

Molecular phylogeny and intraspecific structure of loaches (genera *Cobitis* and *Misgurnus*) from the Far East region of Russia and some conclusions on their systematics

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Abstract Russian Far East loaches of the genera *Cobitis* and *Misgurnus* are among members of the family Cobitidae with poorly understood systematics. In this study we present phylogenetic hypotheses based on mitochondrial (cytochrome *b*) and nuclear (RAG-1) sequences. All analyses recovered comparable topological phylogenies, and all data sets supported the non-monophyly of the genera *Cobitis* and *Misgurnus*. Both genera are represented by multiple lineages that in some cases do not correspond to the species described. We found some phylogenetic incongruities for the genus *Misgurnus* (*M. mohoity* and *M. anguillicaudatus*) that are explained by ancient hybridization, as was suggested previously for *M. anguillicaudatus*. The revealed phylogenetic relationships suggest that *Paramisgurnus* should be treated as a synonym of *Misgurnus* and *M. bipartitus* as a synonym of *M. mohoity*. All analyses recovered *C. choui* as a member of the genus *Cobitis*, confirming previous taxonomic conclusions. Most of the molecular lineages found follow currently recognized taxa with some exceptions, such as

M. anguillicaudatus and *C. lutheri*. Phylogenetic relationships recover several unrelated lineages of *M. anguillicaudatus* and suggest additional studies to solve current taxonomic uncertainty. We found that *C. lutheri* is a non-natural group that contains two unrelated lineages: specimens of *C. lutheri* from the Far East of Russia collected close to the type locality and a second lineage with specimens of *C. lutheri* from Korea, the identification of which must be revised. The study provides evidence of the presence of the Far East species *M. nikolskyi* in Sakhalin Island, but simultaneously shows conspicuous genetic distinctiveness between the island and the mainland populations.

Keywords Cobitidae · Cytochrome *b* · RAG1 · Taxonomy · Amur River · Sakhalin Island

Introduction

The Far East Region of Russia represents the northeastern extreme of Asia with river drainages emptying towards the Pacific Ocean. It is located east of the Siberian and Baikal Lake areas and includes Sakhalin, Kuril, Wrangel, the Commander, and Shantarskiye islands. This region represents an area of faunal transition without major faunal breaks between Asian (southern) and Palearctic (northern) realms with a specific taxonomic composition of freshwater fish fauna (Warpachowski and Herzenstein 1887; Berg 1909; Taranetz 1938; Chereshev 1998; Bogutskaya et al. 2008). The Amur River with more than 200 tributaries is the main river system of the Far East region of Russia, covering also territories of northern China and northeastern Mongolia. Although recent studies have solved taxonomic problems in several taxa (Vasil'eva and Kozlova 1989; Vasil'eva 2001, 2007; Stevenson 2002; Shedko and Shedko

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2003; Vasil'eva and Makeyeva 2003; Vasil'eva et al. 2003; Shedko and Chereshev 2005; Shedko et al. 2005, 2008; Knizhin et al. 2006, 2007; Yamazaki et al. 2006; Vasil'ev and Vasil'eva 2008a, b), there are several groups of freshwater fishes with uncertain taxonomy and distribution limits in the Far East of Russia.

Recent studies on loaches of the genera *Cobitis* and *Misgurnus* have shown that three species of the genus *Cobitis*, namely *Cobitis choui*, *Cobitis lutheri*, and *Cobitis melanoleuca*, and two *Misgurnus* species, *Misgurnus mohoity* and *Misgurnus nikolskyi*, occur in the Amur River drainage and coastal waters of the Far East region of Russia (Vasil'eva 1998, 2001; Vasil'eva et al. 2003; Vasil'ev and Vasil'eva 2008a, b). Most of these loach species are restricted to East Asia, while *C. melanoleuca* is the only spined loach with continuous distribution from the Russian Far East and China to Europe with the western populations in the Don and Kuban river basins (Vasil'eva 1998). In the Far East region of Russia, *C. melanoleuca* is represented by the nominotypical subspecies *C. m. melanoleuca* that differs in the karyotype structure from the Siberian (*C. m. granoei*) and European (*C. m. gladkovi*) subspecies (Vasil'ev and Vasil'eva 2008a). Despite great progress in the taxonomy of loaches from the Far East of Russia, several systematic issues at the specific and population levels remain unsolved and need to be clarified. In recent years, molecular studies have elucidated the phylogenetic relationships of loaches of the family Cobitidae (Tang et al. 2006; Šlechtová et al. 2008). Their major conclusions showed many genera of the Cobitidae as non-monophyletic groups, e.g., *Cobitis* and *Misgurnus*, and in many taxa there was significant disparity between morphological and molecular results. These conclusions reinforce the idea that early classifications that relied on morphological characters, e.g., barbels, scale of Canestrini, scale, and pigmentation, to define taxonomic boundaries were not phylogenetic and in many cases were based on subjective identification of taxonomic differentiation. The other major conclusion was the identification of introgressed mtDNA in different loach groups, such as the genus *Misgurnus*, based on the incongruence of molecular markers (Šlechtová et al. 2008).

In this study, we used mitochondrial (cytochrome *b*) and nuclear (RAG-1) genes of all Russian Far East loaches of the genera *Cobitis* and *Misgurnus*, and other related genera to infer their phylogenetic relationships. We used range-wide populations of the different species of *Cobitis* and *Misgurnus* from the Far East of Russia and adjacent areas to evaluate their genetic variability and to identify their intraspecific structure. We analysed several morphologically atypical populations to provide a wide framework for evaluating contentious loach taxa, and we suggested an alternative hypothesis for the systematics of the genus *Misgurnus*.

Materials and methods

Specimens analysed. All loach species were collected by hand nets in 24 localities of the Far East region of Russia from 1996 up to 2007 (Fig. 1; Table 1). Thirty-one new specimens of *Cobitis* and 21 new *Misgurnus* were sequenced for cytochrome *b* (*cyt b*) and RAG-1. We included groups phylogenetically related with the Far East loaches (Šlechtová et al. 2008): *Paramisgurnus*, *Koreocobitis*, *Niwaella*, and *Sabanejewia* plus a wide representation of *Misgurnus*—*Misgurnus anguillicaudatus*, *Misgurnus bipartitus*, *Misgurnus fossilis*, and *Misgurnus mizolepis*, as well as *Misgurnus* sp. 1, *Misgurnus* sp. 2, and *Misgurnus* sp. 3 (as defined in the study of Šlechtová et al. 2008), and *Misgurnus* sp. (as in Wang and Tzeng, unpublished data), and *Cobitis*—*Cobitis biwae*, *Cobitis hankagensis*, *Cobitis rara*, *Cobitis shikokuensis*, *Cobitis sinensis*, *Cobitis striata*, *Cobitis takatsuensis*, and *Cobitis misgurnoides*. Currently *C. misgurnoides* is not considered a member of the genus *Cobitis*, and it was erected as the new genus *Microcobitis* by Bohlen and Harant (2010). *Microcobitis* was used as a close outgroup, whereas *Pangio* and *Sabanejewia* were phylogenetically more distant outgroups (Šlechtová et al. 2008). These sequences were recovered from GenBank [see Electronic Supplementary Material (ESM) Table S1] and new sequences were deposited in GenBank. Accession numbers (JN858807–858899) and a list of specimens sequenced with localities and collection numbers are provided in Table 1.

PCR analysis. Total DNA was extracted from ethanol-preserved fin tissue using Charge Switch gDNA Microtissue Kit (Invitrogen Inc., Carlsbad, CA, USA). The entire *cyt b* (1,140 base pair; bp) was PCR amplified using the primers GluDGL (Palumbi 1996) and H16460, or the primers Glu-L.Ca14337–14359 and Thr.-H.Ca15568–15548 (Šlechtová et al. 2006). Nuclear RAG-1 (897 bp) was PCR amplified using the primers RAG1-1F (Quenouille et al. 2004) and RAGRV1 (Šlechtová et al. 2007). Both genes were amplified in 25- μ l PCR reactions using the conditions described in Perdices et al. (2008). Primers for sequencing the purified PCR were the same as those used for PCR amplifications. Chromatograms and alignments were revised using Sequencher ver. 4.8 (Gene Codes Corporation Inc., Ann Arbor, MI, USA). The final data set for *cyt b* contained 1,114 bp to make comparable sequence alignments with some published sequences. None of the genes exhibited stop codons or gaps for *cyt b* or RAG-1, and all positions were used in the phylogenetic analyses. Nucleotide composition and base frequencies for all positions were checked using PAUP* 4.0b10 (Swofford 2002). Sequence divergences were calculated using Sequencer 6.1 (written by B. Kessing).

Fig. 1 Map indicating locations of sampling sites (numbered 1–24) for studied *Misgurnus* and *Cobitis* species from the Far East of Russia. Letters designate species according to the codes from Table 1

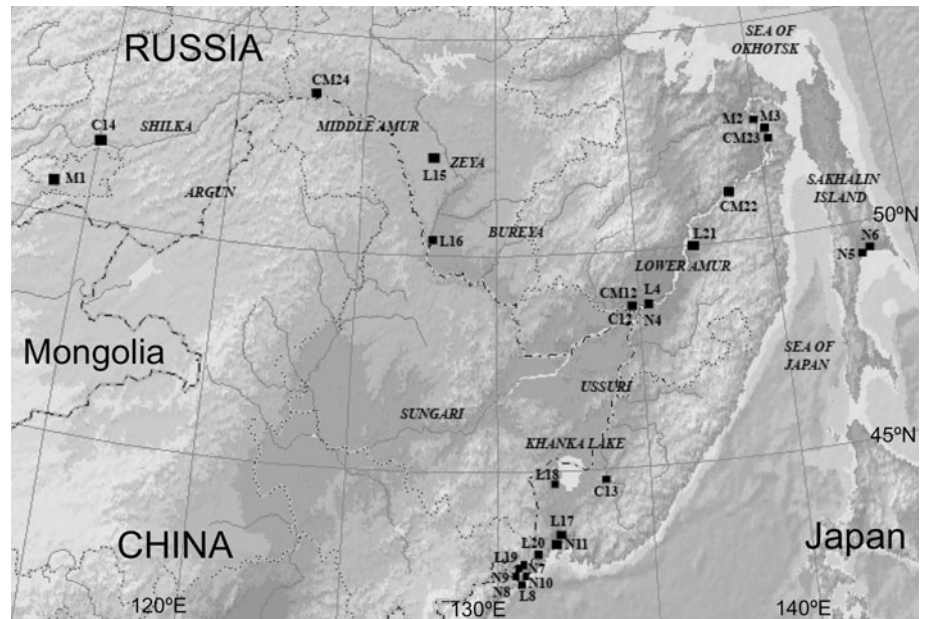


Table 1 Russian Far East loaches sequenced with sampling localities from Russia

Species	Sample site (river, tributary, drainage, locality, district)	Museum no.	Map code	ID	Accession no. RAG-1	Accession no. cyt <i>b</i>
<i>Misgurnus mohoity</i>	Ilya River, Onon R. tributary, Upper Amur drainage, Chita District	P-21540	M1	3603APT 3603BPT	JN858807 JN858808	JN858850 JN858851
	Amur R., Lower Amur drainage, at Susanino village, Khabarovsk District	–	M2	3604APT	JN858809	JN858852
	Unnamed spring, Lower Amur drainage, at Bogorodskoye village, Khabarovsk District	–	M3	3605PT	JN858810	JN858853
<i>Misgurnus nikolskyi</i>	Kupriyanikha R., Lower Amur drainage, Khabarovsk District	–	N4	3580APT 3580BPT	JN858811 JN858812	JN858854 JN858855
	Bol'shoye Chibisanskoye Lake, Aniva Bay, Sakhalin Island	P-21678	N5	3583APT 3583BPT	JN858813 –	JN858856 JN858857
	Maloye Vavayskoye Lake, Aniva Bay, Sakhalin Island	P-21679	N6	3584APT 3584BPT	JN858814 JN858815	JN858858 JN858859
	Lotos Lake, Posyet Bay, Primorye District	P-21930	N7	3626PT 3627PT	JN858816 JN858817	JN858860 JN858861
	Karasik R., Tumannaya R. drainage, Primorye District	P-21929	N8	3630PT 3631PT	– JN858878	JN858862 JN858863
	Grishka' channel, Tumannaya R. drainage, Primorye District	P-21927	N9	3633PT 3634PT 3636PT	JN858819 JN858820 JN858821	JN858864 JN858865 JN858866
	Mramornaya Bay, Posyet Bay, Primorye District	P-21928	N10	3637PT	JN858822	JN858867
	Channel between Amur Lagoon and unnamed lake at Beregovoye village, Primorye District	P-22499	N11	3640PT 3643PT 3646PT	JN858823 JN858824 JN858825	JN858868 JN858869 JN858870

Table 1 continued

Species	Sample site (river, tributary, drainage, locality, district)	Museum no.	Map code	ID	Accession no. RAG-1	Accession no. <i>cyt b</i>			
<i>Cobitis choui</i>	Tunguska R., Lower Amur drainage, Khabarovsk Dist.	P-21542	C12	3585DPT	JN858826	JN858871			
	Arsenyevka R., Ussury R. drainage, at Ozernoye village, Primorye District	P-21934	C13	3586APT	JN858827	JN858872			
				3586BPT	JN858828	JN858873			
				3653PT	JN858829	JN858874			
				3654PT	JN858830	JN858875			
<i>Cobitis lutheri</i>	Shilka R., Upper Amur drainage, at Borshevka village, Chita District	P-21544	C14	3600APT	JN858831	JN858876			
				3600BPT	–	JN858877			
<i>Cobitis lutheri</i>	Tygda R., Zeja R. tributary, Middle Amur drainage, Amur District	P-21545	L15	3590APT	JN858832	JN858878			
				3590BPT	–	JN858879			
	Kupriyanikha R., Lower Amur drainage, Khabarovsk District	P-21546	L4	3591APT	JN858833	JN858880			
				3591BPT	–	JN858881			
	Zeya R., Middle Amur drainage, at Blagoveshchensk city, Amur District	P-21547	L16	3592APT	JN858834	JN858882			
				3592BPT	JN858835	JN858883			
				3592CPT	JN858836	JN858884			
	Razdol'naya R. drainage, Amur Bay basin, Primorye Dist.	P-20041	L17	3602APT	–	JN858885			
	Man'chzhurka R., Khanka Lake drainage, Primorye Dist.	P-20050	L18	3604BPT	JN858837	–			
				Tsukanovka R., Posyet Bay, Primorye District	P-21920	L19	3608PT	JN858838	JN858886
							3609PT	JN858839	JN858887
3613PT							JN858840	JN858888	
				3614PT	JN858841	JN858889			
Karasik R., Tumannaya R. drainage, Primorye District	p-21919	L8	3620PT	JN858842	JN858890				
			3621PT	JN858843	JN858891				
Poyma R., Amur Bay basin, Primorye District	–	L20	3625PT	–	JN858892				
Gur R., Lower Amur drainage, at Voskresensk village, Khabarovsk District	P-21935	L21	3649PT	JN858844	JN858893				
			3650PT	JN858845	JN858894				
<i>Cobitis melanoleuca</i>	Tunguska R., Lower Amur drainage, Khabarovsk District	P-21551	CM12	3585APT	JN858846	JN858895			
				3585BPT	–	JN858896			
	Shelekhovka R., Lower Amur drainage, at Vozdvizhenskoye village, Khabarovsk District	P-21939	CM22	3587BPT	JN858847	JN858897			
				Amur R., Lower Amur drainage, at Savinskoye village, Khabarovsk District	P-21937	CM23	3588APT	–	JN858898
							3588BPT	JN858848	–
Ol'doi R., Middle Amur drainage, Amur District	P-21552	CM24	3596APT	JN858849	JN858899				

Museum numbers for voucher specimens stored at the Zoological Museum of the Moscow State University; Russia map code refers to locality numbers in Fig. 1, and identification (ID) refers to numbers for individuals on cytochrome *b* (*cyt b*) and RAG-1 phylogenies (Figs. 2, 3)

Phylogenetic analysis. Phylogenetic analyses were conducted for each aligned data set using the Bayesian inference (BI) method as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001), neighbour joining (NJ), and maximum parsimony (MP) methods using PAUP*. MP analysis was performed by heuristic searches with TBR branch swapping and ten replicates of random addition of taxa. Only minimal trees were retained and the zero branch length collapsed. For the NJ method, we selected the best fit nucleotide substitution model for each individual data set using the Akaike information criterion (AIC) by Modeltest ver. 3.7 (Posada and Crandall 1998). For the BI method, we ran 3,000,000 cycles of four simultaneous Monte Carlo Markov chains, sampling the Markov chains at intervals of 100 generations. Log-likelihood stability was attained after 80,000 generations, and we excluded the first 800 trees as burn-in. The remaining trees were used to compute a 50% majority rule consensus tree. Robustness of the inferred trees in the MP and NJ analyses was assessed by bootstrapping (1,000 replicates) (Felsenstein 1985), and by posterior probability values (ppb) in the BI procedure.

Results

Sequence diversity. The cytochrome *b* (1,114 bp) and nuclear RAG-1 (897 bp) nucleotide sequences were obtained from 52 new individuals (Table 1). All *cyt b* and RAG-1 sequences showed similar base composition each. Cytochrome *b* sequences had a strong bias against guanine (15.0%), a situation not observed in the nuclear gene (27.5%). Plots of transitions and transversions against uncorrected *p*-distances showed an absence of nucleotide saturation for *cyt b* and RAG-1. Of the 1,114 bp for *cyt b*, 491 were variable (11.7% 1st position, 2.5% 2nd, 32.4% 3rd) and 452 parsimony informative; for RAG-1, 178 positions were variable (19.8%) and 135 parsimony informative (15.1%) (excluding outgroups). Mean uncorrected *p*-distances found among congeneric species were $14.0 \pm 2.3\%$ for *cyt b* and $2.5 \pm 0.5\%$ for RAG-1 for *Cobitis*, and $14.7 \pm 3.0\%$ for *cyt b* and $2.1 \pm 0.8\%$ for RAG-1 for *Misgurnus*. Modeltest identified the GTR + G (1.1276) + I (0.5085) model as the most appropriate model for *cyt b*, and the SYM + G (0.8143) + I (0.6280) model for the RAG-1.

Phylogenetic relationships. All analyses (BI, MP, and NJ) were highly congruent for the separate *cyt b* and RAG-1 data sets. Although we found some incongruities between mitochondrial and nuclear topologies, both data sets supported the non-monophyly of the genera *Cobitis* and *Misgurnus* (Figs. 2, 3). Both *Cobitis* and *Misgurnus* are represented by multiple lineages that in some cases do not correspond to species described for both genera.

Mitochondrial analyses showed two major clades well supported in BI (98% ppb) (Fig. 2). One major clade, Clade A (100% ppb, >66% bootstrap MP, NJ), included all *Cobitis* species, *Niwaella delicata*, *N. multifasciata*, and some species of *Misgurnus* (*M. anguillicaudatus*, *M. bipartitus*, *M. mohoity*, *Misgurnus* sp., *Misgurnus* sp. 2, *Misgurnus* sp. 3) (Fig. 2). Samples of *Niwaella* were related with different *Cobitis* species. The specimens of *C. lutheri* from the Far East of Russia and Korea were not recovered as a monophyletic group. They were separated in two independent lineages with geographical structure and high genetic distances (mean uncorrected *p*-distances $11.3 \pm 0.25\%$). All specimens of *C. lutheri* from the Far East of Russia and one specimen from China were recovered in one lineage, while all specimens from Korea formed a different lineage closely related to *C. striata* (Biwa small race). Other Russian Far East loaches, *C. melanoleuca* and *C. choii*, were always recovered as monophyletic with low genetic intraspecific divergences (0.2 ± 0.2 and $0.2 \pm 0.2\%$, respectively).

The species *C. biwae*, *C. striata*, and *M. anguillicaudatus* were represented by multiple lineages, not related in a monophyletic group. These three taxa are documented as hybrid species with different ploidy (Kitagawa et al. 2003; Morishima et al. 2008; Šlechtová et al. 2008; Saitoh et al. 2010). Their phylogenetic relationships, as already shown in previous studies, varied on their ploidy and on the mitochondrial or nuclear gene used (see Saitoh et al. 2000, 2010; Kitagawa et al. 2001, 2003, 2005). Specimens of *M. mohoity*, *M. bipartitus*, and *Misgurnus* sp. 3 were recovered in a monophyletic group, and closely related to some specimens of *M. anguillicaudatus* and *Misgurnus* sp. 2.

The second major clade, Clade B, supported in BI included together *Koreocobitis*, *Paramisgurnus*, and the rest of the *Misgurnus* species analysed. Samples of *M. mizolepis* were recovered among specimens of *P. dabryanus* with relatively low genetic distances (uncorrected *p*-distances $2.5 \pm 1.3\%$). We found high intraspecific genetic diversity in *M. nikolskyi* specimens (*p*-distances $6.3 \pm 4.4\%$) with at least two well-differentiated mitochondrial lineages. One of them included all individuals collected on Sakhalin Island, and some specimens identified as *M. anguillicaudatus* from Japan, and another lineage that related *M. nikolskyi* specimens collected in the Far East of Russia, *Misgurnus* sp. 1 (Šlechtová et al. 2008), and one specimen from the Amur River in China (Saitoh et al. 2006). The species *M. fossilis* was recovered as monophyletic with low genetic divergences among specimens ($0.2 \pm 0.3\%$). In MP and NJ analyses, the second major clade, Clade B, was not supported, and it was subdivided into three independent lineages: one lineage included *Paramisgurnus* and *Misgurnus mizolepis*, the second

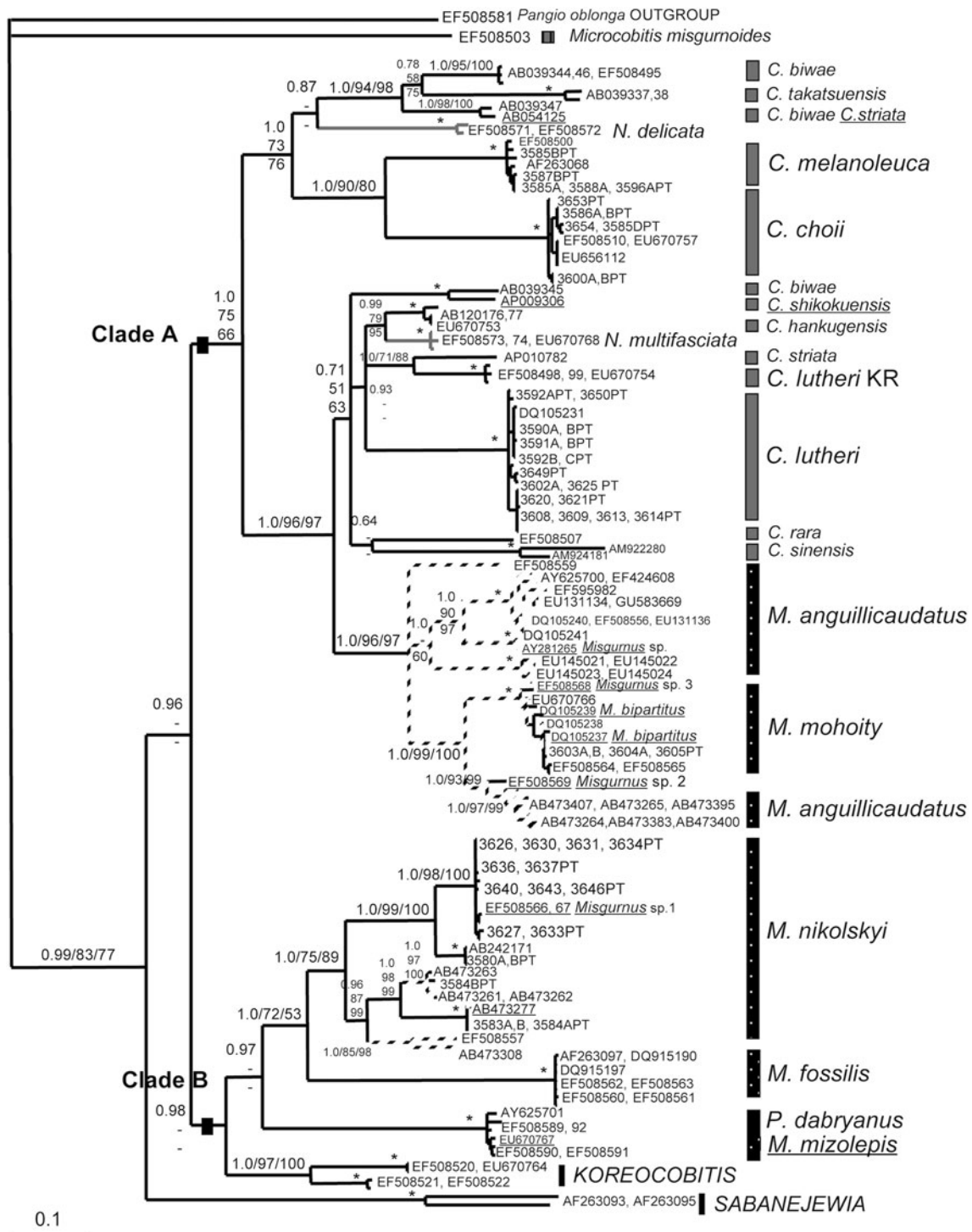


Fig. 2 Phylogenetic relationships based on *cyt b* sequences. Bayesian tree (50% majority rule consensus) using the GTR + I + G model, with values on branches corresponding to Bayesian posterior probabilities, and NJ and MP bootstrap values. An asterisk appears

when all values were 100%, and a dash appears when the branch is not supported. Dotted branches identified specimens of *Misgurnus anguillicaudatus*, and grey branches identified specimens of *Niwaella*

lineage corresponded to *Koreocobitis*, and the third lineage included the rest of the *Misgurnus* species.

In general, nuclear phylogenies are less resolved than mitochondrial topologies (Fig. 3). We found two major

clades with a moderate-high bootstrap value (>56%) and ppb (98%), which do not correspond exactly with the mitochondrial phylogeny. Clade A included exclusively *Cobitis* species and *Niwaella delicata* and *N. multifasciata*.

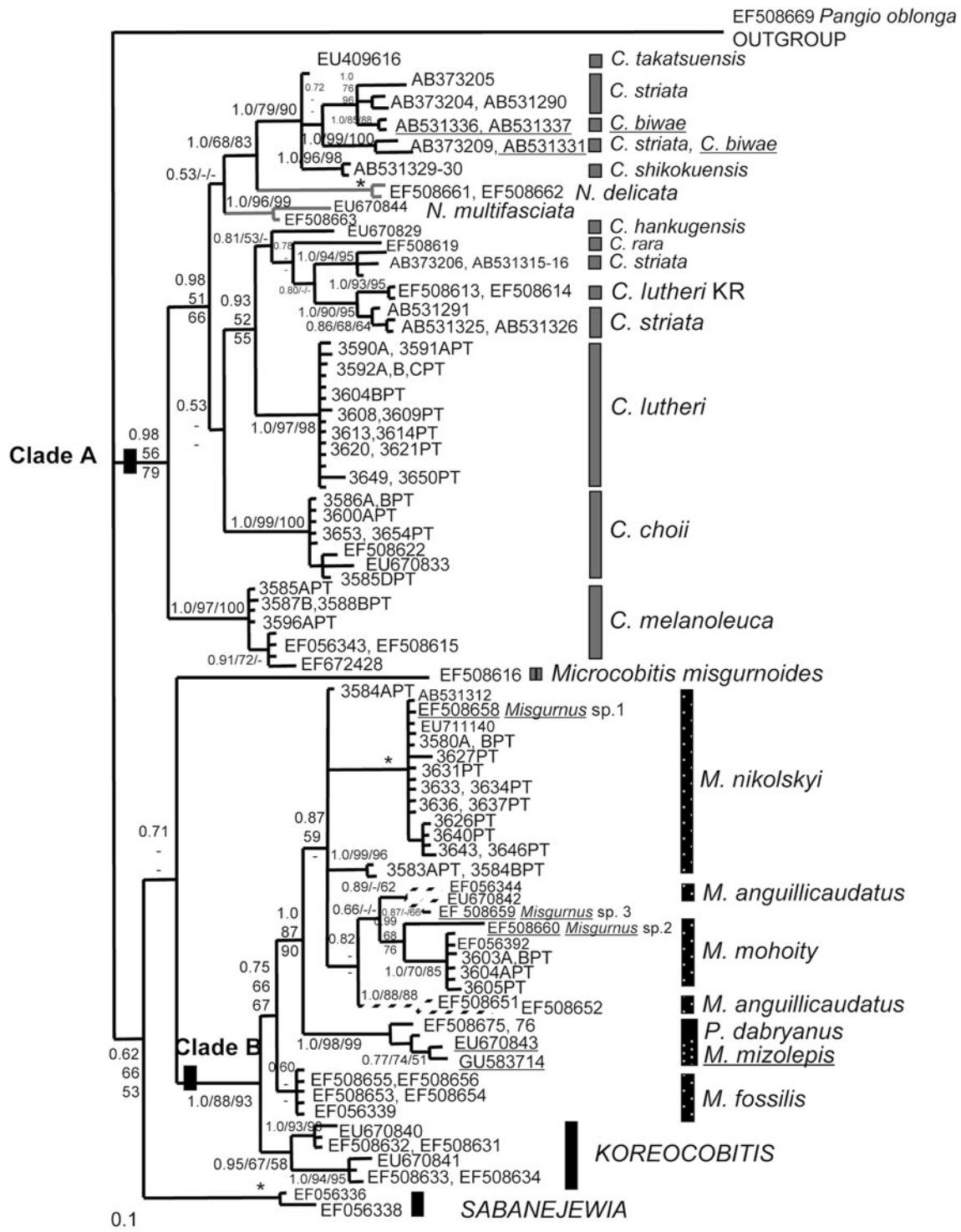


Fig. 3 Phylogenetic relationships based on RAG-1 sequences. Bayesian tree (50% majority rule consensus) using SYM + I + G model, with values on branches corresponding to Bayesian posterior probabilities, and NJ and MP bootstrap values. An asterisk appears

when all values were 100%, and a dash appears when the branch is not supported. Dotted branches identified specimens of *Misgurnus anguillicaudatus*, and grey branches identified specimens of *Niwaella*

Similarly to the mitochondrial results, *C. lutheri* was not monophyletic. All *C. lutheri* individuals from the Far East of Russia comprise a well-supported monophyletic clade

distant from *C. lutheri* specimens from Korea ($2.1 \pm 0.2\%$), and *C. lutheri* specimens from Korea were related to *C. striata* (Large and Middle races). The specimens of

Niwaella species were related to different *Cobitis* species as in the mitochondrial phylogeny. Clade B included all species of *Misgurnus* analysed, *Koreocobitis* and *Paramisgurnus*. *Koreocobitis* was always basal, and *M. mizolepis* was always embedded within *Paramisgurnus* as in the mitochondrial phylogeny (mean p -distances $0.2 \pm 0.1\%$). *Misgurnus mohoity* was recovered as monophyletic, and it was related to *Misgurnus* sp. 2 from Korea, and to some samples identified as *M. anguillicaudatus* from Korea and Japan and *Misgurnus* sp. 3 from Korea, as in the mitochondrial topology. *Misgurnus fossilis* was recovered as non-monophyletic in MP and NJ analyses, and with low support in Bayesian analysis for RAG-1 (Fig. 3).

Discussion

Genera *Cobitis* and *Misgurnus* and related groups.

Mitochondrial and nuclear analyses of the Russian Far East loaches and close relatives produced, at some levels, incongruent phylogenetic results. In all analyses, the genera *Misgurnus* and *Cobitis* were paraphyletic. These results indicate that morphological variation used for previous taxonomic hypotheses does not reflect phylogenetic relationships among members of these genera. Relationships described by *cyt b* and RAG-1 phylogenies always produced non-monophyletic groups for the genera *Cobitis* and *Misgurnus*. In our analyses, some species of *Niwaella* and *Paramisgurnus* were always intimately related to *Cobitis* and *Misgurnus*, respectively. *Niwaella delicata* was always phylogenetically more closely related to *Cobitis* species than to other *Niwaella* species; *N. multifasciata*. *Misgurnus mizolepis* was embedded in the *Paramisgurnus dabryanus* lineage. Therefore, neither *Cobitis* nor *Misgurnus* are natural groups as currently recognised, as has been suggested in other studies (Tang et al. 2006; Šlechtová et al. 2008).

Mitochondrial and nuclear incongruities were especially relevant in the relationships of some *Misgurnus* species with the *Cobitis* group. The close mitochondrial relation found among *M. mohoity* and *M. anguillicaudatus* and some *Cobitis* species suggests that Russian Far East *M. mohoity* is also introgressed at the mitochondrial level, as was previously suggested for *M. anguillicaudatus* (Šlechtová et al. 2008). On the basis of the strong differences of the mitochondrial and nuclear relationships observed, Šlechtová et al. (2008) suggested a past hybridization of *M. anguillicaudatus* and a member of *Cobitis* with subsequent backcrosses of the hybrid with the parental species. Our results also indicated strong phylogenetic differences at the mitochondrial and nuclear levels for *M. mohoity* and *M. anguillicaudatus*, and we suggest the hybridization as a possible mechanism for explaining these incongruities.

At the nuclear level, all *Misgurnus* and *Paramisgurnus* species were grouped in a monophyletic clade that related all Asiatic species with the Central European *M. fossilis* as the basal member of all *Misgurnus* and *Paramisgurnus*. In all nuclear topologies *Koreocobitis* was the sister group to this clade. The recovery of *M. mizolepis* embedded within the *P. dabryanus* lineage is consistent with the argument that *M. mizolepis* represents a junior synonym of *P. dabryanus* (Vasil'eva 2001).

Concerning the systematics of the genus *Misgurnus*, two alternative hypotheses could be proposed. (1) The relationships of *P. dabryanus* with other *Misgurnus* species suggest that *Paramisgurnus* should be considered as a member of the genus *Misgurnus* (Šlechtová et al. 2008). The inclusion of *P. dabryanus* in the genus *Misgurnus* will convert this genus in a monophyletic group, as was shown in the molecular phylogenies (Fig. 3). (2) According to our results, the acceptance of the monotypic genus *Paramisgurnus* implies the restriction of the genus *Misgurnus* to its type species, *M. fossilis*, and therefore, Asian *Misgurnus* must be considered a new genus. *Misgurnus fossilis* is the only non-Asiatic member of the genus *Misgurnus*, and it was suggested that this species was a Pliocene immigrant from East Asia (Bănărescu 1990). Our molecular phylogenies indicate that the *M. fossilis* lineage is deeply divergent from Asian *Misgurnus* and *Paramisgurnus*, and therefore it is difficult to concur with the hypothesis of a Pliocene immigrant. Further work based on multiple types of evidence (genetics, morphology, karyology) must resolve current systematic delimitation of the genus and species of *Misgurnus*.

Systematic implication. The molecular characterization of the Russian Far East species of the genus *Misgurnus* showed that all specimens of *M. mohoity* from the middle and lower Amur drainage had low intraspecific molecular divergence. At the mitochondrial level *M. mohoity* showed low genetic divergence from mud loaches identified as *M. bipartitus* by Tang et al. (2006), and some mud loaches from China and Korea identified as *M. anguillicaudatus* (Fig. 2; ESM Table S1). Earlier karyological and morphological studies have considered *M. bipartitus* a synonym of *M. mohoity* (Vasil'eva 2001; Vasil'eva et al. 2003). Our results confirm this conclusion and permit extending the range of *M. mohoity* to Korea from its known distribution in the Russian part of the Amur River drainage (except Khanka Lake), northeastern Mongolia, and northeastern China (south to the upper stream of the Liao River) (Vasil'eva et al. 2003). Although some *M. anguillicaudatus* from China should be treated as *M. mohoity*, *M. anguillicaudatus* continues to be paraphyletic with different well-defined lineages that probably correspond to different species. It has already been suggested that *M. anguillicaudatus* represents more than a single species with several

phylogenetic lineages grouped under the same name (Khan and Arai 2000; Tang et al. 2006; Šlechtová et al. 2008; Vasil'ev and Vasil'eva 2008a, b). All recent evidence warrants the systematic revision of mud loaches currently considered as *M. anguillicaudatus* as well as other members of the genus *Misgurnus*.

The recovery of *M. nikolskyi* specimens from the mainland and those from Sakhalin Island as a distinct evolutionary lineage, respectively (see Table 1; Fig. 1) supports previous studies that have found some karyological and ecological variability for this species (Vasil'eva 2001; Vasil'eva et al. 2003). However, we found more genetic differentiation within this clade than expected. At the mitochondrial level, we found a core group of *M. nikolskyi* specimens from nearly all Far East localities of Russia and *Misgurnus* sp. 1 from Russia. This lineage is closely related to some individuals of *M. nikolskyi* recovered from GenBank from the Amur River in China. We recovered another well-differentiated lineage of *M. nikolskyi* with individuals from Sakhalin Island, mitochondrially related to some Japanese individuals identified as *M. anguillicaudatus*. At the nuclear level, the *M. nikolskyi* specimens from Sakhalin Island were not related in a monophyletic group with the continental specimens.

Therefore, the considerable genetic divergence of the Sakhalin lineage and its relationship with some Japanese mud loach individuals must be verified by more representative phylogeographic analyses. Morphological characters supported the identification of *M. nikolskyi* individuals analysed; however, a taxonomic study of Japanese mud loaches *M. anguillicaudatus* and *M. nikolskyi* is needed, as several of the specimens of both species are intimately related. This conclusion was also supported in previous studies that showed the genetic heterogeneity of Japanese mud loaches (Morishima et al. 2008; Koizumi et al. 2009).

The molecular phylogenies support non-monophyly of the genera *Cobitis* and *Niwaella*, as previously suggested by Šlechtová et al. (2008). All phylogenies show the species of *Niwaella* closely related to different species of *Cobitis*. The nested position of *C. choui* within all other species of *Cobitis*, as previously revealed by Šlechtová et al. (2008), provides support for considering this species as a member of the genus *Cobitis* (Vasil'ev and Vasil'eva 2008a, b; Kim 2009), and not a member of the genus *Iksookimia* (Kim et al. 1999; Kim and Park 2002; Kottelat 2006).

The molecular analysis of the Far East *Cobitis* shows *C. lutheri* from different localities as a non-natural group. Our results support two well-differentiated molecular lineages. One lineage related all *C. lutheri* specimens from the Far East of Russia with low genetic variability. The second lineage was recovered with Korean specimens identified as *C. lutheri* by Šlechtová et al. (2008) and Lee (2009), but

phylogenetically related to *C. striata* from Japan (Figs. 2, 3). Our results support previous karyological results about the non-conspecificity of the Korean spined loaches identified as *C. lutheri* and *C. lutheri s. stricto* described from Khanka Lake (Vasil'ev and Vasil'eva 2008a, b). Therefore, we maintain *C. lutheri s. stricto* for the Russian Far East specimens that according to previous karyological studies also inhabit the rest of the Amur River basin and waters of Primorye district neighboring the type locality represented in our study by the sample from the Khanka Lake basin (locality 18 in Fig. 1). The close phylogenetic relationship of *C. lutheri* from Korea with another Korean species, *C. tetralineata* (Kitagawa et al. 2005), and the similar karyotype of the Korean *C. lutheri* with *C. tetralineata*, might suggest their consideration of local populations of this last species (Vasil'ev and Vasil'eva 2008a, b). Therefore, previous knowledge coupled with our phylogenetic results suggests that Korean specimens should be taxonomically revised.

The obtained molecular phylogenies do not support the taxonomic treatment of *C. melanoleuca* as a complex of three subspecies with karyological differences (Vasil'ev and Vasil'eva 2008a). Our results are more consistent with explanations that show *C. melanoleuca* as rather homogeneous species with very low genetic divergence across a broad geographic range. However, our study includes a restricted number of localities and should be considered as a preliminary result. Undoubtedly, more *C. melanoleuca* specimens need to be studied to support or not the presumed taxonomic distinctiveness of *C. melanoleuca* subspecies.

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