ANIMAL GENETICS

Phylogenetic Relationships of Flounders from the Family Pleuronectidae (Ostichties: Pleuronectiformes) Based on *16S* rRNA Gene

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Abstract—The systematics and phylogeny of flatfish is investigated on the basis of an incomplete nucleotide sequence of *16S* rRNA and the complete *Co-1* and *Cyt-b* sequences. In total 62 specimens of 14 species of our own collections were submitted to the GenBank/BOLD repositories and studied. Four types of gene trees were reconstructed: Bayesian (BA), maximum likelihood (ML), minimum evolution (ME), and neighbor joining (NJ). These trees showed basically similar topology. Two separate branches on the trees support the previously identified subfamilies Hippoglossoidinae and Pleuronectinae with the monophyletic status of these taxa. The subfamily Pleuronectinae can be considered monophyletic if the tribe Microstomini is excluded from it and genus *Lepidopsetta* is moved into the tribe Pleuronectini. Three sets of nucleotide sequences were formed and independently studied. One set included all the obtained *16S* rRNA gene sequences (291 bp), the second set included a sample of longer *16S* rRNA sequences (617 bp), and the third set consisted of three gene sequences: *16S* rRNA, *Co-1*, and *Cyt-b* (2926 bp). All three data sets gave a similar phylogenetic signal, which is consistent with the traditional concept of the taxonomy of the Pleuronectiformes order; however, the second and third sets provided better resolution of topology.

INTRODUCTION

The fish family of true flounder Pleuronectidae, to which the main attention is paid in the article, is one of the largest in the order Pleuronectiformes, including 59 nominal fish species of right-sided flounders common in the sea waters of the Northern Hemisphere [1, 2]. In their analysis, J. Cooper and F. Chapleau [1] considered the Pleuronectidae family as a monophyletic taxon, based on ten synapomorphies by morphological characters. An important result obtained by the aforementioned authors is generally consistent with the topology of the branches of the family established in several studies of molecular phylogenetics [3–10]. According to [1], this family includes the subfamilies Hippoglossinae, Eopsettinae, Lyopsettinae, Hippoglossoidinae, and Pleuronectinae, which are represented by genera usually consisting of species with high commercial value (for example, species of the genus of halibut-like flounders, *Hippoglossoides*). In connection with the fishery importance of these and other flounders and the need to manage such valuable fishery resources, both the precise classification of specimens of individuals and

species within the genus and the entire systematics' relationships among taxa in this family are very important.

Taxonomic studies of the Pleuronectidae have traditionally been based on morphological characters, as follows from the above paragraph. However, the frequent absence of clear evidence of the homology of species' characters even at low taxonomic levels (within a genus) makes the postulated taxonomic and phylogenetic relationships of many groups of flounders not always convincing if they are justified only by morphology. There are several versions of the classification of flounders, which were proposed by different authors [1, 11–13]. Certain disagreements are also noted regarding the phylogenetic relationships of flounders obtained on the basis of morphological and molecular genetic data [1, 4, 14, 15]. Development of new nuclear and mitochondrial markers based on DNA makes it possible to better identify morphologically similar fish species [16], including many species of flounders. Therefore, it is relevant to search for new or already known, but insufficiently developed, molecular markers for the reconstruction of gene

trees, as well as combined or species phylogenetic trees for flounders of the Pleuronectidae family.

In this study, taking into account the above, we present a comparative analysis of incomplete nucleotide sequences (hereinafter referred to as sequences) of the gene *16S* rRNA for 14 species belonging to Pleuronectidae, previously not used in such a volume for flounders, in order to assess the success of taxonomic identification of specimens and to establish phylogenetic and taxonomic relationships in this family of flounders. The novelty of this study lies in the fact that the systematics of this group in the authors' works cited above were not considered on the basis of *16S* rRNA. Accordingly, in the presented article, we considered the potential of this marker on a sufficient number of specimens for taxonomic and evolutionary genetic studies of flounders of the Russian Federation.

MATERIALS AND METHODS

A total of 62 sequences of *16S* rRNA and additionally 24 sequences of genes *16S* rRNA, *Co-1*, and *Cyt-b* for 14 species belonging to seven genera of the Pleuronectidae family were analyzed. Latin names are given in accordance with the classification [1]. Sampled fish (2−5 specimens of muscle tissue subjected to ethanol fixation, 95%) were taken from the existing collection of the Laboratory of Molecular Systematics, and voucher specimens of the fish themselves are kept in safe custody at the museum of the A.V. Zhirmunsky National Scientific Center of Marine Biology. DNA isolation was performed using commercial kits (DNA Extran-2, Syntol, Russia).

A fragment of the sequence of gene *16S* rRNA was amplified by polymerase chain reaction (PCR) using primers 16Sbr-H and 16Sar-L. The PCR reaction was carried out in a volume of 25 µL of a solution containing the following: distilled deionized water—17.8 µL; dNTP (ZAO Evrogen, Moscow, Russia)—0.5 µL; 5× Buffer (Evrogen)—5 µL; primers at a concentration of 10 µM/µL—0.3 µL for each; *Taq* polymerase—0.1 µL. The following heat program was used: denaturation at 93°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min for 33 cycles. To determine the localization and arrangement of nucleotides in the sequences, PCR products (DNA specimens) were subjected to cyclic sequencing using the BrightDye Terminator Cycle Sequencing Kit according to the following program: denaturing at 96°C for 10 s, annealing at 45°C for 10 s, elongation at 60°C for 2 min.

Bidirectional DNA strand sequences of genes *16S* rRNA were obtained for each DNA specimen. These sequences were then pooled together to generate consensus sequences for each specimen (individual). This procedure was performed using the Geneious, Free Trial software package [17].

Since the length of the obtained sequences varied quite significantly, in the range from 355 base pairs (bp) to 642 bp, two sets of sequences were composed for more accurate further analysis. One set represented all the sequences obtained, while the other included only the longest sequences. According to the data transformation, set 1 contained 62, and set 2 contained 27 sequences. After the alignment procedure and removal of gaps (indels), the length of the sequences of the two sets was 291 and 617 bp, respectively.

Sequence alignment for all taxa was performed using the MEGA-X software package, SP (http://megasoftware.net/) [18] based on the ClustalW [19] module as an integrated MEGA product. The penalties for opening of gaps and for lengthening of gaps were set at 15.0 and 5.0, respectively (for other alignment program settings, the default parameters were used). After the first alignment step, large gaps were manually removed and the final alignment in the second step was done with reduced penalty levels (5.0 and 0.5 for the two options, respectively). All spaces were then manually removed again.

To increase the information capacity, besides the gene *16S* rRNA, the analysis included sequences of the genes *Co-1* and *Cyt-b* previously used in the analysis [10]; taken together, these data constituted the third set of sequences, including a total of 24 specimens with a length of 2926 bp.

For further analysis of sequences and construction of gene trees, an optimal model of nucleotide substitutions for the obtained set of sequences was defined. The best evolutionary model that fit the data obtained was evaluated by means of a special module of the MEGA program. For the set of gene *16S* rRNA with short sequences (291 bp), the best model was K2P + G (two-parameter model of M. Kimura with a gamma distribution of substitutions) [20]; for the set of this gene with long sequences (617 bp), the best model was JC + G (Jukes−Cantor model with a gamma distribution of substitutions) [21]; for the set of sequences of three genes, the best model was HKY + G (Hasegawa−Kishino−Yano model with a gamma distribution of substitutions) [22].

Gene trees were constructed using four reconstruction methods: Bayesian Analysis (BA), Maximum Likelihood (ML), Neighbor Joining (NJ), and Minimum Evolution (ME). They were implemented in MrBayes 3.2.7 (http://nbisweden.github.io/MrBayes/download.html) [23, 24] and MEGA-X [18]. The process of tree reconstruction in BA was simulated for one million generations, n ($n = 10^6$). The three other reconstructions ML, NJ, and ME were performed with replicates equal to k = 1000 copies of bootstrap (bootstrap support).

The sequence of *Platichthys stellatus* was chosen as an outgroup when rooting trees, a representative of which, according to the data for the complete mitoge-

nome (mtDNA), was previously assigned to an external branch in the Pleuronectidae family [10]. Phylogenetic trees were visualized and edited, if necessary, using the FigTree software [25] and MEGA-X [18].

All obtained sequences by gene *16S* rRNA were registered in GenBank (https://www.ncbi.nlm.nih.gov/); part of previously unpublished sequences of *Co-1* and *Cyt-b* are also included in the article (Table 1).

Statistical analysis of the nucleotide composition was performed using SP MEGA-X. Additionally with the Statistica 6 SP [29], one-way analysis of variance (ANOVA) of the nucleotide composition was conducted separately for each gene.

RESULTS

The analysis was constructed on the basis of the sequences of the three previously presented data sets. The full length of the segment of gene *16S* rRNA (full-length sequences "from primer to primer") is 596–631 bp. The numbers in GenBank of the full-length regions of gene *16S* rRNA are as follows: MN888911, MN888895, MN888908, MN888877, MN888901, MN888894, MN888893, MN888892, MN888917, MN888916, MN888915, MN888903, MN888924, MN888918, MN888912, MN888904, MN888905, MN888868, MN888873, MN888898, MN888899, MN888902, MN888927, MN888909, MN888907, MN888884, MN888883, MN888876.

However, not all of the sequences obtained reached their full size. The difference in the length of the sequences is due to poor-quality sequencing of some specimens, which led to a greater "cutoff" of regions near the primers when forming consensus sequences. These sequencing errors are possible owing to the fact that some of the tissue specimens were stored for several years before analysis. However, short fragments are not necessarily bad for assessing variability in closely related taxa and comparing the degree of their similarity-difference for gene tree reconstruction. In this regard, for analysis, the material was divided as explained before into two groups, including long (1) and short (2) gene sequences. In accordance with the outlined approaches (see the Materials and Methods section), four types of trees were constructed: BA, ML, NJ, and ME.

Analysis of all Sequences of 16S rRNA

Figure 1 shows a rooted ML tree derived from a set of sequences of gene *16S* rRNA 291 bp in length. The tree nodes support are listed in the following order: BA/ME/NJ/ML.

The branch with specimens of *Limanda sakhalinensis* became part of the subfamily Hippoglossoidinae, forming a separate, topologically unresolved branch (node) together with *Cleisthenes pinetorum* of subfamily Hippoglossoidinae. A separate, also unauthorized branch was formed by representatives of three nominal species of halibut-like flounders of the genus *Hippoglossoides*. *Lepidopsetta mochigarei*, a representative of the tribe Microstomini, has for all five corresponding branches on the tree unresolved topology. A separate node on the tree is formed by representatives of the Pacific halibut *Hippoglossus stenolepis* from subfamily Hippoglossinae.

Analysis of Longer Sequences of 16S rRNA

Figure 2 shows a rooted NJ tree derived from a set of gene sequences of *16S* rRNA 617 bp in length. The branch *Limanda sakhalinensis* is included in the subfamily Hippoglossoidinae, located in the same cluster with *Cleisthenes pinetorum*. The sequence representing the subfamily Hippoglossinae forms a separate node. *Lepidopsetta mochigarei*, as before for a short set, forms an unresolved node, but topologically it belongs to the tribe Microstomini.

Analysis of the Reconstruction of Gene Trees from the Combined Sequences of Three Genes

For this analysis, the aligned sequences of the gene *16S* rRNA region were compared, jointly with genes *Co-1* and *Cyt-b*. The sequences were concatenated using special SP MEGA-X utility and then subjected to further analysis. According to the data obtained, the branch *Limanda sakhalinensis* is included in the subfamily Hippoglossoidinae (Fig. 3). The branch *Cleisthenes pinetorum* is also included in the subfamily Hippoglossoidinae (Fig. 3).

Nucleotide Composition

The ratio of pyrimidines (T, C) and purines (A, G) in genes *16S* rRNA, *Co-1*, and *Cyt-b* deviated from the ratio of 50 : 50 (Appendix, Fig. 4). In sequences of *16S* rRNA, there are no large differences in the ratio of pyrimidines (T, C) and purines (A, G), but general heterogeneity of the nucleotide composition with a predominance of C and A nucleotides can be observed (Fig. 4a). For *Co-1* and *Cyt-b*, there is a statistically significant deviation in the ratio of pyrimidines to purines with a predominance of pyrimidines (Figs. 4b, 4c).

ANOVA for each gene found that the differences for four nucleotides were statistically significant: for *16S* rRNA, $F = 2147.9$, d.f. = 3; 92, $P < 0.0001$; for *Co-1*, $F = 3673.3$, d.f. = 3; 92, $P < 0.0001$; for *Cyt-b*, $F = 4320.7$, d.f. = 3; 92, $P < 0.0001$. The ratio of $(T + C) : (A + G)$ for *16S* rRNA, *Co-1*, and *Cyt-b* was 45.4 : 54.6, 56.5 : 43.5, and 61.1 : 38.9%, respectively.

DISCUSSION

As noted in the Introduction, the largest subfamily in the family is Pleuronectinae. This subfamily is represented by two tribes, Microstomini and Pleuronec-

Table 1. List of species and their assigned numbers in the GenBank

Species name with laboratory number	NCBI access number		
	16S	*Co-1*	*Cyt-b*
Pseudopleuronectes herzensteini 7k	MN888867	KF386364	KF445172
Pseudopleuronectes herzensteini 05-07	MN888868	KF386361	KF445169
Pseudopleuronectes herzensteini 6k	MN888869	KF386363	KF445171
Pseudopleuronectes schrenki 119-07	MN888870		
Liopsetta pinnifasciata 90-07	MN888871		
Liopsetta pinnifasciata 40-07	MN888872		
Liopsetta pinnifasciata 39-07(2)	MN888873		
Liopsetta pinnifasciata 39-07	MN888874		
Liopsetta pinnifasciata 21-07	MN888875		
Liopsetta pinnifasciata 20-07	MN888876	KF386379	KF445186
Liopsetta pinnifasciata 2	MN888877		
Liopsetta pinnifasciata 19-07	MN888878	KF386378	KF445185
Liopsetta pinnifasciata 13	MN888879	KF386377	KF445184
Pseudopleuronectes yokohamae 46	MN888880	KF386355	KF445163
Pseudopleuronectes yokohamae 45	MN888881	KF386354	KF445162
Pseudopleuronectes obscurus 42-07	MN888882		
Pseudopleuronectes obscurus 36	MN888883		
Pseudopleuronectes yokohamae 09-07	MN888884	KF386353	KF445161
Pseudopleuronectes obscurus 08-08(08-07)	MN888885		
Limanda punctatissima 89-07	MN888886		
Limanda punctatissima 86-07	MN888887	KF386388	KF445195
Limanda punctatissima 58-07	MN888888		
Limanda punctatissima 50-07	MN888889		
Limanda punctatissima 37	MN888890	KF386386	KF445193
Lepidopsetta mochigarei LMO12-5	MN888891		
Lepidopsetta mochigarei LMO12-4	MN888892		
Lepidopsetta mochigarei LMO12-3	MN888893		
Lepidopsetta mochigarei LMO12-2	MN888894		
Lepidopsetta mochigarei LMO12-1	MN888895		
Platichthys stellatus 11-07	MN888896		
Platichthys stellatus 4k	MN888897	KF386371	KF445178
Platichthys stellatus PS6-011	MN888898		
Platichthys stellatus PS5-011	MN888899		
Platichthys stellatus PS4-011	MN888900		
Platichthys stellatus Ps3-011	MN888901		
Platichthys stellatus Ps2-011	MN888902		
Platichthys stellatus 18-07	MN888903	KF386375	KF445182
Platichthys stellatus 16-07	MN888904		
Platichthys stellatus 15-07	MN888905		
Liopsetta pinnifasciata PG1-011	MN888906		
Liopsetta pinnifasciata Pc2-011	MN888907		
Liopsetta pinnifasciata Pc1-011	MN888908		
Liopsetta pinnifasciata 2k	MN888909	KF386376	KF445183
Limanda sakhalinensis 72(2012)	MN888910	KF386382	KF445189

Table 1. (Contd.)

Species name with laboratory number	NCBI access number		
	16S	*Co-1*	*Cyt-b*
Limanda sakhalinensis 71 (2012)	MN888911	KF386381	KF445188
Limanda sakhalinensis 69 (2012)	MN888912		
Limanda sakhalinensis 68 (2012)	MN888913		
Hippoglossus stenolepis HST12-4	MN888914		
Hippoglossus stenolepis HST12-3	MN888915		
Hippoglossus stenolepis HST12-2	MN888916		
Hippoglossus stenolepis HST12-1	MN888917		
Hippoglossoides robustus 289	MN888918	KF386414	KF445220
Hippoglossoides robustus 288	MN888919	KF386413	KF445219
Hippoglossoides robustus 286	MN888920	KF386411	KF445217
Hippoglossoides elassodon 35	MN888921		
Hippoglossoides elassodon 34	MN888922	KF386418	KF445223
Hippoglossoides elassodon 33	MN888923	KF386417	KF445222
Hippoglossoides robustus 31	MN888924	KF386410	KF445216
Hippoglossoides dubius 5k	MN888925		
Cleisthenes pinetorum 79-07	MN888926	KF386409	KF445215
Cleisthenes pinetorum 78-07	MN888927	KF386408	KF445214

tini. According to the data obtained by *16S* rRNA, they do not form monophyletic branches (see Figs. 1, 2). Thus, the taxonomy at the subfamily level needs further clarification. For example, for a larger information signal, it is necessary to increase the number of both nuclear and mitochondrial markers in the study. This will help reduce the number of topologically unresolved nodes in the resulting trees.

As noted above, J. Cooper and F. Chapleau [1], in their revision of this family based on traditional morphological characters, substantiated that Pleuronectidae is a monophyletic group. The monophyly of flounders established on the basis of the classical approach corresponds in many cases to molecular phylogenetic reconstructions in studies of this family for such markers as *12S* rRNA and *16S* rRNA, as well as for genes *Co-1* and *Cyt-b* [4–6, 8, 9] and the complete mitogenome [10].

The subfamily Hippoglossoidinae, in the most representative material in the work, included two of the three genera *Cleisthenes* (*C. pinetorum*) and *Hippoglossoides* (*H. dubius* Schmidt, 1904, *H. elassodon* Jordan & Gilbert, 1180, *H. robustus* Gill & Townsend, 1897) (Fig. 3). Species of the genus *Hippoglossoides* form a mixed cluster on the BA tree (Fig. 3). On the basis of this, it can be assumed that two taxa *H. elassodon* and *H. robustus* are synonyms of a single species. Synonymy of *H. elassodon* and *H. robustus* was already proposed earlier on the basis of morphological and

molecular-phylogenetic data [4, 27–31]. By the principle of seniority, a taxon of the species rank, *H. elassodon* Jordan & Gilbert, 1880 can be accepted as valid, and *H. robustus* Gill & Townsend, 1897 can be considered a junior synonym for this species. The proposal of synonymy of *H. elassodon* and *H. robustus* has was already made previously, as noted above. However, K.A. Vinnikov et al. [31] in their two-sided analysis (morphology + genetics) propose to reintroduce synonymy. But synonymy was never introduced. In the databases, these two taxa still appear as separate species. One of the tasks of the article is to sharpen this issue in order to finally resolve this case in a special publication.

The data for the genus *Limanda* deserve a separate discussion. Sequences of the species *Limanda sakhalinensis* occur, as noted in the results and shown in Figs. 1–3, and are included in the branch of the subfamily Hippoglossoidinae. In a comparative anatomical study by J. Cooper and F. Chapleau [1], monophyly of this genus was not confirmed. In our study, as in previous molecular phylogenetic studies [4, 5, 10], *Limanda sakhalinensis* Hubbs, 1915 was included in the subfamily Hippoglossoidinae. Considering all these data, it is quite appropriate to recommend revising the position of *Limanda sakhalinensis* Hubbs, 1915, moving it to the genus *Hippoglossoides* with the name *Hippoglossoides* (Limanda) *sakhalinensis* in the composition of subfamily Hippoglossoidinae. Accord-
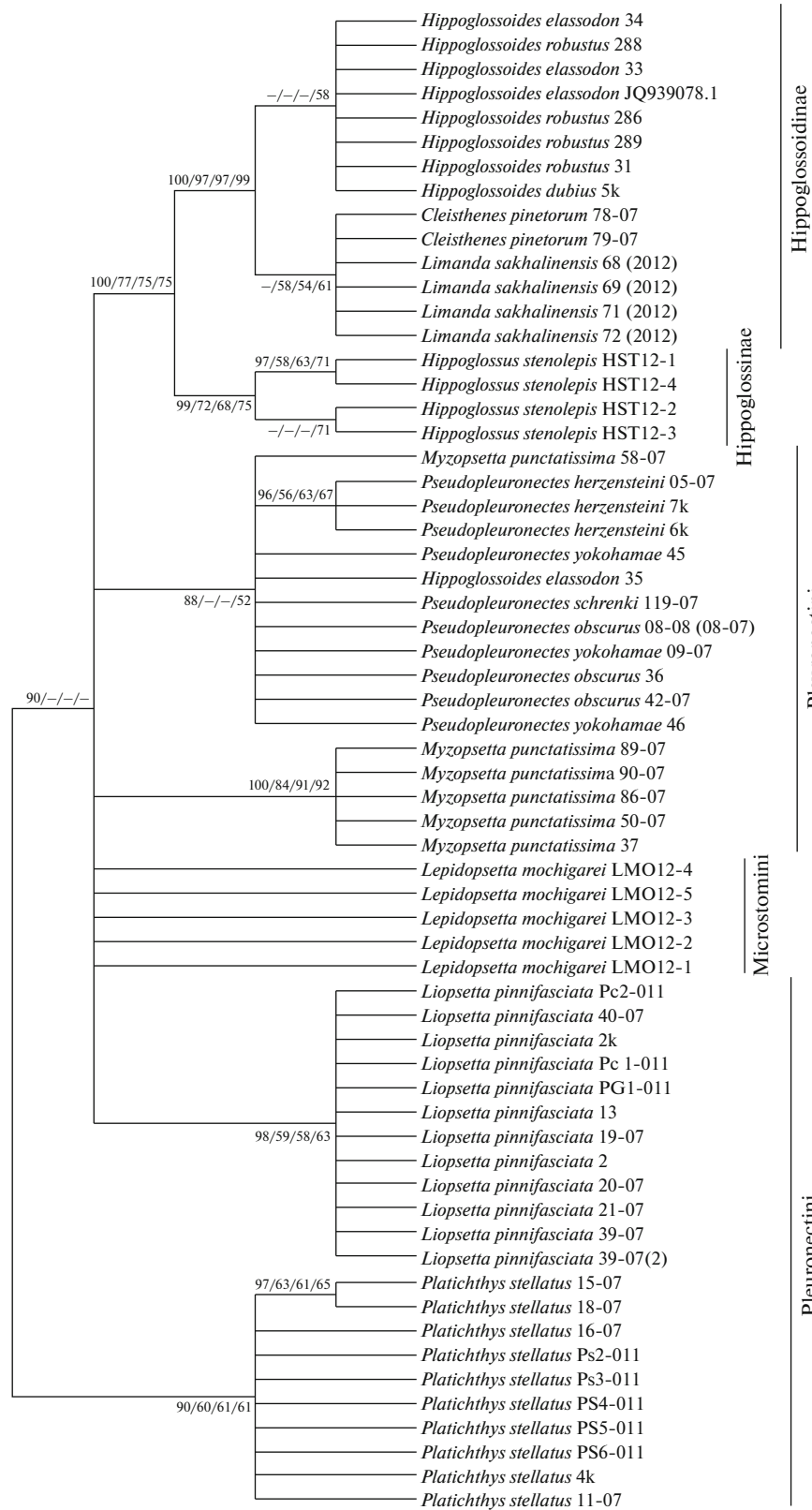
Fig. 1. Rooted gene tree showing phylogenetic relationships based on 62 short nucleotide sequences of *16S* rRNA. The topology is presented on the basis of ML reconstruction. The nodes give support values for four tree reconstruction methods in the following order: BA/ME/NJ/ML. For the BA tree, a posteriori probabilities (%, $n = 10^6$ generations) are shown, and bootstrap supports are given for the other three reconstructions (k = 1000 replicas).
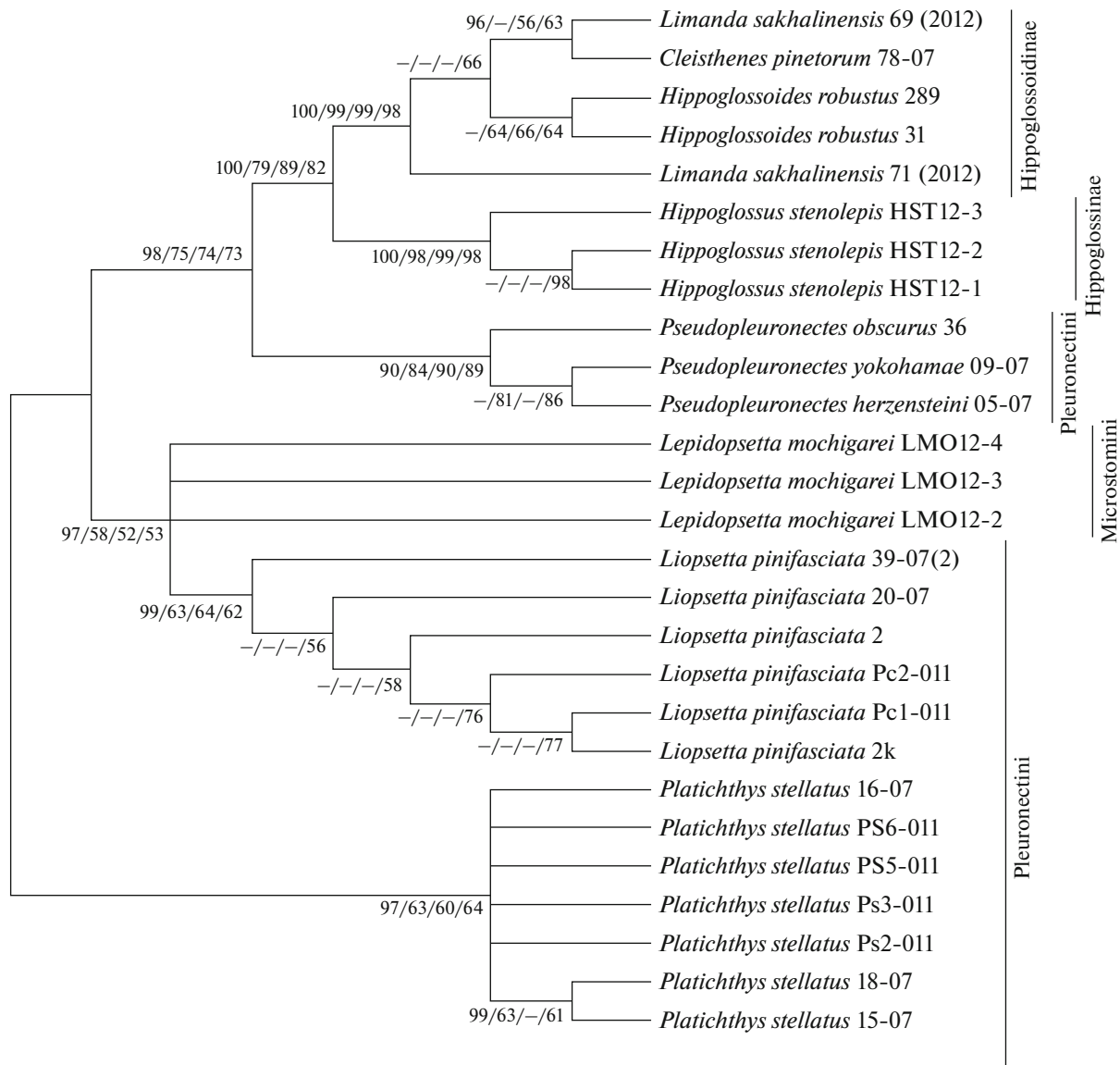
Fig. 2. Rooted NJ tree topology showing phylogenetic relationships based on data from 27 sequences of a region of gene *16S* rRNA. The nodes give the values of supports for the BA tree ($n = 10^6$ generations) and the three other reconstructions in the following general order: BA/ME/ML/NJ (k = 1000 bootstrap replicas for the last three reconstruction methods).

ingly, it is necessary to revise the morphology and species characteristics, as well as diagnostic keys, which is supposed to be done in independent work.

Genus *Lepidopsetta* (*L. mochigarei*) was included in the tribe Pleuronectini, subfamily Pleuronectinae by our data, while in [1] this genus was considered exclusively within the tribe Microstomini of the subfamily Pleuronectinae. In molecular phylogenetic studies based on *Co-1* and *Cyt-b* [4, 5, 10], genus *Lepidopsetta* was considered within the tribe Pleuronectini. Thus, it is preferable to consider the genus *Lepidopsetta* as a part of the tribe Pleuronectini. However, this question, given the weak topological signal for this branch

by the marker *16S* rRNA in the work, requires further refinement using a larger number of genes.

Deviation in the equality in ratio (T + C): (A + T) is well described in the literature for many protein-coding genes [4, 32]. The presented analysis (Fig. 4, Appendix) shows that the bias for genes *Co-1* and *Cyt-b* in the ratio of purines to pyrimidines is significantly different from the bias for the gene *16S* rRNA. Obviously, the detected displacement of the nucleotide composition for the structural genes studied in this work reflects the hydrophobic properties of the proteins encoded by them [33]. Discovery of the cause of the heterogeneity of the nucleotide composition in the sequences of *16S* rRNA requires further research.
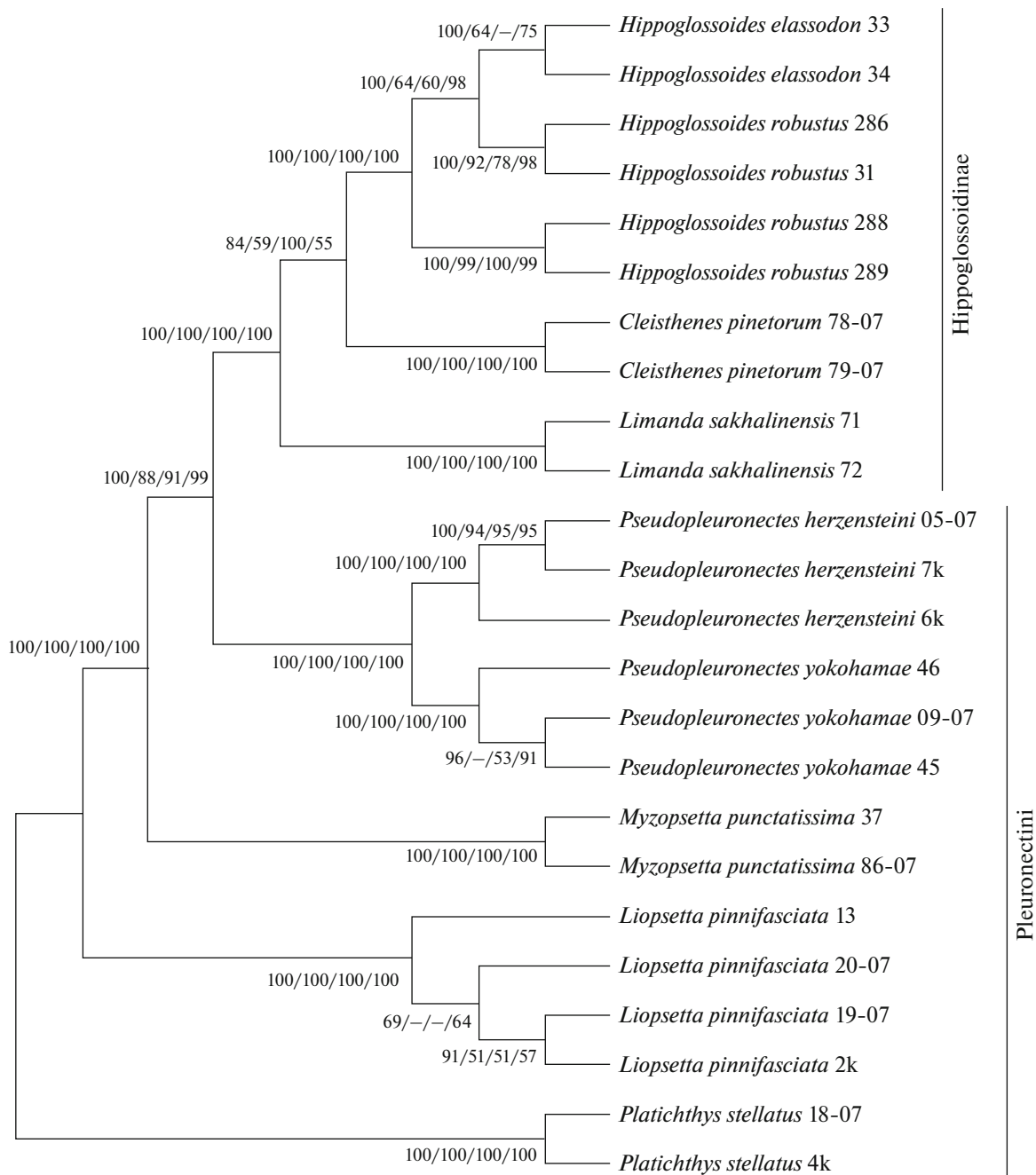
Fig. 3. Rooted ML tree showing phylogenetic relationships based on data from 24 concatenated sequences of gene *16S* rRNA, *Co-1*, and *Cyt-b*. The nodes give the values of supports for the BA tree ($n = 10^6$ generations) and for the three other reconstructions in the following general order: BA/NJ/ME/ML (k = 1000 bootstrap replicas for the last three reconstruction methods).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.
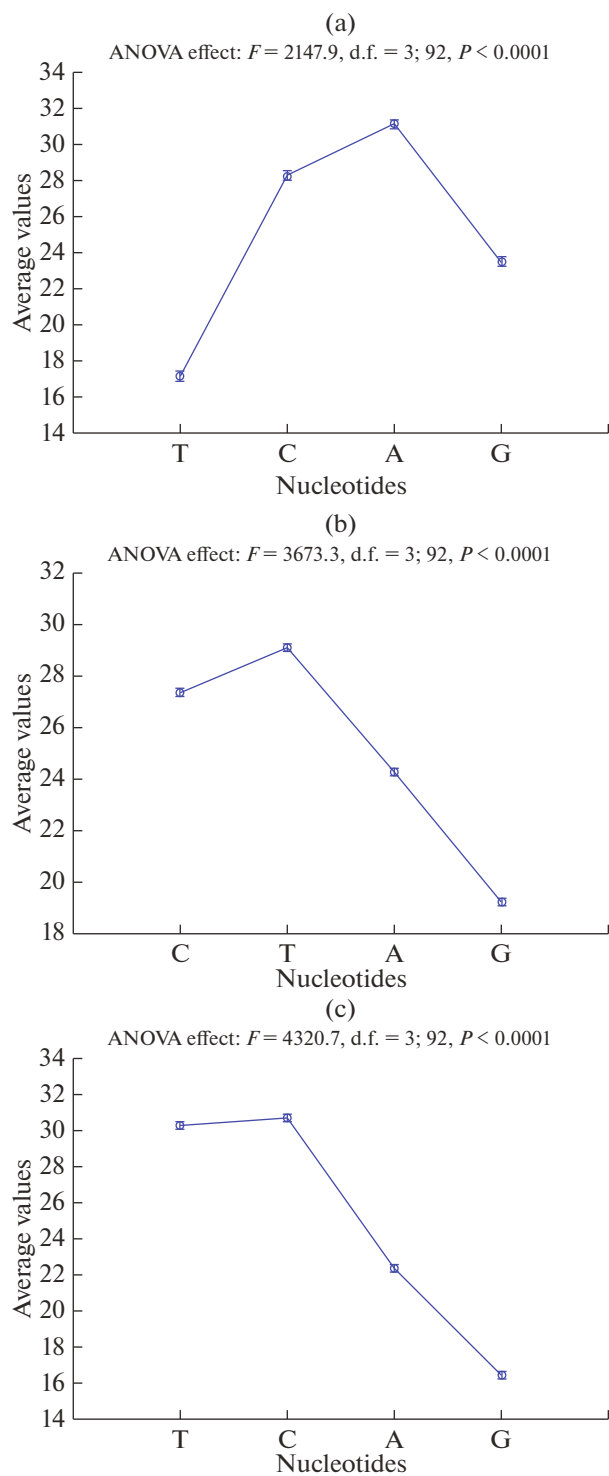
Fig. 4. Average values of the composition (%) of four nucleotides in 24 studied sequences of genes *16S* rRNA (a), *Co-1* (b), and *Cyt-b* (c). According to the results of one-way analysis of variance (ANOVA). The vertical lines represent the 95% confidence interval.

Nucleotide composition for genes *16S* rRNA, *Co-1*, and *Cyt-b* in 24 flounder fish sequences

Species/Mean	Nucleotides, all positions (% of total)				
	T	C	A	G	cumulative (bp)
16S rRNA					
Cleisthenes pinetorum 78-07	16.8	28.9	29.9	24.4	291
Cleisthenes pinetorum 79-07	16.8	28.9	29.9	24.4	291
Hippoglossoides elassodon 33	16.8	28.9	30.2	24.1	291
Hippoglossoides elassodon 34	16.8	28.9	29.9	24.4	291
Hippoglossoides robustus 286	16.8	28.9	30.2	24.1	291
Hippoglossoides robustus 288	17.2	28.5	30.2	24.1	291
Hippoglossoides robustus 289	16.8	28.9	30.2	24.1	291
Hippoglossoides robustus 31	16.8	28.9	30.2	24.1	291
Limanda sakhalinensis 71	16.8	28.9	29.9	24.4	291
Limanda sakhalinensis 72	16.8	28.9	29.9	24.4	291
Liopsetta pinnifasciata 13	17.5	27.8	31.6	23.0	291
Liopsetta pinnifasciata 19-07	17.5	27.8	31.6	23.0	291
Liopsetta pinnifasciata 20-07	17.5	27.8	31.6	23.0	291
Liopsetta pinnifasciata 2k	17.5	27.8	31.6	23.0	291
Myzopsetta punctatissima 37	17.2	27.5	32.3	23.0	291
Myzopsetta punctatissima 86-07	17.2	27.5	32.3	23.0	291
Platichthys stellatus 18-07	17.2	27.8	31.3	23.7	291
Platichthys stellatus 4k	17.2	27.8	31.6	23.4	291
Pseudopleuronectes herzensteini 05-07	17.5	27.8	32.0	22.7	291
Pseudopleuronectes herzensteini 6k	17.5	27.8	32.0	22.7	291
Pseudopleuronectes herzensteini 7k	17.5	27.8	32.0	22.7	291
Pseudopleuronectes obscurus 09-07	17.2	28.2	32.0	22.7	291
Pseudopleuronectes obscurus 45	16.8	28.2	32.0	23.0	291
Pseudopleuronectes obscurus 46	17.2	28.2	32.0	22.7	291
Mean	**17.14 ± 0.13**	**28.26 ± 0.13**	**31.1 ± 0.13**	**23.5 ± 0.13**	–
Co-1					
Cleisthenes pinetorum 78-07	29.4	27.5	23.6	19.5	1540
Cleisthenes pinetorum 79-07	29.4	27.3	23.7	19.5	1540
Hippoglossoides elassodon 33	29.3	27.6	24.0	19.2	1540
Hippoglossoides elassodon 34	29.1	27.6	24.0	19.4	1540
Hippoglossoides robustus 286	29.0	27.8	24.0	19.2	1540
Hippoglossoides robustus 288	29.2	27.5	24.0	19.3	1540
Hippoglossoides robustus 289	29.2	27.4	24.1	19.3	1540
Hippoglossoides robustus 31	29.0	27.8	24.0	19.2	1540
Limanda sakhalinensis 71	28.8	27.1	24.4	19.7	1540
Limanda sakhalinensis 72	28.6	27.3	24.4	19.7	1540
Liopsetta pinnifasciata 13	29.4	27.0	24.8	18.8	1540
Liopsetta pinnifasciata 19-07	29.4	27.0	24.8	18.8	1540
Liopsetta pinnifasciata 20-07	29.4	27.0	24.8	18.8	1540
Liopsetta pinnifasciata 2K	29.4	27.0	24.8	18.8	1540
Myzopsetta punctatissima 37	28.8	27.2	24.7	19.2	1540
Myzopsetta punctatissima 86-07	28.8	27.2	24.7	19.2	1540

Appendix. (Contd.)

Species/Mean	Nucleotides, all positions (% of total)				
	T	C	A	G	cumulative (bp)
Platichthys stellatus 18-07	29.0	27.2	24.8	19.0	1540
Platichthys stellatus 4K	28.8	27.3	24.8	19.0	1540
Pseudopleuronectes herzensteini 05-07	28.8	28.0	23.7	19.5	1540
Pseudopleuronectes herzensteini 6K	28.7	28.0	23.7	19.6	1540
Pseudopleuronectes herzensteini 7K	28.7	28.0	23.6	19.7	1540
Pseudopleuronectes obscurus 09-07	29.3	26.9	24.5	19.2	1540
Pseudopleuronectes obscurus 45	29.3	27.0	24.5	19.2	1540
Pseudopleuronectes obscurus 46	29.4	27.1	24.5	19.1	1540
Mean	**29.09 ± 0.07**	**27.38 ± 0.07**	**24.29 ± 0.07**	**19.25 ± 0.07**	–
Cyt-b					
Cleisthenes pinetorum 78-07	30.8	30.9	22.5	15.9	1095
Cleisthenes pinetorum 79-07	30.8	30.8	22.6	15.8	1095
Hippoglossoides elassodon 33	30.3	31.2	22.2	16.3	1095
Hippoglossoides elassodon 34	30.3	31.2	22.2	16.3	1095
Hippoglossoides robustus 286	30.3	31.3	22.2	16.2	1095
Hippoglossoides robustus 288	30.6	31.1	22.1	16.3	1095
Hippoglossoides robustus 289	30.6	31.1	22.2	16.2	1095
Hippoglossoides robustus 31	30.4	31.3	22.1	16.2	1095
Limanda sakhalinensis 71	30.8	30.1	23.4	15.7	1095
Limanda sakhalinensis 72	30.8	30.2	23.3	15.7	1095
Liopsetta pinnifasciata 13	29.9	30.9	22.9	16.3	1095
Liopsetta pinnifasciata 19-07	29.7	31.0	22.9	16.4	1095
Liopsetta pinnifasciata 20-07	29.5	31.3	23.1	16.1	1095
Liopsetta pinnifasciata 2K	29.8	30.9	23.0	16.3	1095
Myzopsetta punctatissima 37	30.8	29.8	22.0	17.4	1095
Myzopsetta punctatissima 86-07	30.8	29.9	22.1	17.3	1095
Platichthys stellatus 18-07	30.0	30.6	22.6	16.7	1095
Platichthys stellatus 4K	29.8	30.8	22.9	16.5	1095
Pseudopleuronectes herzensteini 05-07	29.9	31.4	21.4	17.4	1095
Pseudopleuronectes herzensteini 6K	30.0	31.0	21.6	17.4	1095
Pseudopleuronectes herzensteini 7K	30.0	31.2	21.4	17.4	1095
Pseudopleuronectes obscurus 09-07	30.5	30.1	22.6	16.7	1095
Pseudopleuronectes obscurus 45	30.4	30.3	22.2	17.1	1095
Pseudopleuronectes obscurus 46	30.4	30.1	22.6	16.9	1095
Mean	**30.29 ± 0.1**	**30.77 ± 0.1**	**22.42 ± 0.1**	**16.52 ± 0.1**	–

Mean values are shown with standard error.

REFERENCES

1. Cooper, J.A. and Chapleau, F., Monophyly and intrarelationships of the family Pleuronectidae (Pleuronectiformes), with a revised classification, *Fish. Bull.*, 1998, vol. 96, no. 4, pp. 686—726.

2. Keast, A. and Chapleau, F., A phylogenetic reassessment of the monophyletic status of the family Soleidae, with comments on the suborder Soleoidei (Pisces; Pleuronectiformes), *Canad. J. Zool.*, 1988, vol. 66, no. 12, pp. 2797—2810.
https://doi.org/10.1139/z88-408

3. Berendzen, P.B. and Dimmick, W.W., Phylogenetic relationships of Pleuronectiformes based on molecular evidence, *Copeia*, 2002, vol. 3, pp. 642—652.
https://doi.org/10.1643/0045-8511(2002)002[0642:PROPBO]2.0.CO;2

4. 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.*, 2007, vol. 152, no. 4, pp. 757—773.
https://doi.org/10.1007/s00227-007-0726-9

5. Kartavtsev, Y.P., Sharina, S.N., Goto, T., et al., Cytochrome oxidase 1 gene sequence analysis in six flatfish species (Teleostei, Pleuronectidae) of Far East Russia with inferences in phylogeny and taxonomy, *Mitochondrial DNA*, 2008, vol. 19, pp. 479—489.
https://doi.org/10.1080/19401730802570934

6. Pardo, B.G., Machordom, A., Foresti, F., et al., Phylogenetic analysis of flatfish (order Pleuronectiformes) based on mitochondrial 16S rDNA sequences, *Sci. Mar.*, 2005, vol. 69, no. 4, pp. 531—543.

7. Sharina, S.N. and Kartavtsev, Y.P., Phylogenetic and taxonomic analysis of flatfish species (Teleostei, Pleuronectiformes) inferred from the primary nucleotide sequence of cytochrome oxidase 1 gene (*Co-1*), *Russ. J. Genet.*, 2010, vol. 46, no. 3, pp. 356—361.
https://doi.org/10.1134/S1022795410030130

8. Betancur, R.R., Munroe, T.A., Orti, G., et al., Addressing gene tree discordance and non-stationarity to resolve a multi-locus phylogeny of the flatfishes (Teleostei: Pleuronectiformes), *Syst. Biol.*, 2013, vol. 62, pp. 763—785.
https://doi.org/10.1093/sysbio/syt039

9. Betancur, R.R. and Ortí, G., Molecular evidence for the monophyly of flatfishes (Carangimorpharia, Pleuronectiformes), *Mol. Phylogenet. Evol.*, 2014, vol. 73, pp. 18—22.
https://doi.org/10.1016/j.ympev.2014.01.006

10. Kartavtsev, Y.Ph., Sharina, S.N., Saitoh, K., et al., Phylogenetic relationships of Russian Far Eastern flatfish (Pleuronectiformes, Pleuronectidae) based on two mitochondrial gene sequences, *Co-1* and *Cyt-b*, with inferences in order phylogeny using complete mitogenome data, *Mitochondrial DNA*, 2014, vol. 27, no. 1, pp. 667—678.
https://doi.org/10.3109/19401736.2014.913139

11. Norman, I.R., *A Systematic Monograph of the Flatfishes (Heterosomata)*, vol. I: *Psettodidae, Bothidae, Pleuronectidae*, London: British Museum, 1934.

12. Sakamoto, K., Interrelationships of the family Pleuronectidae (Pisces: Pleuronectiformes), *Mem. Fac. Fish., Hokkaido Univ.*, 1984, vol. 31, pp. 95—215.

13. Lindberg, G.U. and Fedorov, V.V., *Fishes of Japan Sea and Nearby Parts of Okhotsk and Yellow Seas*, part 6: *Teleostomi. Osteichthyes. Actinopterigii*, XXXI: *Pleuronectiformes*, St. Petersburg: St. Petersburg Univ. Press, 1993.

14. Chapleau, F., Pleuronectiform relationships: a cladistic reassessment, *Bull. Mar. Sci.*, 1993, vol. 52, no. 1, pp. 516—540.

15. Vernau, O., Moreau, C., Catzeflis, F.M., and Renaud, F., Phylogeny of flatfishes (Pleuronectiformes): comparisons and contradictions of molecular and morpho-anatomical data, *J. Fish Biol.*, 1994, vol. 45, no. 4, pp. 685—696.
https://doi.org/10.1111/j.1095-8649.1994.tb00934.x

16. Keith, P., Lord, C., Lorion, J., et al., Phylogeny and biogeography of Sicydiinae (Teleostei: Gobiidae) inferred from mitochondrial and nuclear genes, *Mar. Biol.*, 2011, vol. 158, pp. 311—326.
https://doi.org/10.1007/s00227-010-1560-z

17. Kearse, M., Moir, R., Wilson, A., et al., Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data, *Bioinformatics*, 2012, vol. 28, no. 12, pp. 1647—1649.
https://doi.org/10.1093/bioinformatics/bts199

18. Tamura, K., Peterson, D., Peterson, N., et al., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.*, 2011, vol. 28, pp. 2731—2739.
https://doi.org/10.1093/molbev/msr121

19. Thompson, J.D., Higgins, D.G., and Gibson, T.J., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.*, 1994, vol. 22, no. 22, pp. 4673—4680.
https://doi.org/10.1093/nar/22.22.4673

20. Kimura, M., A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, *J. Mol. Evol.*, 1980, vol. 16, pp. 111—120.
https://doi.org/10.1007/bf01731581

21. Jukes, T.H. and Cantor, C.R., Evolution of protein molecules, in *Mammalian Protein Metabolism*, New York: Academic, 1969, vol. III, pp. 21—132.
https://doi.org/10.1016/B978-1-4832-3211-9.50009-7.

22. Hasegawa, M., Kishino, H., and Yano, T., Dating of the human—ape splitting by a molecular clock of mitochondrial DNA, *J. Mol. Evol.*, 1985, vol. 22, pp. 160—174.
https://doi.org/10.1007/bf02101694

23. Hulsenbeck, J.P. and Rondquist, F., MrBAYES: Bayesian inference of phylogenetic trees, *Bioinformatics*, 2001, vol. 17, no. 8, pp. 754—755.
https://doi.org/10.1093/bioinformatics/17.8.754

24. Ronquist, F. and Huelsenbeck, J.P., MrBayes 3: Bayesian phylogenetic inference under mixed models, *Bioinformatics*, 2003, vol. 19, no. 12, pp. 1572—1574.
https://doi.org/10.1093/bioinformatics/btg180

25. http://tree.bio.ed.ac.uk/software/figtree/.

26. *STATISTICA for Windows (Computer Program Manual)*, Tulsa: StatSoft, 1999. http://statsoft.ru/products/STATISTICA_Base/.

27. Ivankov, V.N., Vinnikov, K.A., Borisovets, E.E., and Kartavtsev, Y.P., Taxonomic relations between *Pseudopleuronectes yokohamae* and *P. schrenki, Conference for Educated Students—Abstracts*, Vladivostok: Far Eastern University Press, 2002, pp. 69—70.

28. Kartavtsev, Yu.F., Sviridov, V.V., Hanzawa, N., and Sasaki, T., Genetic divergence of Far Eastern dace species of the genus *Tribolodon* (Pisces: Cyprinidae) and closely related taxa, *Russ. J. Genet.*, 2002, vol. 38, no. 11, pp. 1285—1297.

29. Vinnikov, K.A., Taxonomic status of flatfishes, *Hippoglossoides elassodon* and *H. robustus* (morphometric analysis), *VI Regional'naya konferentsiya po aktual'nym problemam ekologii, morskoy biologii i biotekhnologii studentov, aspirantov, molodykh prepodavateley i sotrudnikov vuzov i nauchnykh organizatsiy Dal'nego Vostoka Rossii: tezisy dokladov* (VI Regional Conference on Current Problems of Ecology, Marine Biology and Biotechnology for Ordinary Students, Graduate Students, Young Teachers and Employees of Universities and Scientific Organizations of the Russian Far East: Abstracts), Vladivostok: Dal'nevost. Univ., 2003, pp. 25—26.

30. Vinnikov, K.A., Ivankov, V.N., and Pitruk, D.L., Taxonomic status of *Pseudopleuronectes yokohamae* and *P. schrenki* (Pleuronectidae sensu Cooper and Chapleau, 1998), *J. Ichthyol.*, 2006, vol. 46, no. 3, pp. 301—310.
https://doi.org/10.1134/S0032945206040035

31. Vinnikov, K.A., Thomson, R.C., and Munroe, T.A., Revised classification of the righteye flounders (Teleostei: Pleuronectidae) based on multilocus phylogeny with complete taxon sampling, *Mol. Phylogenet. Evol.*, 2018, vol. 125, pp. 147—162.
https://doi.org/10.1016/j.ympev.2018.03.014

32. Kim, I.C., Kweon, H.S., Kim, Y.J., et al., The complete mitochondrial genome of the javeline goby *Acanthogobius hasta* (Perciformes, Gobiidae) and phylogenetic considerations, *Gene*, 2004, vol. 336, no. 2, pp. 147—153.
https://doi.org/10.1016/j.gene.2004.04.009

33. Nailor, G.J., Collins, T.M., and Brown, W.M., Hydrophobicity and phylogeny, *Nature*, 1995, vol. 373, no. 6515, pp. 565—566.
https://doi.org/10.1038/373565b0