Phoxinus, respectively.

Fig. 4. Maximum-Likelihood tree (ML) of Rhynchocypris species, northern Far East Phoxinus phoxinus, two species of North American Phoxinus, Leuciscus, Pseudaspius, Tribolodon species, and Cyprinus carpio (as the outgroup). Bootstrap probabilities in % for 1000 replications (ML/Maximum-Parsimony method, MP/Neighbor-Joining method, NJ) are indicated at major nodes. "-" indicates less than 5%.









0.01 substitutions/site

Table 1. Nominal species of phoxinin minnows (Cyprinidae) reported in the northern Far East

Present study	Japan		Korea		Russia		Howes (1985)
	Nakamura (1969)	Hosoya (2002)	Uchida (1939)	Kim (1997)	Berg (1949)	Chereshnev (1998)	
<u>Rhynchocypris oxycephalus</u> (Sauvage & Dabry)	<u>Moroco juyi</u> (Jordan & Snyder)	<u>Phoxinus oxycephalus juyi</u>	<u>M. oxycephalus</u>	<u>R. oxycephalus</u>	<u>P. lagowskii oxycephalus</u>	<u>P. lagowskii oxycephalus</u>	<u>Tribolodon juyi</u> and <u>R. oxycephalus</u>
<u>R. lagowskii</u> * (Dybowski)	<u>M. steindachneri</u> (Sauvage)	<u>P. lagowskii steindachneri</u>	<u>M. lagowskii</u>	<u>R. steindachneri</u>	<u>P. lagowskii lagowskii</u>	<u>P. lagowskii lagowskii</u>	<u>Lagowskiella lagowskii</u> and <u>R. steindachneri</u>
<u>R. perenurus</u> ** (Pallas)	<u>M. percnurus sachalinensis</u> (Berg)	<u>P. percnurus sachalinensis</u>	<u>M. percnurus</u>	<u>R. percnurus</u>	<u>P. percnurus</u> 6 subspecies	<u>P. perenurus</u> ** 2 subspecies	<u>Eupallasella percnurus</u>
<u>R. czekanowskii</u> (Dybowski)	-	-	-	-	<u>P. czekanowskii</u> 5 subspecies	<u>P. czekanowskii</u> 3 subspecies	<u>L. czekanowskii</u>
<u>R. kumgangensis</u> (Kim)	-	-	<u>Moroco sp.</u>	<u>R. kumgangensis</u>	-	-	-
<u>R. semotilus</u> (Jordan & Starks)	-	-	<u>M. semotilus</u>	<u>R. semotilus</u>	-	-	<u>P. semotilus</u>
<u>Phoxinus phoxinus</u> (Linnaeus)	-	-	<u>P. phoxinus</u>	<u>P. phoxinus</u>	<u>P. phoxinus</u> 3 subspecies	<u>P. phoxinus</u>	<u>P. phoxinus</u>

*:Rhynchocypris lagowskii includes two species, lagowskii 1 and 2, in the present study.

**:The correct spelling is “perenurus” and not “percnurus” (Eschmeyer, 1998).

Table 2. Sampling locality, abbreviation, date of collection and sample size of 40 populations of 6 *Rhynchocypris* and 1 *Phoxinus* species

Number	Abbreviation	Locality	Date	Sample size***
<u>Rhynchocypris oxycephalus</u>				
1	Asahi	Asahi R., Japan	June 1999	20
2	Koya	Koya R., Japan	June 1999	20
3	Kawatana	Kawatana River, Japan	December 1999	20
4	Chikugo	Chikugo R., Japan	September 1999	20
5	Matsuura	Matsuura R., Japan	January 2000	20
6	Tsushima	Sago R., Tsushima Island, Japan	April 1999	20
7	Jeju	A small river of Jeju Island, Korea	August 1999	20
8	Somjin	Somjin R., Korea	May 1996	11
9	Sukhodol	Sukhodol R., Russia	August 1997	22
10	Manoma	Manoma R., Amur R. system, Russia	August 1997	20
<u>R. lagowskii</u>				
11*	Mogami	Mogami R., Japan	October 1997	20
12*	Chikuma	Chikuma R., Japan	July 1997	10
13*	Sagami	Sagami R., Japan	January 1998	20
14**	Sampo	Sampo R., Korea	May 1996	20
15**	Nakpung	Nakpung R., Korea	May 1996	20
16**	Jeon	Jeon R., Korea	May 1996	20
17**	Artyomovka	Artyomovka R., Russia	June 2001	15
18**	Chemaya	Chemaya R., Russia	June 2001	20
19**	Razdolnaya	Razdolnaya R., Russia	June 2000	19
20*	Ussuri	Ussuri R., Amur R. s., Russia	August 1997	14
21*	Chita	Chita R., Amur R. s., Russia	July 2001	12
<u>R. perenurus</u>				
22	Abira	Abira R., Japan	June 1996	13
23	Tonbetsu	Tonbetsu R., Japan	June 1993	20
24	Tym	Tym R. Sakhalin, Russia	August 2000	6
25	Tumnin	Tumnin R., Russia	August 1998	4
26	Bogataya	Bogataya R., Russia	July 2001	20
27	Iruma	Iruma R., Amur R. s., Russia	August 1997	20
28	Lena	Lena R., Russia	August 1998	20
29	Anadyr	Anadyr R., Russia	August 1998	20
<u>R. czekanowskii</u>				
30	Tym	Tym R. Sakhalin, Russia	August 2000	9
31	Tumnin	Tumnin R., Russia	August 1998	4
32	Chemigovka	Chemigovka R., Amur R. s., Russia	August 1998	22
33	Chita	Chita R., Amur R. s., Russia	July 2001	5
<u>R. kumgangensis</u>				
34	Bukhan	Bukhan R., Korea	May 1996	20
<u>R. semotils</u>				
35	Songhyon	Songhyon R., Korea	July 1999	16
<u>Phoxinus phoxinus</u>				
36	Namhan	Namhan R., Korea	April 1996	20
37	Samchokoship	Samchokoship R., Korea	May 1996	20
38	Manoma	Manoma R., Amur R. S., Russia	August 1997	20
39	Chita	Chita R., Amur R. s., Russia	July 2001	4
40	Anadyr	Anadyr R., Russia	August 1998	20

* and **: *R. lagowskii* 1 and 2, respectively.

***: Used for allozyme analyses, and each one specimen for mitochondrial DNA analyses except for 9. Sukhodol (3 individuals), 10. Manoma (3 individuals), and 29. Anadyr (5 individuals).

Table 3. Number of diagnostic allozyme loci out of 18 loci examined (above diagonal) and Nei's (1972) genetic distance (below diagonal: mean with standard deviation in parentheses) between pairs of 8 OTUs (operational taxonomic units) of 6 *Rhynchoypris* and 1 *Phoxinus* species, and intra-OTU values (on diagonal); *R. lagowskii* is divided according to the genetic relationship into 2 OTUs, *lagowskii*1 from Japan and the Amur River and *lagowskii* 2 from Vladivostok to Korea (see Fig. 2).

	<i>Rhynchoypris</i>							<i>Phoxinus</i>
	<i>oxycephalus</i>	<i>lagowskii</i> 1	<i>lagowskii</i> 2	<i>perenus</i>	<i>czekanowskii</i>	<i>kumgangensis</i>	<i>semotilus</i>	<i>phoxinus</i>
<i>R. oxycephalus</i>	0.074(0.061) (0-2)	5-7	4-7	6-9	5-8	11-12	8-11	14-15
<i>R. lagowskii</i> 1	0.389(0.072)	0.002(0.002) (0)	5-6	8-10	7-9	10-11	9-11	15
<i>R. lagowskii</i> 2	0.439(0.075)	0.365(0.040)	0.220(0.164) (1-5)	8-11	6-10	9-12	8-10	15
<i>R. perenus</i>	0.579(0.076)	0.771(0.065)	0.725(0.084)	0.148(0.074) (0-4)	2-6	10-13	10-12	16
<i>R. czekanowskii</i>	0.489(0.077)	0.630(0.055)	0.682(0.114)	0.280(0.069)	0.088(0.038) (1-2)	11-12	10-11	15
<i>R. kumgangensis</i>	1.004(0.087)	0.832(0.008)	0.843(0.209)	1.143(0.072)	1.081(0.095)	-	7	11
<i>R. semotilus</i>	0.826(0.080)	0.699(0.007)	0.712(0.114)	0.922(0.073)	0.895(0.064)	0.481	-	16
<i>P. phoxinus</i>	1.924 (0.143)	1.894(0.063)	1.663(0.185)	2.487(0.176)	1.916(0.072)	0.846(0.019)	2.162(0.011)	0.019(0.009) (0)

Table 4. Number of nucleotide substitutions (above diagonal: transition/transversion in 1277 bp) and Kimura's (1980) evolutionary distance (two-parameter model) (below diagonal: mean with standard deviation in parentheses) between pairs of 8 OTUs (operational taxonomic units) of 6 *Rhynchocypris* and 1 *Phoxinus* species, and intra-OTU values (on diagonal); *R. lagowskii* is divided according to the genetic relationship into 2 OTUs, *lagowskii* 1 from Japan and the Amur River and *lagowskii* 2 from Vladivostok to Korea (see Fig. 2). Data for *R. oxycephalus* and *R. perenurus* individuals that have haplotypes of different species were excluded from the calculation.

	<i>Rhynchocypris</i>							<i>Phoxinus</i>
	<i>oxycephalus</i>	<i>lagowskii</i> 1	<i>lagowskii</i> 2	<i>perenurus</i>	<i>czekanowskii</i>	<i>kumgangensis</i>	<i>semotilus</i>	<i>phoxinus</i>
<i>R. oxycephalus</i>	0.012(0.006) (1-24/0-3)	23-30/7-11	22-46/5-10	30-39/10-13	32-43/7-8	43-53/22-23	46-56/10-11	78-88/24-27
<i>R. lagowskii</i> 1	0.030(0.002)	0.010(0.005) (2-16/0-1)	24-33/5-8	23-37/10-12	32-38/8-9	49-51/19-20	43-46/7-8	80-90/23-26
<i>R. lagowskii</i> 2	0.036(0.006)	0.030(0.002)	0.012(0.006) (1-20/0-4)	31-42/9-13	36-41/7-10	50-53/18-21	47-52/6-9	83-93/22-27
<i>R. perenurus</i>	0.036(0.002)	0.031(0.003)	0.039(0.002)	0.006(0.005) (0-16/0-1)	36-45/10-11	51-58/23-24	47-51/11-12	81-87/27-30
<i>R. czekanowskii</i>	0.035(0.002)	0.036(0.003)	0.038(0.002)	0.040(0.002)	0.003(0.002) (0-7/0)	53-56/21	46-49/9	82-89/21-23
<i>R. kumgangensis</i>	0.059(0.002)	0.059(0.003)	0.058(0.002)	0.064(0.002)	0.063(0.002)	-	52/14	94-96/28-32
<i>R. semotilus</i>	0.050(0.003)	0.043(0.002)	0.046(0.002)	0.049(0.001)	0.046(0.001)	0.054	-	84-87/18-22
<i>P. phoxinus</i>	0.092(0.002)	0.094(0.003)	0.096(0.002)	0.096(0.002)	0.091(0.002)	0.107(0.003)	0.089(0.003)	0.017(0.011) (0-25/0-8)