

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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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; Chereshevnev, 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, *Eupalasella*, *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

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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)	
<i>Rhynchocypris oxycephalus</i> (Sauvage & Dabry)	<i>Moroco jouyi</i> (Jordan & Snyder)	<i>Phoxinus oxycephalus jouyi</i>	<i>M. oxycephalus</i>	<i>R. oxycephalus</i>	<i>P. lagowskii oxycephalus</i>	<i>P. lagowskii oxycephalus</i>	<i>Tribolodon jouyi</i> and <i>R. oxycephalus</i>
<i>R. lagowskii</i> * (Dybowski)	<i>M. steindachneri</i> (Sauvage)	<i>P. lagowskii steindachneri</i>	<i>M. lagowskii</i>	<i>R. steindachneri</i>	<i>P. lagowskii lagowskii</i>	<i>P. lagowskii lagowskii</i>	<i>Lagowskiella lagowskii</i> and <i>R. steindachneri</i>
<i>R. perenurus</i> ** (Pallas)	<i>M. percnurus sachalinensis</i> (Berg)	<i>P. percnurus sachalinensis</i>	<i>M. percnurus</i>	<i>R. percnurus</i>	<i>P. percnurus</i> 6 subspecies	<i>P. perenurus</i> * 2 subspecies	<i>Eupallasea percnurus</i>
<i>R. czekanowskii</i> (Dybowski)	–	–	–	–	<i>P. czekanowskii</i> 5 subspecies	<i>P. czekanowskii</i> 3 subspecies	<i>L. czekanowskii</i>
<i>R. kumgangensis</i> (Kim)	–	–	<i>Moroco sp.</i>	<i>R. kumgangensis</i>	–	–	–
<i>R. semotilus</i> (Jordan & Starks)	–	–	<i>M. semotilus</i>	<i>R. semotilus</i>	–	–	<i>P. semotilus</i>
<i>Phoxinus phoxinus</i> (Linnaeus)	–	–	<i>P. phoxinus</i>	<i>P. phoxinus</i>	<i>P. phoxinus</i> 3 subspecies	<i>P. phoxinus</i>	<i>P. phoxinus</i>

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

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

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 Dabry, 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**), phosphoglucosmutase (E. C. 5.4.2.2, *PGM**), L-iditol 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'-CACAAGCCTCGCTGTTTACCA-3') - H3058 (5'-TCCGGTCTGAACCTCAGATCACGTA-3') (Watanabe *et al.*, 2000) with a thermal cycler (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, 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.

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***
<i>Rhynchocypris oxycephalus</i>				
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
<i>R. lagowskii</i>				
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**	Chernaya	Chernaya 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
<i>R. perenurus</i>				
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
<i>R. czekanowskii</i>				
30	Tym	Tym R. Sakhalin, Russia	August 2000	9
31	Tumnin	Tumnin R., Russia	August 1998	4
32	Chernigovka	Chernigovka R., Amur R. s., Russia	August 1998	22
33	Chita	Chita R., Amur R. s., Russia	July 2001	5
<i>R. kumgangensis</i>				
34	Bukhan	Bukhan R., Korea	May 1996	20
<i>R. semotils</i>				
35	Songhyon	Songhyon R., Korea	July 1999	16
<i>Phoxinus phoxinus</i>				
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 analysis, and each one specimen for mitochondrial DNA analysis except for 9. Sukhodol (3 individuals), 10. Manoma (3 individuals), and 29. Anadyr (5 individuals).

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 *ezoë* 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 *Cypripinus carpio* (X61010, Chang *et al.*, 1994) as an out group, in order

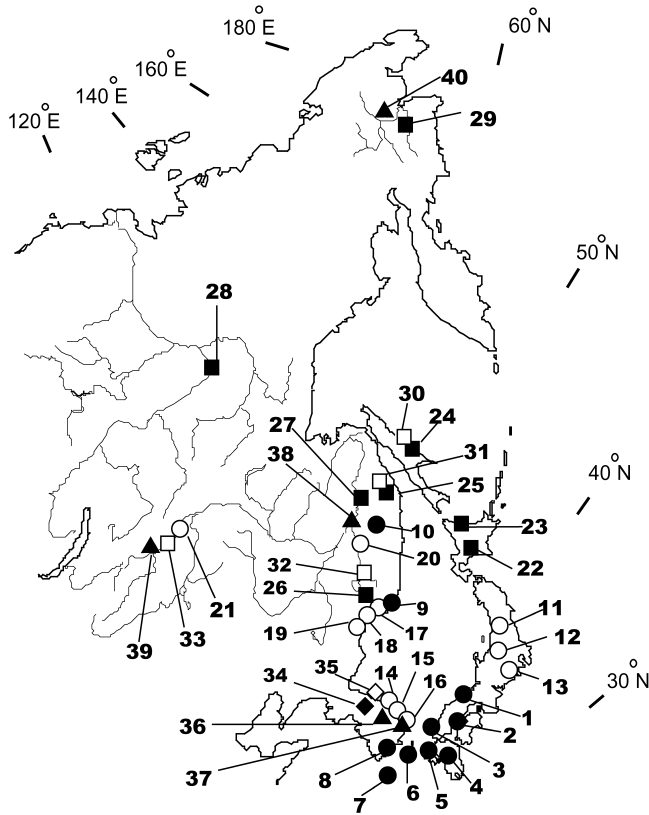


Fig. 1. Sampling localities of phoxinin minnows from the northern Far East region: *Rhynchocypris oxycephalus* (closed circles), *R. lagowskii* group (open circles; populations 11–13, 20 and 21 are *R. lagowskii* 1, and populations 14–19 are *R. lagowskii* 2), *R. perenurus* (closed squares), *R. czekanowskii*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

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 $D=0.846\text{--}2.487$, 11–16 loci being displaced), with *Rhynchocypris kumgangensis* and *R. semotilus* being next distant from the remaining species ($D=0.699\text{--}1.143$, 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 ($D=0.365\text{--}0.439$, 4–7 loci being displaced), but were somewhat far from the remaining species, *R. perenurus* and *R. czekanowskii* ($D=0.630\text{--}0.725$, 6–11 loci displaced). *Rhynchocypris oxycephalus*, *R. perenurus* and *R. czekanowskii* were relatively close to one another ($D=0.280\text{--}0.579$, 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*

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 *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).

	<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>	0.074(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. perenurus</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)

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

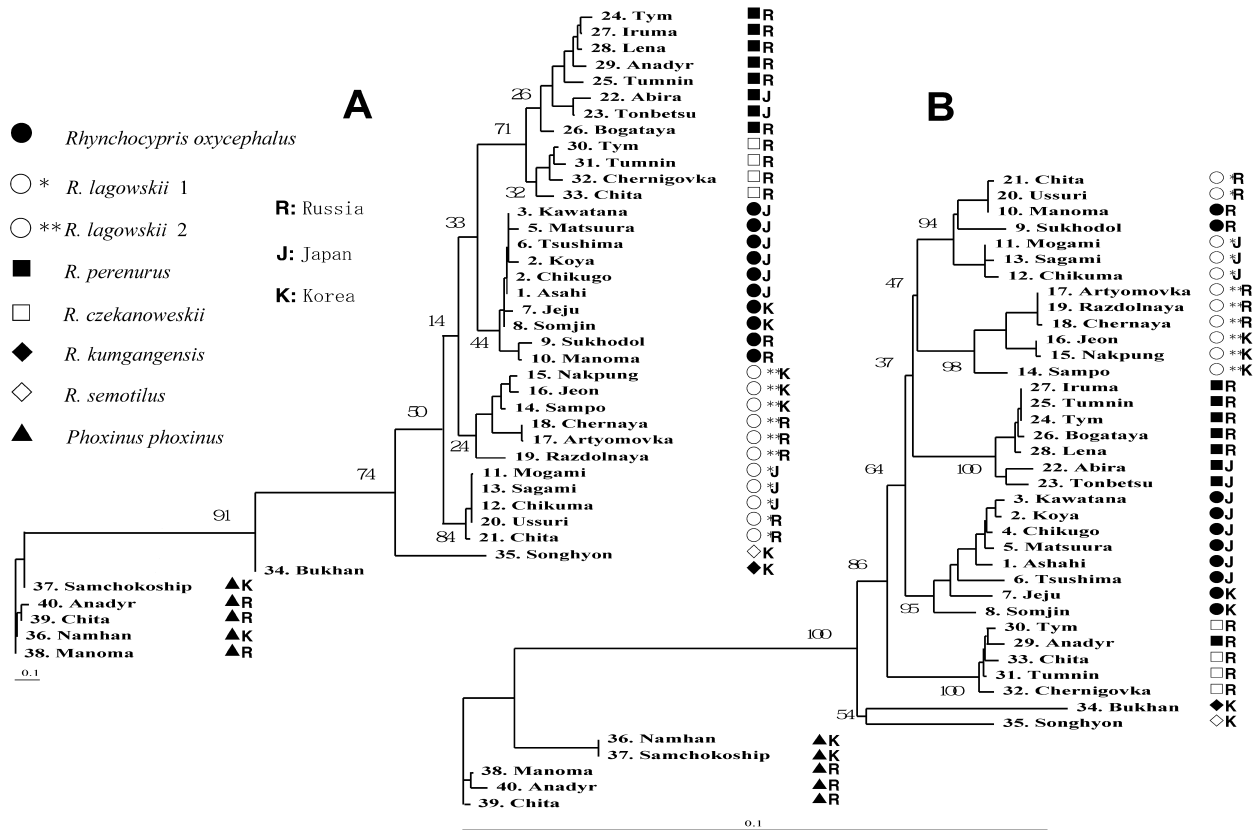


Fig. 2. Neighbor-joining tree of 40 populations of phoxinin minnows from the northern Far East region based on Nei's (1972) genetic distance for allozyme data (A), and that of 40 individuals representative of the 40 populations based on Kimura's (1985) two parameter distance for mtDNA 16S rRNA gene data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

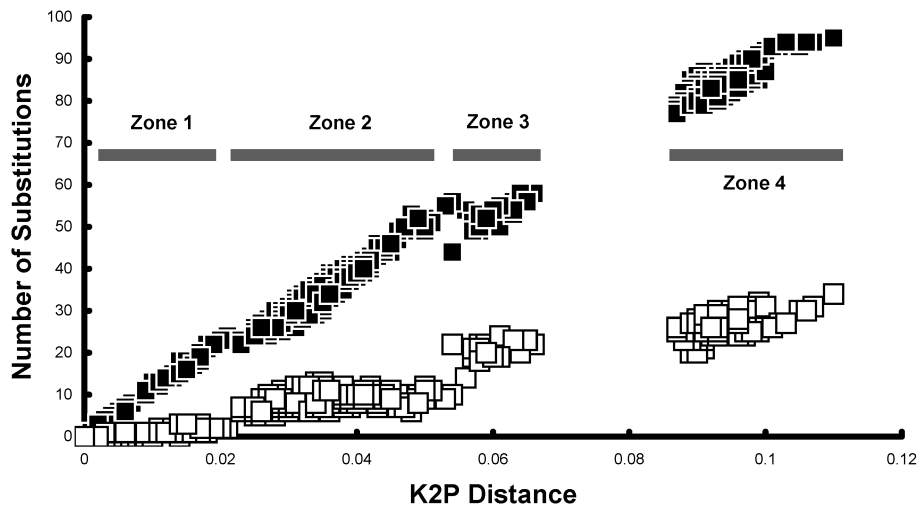


Fig. 3. Absolute number of substitutions in transitions (closed squares) and transversions (open squares) against Kimura's (1985) two parameter distance for all pairwise comparisons between 40 individuals of northern Far East phoxinin minnow. Zones 1, 2, 3, and 4 show intra-specific data, inter-specific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

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)

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

Fig. 4 represents the ML dendrogram with bootstrap

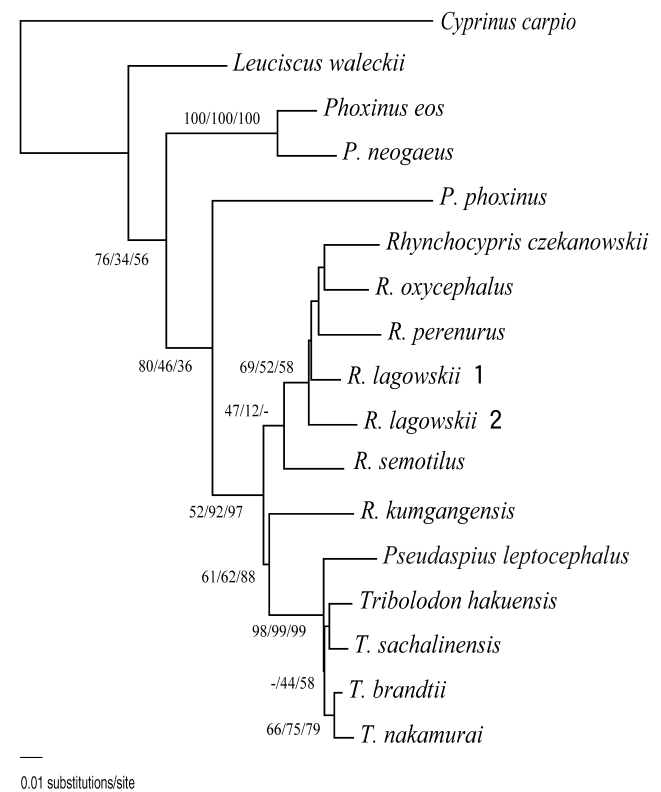


Fig. 4. Maximum-Likelihood tree (ML) of *Rhynchocypris* species, northern Far East *Phoxinus phoxinus*, two species of North American *Phoxinus*, *Leuciscus*, *Pseudaspius*, *Tribolodon* species, and *Cyprinus carpio* (as the outgroup). Bootstrap probabilities in % for 1000 replications (ML/Maximum-Parsimony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

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 (Chereshev, 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* (Chereshev, 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 *manschuricus* (Berg, 1907) from Siberia (e.g. Nakamura, 1969; Chereshevnev, 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*.

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