



Phylogenetic Relationships of Sculpin *Cottus volki* Taranetz, 1933 (Scorpaeniformes, Cottidae) according to the Results of Analysis of Control Region in Mitochondrial DNA

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Abstract—Sequencing of the fragment of control region in mitochondrial DNA in sculpin *Cottus volki* and the comparison of obtained data with homologous nucleotide sequences in the other species from genus *Cottus* demonstrated that *C. volki* occupies the basal position in the “*poecilopus*” group, which includes also typical sculpin *C. poecilopus* Heckel, 1837 from water bodies of Europe, sculpin *C. szanaga* Dybowski, 1869 from the Amur River basin, and sculpin *Cottus* cf. *poecilopus* from the Lena River basin. Early separation of *C. volki* line from common trunk of “*poecilopus*” group explains the presence in *C. volki* of several primitive (plesiomorphic for this group) morphological characters: long interior ray of ventral fin, practically complete lateral body line, and well-developed dentition of palatine bones.

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Sculpin *Cottus volki* Taranetz, 1933 has a small area and is distributed only in the part of the Primor’e coast from the Velikaya Kema River (approximately 45° N) in the north to the Partizanskaya River (approximately 42° N) in the south. In some morphological characteristics, *C. volki* is close to the group of species “*poecilopus*” including additionally to typical *C. poecilopus* Heckel, 1837 from water bodies of Europe and sculpin *C. szanaga* Dybowski, 1869 from the Amur River basin, the northern part of the Primor’e coast, continental rivers of the Tatarian Strait and the rivers of the northern Sakhalin, sculpin *Cottus* sp. (Chereshnev, 1982) from water bodies of northeastern Asia (Chukot Peninsula, Kolyma, rivers of the northern part of the Sea of the Okhotsk coast), and sculpins from West and East Siberia described under the names *C. poecilopus altaicus* Kashchenko, 1899 (the Katun River in the Ob River basin) and *C. kuznetzovi* Berg, 1903 (the Anukit River in the Lena River basin). All these forms of sculpin are similar and simultaneously differ from the other groups of genus *Cottus*, first of all, in a typical ventral fin reaching anus (in males reaching behind the beginning of the anal fin) with short (less than 60% of the fin length) or a sometimes absent interior ray streaked with numerous (usually 5–15) dark transverse stripes and with an intense yellow-orange color of its interray webs in mature males (Kashchenko, 1899; Berg, 1909, 1949; Taranets, 1933, 1936; Chereshnev, 1982; Shedko and Shedko, 2003; Freyhof et al., 2005).

Among the species and forms of group “*poecilopus*”, sculpin *C. volki* is distinguished with an unpaired pore at the end of the chin (canalis praeoperculo-man-

dibularis CPM of the left and right sides of the head are connected with each other), a usually double 5th CPM pore, and a high (as a rule 19–20) number of rays in the second dorsal fin (see: Taranets, 1933, 1936; Chereshnev, 1982; Shedko and Shedko, 2003; Fujii et al., 2005). Additionally, it distinguishes from the other representatives of this group in the conditions of several other characters: practically complete lateral line (reaching, though having been separated into fragments in the place of its bending at the beginning of the caudal peduncle, the base of the caudal fin), a relatively long interior ray of the ventral fin (usually more than 40% of the whole fin length) and well-developed dentition of palatine bones, and these features can be considered as evidence of early divergence of *C. volki* from the common ancestor of the “*poecilopus*” line (assuming that these are primitive or plesiomorphic characteristics) or as the reason to exclude it from the “*poecilopus*” group.

This work was aimed at solving the problem of phylogenetic position of *C. volki* on the basis of the comparison of nucleotide sequences in the control region (CR) of mitochondrial DNA (mtDNA) in this and other species of sculpins from the *Cottus* genus.

MATERIAL AND METHODS

The preparations of mtDNA obtained in April 1989 with the alkaline method (Palva and Palva, 1985) from the fresh liver and eggs taken from 5 specimens of *C. volki* from the Chernaya River (southern Primor’e, approximately 43°13′ N, 134°17′ E).

The fragment of the mitochondrial genome of *C. volki*, completely including its control region, was amplified with the method of the polymerase chain reaction (PCR) using direct LRBT-25 (5'-AGA-GCG-CCG-GTG-TTG-TAA-TC-3') and back LRBT-1195 (5'-GCT-AGC-GGG-ACT-TTC-TAG-GGT-C-3') primers (Uiblein et al., 2001) specific to the genes of transport RNA, flanking it (tRNA-Thr and tRNA-Phe). Amplification was carried out in the device of Biometra Co. (UNO-Thermoblock 40) in 50 µl of reaction mixture including 10–20 ng mtDNA, 5 µl of buffer solution (0.6 M Tris-HCl, pH8.5; 0.015 M MgCl₂; 0.25 M KCl; 0.1 M 2-mercaptoethanol; 1% Triton X-100), 5 µl of the sum of desoxytriphosphates from 8 mM solution, 5 µl of both primers from 2 µM solutions, 2.0–2.5 e.a. Taq-polymerase (SibEnzim, city of Novosibirsk), and deionized water. PCR reaction was carried out according to the scheme: initial denaturation (96°C, 60 s), 30 cycles of amplification (96°C, 90 s; 56°C, 60 s; 72°C, 90 s), and completion of the chains (72°C, 300 s). The products of amplification were purified in the columns Quantum Prep (Bio-Rad Laboratories, USA) and subjected to cyclic sequencing with the help of the Big Dye Terminator set, version 3.1 (Applied Biosystems, USA) using the same primers.

Nucleotide sequences were determined in automatic sequencer ABI Prizm 310 (Applied Biosystems, USA) at the base of the Institute of Biology and Soil Sciences, Far East Division, Vladivostok). Every sample was amplified and then sequenced in one direction twice. ABI-chromatograms were analyzed using the programs from the Staden package (Staden et al., 1999).

The variants of sequenced sequences were deposited in the database Genbank/NCBI under the access numbers DQ367847–DQ367849. Additionally, the data obtained earlier (Kontula et al., 2003; Miya et al., 2003; Slechtova et al., 2004; Yokoyama and Goto, 2005) on the sequences in homologous sections of mtDNA in sculpins from genus *Cottus* and in other representatives of the Cottinae subfamily included to the analysis as an external group (species from genera *Leptocottus*, *Trachidermus*, and *Rheopresbe*) were used in the work.¹ Nucleotide sequences were aligned into a united matrix with the help of ClustalX (Thompson et al., 1997) interface and a subsequent hand treatment.

Phylogenetic analysis was carried out using three approaches: (1) the maximum likelihood method (the

¹ Because *Cottus kazika* Jordan et Starks, 1904 differs sharply from the other species of genus *Cottus* in genetic characteristics, ecology (it is a catadromous species, whereas all other species of this genus are freshwater or amphidromous), and morphology (it has 4 well-expressed spines on preoperculum, whereas other species of genus *Cottus* have, as a rule, no more than 3 spines; see Watanabe, 1960; Kinziger et al., 2005; Yokoyama and Goto, 2005), this species should be excluded from genus *Cottus*. As Watanabe (1960) synonymized *Cottus kazika* and *Rheopresbe fujiyamae* Jordan et Starks, 1904, so the status of *Rheopresbe* would be returned, and the only species of this genus would be known as *Rheopresbe kazika* Jordan et Starks, 1904, comb. n., and we were guided by this idea in our work.

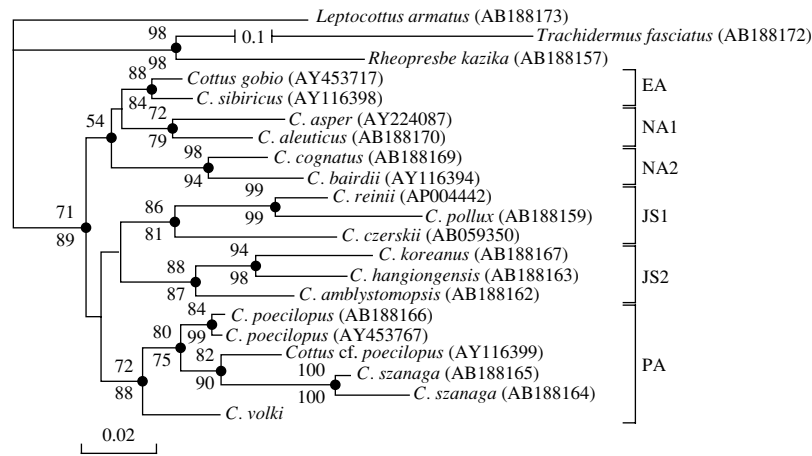
model was used of nucleotide substitutions HKY + I, which accounted the main features of evolution of CS mtDNA: unequal portions of four types of nucleotides, predomination of transition nucleotide substitutions over transverse ones, and the presence of blocks composed of highly conservative sequences of nucleotides); (2) maximum parsimony (the trees were constructed by unweighed matrix of data including the positions with insertions/deletions); (3) Bayesian analysis on the basis of the model of nucleotide substitutions HKY + I.

Heuristic search of the most likelihood or maximum parsimonious trees (ML- and MP-trees) was carried out using PAUP 4.0d81 (Swofford, 2002) software in 30 replications with random addition of sequences into analysis and rearrangements according to the TBR-algorithm. The stability of the order of clustering was evaluated with the bootstrap method in 1000 repeated random samples.

Monte Carlo analysis using Markov Chain Methods (Bayesian analysis of phylogeny) was carried out using MrBayes program version 3.1.1 (Ronquist, Huelsenbeck, 2003) by simultaneous launching of four chains (three "hot" and one "cold") during 2×10^6 cycles with sampling every hundredth generated tree. Of the 20 000 obtained trees, the first 2000 trees were rejected, and the remaining trees, characterized by a stabilized level of variation of likelihood values (LnL) of the parameters nucleotide substitutions model and tree lengths, were used for constructing the consensual phylogenetic tree and obtaining a posteriori the probability of its branching.

RESULTS AND DISCUSSION

Sequencing of mtDNA of *C. volki* was carried out in two stages. First, using direct primer LRBT-25, the nucleotide sequence was determined in mtDNA fragment 441 bp in length homologous to the fragment of the mitochondrial genome of *Cottus reinii* Hilgendorf, 1879, which begins from position 15657 and finishes with position 16097 (No. AP004442, Genbank/NCBI). The sequence of a sequenced fragment included 50 bp 3' of the gene end of tRNA Pro and 391 bp of CR of mtDNA from its beginning to conservative block CSB-D inclusive (so-called left domain or the first hypervariable region). The sum total for three variants of haplotypes were found in 5 specimens of *C. volki*. One type was recorded in three individuals, two other types were found in a single individual and differed from the first type in one and from each other in two nucleotide positions. Because the level of variability in the studied sample of *C. volki* appeared to be low, additionally 305 bp from the right domain of CR of mtDNA beginning from pyrimidine tract (positions from 16232 to 16535 of the mitochondrial genome of *C. reinii*) were sequenced using back primer LRBT-1195 only in one individual with domination by the frequency variant of the haploid. In all, the sequences were determined, in such a way, in two regions of the mitochondrial genome



ML-tree of sculpins from the “*poecilopus*” group and other representatives of sculpins from subfamily Cottinae based on the analysis of CR of mtDNA. The numbers at the bases of clusters are the bootstrap estimates (percents from 1000 replicates) of stability of branching nodes of 50% ML (above the line) and MP (under the line) consensual trees. Boldface dots designate branching nodes with Bayesian estimates of a posterior probability above 0.95. (Digits in parentheses after species names are the numbers of nucleotide sequences taken from the genetic base of Genbank/NCBI.

of *C. volki* of the summary length 746 bp. After the procedure of their alignment together with homologous sequences of 20 other representatives of Cottinae taken from the genetic databank Genbank/NCBI, the matrix 21 × 761 was obtained and served the basis for phylogenetic analysis.

In the tree (figure) constructed with the maximum likelihood method on the base of the model of nucleotide substitutions HKY + I (parameters of the tree and the model: -LnL = 4063.41; total tree length 0.94; nucleotide frequencies: A = 0.316, C = 0.195, G = 0.179, T = 0.310; the ratio between the rates of transition and transversion mutations 2.515; the proportion of invariable sites 0.581), sculpins from genus *Cottus* formed compact clade composed of six clearly expressed phylogenetic groups (phylogroups) received moderately high level of support: EA, NA1–2, JS1–2, and PA. One of them (phylogroup PA) was formed by sculpins from the group “*poecilopus*.” Typical sculpin *C. poecilopus* occupied in this group the sister position relative to clade formed by the Amur sculpin *C. szanaga* and *C. cf. poecilopus* from the Lena River. Sculpin *C. volki* occupied the basal position relative to other species in phylogroup PA.

Heuristic search of maximum parsimonious trees on the basis of analysis of the matrix from 160 phylogenetically informative characters revealed five equally parsimonious (tree length 499 steps; correspondence index (CI) 0.50; Homoplasy index (HI) 0.50; retention index (RI) 0.53) variants characterized by principal similarity with ML-tree in the number and composition of the main clades of sculpins from genus *Cottus* in the order of branching within these clades, the levels of their bootstrap support (the trees are not presented, but can be obtained from the authors by request). The differences of these two variants of MP-trees from each other

and from ML-tree was only in the order of the combining of six phylogroups shown in the figure, and this fact was expected, because this order remained not to be solved in essence in the case of ML-tree also.

The topology of the consensual phylogenetic tree obtained using the Bayesian analysis on the basis of the model of nucleotide substitutions HKY + I (averaged parameters of the tree and model: -LnL = 4079.73; total tree length 1.04; nucleotide frequencies: A = 0.316, C = 0.197, G = 0.178, T = 0.309; the ratio between the rates of transition and transversion mutations 2.546; the proportion of invariable sites 0.559) followed in details the topology of ML-tree and because of this is not presented. Practically all branching nodes, which were reproduced in more than 50% of bootstrap replicates in the process of testing the stability of ML- and MP-trees, also obtained a posteriori high values of probability in the Bayesian tree (figure).

Hence, inclusion of *C. volki* to the “*poecilopus*” group can be considered to be well supported, taking into consideration the well-known conservative character of bootstrap estimates (see Felsenstein, 2004). Respectively, the presence in *C. volki* of primitive (plesiomorphic for this group) characteristics can be explain by early separation of *C. volki* line from the common stem of this group of sculpins *Cottus*.

It should be noted in this relation that recently obtained (Kinziger et al., 2005) mtDNA data for “*C. poecilopus volki*” (the place of collecting and the collector of material were not given in this work, only the inventorial number from the collection of the University of Alabama Museum of Natural History, UAIC) characterize most probably not *C. volki*, but *C. koreanus* Fujii, Choi et Yabe, 2005 from South Korea, because “*C. poecilopus volki*” in the work of Kinziger et al. occupied exactly the same (sister relative

to *C. hangiongensis* Mori, 1930) position as *C. koreanus* in the work of Yokoyama and Goto (2005) and in our work (see figure). Moreover, the closeness of *C. volki* to *C. hangiongensis* seems to be improbable according to the Kishino–Hasegawa (1989) test, because ML-tree ($-\ln L = 4121.32$) and MP-trees (the length 523 steps) depicted on the basis of sequences in the CR of mtDNA providing that sister relationships between *C. volki* and *C. hangiongensis* were recorded earlier were worse with high probability (in both cases $p < 0.001$; the calculations were performed with the help of the PAUP program) than those obtained in the result of heuristic search without prior assumptions about probable variants of tree topologies. *C. koreanus* resembles in appearance *C. volki* and other representatives of the “*poecilopus*” group, with which it was long identified (Fujii et al., 2005). It is likely the cause of incorrect identification of the samples from the UAIC collection.

It is our opinion that a revealed high level of genetic differences between the representatives of PA phylogroup, comparable or exceeded to that observed within other phylogroups combined some “good” species from genus *Cottus*, confirms indirectly the validity of separating the line *C. poecilopus* to several independent species: *C. poecilopus*, *C. szanaga*, and *C. volki* (Holčík and Pivnická, 1969; Chereshevnev, 1982; Shedko and Shedko, 2003).

Unfortunately, poor knowledge of sculpins from water bodies of West and East Siberia makes their exact species identification impossible. Relatively high genetic differences of *C. cf. poecilopus* from the Lena River simultaneously from both *C. poecilopus* and *C. szanaga* (which are approximately two times higher than those found for the pair of Eurasian species *C. gobio* L., 1758 and *C. sibiricus* Warpachowski, 1889, see figure) suggest that comprehensive study is necessary of the morphology of Siberian sculpins in order to determine their species belonging and to assess their taxonomic status.

According to Chereshevnev (1982), one more species from the “*poecilopus*” group, not legalized taxonomically, inhabits water bodies in northeastern Asia (Chukot Peninsula, Kolyma, the rivers of the northern part of the Sea of Okhotsk coast). Judging by the relatively regular presence of teeth on palatal bones and a simultaneously high degree of reduction of the interior ray in the ventral fin (usually less than 40% of the whole fin length), its separating from the general ancestral line “*poecilopus*” could occur after the separating of the *C. volki* line, but prior to the moment of divergence of ancestral lines *C. poecilopus* and *C. szanaga*. There is a good probability that it was eastern Asia (in its continental part belonging to the Pacific Ocean basin), where the separation occurred of the total ancestral trunk of the “*poecilopus*” group species, and then they distributed to Siberia and Europe from this region. It is our opinion that, in such a way, obtained pattern of

phylogenetic relationships can be connected with morphological features and the character of geographical distribution of the members of this group.

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