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EXPERIMENTAL ARTICLES

On the Systematics and Phylogeography of Eight-Barbel Loaches of the Genus *Lefua* (Cobitoidea: Nemacheilidae): mtDNA Typing of *L. pleskei*

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Abstract—Comparative analysis of own sequence data for the mtDNA control region (926 to 928 bp) from eight-barbel loaches inhabiting eight localities in the Amur River basin (4) and the Sea of Japan (4) and the GeneBank/NCBI data for the *Lefua* individuals from the other regions of East Asia showed that eight-barbel loaches from Primorskii krai water basins were marked by a specific group of mtDNA haplotypes. This finding is considered as supporting the species status of *L. pleskei*. Genetic distances within *L. pleskei* are small (on average 0.35%) and close to those within *L. nikkonis* (on average 0.48%). The distances between this species pair are the least (on average 2.15%) among all other pairs compared. In MP, ML, and Bayesian trees, *L. pleskei* and *L. nikkonis* haplotypes formed a common clade with high statistically significant support. In all tree variants, *L. costata* mtDNA haplotypes were located out of the group of interest. A clade consisting of highly diverged lineages of *Lefua* sp. and *L. echigonia* haplotypes occupied a basal position. The mtDNA haplotypes of *L. pleskei* and *L. costata* from the Amur River basin were evolutionary young and derived from the haplotypes found in these species from the Sea of Japan (*L. pleskei*) or the Yellow Sea (*L. costata*) basins. It is thereby suggested that both species rather recently migrated into the Amur River system. According to the molecular clock data, basal diversification of the eight-barbel loach lineages took place at the end of middle Miocene (about 11 to 12 Myr ago), while divergence of *L. pleskei* and *L. nikkonis* ancestral forms probably occurred approximately, 5 Myr ago. Since all main lineages of eight-barbel loaches were found in the Sea of Japan basin (continental coastline and the islands), the divergence order and dispersal patterns of the *Lefua* species might have been largely determined by the geological development pattern of this water body and the adjacent territories.

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INTRODUCTION

Eight-barbel loaches of the genus *Lefua* Herzenstein, 1888 are small fresh-water fish, inhabiting the rivers and lakes of the eastern part of Asia. It is accepted that the genus consists of four species [1–3]. One of these species, *L. costata* (Kessler, 1876), is widely distributed on the continent (from the Amur River basin to the Chinese province Shandong in the south). The other species are found on the islands: *L. echigonia* Jordan et Richardson, 1907, on Honshu Island; the taxonomically not yet legalized *Lefua* sp., in the southwest of Honshu Island and on Shikoky Island; *L. nikkonis* (Jordan et Flower, 1903), on Hokkaido Island, northern part of Honshu Island (Prefecture Aomori), and the southern part of Sakhalin Island*. Recent studies of genetic differentiation of *Lefua* from the islands of Japanese Archipelago [5–7] generally confirmed the accepted structure of the genus. According to these studies, *L. nikkonis* was close to *L. constata* (the latter species was represented by samples from the south of the Korea Penninsula), while *L. echigonia* and *Lefua* sp. were well-differentiated lineages [5–7].

Continental eight-barbel loaches were less subject to recent taxonomic or evolutionary investigations than the loaches from the insular part of the genus range. However, continental *Lefua* are morphologically heterogeneous. Earlier, a number of eight-barbel loach species from the continent were described, including *Octonema pleskei* Hezenstein, 1887; *Nemacheilus dixoni* Fowler, 1899; *Elxis coreanus* Jordan et Starks, 1905; and *Lefua andrewsi* Fowler, 1922. Later, validity of these species was considered groundless, and they were reduced to synonyms of *L. costata* [1, 2, 8–10]. This idea dominated until recently, when it became clear that at least some of these nominative species can be recognized as valid. Recently, it was demonstrated that eight-barbel loaches from the south of Primorskii krai were not morphologically identical to typical *L. costata* from the northeast of China, and should be considered a distinct species, *Lefua pleskei* (Herzenstein, 1887) [11]. Analysis of the mitochondrial DNA

* Revision of the material available showed that the indication on the presence of eight-barbel loaches in the system of Vavai lakes of Southern Sakhalin [4] should be attributed specifically to *L. nikkonis*.

Table 1. Brief geographic characteristics of our *Lefua* eight-barbel loach samples and the data from the GenBank/NCBI database [6, 7, 15]

No.	The origin of our samples (sample nos. 1–8, see Fig. 1) and those from the GenBank/NCBI database (nos. 9–16)	Names of the haplotypes identified or the GenBank/NCBI accession numbers
	L. pleskei	
1	Russia, Lake Khanka basin (Amur River), Ilistaya River (n = 2)	PL1
2	Russia, Lake Khanka basin (Amur River), Komissarovka River (n = 1)	PL2
3	Russia, Ussuri River basin (Amur River), Chirki River (n = 1)	PL3
4	Russia, Sea of Japan basin, brook in Lazurnaya Bay (n = 3)	PL4
5	Russia, Sea of Japan basin, Golubichnaya River (n = 3)	PL8
6	Russia, Sea of Japan basin, Velikaya Kema River (n = 2)	PL6, PL7
7	Russia, Sea of Japan basin, Lake Solenoe (n = 4)	PL5 (1), PL6 (3)
	L. costata	
8	China, Amur River basin, Sungari River (n = 1)	COS
9	China, Liaoning Province, Liaohe River basin	DQ105257
10	Korea, Yellow Sea basin	AB102814, AB177661
11	Korea, Sea of Japan basin	AB102815, AB177662, AB177663
12	Japan, Honshu Island*	AB102811, AB102813
	L. nikkonis	
13	Japan, Hokkaido Island	AB102810, AB177654, AB177655, AB177657
	Lefua sp.	
14	Japan, Honshu Island	AB177669, AB177673
15	Japan, Hokkaido Island	AB177675
	L. echigonia	
16	Japan, Honshu Island	AB102823, AB102828, AB102843, AB177682, AB177695, AB177708

Note: * Mihara et al. [7] suggest artificial origin of *L. costata* populations on the Honshu Island, which, as they think, appeared there as a result of unintended introduction of these fish from the Korea waters.

(mtDNA) variation is a powerful tool of molecular systematics, which showed its effectiveness in addressing the status of different forms of *Lefua* [5–7]. For these reasons, in the present study genetic isolation and phylogenetic position of eight-barbel loaches from different localities of Primorye (including the type locality of *L. pleskei*, Ilistaya River) among the other groups of the genus *Lefua* was evaluated based on sequence analysis of the mtDNA control region (CR).

MATERIALS AND METHODS

A total of 16 *L. pleskei* individuals from the two main freshwater systems of Primorskii krai, Ussuri River basin and Lake Khanka (in the broad sense, the Amur River basin) and the Sea of Japan were examined (Fig. 1 and Table 1). In addition, one eight-barbel loach individual, morphologically corresponding to the descriptions of typical *L. castata* [11, 12] and caught in the Sungari River, was examined.

Total DNA was extracted from the ethanol-fixed muscle or fin tissues according to a standard phenol–chloroform method [13]. The eight-barbel loach mitochondrial genome region including the entire control region was amplified in polymerase chain reaction (PCR) with forward ProS and reverse PheAS primers [6]. The primer sequences were specific to the tRNA genes (tRNA-Pro and tRNA-Phe), flanking the control region. The reactions were performed on the Biometra (UNO-Thermoblock 40) thermal cycler in 50 μl of the reaction mixture containing 1 to 2 μg of total DNA template; 5 μl buffer (0.6 M Tris–HCl, pH 8.5; 0.015 M $MgCl_2$; 0.25 M KCl; 0.1 M 2-mercaptoethanol; and 1% Triton X-100); 5 μl of four deoxytriphosphate mixture from 8 mM solution 5 μ of each primer from 2 μl solutions; 2 to 2.5 units of *Taq* polymerase (Sibenzim, Novosibirsk); and deionized water. PCR reaction was carried out according to a following scheme: initial denaturing (94°C for 120 s); 30 cycles of amplification (94°C for 30 s; 58°C for 10 s; and 72°C for 30 s); and final extension (72°C for 480 s). Amplification products

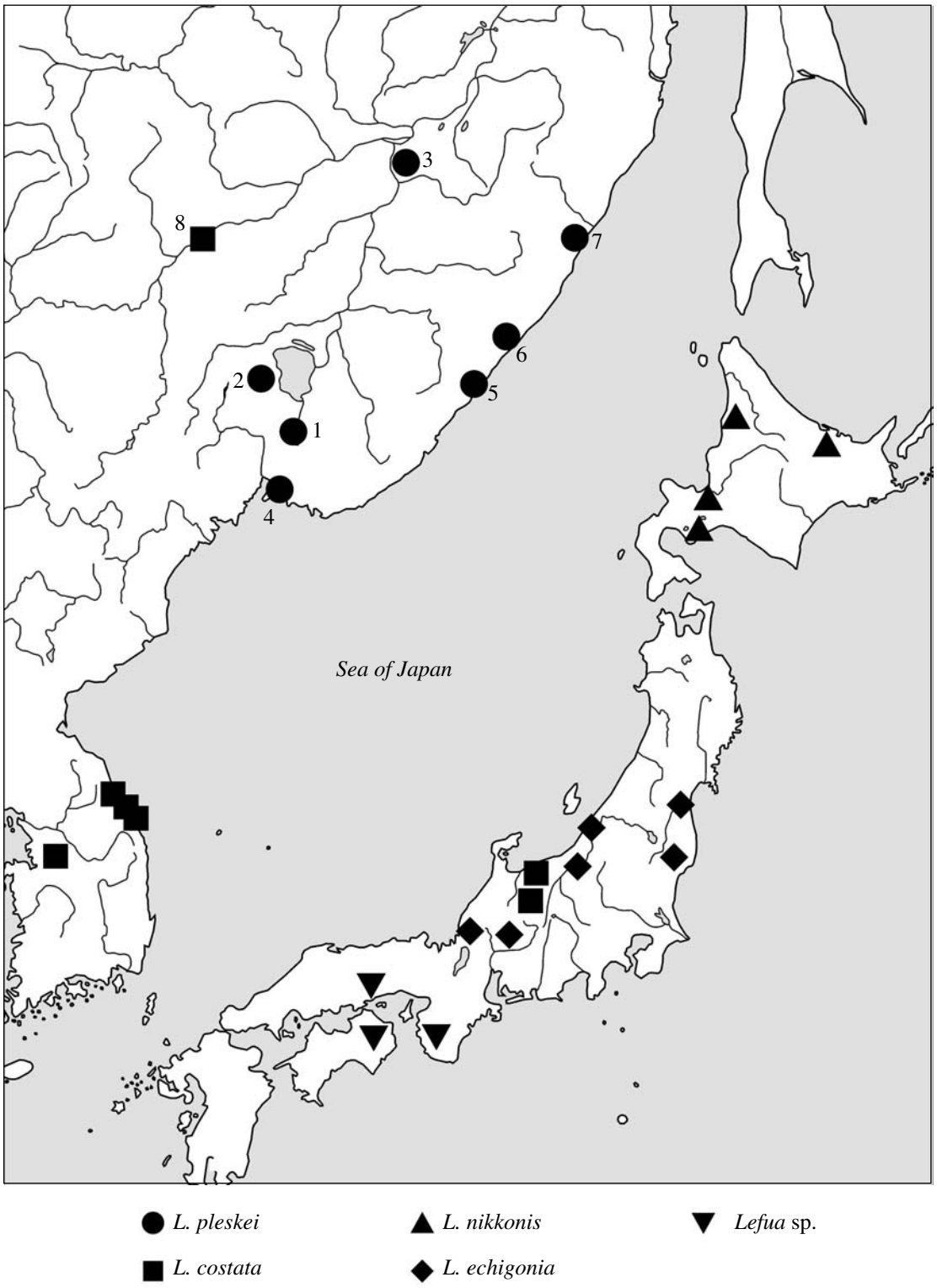

Fig. 1. Collection site map demonstrating the distribution of sampling sites for *Lefua*, obtained in the present study (enumerated), as well as geographic affiliation of the data from the GenBank/NCBI (see Table 1; the place of origin of *L. costata* with haplotype DQ105257 is out of the map limits).

were purified on the Quantum Prep (Bio-Rad Laboratories, United States) columns and subjected to cyclic sequencing using the Big Dye Terminator version 3.1 (Applied Biosystems,United States) reagent kit and two the same, and two additional primers, 296S and 65IS [6]. The sequencing products were separated on the ABI Prizm 310 (Applied Biosystems, United States) automated sequencer at the Institute of Biology and Soil Science, Russian Academy of Sciences, Vladivostok. ABI chromatograms were treated using the STADEN software package [14].

The mtDNA CR sequences from the *Lefua* species determined were deposited in the GenBank/NCBI database under the accession numbers EU150372 to EU150380. For comparison, the sequences of homologous mitochondrial genome regions from the representatives of different *Lefua* species [6, 7, 15] (see Table 1), as well as from two relative species, *Orenectes platycephalus* and *Micronemachelus pulcher* (DQ105258-9, GenBank/NCBI) from the same database were used.

Multiple progressive alignment of nucleotide sequences was performed using the MAFFT v. 6.240 software program with iterative refinement method FFT-NS-i; gap open penalty, 0.6; and other parameters taken by default). The total number of nucleotide positions in the aligned sequence array was 999.

For phylogenetic reconstructions, the 32×929 data matrix was used. This matrix was constructed by removing 70 positions, including uninformative insertions and the regions with ambiguous alignment, from the aligned starting sequence array. Phylogenetic analysis was performed using three methods: (1) maximum likelihood based on nucleotide substitution model, HKY + G_4, taking into consideration the main features of the mtDNA CR evolution, namely, unequal proportion of four types of nucleotides, prevalence of transition type substitutions over transversions, and heterogeneous substitution rates at different nucleotide positions of the mtDNA CR; (2) maximum parsimony (the trees were generated based on unweighted data matrix with the inclusion of the insertion/deletion positions; (3) Bayesian analysis based on the nucleotide substitutions model, HKY + G_4.

Heuristic search for maximum likelihood or maximum parsimonious trees (ML- and MP trees) was performed with the PAUP 4.0b10 software program package [17] and consisted of 30 random addition sequence replicates using the TBR branch-swapping algorithm. Robustness of the clustering order was evaluated by bootstrap analysis in 1000 pseudoreplicates.

Markov chain Monte-Carlo analysis (Bayesian phylogenetic analysis) was performed with the MrBayes version 3.1.2 program [18] by means of simultaneous running of four chains (three "heated" and one "cold" chain) during 2×10^6 cycles with the selection of each hundredth tree among the generated ones. Among the 20001 trees generated, the first 2001 trees were discharged. The remaining trees, characterized by stabilized variation levels of maximum-likelihood estimates (Ln*L*), as well as the parameters of nucleotide substitution model and the tree lengths, were used for generation of a consensus phylogenetic tree and obtaining the posterior probability estimates of tree branching.

RESULTS

According to the sequence data, the total length of the mitochondrial genome control region in the eight-barbel loaches examined constituted 926 bp for *L. costata*, and 928 bp in *L. pleskei*. A total of nine different variants of the mtDNA CR were detected, including one haplotype in *L. costata* (COS), and eigh haplotypes in *L. pleskei* (PL1–8).

Haplotypes of *L. pleskei* differed by one to five nucleotide positions. In each of three sampling sites in the Lake Khanka and Ussuri River basins (Amur) specific haplotype variants were discovered, including PL1 in *L. pleskei* from Ilistaya River, and PL2 and PL3 in the individuals of the same species from Komissarovka and Chirki rivers. Unique haplotype variants were also detected in *L. pleskei* from the Sea of Japan basin: PL4 in the individuals from the brook in Lazurnaya Bay; PL5, in Solenoe Lake; PL7, in Velikaya Kema River; and PL8, in Golubichnaya River. One of the haplotypes (PL6) was common to the *L. pleskei* individuals caught in two localities of the northern coast of Primorye, Solenoe Lake and Velikaya Kema River (see Table 1 and Fig. 1).

Haplotype of *L. costata* from Sungari River was remarkably different from the haplotypes of *L. pleskei* by 49 to 53 nucleotide substitutions and two single deletions. The differences of this haplotype from the *L. costata* haplotypes from the GenBank database were substantially lower, constituting 5 to 17 nucleotide positions. Moreover, relative to the nucleotide substitutions number, the closest haplotype to COS was AB102811, found in *L. castata* from the Honshu Island. Taken together, the differences between the haplotype of *L. costata* constituted from 1 to 26 nucleotide positions.

Among the *Lefua* species investigated, *L. pleskei* demonstrated the lowest level of the mtDNA CR intraspecific variation, which was most similar to that within *L. nikkonis* (on average, 0.0035 and 0.0048 substitutions/position, respectively) (Table 2).

The mean divergence level of the mtDNA CR sequences within *L. costata* was several times higher, constituting 0.0147 substitutions/position. Even higher level of the mean differences was typical of the haplotypes within *L. echigonia* and *Lefua* sp. from the islands of Japanese Archipelago (Table 2).

The mean level of interspecific divergence of the mtDNA CR sequences for the pair of *L. pleskei* and *L. nikkonis* was also the lowest among all other *Lefua* pairs compared, 0.0215 versus 0.0496 to 0.1375 (Table 2). The mtDNA haplotypes of this pair were

Table 2. The mean number of nucleotide substitutions (in %) between the mtDNA CR haplotypes of *M. pulcher*, *O. platycephalus* and *Lefua* species

No.	Species	1	2	3	4	5	6	7
1	*M. pulcher*	–						
2	*O. platycephalus*	16.96	–					
3	*L. echigonia*	19.00	16.70	6.37				
4	*L. costata*	18.59	15.63	13.75	1.47			
5	*L. pleskei*	18.75	15.05	13.44	5.24	0.35		
6	*L. nikkonis*	18.92	15.28	13.25	4.96	2.15	0.48	
7	*Lefua* sp.	17.65	16.37	11.56	10.78	11.09	10.93	4.40

most close to the haplotypes found in *L. castata* (the average distances upon comparison with *L. pleskei* and *L. nikkonis*, 0.0524 and 0.0496, respectively). In general, interspecific distances between *L. costata*, *L. pleskei*, and *L. nikkonis* do not exceed the mean divergence limits within the eight main mtDNA regional groups (mtDNA phylogroups), identified by Mihara et al. [7] in *L. echigonia* and *Lefua* sp. (0.0637 and 0.0440; see also [7])**.

Thus, relative to the distance estimates, the mtDNA CR haplotypes of *Lefua* fall into three well-shaped groups differing, on average, by 10.93 to 13.54% of nucleotide positions upon the average intragroup variation level of 3.29 to 6.37%.

Using the maximum likelihood method and nucleotide substitution model HKY + G_4, a tree was generated (the tree and model parameters: –Ln*L* = 4560.44; nucleotide frequencies, A = 0.362, C = 0.180, G = 0.122, T = 0.336; ratio between the transition and transversion rates, 5.041; α parameter of γ distribution, 0.390), in which haplotypes of the mtDNA CR from *Lefua* formed a monophyletic group, which contained three distinct clades, (1) *L. echigonia*, (2) *Lefua* sp., and a clade (3), formed by the haplotypes found in *L. costata*, *L. pleskei*, and *L. nikkonis* (Fig. 2). Within the latest group, clade of the *L. costata* haplortypes had the position of an outgroup relative to the clades formed by the haplotypes of *L. pleskei* and *l. nikkonis*, which demonstrated sister relationships. In the clade of *L. costata*, haplotype COS from the Sungari River formed a clade together with haplotype DQ105257 from the Liaohe River basin. Haplotypes of *L. costata* from Honshu Island and the water bodies of Korea were located out of this clade. In the clade of *L. pleskei*, haplotypes of the individuals from the Sea of Japan basin (PL4–8) were basal in relation to the clade consisting of three haplotypes from the Amur River basin (PL1–3).

Heuristic search for most parsimonious trees based on the analysis of the matrix consisting of 256 phylogenetically informative characters revealed two equally parsimonious variants (tree length, 781 steps; homoplasy index HI, 0.31; retention index RI, 0.84), which were principally similar to the ML tree in the branching order, the number and composition of the main clades of mtDNA CR haplotype of *Lefua* (trees are not shown, but can be received by request). The main difference between the MP tree variants described and the ML tree was the position of haplotypes AB102823 and AB177682, which in MP trees occupied position of an outgroup in relation to the other haplotypes of *L. echigonia*.

Topology of the consensus tree, obtained in Bayesian analysis using the HKY + G_4 model (averaged parameters of the tree and model: –Ln*L* = 4630.62; nucleotide frequencies, A = 0.359, C = 0.183, G = 0.122, and T = 0.336; the ratio between transition and transversion rates, 5.227; α parameter of γ distribution, 0.402) was quite similar to ML tree topology and is not presented.

According to the bootstrap values and the values of a posteriori probability obtained, the data matrix used produced sufficiently strong and closely unambiguous phylogenetic signal, providing consideration of the phylogenetic hypothesis on the relationships between the *Lefua* mtDNA CR haplotyopes, presented in Fig. 2, as statistically significantly supported in its most part.

Taking into consideration a well-known fact that time-referenced mutation accumulation pattern in mtDNa is close to linear, obtaining either absolute or relative time estimates for certain events in the evolution of the *Lefua* eight-barbel loaches seemed interesting.

High length variability of the adjacent tree branches in Fig. 2 suggests different rates of evolution of mtDNA CR in different tree branches. Indeed, likelihood ratio test rejected the assumption on the identical rates of the mutation accumulation in different parts of the tree at the high level of statistical significance. Specifically, maximum likelihood estimator for the ML tree with constant evolution rate (–Ln*L* = 4603.060) was highly statistically significantly different ($P < 0.001$) from that for the tree generated without this condition. Because

**In the present study, six mtDNA phylogroups of the first species, and two mtDNA phylogroups of the second species are represented by six and three most typical, to our opinion, sequences, respectively (see Table 1).

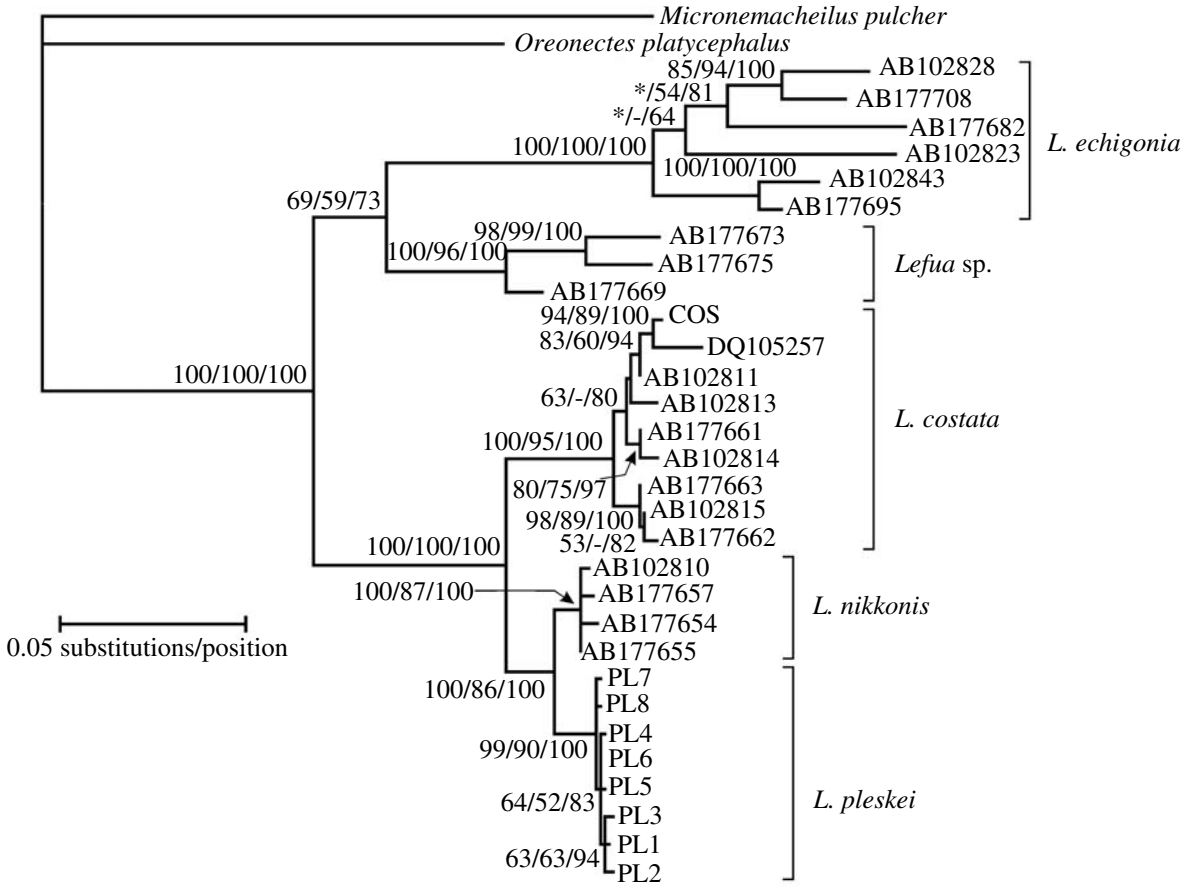

Fig. 2. ML tree for *Lefua* eight-barbel loaches and two outgroups based on the analysis of the mtDNA CR. Figures at the cluster bases separated by slashes are the estimates of branching nodes robustness in 50% MP, ML (in % from 1000 bootstrap replicates), and Bayesian (a posteriori values multiplied by 100) consensus trees, respectively. Blank means reproduction of the given branching node in less than one half of all bootstrap replicates; asterisk, another clustering pattern of *L. echigonia* haplotypes in case of MP tree (see the text).

of this, to transform the tree in Fig. 2 into mtDNA chronogram, a method assuming nonstrict molecular clock [19, 20] and realized within the Bayesian approach in the Estbranches and Multidivtime programs (http://statgen.ncsu.edu/thorne/multidivtime.html) was used. Unfortunately, no fossil records of *Lefua, Oreonectes*, and *Micronemacheilus* are available. For this reason, the chronogram scale can be expressed only in relative units, i.e., the time relative to the moment of radiation of *Lefua* and *Oreonectes* ancestral lineages.

Based on the chronogram (Fig. 3) constructed using the sequence evolution model F84 + G_5, according to the recommendation of the designer of the method stated in the guide to the Estbranches and Multidivtime programs, it can be suggested that radiation of the main mtDNA haplotype lineages in *L. echigonia*, *Lefua* sp. and *L. costata*, as well as of the ancestral lineages of the haplotype groups of *L. pleskei* and *L. nikkonis* occurred, approximately, at one time. In turn, the haplotype divergence time within *L. pleskei* and *L. nikkonis* was twofold lower and almost identical to the haplotype divergence time from the geographically close populations in the other three *Lefua* species.

DISCUSSION

The discovery of specific group of mtDNA haplotypes in eight-barbel loaches from Primorye can be considered as a confirmation of the legitimacy of suggestion on exclusion of *L. pleskei* from *L. costata* and the recognition of this form as an independent species [11]. Note that this suggestion was made based on analysis of morphological characters.

According to the results obtained, *L. pleskei* and *L. nikkonis* form a sister group. The level of genetic differences between these species is relatively low, not exceeding the level of differences between the geographically isolated populations in the other *Lefua* species. However, *L. pleskei* and *L. nikkonis* are well distinguished from each other by the numbers of scales along the median body line, 56 to 75 versus 90 and more ([3, 9], and our unpublished data), as well as by

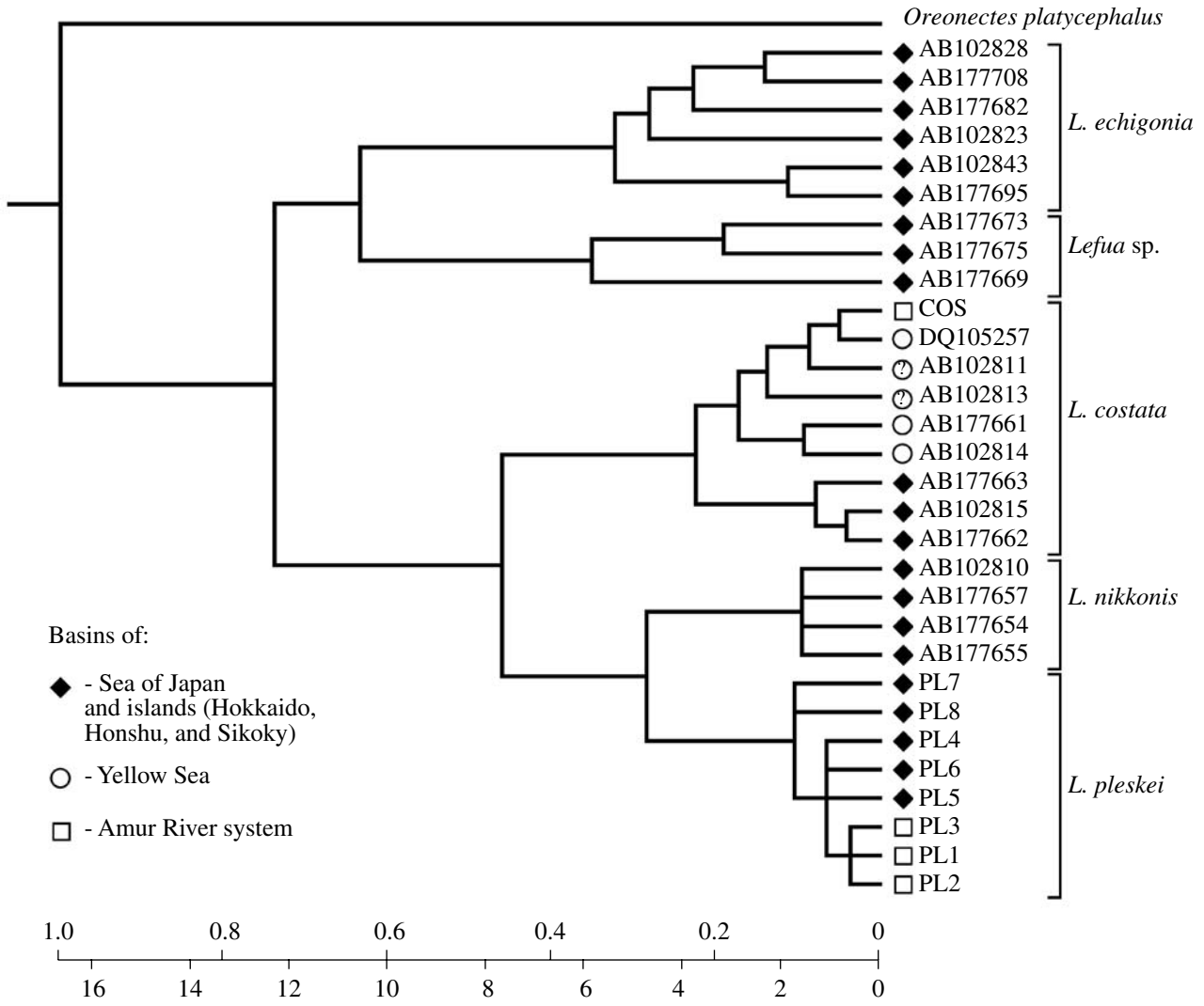

Fig. 3. Bayesian chronogram of mtDNA haplotype divergence in *Oreonectes* and *Lefua* expressed in relative scale (the divergence time of *Oreonectes* and *Lefua* ancestral lineages is taken as unity). Lower scale, "time" in conditionary units (posteriors of expected substitution numbers per 100 nucleotide pairs).

the vertebrate numbers, 38 to 39 versus 41 to 42 [3, 11]. These differences suggest independence of the species.

Molecular data available make it possible to shed the light on the divergence time of the ancestral forms of these species. Saka et al. [5] analyzed the sequences of the mitochondrial cytochrome b gene in three Japanese *Lefua* species. Using the evolutionary rate estimates of this gene for a number of other fish species, it was demonstrated that basal divergence in the *L. echigonia* clade occurred 3.4 to 7.7 Myr ago. According to the data of Bayesian timing (see Fig. 3), the expected number of substitutions from the moment of basal dichotomy in *L. echigonia* clade constitutes 5.4 substitutions per 100 bp of the mtDNA CR. If we assume the middle of the time interval calculated by Saka et al. (5.6 Myr ago) as a basis, then the expected evolutionary rate for the mtDNA CR will be, approximately, one substitution per 100 bp per one Myr. This means, that the lower scale in Fig. 3 can be also considered as the time expressed in million years. From here, it can be suggested that radiation of *L. pleskei* and *L. nikkonis* (as a result of the dispersal of their ancestral populations among different fresh water basins, or the appearance of some obstacle, which caused subsequent species isolation) occurred, approximately, 4.8 Myr ago. The same estimate (4.6 Myr ago) was produced in the the Bayesian dating variant, in which the chronogram (not demonstrated because of almost complete coincidence with the chronogram in Fig. 3) was calculated in absolute time scale upon the condition of basal dichotomy in the *L. echigonia* clade within the interval of 3.4 to 7.7 Myr ago.

In general, according to the time scale presented, initial diversification of the *Lefua* lineages could have taken place during Middle Miocene (about 11 to 12 My rago). Since all main lineages of eight-barbel loaches

were found in the Sea of Japan basin (continental coastline and the islands), the evolution of these species might be closely associated with the geological development pattern of this water body. At the end of Middle Miocene, development of the Sea of Japan basin was characterized by the phase change. The period of the sea enlargement and deepening changed for the period of contraction and rising of the coastal regions along its perimeter [21, 22]. It seems likely that at this time eight-barbel loaches could invade the ancient islands of the Japanese chain from the south. About 5 Myr ago, a dramatic increase of the rate of the coastline rise and deformation of the Honshu and Hokkaido islands, and the south of the Sakhalin Island took place [21]. It may well be that this change of the situation in the region is reflected in the approximate coincidence of the periods of basal diversification in the clades of *L. echigonia* and *Lefua* sp. and divergence of the *L. pleskei* and *L. nikkonis* lineages (see Fig. 3).

Based on the relative location of the *L. pleskei* and *L. nikkonis* ranges, eight-barbel loaches of this group must have colonized the mainland parts located in the place of the modern Sakhalin Island [23]. At present, eight-barbel loaches are absent from the most part of Sakhalin, excluding some water pools in the northwestern part of the island, adjacent to the Amur estuary [24], as well as in the Vavai lakes system at the south [4]. Wide gap in the distribution of *L. pleskei* and *L. nikkoinis* with relatively low level of genetic variation within both of the species suggests that in the course of evolution the species experienced the stage of the range shrinking and decrease of the effective population number. It should be noted that eight-barbel loaches possess rather high abilities to dispersal and overcoming obstacles (vagility). For instance, neither the watershed between the Amur River system and the Sea of Japan basin, nor the isolation of the freshwater bodies of the Primorye coastline from one another were the insuperable obstacles for dispersal of *L. pleskei*. Because of this, it is unclear why eight-barbel loaches could not recolonize the Sakhalin Island.

From the data of Naseka and Bogutskaya [11], it follows that *L. pleskei* and *L. costata* form the sympatry region in the Sungari River system. Since haplotype COS of *L. costata* from the Sungari River was most close to the haplotype of *L. costata* from the Liaohe River basin, it can be suggested that eight-barbel loaches from the latter basin penetrated into the Sungari River basin. This was probably favored by the weak watershed between the Sungari River (Amur River system) and the Liaohe River (Yellow Sea basin).

The young evolutionary age of mtDNA haplotypes of eight-barbel loaches from the Amur River basis is questionable. In both species from this region haplotypes that were derived from those found in the Sea of Japan (*L. pleskei*), or in the Yellow Sea (*L. costata*) were detected. It is unclear, whether the Amur populations of these species became extinct, or *L. pleskei* and *L. costata* never inhabited the Amur River basin. In case of *L. costata* the latter suggestion seems quite probable, while relative to the other species, the suggestions still should be confined to the indication that according to Naseka and Bogutskaya [11], eight-barbel loaches from the Sungari River basin, attributed by these authors to *L. pleskei*, were somewhat different from typical *L. pleskei* from the Khanka Lake and Peter the Great Bay by the number vertebrates and coloration. It can be thereby suggested, that in this study, only one from a number of genetically different groups of *L. pleskei*, inhabiting the Amur River, was investigated. Furthermore, it seems likely that the level of genetic subdivision of *L. pleskei* is higher than that described in the present study. To solve this problem, additional investigations with the involvement of larger samples from different parts of the Amur River basin are required.

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