# Molecular Phylogenetic Study of Several Eelpout Fishes (Perciformes, Zoarcoidei) from Far Eastern Seas on the Basis of the Nucleotide Sequences of the Mitochondrial Cytochrome Oxidase 1 Gene (Co-1) 

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#### Abstract

A total of 95 nucleotide sequences of a Co-1 gene fragment of approximately 650 bp were analyzed for fishes of the orders Perciformes and Scorpaeniformes (outgroup). Gene trees based on four algorithms (BA, NJ, MP, and ML) were similar in topology of solved branches. An emphasis was placed on the species and generic levels, but a significant phylogenetic signal was obtained for higher taxonomic ranks as well. For instance, a monophyletic origin was confirmed for the family Zoarcidae and the subfamily Opisthocentrinae (Stichaeidae). The proportion of different nucleotides in the sequences compared ( $p$-distances) significantly increased with increasing taxonomic rank. The $p$-distances were estimated for four hierarchic levels and were (1) $0.15 \pm 0.06 \%$ for the within-species hierarchic level, (2) $6.33 \pm 0.37 \%$ for the within-genus level, (3) $11.83 \pm$ $0.06 \%$ for the within-family level, and (4) $15.22 \pm 0.05 \%$ for the within-order level. The difference in the Co- 1 gene fragments between levels (1) and (2) allows almost errorless species identification on the basis of this kind of a molecular bar code.


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## INTRODUCTION

Molecular phylogenetic analysis of taxa at the spe-cies-family level is most often based on the nucleotide sequences (hereafter referred to as sequences) of the mitochondrial genes for subunit 1 cytochrome oxidase $c$ (cytochrome oxidase 1, Co-1) and cytochrome $b[1$, 2]. When taxa of higher ranks are studied, analysis of these genes may yield inadequate results because of mutational saturation due to repeated substitutions or the homoplasy effect. The sequences of the two genes are still useful for species identification and reconstruction of phylogenetic relationships in various taxa [3-5], including fishes, up to the order level [1, 4, 6, 7]. In addition, genetic distances ( $p$-distances) provide information on the taxonomic position of individuals because they characterize a certain level well and substantially increase from lower to higher hierarchic or taxonomic ranks [1, 2, 4-7].

Fish taxonomy still has many disputable issues, in particular, those related to the order Perciformes. The order includes approximately 6,000 species and is one of the largest in fish [8]. Phylogenetic studies are topical for such a numerous group. The taxonomic positions of many families in suborders are still unclear. Moreover, a monophyletic origin cannot be established for certain for both families and suborders, as is required by modern taxonomy [9]. Particular interest is attracted by the family Stichaeidae, which belongs
to the suborder Zoarcoidei and currently includes 80 species of six subfamilies and 38 genera [10-12]. Many taxonomic problems are still unsolved at the levels of genera and species for other suborders and families, and monophyly of many taxa remains questionable [8].

Molecular phylogenetic analysis of Stichaeidae has already been performed in several studies [7, 13, 14]. Stepien et al. [13] examined only few individuals to study the evolution of Blennioidei, and their information was insufficient for phylogenetic inferences. Radchenko et al. [14] investigated the total suborder Zoarcoidei and noted a distinctness between the subfamilies Opisthocentrinae and Chirolophinae and between Lumpeniinae and Stichaeinae. According to Kartavtsev et al. [7], Opisthocentrinae similar group with Chirolophinae, but Stichaeinae is a daughter branch of Chirolophinae, which agrees with the conventional taxonomy of the group [15].

The objective of this work was to carry out molecular phylogenetic analysis of several perch-like fishes (Zoarcoidei) on the basis of data on the $\mathrm{Co}-1$ gene sequences.

Accordingly, the three main tasks of our work were (1) to study genetic divergence ( $p$-distances) within species and in taxa of higher hierarchical ranks on the basis of the Co-1 gene; (2) to analyze the nucleotide composition of the sequences of the perch-like fishes

Table 1. Species under study with their voucher identification numbers, GenBank accession numbers, and collection sites

| No. | Species | Individual identification number | GenBank accession no. | Source of the individual or sequence |
| :---: | :---: | :---: | :---: | :---: |
| 1 | Acantholumpenus mackayi (Gilbert, 1896) | AM3 | HQ704734 | Sea of Japan, Russia |
| 2 | Alectrias alectrolophus (Pallas, 1814) | 317 | FJ932610 | NCBI GenBank |
| 3 | Ammodytes hexapterus Pallas, 1814 | 308 | HQ704752 | Bering Sea, Russia |
| 4 | A. hexapterus Pallas, 1814 | 309 | HQ704753 | The same |
| 5 | A. hexapterus Pallas, 1814 | 310 | HQ704754 | " |
| 6 | Anisarchus medius (Reinhardt, 1837) | 281 | HQ704777 | " |
| 7 | A. medius (Reinhardt, 1837) | 282 | HQ704778 | " |
| 8 | A. medius (Reinhardt, 1837) | 283 | HQ704779 |  |
| 9 | A. medius (Reinhardt, 1837) | 284 | HQ704780 | " |
| 10 | A. medius (Reinhardt, 1837) | 285 | HQ704781 | " |
| 11 | Anoplarchus purpurescens Gill, 1861 | 5846 | FJ164282 | NCBI GenBank |
| 12 | A. purpurescens Gill, 1861 | 5847 | FJ164281 | The same |
| 13 | A. purpurescens Gill, 1861 | 5848 | FJ164280 | " |
| 14 | Askoldia variegata Pavlenko, 1910 | AV1 | JF343542 | Tatar Strait, Russia |
| 15 | A. variegata Pavlenko, 1910 | AV2 | JF343543 | The same |
| 16 | A. variegata Pavlenko, 1910 | AV3 | JF343544 | " |
| 17 | Bathymaster signatus Cope, 1873 | 141 | HQ704765 | Bering Sea, Russia |
| 18 | B. signatus Cope, 1873 | 142 | HQ704766 | The same |
| 19 | B. signatus Cope, 1873 | 143 | HQ704767 | " |
| 20 | B. signatus Cope, 1873 | 144 | HQ704768 | " |
| 21 | B. signatus Cope, 1873 | 145 | HQ704769 |  |
| 22 | Bothrocara zestum Jordan \& Fowler, 1902 | 102 | HQ704771 | " |
| 23 | B. zestum Jordan \& Fowler, 1902 | 105 | HQ704772 | , |
| 24 | Cebidichthys violaceus (Girard, 1854) | MFC387 | GU440267 | NCBI GenBank |
| 25 | Chirolophis decoratus (Jordan \& Snyder, 1902) | MFC239 | GU440277 | The same |
| 26 | Chirolophis japonicus Herzenstein, 1890 | CJ3 | HQ704732 | Sea of Japan, Russia |
| 27 | Chirolophis nugator (Jordan \& Williams, 1895) | MFC338 | GU440279 | NCBI GenBank |
| 28 | Dasycottus setiger Bean, 1890 | 62 | HQ704756 | Bering Sea, Russia |
| 29 | Ernogrammus hexagrammus (Schlegel, 1845) | 1196 | FJ932611 | NCBI GenBank |
| 30 | E. hexagrammus (Schlegel, 1845) | E1 | HQ704722 | Sea of Japan, Russia |
| 31 | E. hexagrammus (Schlegel, 1845) | E2 | HQ704723 | The same |
| 32 | E. hexagrammus (Schlegel, 1845) | E3 | HQ704724 | " |
| 33 | E. hexagrammus (Schlegel, 1845) | E4 | HQ704725 | " |
| 34 | E. hexagrammus (Schlegel, 1845) | E5 | HQ704726 | " |
| 35 | Esselenichthys carli (Follett \& Anderson, 1990) | MFC200 | GU440318 | NCBI GenBank |
| 36 | Kasatkia seigeli Posner \& Lavenberg, 1999 | SIO03-92 | HQ010045 | The same |
| 37 | Leptoclinus maculatus (Fries, 1838) | 263 | HQ704750 | Bering Sea, Russia |
| 38 | L. maculatus (Fries, 1838) | 264 | HQ704751 | The same |
| 39 | Lobotes surinamensis (Bloch, 1790) | LOS 1 | HQ704735 | Sea of Japan, Russia |
| 40 | Lumpenella longirostris (Evermann \& Goldsborough, 1907) | 441 | FJ164727 | NCBI GenBank |
| 41 | L. longirostris (Evermann \& Goldsborough, 1907) | 442 | FJ164730 | The same |
| 42 | L. longirostris (Evermann \& Goldsborough, 1907) | 448 | FJ164729 |  |
| 43 | Lumpenus sagitta Wilimovsky, 1956 | 302 | HQ704782 | Bering Sea, Russia |
| 44 | L. sagitta Wilimovsky, 1956 | 303 | HQ704783 | The same |
| 45 | L. sagitta Wilimovsky, 1956 | 304 | HQ704784 |  |
| 46 | L. sagitta Wilimovsky, 1956 | LS1 | HQ704731 | Sea of Japan, Russia |
| 47 | Lycenchelys crotalinus (Gilbert, 1890) | 231 | HQ704760 | Bering Sea, Russia |

Table 1. (Contd.)

| No. | Species | Individual identification number | GenBank accession no. | Source of the individual or sequence |
| :---: | :---: | :---: | :---: | :---: |
| 48 | L. crotalinus (Gilbert, 1890) | 234 | HQ704761 | The same |
| 49 | Lycodes brevipes Bean, 1890 | 108 | HQ704757 | " |
| 50 | L. brevipes Bean, 1890 | 109 | HQ704758 | " |
| 51 | L. brevipes Bean, 1890 | 110 | HQ704759 | " |
| 52 | L. concolor Gill \& Townsend, 1897 | 226 | HQ704773 | " |
| 53 | L. concolor Gill \& Townsend, 1897 | 227 | HQ704774 | " |
| 54 | L. concolor Gill \& Townsend, 1897 | 228 | HQ704775 | " |
| 55 | L. concolor Gill \& Townsend, 1897 | 230 | HQ704776 | " |
| 56 | Lycodes beringi Andriashev, 1935 | 59 | HQ704755 | " |
| 57 | Lycodes palearis Gilbert, 1896 | 236 | HQ704762 | " |
| 58 | L. palearis Gilbert, 1896 | 237 | HQ704763 | " |
| 59 | L. palearis Gilbert, 1896 | 238 | HQ704764 | " |
| 60 | Lycodes raridens Taranetz \& Andriashev, 1937 | 311 | HQ704785 | " |
| 61 | L. raridens Taranetz \& Andriashev, 1937 | 312 | HQ704786 | " |
| 62 | L. raridens Taranetz \& Andriashev, 1937 | 313 | HQ704787 | " |
| 63 | L. raridens Taranetz \& Andriashev, 1937 | 314 | HQ704788 | " |
| 64 | Opisthocentrus ocellatus (Tilesius, 1811) | OO1 | HQ704736 | Sea of Japan, Russia |
| 65 | O. ocellatus (Tilesius, 1811) | 0011 | HQ704741 | The same |
| 66 | O. ocellatus (Tilesius, 1811) | OO2 | HQ704737 | " |
| 67 | O. ocellatus (Tilesius, 1811) | 0 O 3 | HQ704738 | " |
| 68 | O. ocellatus (Tilesius, 1811) | 005 | HQ704740 | " |
| 69 | Opisthocentrus tenuis Bean \& Bean, 1897 | OT5 | HQ704739 | " |
| 70 | O. tenuis Bean \& Bean, 1897 | OT4 | EU200481 | " |
| 71 | Opisthocentrus zonope Jordan \& Snyder, 1902 | OZ1 | HQ704727 | " |
| 72 | O. zonope Jordan \& Snyder, 1902 | OZ2 | HQ704728 | " |
| 73 | O. zonope Jordan \& Snyder, 1902 | OZ3 | HQ704729 | " |
| 74 | O. zonope Jordan \& Snyder, 1902 | OZ4 | HQ704730 | " |
| 75 | Ph. dybowskii (Steindachner, 1880) | PhD1 | HQ704746 | " |
| 76 | Ph. dybowskii (Steindachner, 1880) | PhD2 | HQ704747 | " |
| 77 | Ph. dybowskii (Steindachner, 1880) | PhD3 | HQ704748 | " |
| 78 | Plectobranchus evides Gilbert, 1890 | 04HBL008132 | FJ165022 | NCBI GenBank |
| 79 | Poroclinus rothrocki Bean, 1890 | 533 | FJ165043 | The same |
| 80 | P. rothrocki Bean, 1890 | 534 | FJ165039 | " |
| 81 | P. rothrocki Bean, 1890 | 622 | FJ165040 | " |
| 82 | Ronquilus jordani (Gilbert, 1889) | TZ-06-RICKER-774 | FJ165103 | " |
| 83 | R. jordani (Gilbert, 1889) | TZ-06-RICKER-775 | FJ165104 | " |
| 84 | R. jordani (Gilbert, 1889) | TZ-06-RICKER-779 | FJ165105 | " |
| 85 | R. jordani (Gilbert, 1889) | TZ-06-RICKER-785 | FJ165106 | " |
| 86 | R. jordani (Gilbert, 1889) | TZ-06-RICKER-830 | FJ165107 | " |
| 87 | Stichaeopsis nevelskoi (Schmidt, 1904) | SN1 | HQ704733 | Sea of Japan, Russia |
| 88 | Stichaeus ochriamkini Taranetz, 1935 | SO1 | HQ704742 | The same |
| 89 | S. ochriamkini Taranetz, 1935 | SO3 | HQ704743 | " |
| 90 | S. ochriamkini Taranetz, 1935 | SO4 | HQ704744 | " |
| 91 | S. ochriamkini Taranetz, 1935 | SO5 | HQ704745 | " |
| 92 | Stichaeus punctatus (Fabricius, 1780) | 210 | HQ704770 | Bering Sea, Russia |
| 93 | Xiphister mucosus (Girard, 1858) | 5073 | FJ165465 | NCBI GenBank |
| 94 | X. mucosus (Girard, 1858) | 5074 | FJ165466 | The same |
| 95 | X. mucosus (Girard, 1858) | 5075 | FJ165467 | " |



Fig. 1. Map with collections sites indicated. The sites where individuals under study were fished in the Japanese and Bering seas are encircled.
examined; and (3) to study the peculiarities of molecular genetic divergence and phylogenetic relationships among the perch-like fishes, with a special emphasis on Stichaeidae and Zoarcidae.

## MATERIALS AND METHODS

A total of $95 \mathrm{Co}-1$ gene sequences were used in comparative phylogenetic analysis (Table 1), including 70 original sequences and 25 sequences from GenBank (NCBI, http://www.ncbi.nlm.nih.gov/) [16]. The analysis included 36 species of five perch-like families: Stichaeidae ( 25 species), Zoarcidae (seven species), Bathymasteridae (two species), Lobotidae (one species), and Ammodytidae (one species), and one species (Dasycottus setiger Bean, 1890) of the order Scorpaeniformes. Material for the study was collected from three trawling catches in the Bering Sea during an expedition onboard the TINRO research chip in 2008. In the Japanese See, material was collected from 2009 to 2010 with the use of gill nets, fry nets, and shrimp traps (Fig. 1). Species identification followed Lindberg and Krasyukova [17]; species of the genera Pholidapus and Opistocentrus were identified using Shiogaki criteria [18]. Species names are given as in [19]. Muscle tissue for genetic analysis was taken from each separate individual and fixed with $96 \%$ ethyl alcohol. The majority of the fishes examined were stored in a fixed form in the collection of the Zhirmulsky Institute of Marine Biology (IMB, curator A.A. Balanov) under corresponding catalog (voucher)
numbers; their color photographs and complete information on the samples and genetic data were included in the BOLD database [20] (http://www.boldsystems.org/) of the iBOL global program.

Muscle tissue DNA was isolated via phenol-chloroform extraction [21] with minor modification [7]. A mitochondrial $\mathrm{Co}-1$ gene fragment of approximately 650 bp was amplified in the polymerase chain reaction (PCR) with primers FishF1 (5'-TCAACCAACCA-CAAAGACATTGGCAC-3') and FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') [4]. The reaction mixture $(25 \mu \mathrm{l})$ contained $17.4 \mu \mathrm{l}$ of distilled water, $2.5 \mu 1$ of a $10 \times$ buffer (TaKaRa, Japan), $2.0 \mu$ of a dNTP mixture ( 2.5 mM each triphosphate), $1 \mu \mathrm{l}$ of each primer ( $20 \mathrm{pmol} / \mu \mathrm{l}), 0.1 \mu \mathrm{l}$ of 5 units $/ \mu \mathrm{l}$ Taq polymerase, and $1 \mu l$ of DNA. PCR was run on Eppendorf Mastercycler and BIORAD My Cycler thermal cyclers. Amplification included denaturation at $94^{\circ} \mathrm{C}$ for $5 \mathrm{~min} ; 30$ cycles of denaturation at $94^{\circ} \mathrm{C}$ for 30 s , annealing at $60^{\circ} \mathrm{C}$ for 30 s , and synthesis at $72^{\circ} \mathrm{C}$ for 1 min ; and last synthesis at $72^{\circ} \mathrm{C}$ for 7 min . The PCR products were electrophoresed in $1 \%$ agarose gel, stained with ethidium bromide, and viewed in transmitting UV light. The amplicon size was estimated against a 100-bp DNA marker. The best samples were selected for subsequent manipulations and purified with $96 \%$ and $70 \%$ ethanol according to a standard protocol. Cyclic sequencing was then performed. The nucleotide sequences were identified physically on an ABI-3130 sequencer (Applied Biosystems, United

States) at the IMB and Far East Federal University (Vladivostok).

Two antiparallel sequences were obtained for each individual. Consensus sequences were formed using the ChromasPro editor [22] and deposited in GenBank [16] and, partly, in BOLD [20]. The best-fit model of nucleotide substitution for subsequent construction of gene trees was selected using the Modeltest 3.7 program [23] and the PAUP* 4.0 package [24]. The GTR+I+G model was selected as optimal for the given data set according to the Akaike information criterion. The topology of a Bayesian (BA) tree was calculated using the MrBayes 3.1 software package [25]. Sequence alignments were constructed using the MEGA 5 software package [25] with Clustal-W in two steps. At the first step, the gap opening penalty was 15, and the gap closing penalty was 5 for both pairwise and multiple alignments. At the second step, the penalties were reduced to 5 and 0.5 , respectively. Gaps were removed after each alignment step. Calculation and visualization of neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) trees were carried out using MEGA 5 [26]. Since the program does not utilize the model GTR $+\mathrm{I}+\mathrm{G}$ to construct an NJ tree, we used the similar Tamura-Nei model of nucleotide substitution with the $\gamma$ parameter $\mathrm{G}=0.9$ and the invariable site proportion $\mathrm{I}=0.56$. When the MP tree was constructed, starting trees were searched by the close-neighbor-interchange (CNI) method and search level 1. Random addition of starting trees ( 10 replications) was used in the CNI search. The stability of the tree topology was checked by bootstrap analysis with 1000 replications in the case of the MP and NJ algorithms and 500 replications in the case of the ML algorithm. The replications were used to construct consensus trees with a threshold of $50 \%$. BA trees were obtained for $10^{6}$ model generations. The parameters of the BA program were: MCMCP ngen $=$ 1000000 , printfreq $=1000$, samplefreq $=1000$, nchains $=4$, amd SUMP burnin $=100$; the other parameters were set at default values. Analysis yielded 19,652 trees. A $50 \%$ consensus tree was constructed on their basis and included 9,851 trees.

## RESULTS

The total length of the sequences under study was 524 bp after alignment. The BA, NJ, MP, and ML consensus trees were basically similar in topology at the levels of genera and species, differing only in bootstrap support of nodes. At higher hierarchical levels, the topology of the trees was only partly resolved in most cases and, consequently, differed among the algorithms. However, the branches of the family Zoarcidae and subfamily Opisthocentrinae had a good sup-
port in all of the four reconstruction algorithms. The BA tree was the best resolved topologically and, consequently, was taken as an illustration (Fig. 2).

When necessary, the resulting trees were rooted using Dasycottus setiger as an outgroup. The species grouped with Ammodytes hexapterus and Lobotes surinamensis to form a separate branch, which was supported in different algorithms (98/54/59/72) (Fig. 2). The percent support is hereafter given for the BA, NJ, MP, and ML trees, respectively; a dash indicates a lack of support in the corresponding algorithm. Ammodytes hexapterus individuals separate into two branches, while their common node is supported $(98 / 54 / 59 / 72)$.

At the level of families, a distinct monophyletic branch is formed by representatives of the family Zoarcidae (99/100/99/99). Lycenchelys crotilinus is the first to produce a separate branch in the resulting topology. Then, Bothrocara zestum separates from the main tree $(57 / 67 / 65 /-)$. This is followed by division into two branches $(92 / 54 / 59 / 62)$, of which one is formed by the only representative of Lycodes beringi, and the other combines $L$. brevipes, L. palearis, L. raridens, and L. concolor. In the latter branch, L. concolor $(99 / 89 / 69 / 80)$ and L. raridens $(91 / 51 / 56 / 54)$ consecutively form separate lineages, and then a common branch is formed by L. palearis and L. brevipes ( $91 / 51 / 54 / 54$ ), which are the most similar genetically in the family according to the given topology.

A separate stable branch includes representatives of the subfamily Opisthocentrinae of the family Stichaeidae. One member of the subfamily (Plectobranchus evides) is basal relative to the total subfamily, forming an unresolved branch of the tree. The subfamily is represented by five genera and seven species. Kasatkia seigeli is the first to separate from the main branch (99/91/74/92). The branch then divides into two separate groups $(96 / 87 / 60 / 75)$, which include representatives of the genus Opisthocentrus and the species Ascoldia variegata and Pholidapus dybowskii, which, in turn, form two species branches (51/72/60/75). In the genus Opisthocentrus, O. zonope is the first to separate $(99 / 97 / 84 / 98)$ and is followed by O. ocellatus and $O$. tenius $(90 / 99 / 88 / 54)$.

In addition, several representatives of the subfamilies Stichaeinae, Chirolophinae, and Xiphisterinae of the family Stichaeidae separate to form individual branches, which were not topologically resolved. Separation of several branches at the suprafamily level was noted in the BA tree, but these branches did not have a high statistical support and were not topologically resolved in the other algorithms. A clustering was observed for the species Alectrias alectrolophus and Anoplarchus purpurescens (99/86/69/94) and branches of the genus Chirolophis, with consecutive separation

Fig. 2. Rooted consensus tree of phylogenetic relationships of perch-like fishes as based on the Co- 1 nucleotide sequence. Support is indicated for the nodes that were resolved in more than $50 \%$ of bootstrap replications (ML, MP, and NJ) or had an a posteriori probability of more than $50 \%(B A)$. Statistical support is given for the BA, NJ, MP, and ML trees, respectively.

of Ch.japonicus (100/95/87/92) and Ch. nugatory with Ch. decoratus (99/100/99/100). The other branches included individual species: Stichaeopsis nevelskoi, Ernogrammus hexagrammus, and Xiphister mucosus, and lacked substantial (more than 70-80\%) support.

In the subfamily Lumpeninae of the family Stichaeidae, Leptoclinus maculatus and Anisarchus medius form a common branch (54/-/-/-) and group with representatives of the family Zoarcidae. The branches of Lumpenella longirostris, Poroclinus rothrocki, Acantholumpenus mackayi, and Lumpenus sagitta are unresolved.

The family Bathymasteridae (Bathymaster signatus and Ronquilus jordani) does not form a separate branch, representing a polyphyletic group. The tree includes several unresolved branches; in particular, such branches are formed by the other representatives of the subfamily Xiphisterinae of the family Stichaeidae, which produce a common node with Cebidichthys violaceus and Esselenichthys carli (98/87/90/91).

To estimate divergence at various taxonomic levels, mean $p$-distances were calculated from a matrix of pairwise distances (see Appendix). In this analysis, values were taken from the triangular matrix and divided into four groups according to the taxon rank, an arithmetic mean was calculated for each group, and differences between the means were evaluated by oneway analysis of variance with the use of Statistica 6.0 [27]. This gave a picture of nucleotide diversity at four different hierarchical levels: (1) within species, among individuals of one species; (2) within genus, among individuals of one genus; (3) within family, among individuals of one family; and (4) within order, among individuals of one order (Fig. 3). The mean $p$-distances for the corresponding taxonomic levels were, \% (1) $0.15 \pm$
0.06 , (2) $6.00 \pm 0.37$, (3) $12.00 \pm 0.06$, and (4) $15.00 \pm$ 0.05 (mean $\pm$ standard error).

One-factor analysis of variance (ANOVA) of the variation of $p$-distances within groups and between groups showed that the mean values of the four groups differed significantly: $F=1075.5$, d. $f .=3, P<0.0001$. The above mean $p$-distances and their errors and analysis of variance indicate that different $p$-distances are characteristic of the within-species, within-genus, within-family, and within-order groups in the suborder Zoarcoidei.

Nucleotide proportions were heterogeneous (Table 2), which was mostly due to a predominance of pyrimidines in both our $\mathrm{Co}-1$ sequences (Group I) and GenBank sequences (Group II) (Fig. 4). Two-way analysis of variance (ANOVA) for the variation of purine and pyrimidine proportions in the two groups revealed a significant difference between mean values for the main factor, which was the proportions of the four nucleotides: $F=5.9635$, d.f. $=3, P=0.00056$. The proportions of similar nucleotides did not differ among sequences within groups I and II (Fig. 4).

## DISCUSSION

The topologies of the $\mathrm{Co}-1$ gene trees indicate that the 524-bp sequences under study were not informative enough to allow a resolution of all branches and to reveal the relationships for the majority of taxonomic groups. As was described in Results, many branches of higher hierarchical levels were not topologically resolved in the molecular phylogenetic trees (Fig. 2). At the same time, the $\mathrm{Co}-1$ sequences of individuals from one species clustered close together and displayed minimum $p$-distances within all of the genera. The distances were $0.15 \pm 0.06 \%$ on average (Fig. 3). Sequences from different species of one genus formed


Fig. 3. One-factor analysis of variance of $p$-distances based on the $C o-1$ nucleotide sequences for several hierarchic levels. Mean values were obtained from the triangular matrix of $p$-distances (Appendix) for each of the groups.
$F=5,9635, d_{.} f .=3 ; 380, P=0.00056$


Fig. 4. Two-factor analysis of variance of the nucleotide composition in groups I (original sequences) and II (GenBank sequences).

Table 2. Mean nucleotide composition (\%) of the Co-1 sequences under study (mean $\pm$ standard error)

| Nucleotide | $T(U)$ | $\mathbf{C}$ | $\mathbf{A}$ | $\mathbf{G}$ |
| :--- | :---: | :---: | :---: | :---: |
| GenBank data | $30.5 \pm 0.24$ | $27.6 \pm 0.24$ | $23.2 \pm 0.24$ | $18.7 \pm 0.24$ |
| Original data | $29.7 \pm 0.14$ | $28.5 \pm 0.14$ | $23.0 \pm 0.14$ | $18.9 \pm 0.14$ |

separate branches with a mean $p$-distance of $6.00 \pm$ $0.37 \%$ (Fig. 3). The monophyletic origin of the genera under study was supported well in all four algorithms of tree reconstruction (Fig. 2). These features of the tree and the distance of more than one order of magnitude between the $p$-distances of the within-species and within-genus levels provide a basis for developing a DNA-based bar code of species. In other words, the Co- 1 sequences under study are informative enough for species diagnosis at the level of genera, which agrees with published data $[1,4,6,7]$.

In addition, the topology of the $\mathrm{Co}-1$ tree has a good support at the levels of one family and two subfamilies. The family Zoarcidae and subfamily Opisthocentrinae have the highest support in nodes and form monophyletic branches. With the rather short length of the sequences examined, the finding indicates that the two taxa are the most evolutionarily separate in the group under study. In the subfamily Opisthocentrinae, species branches have a good support and group to produce monophyletic genera. The monophyletic origin of the subfamily has a high support of $74-99 \%$ (Fig. 2). A similar topology was observed in a study of a smaller taxon sample, which included only the genera Opisthocentrus and Pholidapus, and sequences of a slightly different length [7]. At the same time, the topological position of the monotypal genus Plectobranchus in the subfamily could not be resolved on the basis of the $\mathrm{Co}-1$ gene fragment.

The mean genetic distance between genera within the subfamily vary from 7 (between Ascoldia and Pholidapus) to $11 \%$ (between Kasatkia and Opisthocentrus), while the genetic distance between the genus Plectobranchus and the other genera of the subfamily reaches $15-17 \%$, which is higher than the mean $p$-distance within the subfamily ( $12 \%$ ). Gilbert and Thompson [28], who described P. evides, noted its similarity to species of the subfamily Lumpeninae; the Pacific subspecies L. maculates diaphanocarus was described as a species of the genus Plectobranchus [29]; and Jordan and Evermann [30] isolated the species in a separate monotypal subfamily. It is possible that the species appeared to be separate from the other members of the subfamily in our study because we had no data for species of the genus Lumpenopsis, which is close to Plectobranchus. Makushok [15] believes that Plectobranchus combines the features of Lumpeninae and Opisthocentrinae, uniting them in one taxonomic entity. To address these issues, it is necessary to analyze a more representative sample, including species of the genus Lumpenopsis, and to make the reference
sequence more informative by using the full-length Co- 1 sequence (approximately $1,500 \mathrm{bp}$ ) and including other genes in the analysis.

The genus Pholidapus cluster with Ascoldia to produce a separate branch with a good support in our tree, and this finding disagrees with the concept that Pholidapus is a subgenus within the genus Opisthocentrus [31].

The subfamily Chirolophinae similarly has a high statistical support. All of the three its species form a monophyletic branch and belong to one genus (Fig. 2), reflecting the natural position of the group in the system [10]. It is noteworthy, however, that an extremely low $p$-distance of 0.01 was observed between Chirolophis nugator and Ch. decoratus. Such a distance is characteristic of the within-species level in the group under study (see table in the Appendix). This finding makes it possible to assume that the two sequences, which were extracted from GenBank, belong to one species, indicating that the species was misidentified for one of the two individuals used to obtain these sequences.

Sequences of the family Zoarcidae do not group in the $\mathrm{Co}-1$ gene trees in accord with the natural system [32], which suggests that the genus Lycodes is the most primitive in the subfamily Lycodinae. However, only a limited sample of species represented the subfamily in our study, and, consequently, it is impossible to make reliable conclusions on the relationships within the group. The genetic distances between genera within the family Zoarcidae display only a minor variation, from 7 (between Bothrocara and Lycodes) to $8 \%$ (between Bothrocara and Lycenchelys). Radchenko et al. [33] considered the taxonomic relationships of the genera Bothrocara and Lycodes. However, the results cannot be compared with our findings because of the sample specifics (other species were examined).

The topology of our trees disagrees with the common views of the system of Stichaeidae. The subfamilies Lumpeninae, Stichaeinae, and Xiphisterinae formed unresolved branches and, accordingly, their branching was unclear. A high support was obtained only for within-species groups of these taxa. In view of the above, it is possible to conclude either that the system of the family Stichaeidae needs further development or that the phylogenetic signal based on the Co-1 gene fragment is insufficient for reliable conclusions. Interestingly, the sequence of a L. sagitta individual from the Sea of Japan did not cluster closely with individuals from the Bering See. Gene genetic distances between the sequences of these individuals were 0.05 , somewhat higher than usual at the within-species level (0.01). Since the samples were collected at the margins
of the species area, our findings suggest a high interpopulation difference in the $\mathrm{Co}-1$ gene for $L$. sagitta.

The family Bathymasteridae formed two distant groups (Fig. 2). Anderson [32] believes that the family is the most primitive in the suborder Zoarcoidei, and this is supported by molecular data [14]. It seems that genes with a higher extent of conservation should be examined to study the taxonomic relationships within the family. We intend to perform such analysis in our further work.

The families Ammodytidae and Lobotidae were in polytomy relative to Dasycottus setiger, but formed a separate group distant from the other taxa. This result supports the natural positions of the group in the conventional system [8] and indicates that the outgroup was selected adequately.

Since $p$-distances increased with increasing taxonomic rank (Fig. 3), a geographical model of speciation and phyletic evolution may be assumed to dominate in the perch-like taxa under study, as in other groups [3, 6]. However, relative genetic distances are an insufficient basis for identifying the model of speciation, and several parameters or descriptors should be analyzed [1,5]. The above assumption is thereby only preliminary. Extremely low $p$-distances within species testify to a high sequence homology. This is indicative of correct species identification and, as mentioned above, is broadly used to construct a DNA-based bar code of species [20].

The nucleotide composition of the sequences under study was biased from the uniform composition $1: 1: 1: 1$ towards a predominance of pyrimidines (Fig. 4). This may be assumed to reflect the hydrophobic properties of protein-coding genes [34], including Co-1. However, taxonomic differences in nucleotide composition are also possible, pointing to evolutionary specifics of different phyletic lineages [6].

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## REFERENCES

1. Kartavtsev, Y.P. and Lee, J.-S., Analysis of Nucleotide Diversity at Genes Cyt-b and Co-1 on Population, Species, and Genera Levels: Applicability of DNA and

Allozyme Data in the Genetics of Speciation, Russ. J. Genet., 2006, vol. 42, no. 4, pp. 341-362.
2. Johns, G.C. and Avise, J.C., A Comparative Summary of Genetic Distances in the Vertebrates from the Mitochondrial Cytochrome b Gene, Mol. Biol. Evol., 1998, vol. 15, pp. 1481-1490.
3. Avise, J.C., Phylogeography: The History and Formation of Species, Cambridge: Harvard University Press, 2001.
4. Ward, R.D., Zemlak, T.S., Innes, B.H., et al., DNA Barcoding Australia Fish Species, Philos. Trans. R. Soc. London, Ser. Biol. Sci., 2005, vol. 360, pp. 1847-1857.
5. Kartavtsev, Yu.F., Molekulyarnaya evolyutsiya i populyatsionnaya genetika (Molecular Evolution and Population Genetics), Vladivostok: Izd. Dal'nevost. Univ., 2005.
6. Kartavtsev, Y.P., Park, T.-J., Vinnikov, K.A., et al., Cytochrome b (Cyt-b) Gene Sequence Analysis in Six Flatfish Species (Teleostei, Pleuronectidae), with Phylogenetic and Taxonomic Insights, Mar. Biol. (Berlin), 2007, vol. 152, pp. 757-773.
7. Kartavtsev, Y.P., Sharina, S.N., Goto, T., et al., Molecular Phylogenetics of Pricklebacks and Other Percoid Fishes from the Sea of Japan, Aquat. Boil., 2009, vol. 8, pp. 95-103.
8. Nelson, J.S., Fishes of the World, New York: Wiley, 2006, 4th ed.
9. Mayr, E., Principles of Systematic Zoology, New York: McGraw-Hill, 1969.
10. Mecklenburg, C.W. and Sheiko, B.A., Family Stichaeidae Gill 1864 Pricklebacks: Annotation Checklist of Fishes, California Acad. Sci., 2004, no. 35.
11. Kimura, S. and Sato, A., Descriptions of Two New Pricklebacks (Perciformes: Stichaeidae) from Japan, Bull. Natl. Mus. Sci., 2007, ser. A, suppl. 1, pp. 67-79.
12. Shinohara, G. and Yabe, M., A New Genus and Species of Prickleback (Perciformes: Stichaeidae) from Japan, Ichthyol. Res., 2009, vol. 56, no. 4, pp. 394-399.
13. Stepien, C.A., Dillon, A.K., Brooks, M.J., et al., The Evolution of Blennioid Fishes Based on an Analysis of Mitochondrial 12S rDNA, in Molecular Systematics of Fishes, 1997, pp. 245-270.
14. Radchenko, O.A., Chereshnev, I.A., Petrovskaya, A.V., and Balanov, A.A., Molecular Systematics and Phylogeny of the Suborder Zoarcoidei, Vestn. Dal'nevost. Otd. Russ. Akad. Nauk, 2009, no. 3, pp. 40-47.
15. Makushok, V.M., The Morphology and Classification of the Northern Blennoid Fishes and Closely Related Fish Families (Stichaeoidae, Blennioidei, Pisces), Tr. Zool. Inst. Akad. Nauk SSSR, 1958, vol. 25, pp. 3-129.
16. GeneBank, NCBI, www.ncbi.nlm.nih.gov/
17. Lindberg, G.U. and Krasyukova, Z.V., Ryby Yaponskogo morya i sopredel'nykh chastei Okhotskogo i Zheltogo morei (Fishes of the Sea of Japan and the Adjacent Areas of the Sea of Okhotsk and the Yellow Sea), Leningrad: Nauka, 1975, part 4.
18. Shiogaki, M., A Review of the Genera Pholidapus and Opisthocentrus (Stichaeidae), Jpn. J. Ichthyol., 1984, vol. 31, no. 3, pp. 213-224.
19. Froesy, R. and Pauly, D., FishBase, www.fishbase.org.
20. Ratnasingham, S. and Hebert, P.D.N., BOLD: The Barcode of Life Data System, Mol. Ecol. Notes, 2007, vol. 7, no. 3, pp. 355-364, www.barcodinglife.org.
21. Maniatis, T., Fritsch, E.F., and Sambrook, J., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor: Cold Spring Harbor Lab., 1982.
22. ChromasPro, www.technelysium.com.au/chromas.html/
23. Posada, D. and Grandal, K.A., MODELTEST: Testing the Model DNA Substitution, Bioinformatics, 1998, vol. 14, pp. 817-818.
24. Swofford, D.L., PAUP* Phylogenetic Analysis Using Parsimony (and Other Methods): Version 4, Sunderland: Sinauer Associates, 2002.
25. Huelsenbeck, J.P. and Ronquist, F., MrBayes: Bayesian Inference of Phylogeny, Bioinformatics, 2001, vol. 17, pp. 754-755.
26. Tamura, K., Dudley, J., Nei, M., and Kumar, S., MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0, Mol. Biol. Evol., 2007, vol. 24, pp. 1596-1599.
27. STATISTICA for Windows (Data Analysis Software System), Version 6, Tulsa: StatSoft, 2001, www.statsoft.com. 2001.
28. Gilbert, Ch.H. and Thompson, J.C., Notes on the Fishes of Puget Sound, Proc. US Nat. Mus., 1905, vol. 28, pp. 973-987.
29. Shmidt, P.Yu., Ryby vostochnykh morei Rossiiskoi Imperii (Fishes of the Eastern Seas of the Russian Empire), St. Petersburg: Izd. Russ. Imper. Geograf. Obshestva, 1904.
30. Jordan, D.S. and Evermann, B.W., The Fishes of North and Middle America, Bull. US Nat. Mus., 1898, no. 47, part 3, pp. 2183-3136.
31. Rutenko, O.A. and Ivankov, V.N., Morphological Analysis and Taxonomical Status of Four Fish Species of the Genera Opisthocentrus and Pholidapus (Perciformes: Stichaeidae), Biol. Morya, 2009, vol. 35, no. 5, pp. 329-336.
32. Anderson, M.E., Systematics and Osteology of the Zoarcidae (Teleostei: Perciformes), no. 60 of Ichthyology Bulletin, Smith, J.L.B., Ed., Inst. Ichthyology, 1994.
33. Radchenko, O.A., Chereshnev, I.A., and Petrovskaya, A.V., Close Relationships and Divergence among Some Fish Taxa of the Subfamily Lycodinae (Zoarcidae, Pisces) Inferred from Molecular Genetic and Morphological Data, Vopr. Ikhtiol., 2009, vol. 49, no. 5, pp. 603-616.
34. Naylor, G.J., Collins, T.M., and Brown, W.M., Hydrophobicity and Phylogeny, Nature, 1995, vol. 373, pp. 565-566.

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