ISSN 1063-0740, Russian Journal of Marine Biology, 2016, Vol. 42, No. 6, pp. 471–480. © Pleiades Publishing, Ltd., 2016.
Original Russian Text © I.N. Moreva, O.A. Radchenko, S.Yu. Neznanova, A.V. Petrovskaya, S.A. Borisenko, 2016, published in Biologiya Morya.

ORIGINAL PAPERS
Cytogenetics

The Relationships of *Stichaeus nozawae* (Jordan et Snyder, 1902) and *Stichaeus grigorievi* (Herzenstein, 1890) (Pisces: Stichaeidae) Inferred from the Data of Genetic and Karyological Analyses and Ultrastructural Study of Spermatozoa

I. N. Moreva[a], O. A. Radchenko[b], S. Yu. Neznanova[a], A. V. Petrovskaya[b], and S. A. Borisenko[a]

[a]*Zhirmunsky Institute of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, 690041 Russia*

[b]*Institute of Biological Problems of the North, Far Eastern Branch, Russian Academy of Sciences, Magadan, 685000 Russia*

e-mail: irruz@yandex.ru

Received March 31, 2016

Abstract—A complex study, including genetic and karyological approaches, as well as an analysis of the ultrastructural organization of spermatozoa of the Nozawa's prickleback *Stichaeus nozawae* (Jordan et Snyder, 1902) and Grigorev's prickleback *S. grigorievi* (Herzenstein, 1890) from Vostok Bay, Sea of Japan, is considered in the present work. The species exhibit differences in the spermatozoon head width and flagellum length. These characters are species-specific and, along with morphological traits, can be used as a proof of the validity of the species under study. The similarity between *S. nozawae* and *S. grigorievi*, as established based on genetic and karyological analyses ($2n = 48$, NF = 70, localization of nucleolar organizers), does not correspond to inter-species differences in morphological and biological characters. The lack of variations in DNA and the chromosome set that are suitable for species differentiation, between Nozawa's and Grigorev's pricklebacks is an evidence for the evolutionary adolescence of these species.

Keywords: genus *Stichaeus*, Sea of Japan, mitochondrial DNA, nuclear DNA, karyotype, nucleolar organizers, ultrastructural analysis, spermatozoa

DOI: 10.1134/S1063074016060079

INTRODUCTION

The genus *Stichaeus* belongs to the family Stichaeidae; it includes five species. The brown shanny *S. fuscus* [33] is common off the northern coast of Hokkaido Island and in the Peter the Great Bay. *S. fuscus* is considered a very rare species [17] in the Sea of Japan, the same as Okhryamkin's shanny *S. ochriamkini*, which occurs in the Sea of Japan and waters off the Pacific coast of northern Japan and adjacent waters. The Arctic shanny *S. punctatus* [17] inhabits the Chukchi Sea, the Sea of Okhotsk, and the northern Atlantic Ocean [6, 8, 16, 32, 33]. The Nozawa's prickleback *S. nozawae* and the Grigorev's prickleback *S. grigorievi* have been described from coastal waters off Hokkaido: from waters off the Sea of Japan coast (Otaru Bay) and from waters off the Pacific coast (Uchiura Bay), respectively. The ranges of these species overlap in the Sea of Japan, in the southern Sea of Okhotsk, and in the Pacific Ocean off the southern Kuril Islands. *S. grigorievi* is distributed more widely than *S. nozawae*, as it inhabits the Yellow Sea as well [3–5].

The results of genetic studies, according to which *S. nozawae* and *S. grigorievi* almost do not differ [26, 41], are not consistent with the data of morphological analysis that determine them as valid species [5, 17, 21, 31]. It is evident that these species need further study with alternative methods. The aim of this work is to evaluate the inter-species variability and the degree of relationship between *S. nozawae* and *S. grigorievi* using the genetic and karyological methods, as well as analysis of the ultrastructure of their spermatozoa.

MATERIALS AND METHODS

The analyzed fish were caught in Vostok Bay (Peter the Great Bay, Sea of Japan) from a depth of 1.5–2 m on May 1–14, 2010 and 2011 (Table 1). The genetic analysis included a study of variations in nucleotide sequences of the genes of *COI*, cytochrome *b*, 16S rRNA of mitochondrial DNA (mtDNA), RNF213, and rhodopsin of nuclear DNA (nDNA) in *Stichaeus nozawae* and *S. grigorievi*. As an outgroup, we used *Stichaeopsis nevelskoi* of the subfamily Stichaeinae, fam-

Table 1. The materials used in the study

Species (no. of specimen)	Area of collection	mtDNA and nDNA genes (no. in GenBank)				
		16S rRNA	cytochrome *b*	*COI*	RNF213	rhodopsin
*Stichaeus nozawae** (1430)	Vostok Bay, Sea of Japan	KF366331	KF366319	KF366307	KF366343	KF366358
*S. nozawae** (1431)	Same area	KR606575	KR606585	KR606595	KR606605	KR606615
*S. nozawae** (1499)	"	KR606576	KR606586	KR606596	KR606606	KR606616
*S. nozawae** (1572)	"	KR606577	KR606587	KR606597	KR606607	KR606617
*S. grigorievi** (1366)	"	KF366332	KF366320	KF366308	KF366344	KF366359
*S. grigorievi** (1367)	"	KR606578	KR606588	KR606598	KR606608	KR606618
*S. grigorievi** (1368)	"	KR606579	KR606589	KR606599	KR606609	KR606619
*S. grigorievi** (1369)	"	KR606580	KR606590	KR606600	KR606610	KR606620
*S. grigorievi** (1433)	"	KR606581	KR606591	KR606601	KR606611	KR606621
*S. grigorievi** (1498)	"	KR606582	KR606592	KR606602	KR606612	KR606622
Stichaeopsis nevelskoi (1157)	Western Kamchatka shelf	JQ417850	JQ417846	JQ417842	JQ417855	KF366360
S. nevelskoi (1161)	Same area	KR606583	KR606593	KR606603	KR606613	KR606623
S. nevelskoi (1162)	"	KR606584	KR606594	KR606604	KR606614	KR606624

* Karyological and cytological studies.

ily Stichaeidae. Genomic DNA was isolated by the standard method, including lysis of tissue with proteinase K (0.2 mg/mL) in the presence of 1% SDS [30]. Primers for PCR and sequencing of genes were described previously [11–13]. The phylogenetic analysis was based on a combined approach, in which independently aligned sequences of the mtDNA and nDNA genes are combined into a single sequence. For two parts of the data array, mitochondrial and nuclear genes separately, the optimal models for nucleotide substitutions TVM+G and TIMef+I, used in constructing the Bayesian tree, were selected in the Modeltest v3.7 software [37]. A Bayesian analysis was performed in the MrBayes v. 3.1.2 software [38] by running three "hot" chains and one "cold" one during 10^6 cycles with selection of each one-hundredth of the generated tree. The first 1001 of the 10 001 obtained trees were discarded; the consensus tree and the values of *a posteriori* branching probability were built based on the rest of the trees. To assess DNA divergence, *p*-distances were computed in the PAUP 4.0b10 software [39].

For a karyological analysis of *S. nozawae* and *S. grigorievi*, air-dried chromosome mounts were prepared using a suspension of cells from the anterior kidney of fish [27]. To identify nucleolar organizer regions (NOR), the Ag-NOR banding technique was applied [24]. Chromosomes of several morphological types were identified in metaphase plates. Metacentric (M) chromosomes with equal arms and submetacentric (SM) chromosomes with unequal arms were referred to as bi-armed ones; subtelocentric (ST) chromosomes with the very short second arm and acrocentric (A) chromosomes with the invisible second arm were considered uni-armed ones.

The material for the ultrastructural study of fish spermatozoa using transmission (TEM) and scanning electron microscopy (SEM) was prepared by the technique described previously [7]. A total of 43 spermatozoa of each species were examined, photographed, and measured using a Zeiss EVO 40 SEM and the SmartTiff Installation Guide software (Germany), as well as a Zeiss Libra 120 TEM (Germany). Dimensions of spermatozoa were evaluated based on five criteria: length and width of the head, length and width of the middle part, and length of the flagellum. The statistical data processing (ANOVA procedure) was performed on a personal computer via the standard algorithm of the Statistica 6.0 software package [1]. The significance of differences was evaluated at a 95% level.

RESULTS

Genetic Analysis

Certain nucleotide sequences of mtDNA and nDNA genes are deposited in the GenBank/NCBI database (Table 1). The studied regions of the genes for *COI*, cytochrome *b*, and 16S rRNA with a length of 869, 585, and 588–590 base pairs (bp) were located within the 5766–6634, 14732–15316, and 2193–2781 bp ranges according to the numbering of the mitochondrial genome of *Lycodes toyamensis* (accession no. AP004448 in GenBank). A fragment of the RNF213 gene with a length of 628 bp was located within 4318–4947 bp, according to the numbering of the nucleotide sequence of this gene in *Danio rerio*

Table 2. The values of the *p*-distances (in %) based on the data of combined sequences of the genes for *COI*, cytochrome *b*, 16S rRNA of mtDNA, RNF213, and rhodopsin of nDN

No.	Species (no. of specimen)	1	2	3	4	5	6	7	8	9	10	11	12
1	*Stichaeus nozawae* (1430)	–	–	–	–	–	–	–	–	–	–	–	–
2	*S. nozawae* (1431)	0.06	–	–	–	–	–	–	–	–	–	–	–
3	*S. nozawae* (1499)	0.12	0.12	–	–	–	–	–	–	–	–	–	–
4	*S. nozawae* (1572)	0.18	0.18	0.24	–	–	–	–	–	–	–	–	–
5	*S. grigorievi* (1366)	**0.44**	**0.50**	**0.41**	**0.56**	–	–	–	–	–	–	–	–
6	*S. grigorievi* (1367)	**0.44**	**0.44**	**0.41**	**0.50**	0.21	–	–	–	–	–	–	–
7	*S. grigorievi* (1368)	**0.35**	**0.41**	**0.38**	**0.47**	0.15	0.15	–	–	–	–	–	–
8	*S. grigorievi* (1369)	**0.38**	**0.44**	**0.41**	**0.50**	0.12	0.15	0.09	–	–	–	–	–
9	*S. grigorievi* (1433)	**0.47**	**0.47**	**0.38**	**0.53**	0.09	0.15	0.18	0.15	–	–	–	–
10	*S. grigorievi* (1498)	**0.41**	**0.44**	**0.41**	**0.50**	0.15	0.18	0.12	0.15	0.12	–	–	–
11	*S. nevelskoi* (1157)	5.53	5.52	5.49	5.58	5.55	5.52	5.49	5.49	5.49	5.55	–	–
12	*S. nevelskoi* (1161)	5.53	5.52	5.49	5.58	5.55	5.53	5.49	5.49	5.49	5.55	0.06	–
13	*S. nevelskoi* (1162)	5.55	5.55	5.52	5.61	5.58	5.55	5.52	5.52	5.52	5.58	0.15	0.15

Here and in Table 3, the values of *p*-distances between *Stichaeus nozawae* and *S. grigorievi* are highlighted in bold.

Table 3. The values of *p*-distances (in %) based on the data of sequences of the genes for *COI*, cytochrome *b*, 16S rRNA of mtDNA (below the diagonal), RNF213, and rhodopsin of nDNA (above the diagonal)

No.	Species (no. of specimen)	1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Stichaeus nozawae* (1430)	–	0.07	0.07	0.07	**0.44**	**0.66**	**0.37**	**0.44**	**0.59**	**0.37**	1.32	1.40	1.40
2	*S. nozawae* (1431)	0.05	–	0.00	0.00	**0.51**	**0.59**	**0.44**	**0.51**	**0.51**	**0.37**	1.25	1.32	1.32
3	*S. nozawae* (1499)	0.15	0.20	–	0.00	**0.51**	**0.59**	**0.44**	**0.51**	**0.51**	**0.37**	1.25	1.32	1.32
4	*S. nozawae* (1572)	0.20	0.25	0.34	–	**0.51**	**0.59**	**0.44**	**0.51**	**0.51**	**0.37**	1.25	1.32	1.32
5	*S. grigorievi* (1366)	**0.44**	**0.49**	**0.34**	**0.64**	–	0.29	0.07	0.00	0.15	0.07	1.32	1.40	1.39
6	*S. grigorievi* (1367)	**0.29**	**0.34**	**0.29**	**0.49**	0.15	–	0.29	0.29	0.22	0.22	1.32	1.40	1.40
7	*S. grigorievi* (1368)	**0.34**	**0.39**	**0.34**	**0.54**	0.20	0.05	–	0.07	0.22	0.00	1.25	1.32	1.32
8	*S. grigorievi* (1369)	**0.34**	**0.39**	**0.34**	**0.54**	0.20	0.05	0.10	–	0.15	0.07	1.32	1.40	1.39
9	*S. grigorievi* (1433)	**0.39**	**0.44**	**0.29**	**0.59**	0.05	0.10	0.15	0.15	–	0.07	1.25	1.32	1.32
10	*S. grigorievi* (1498)	**0.44**	**0.49**	**0.44**	**0.64**	0.20	0.15	0.20	0.20	0.15	–	1.17	1.25	1.25
11	*S. nevelskoi* (1157)	8.33	8.37	8.33	8.43	8.37	8.33	8.33	8.28	8.33	8.47	–	0.07	0.07
12	*S. nevelskoi* (1161)	8.28	8.33	8.28	8.38	8.33	8.28	8.28	8.23	8.28	8.42	0.05	–	0.15
13	*S. nevelskoi* (1162)	8.33	8.37	8.33	8.43	8.37	8.33	8.33	8.28	8.33	8.47	0.20	0.15	–

(no. XM001920995). The length of the region of the rhodopsin gene was 735 bp; it was located within 133–867 bp (*Tetraodon nigroviridis*, no. AJ293018). The length of sequenced regions of mtDNA genes was 2043 bp; nDNA genes, 1363 bp.

An insignificant intra-species polymorphism was found in pricklebacks on the basis of evaluated *p*-distances (Table 2): the differentiation of combined nucleotide sequences of DNA in *Stichaeus grigorievi* ranged from 0.09 to 0.21%; in *S. nozawae*, from 0.06 to 0.24%. The genetic differences between the combined DNA of *S. nozawae* and *S. grigorievi* varied from 0.35 to 0.56% with an average of 0.44%, which is very low for comparison of the species. An analysis of the variability of the genes belonging to different genetic systems (mitochondrial and nuclear genomes) provided similar values of distances between these species: 0.43% (range 0.29–0.64) by mtDNA genes and 0.49% (0.37–0.66) by nDNA genes (Table 3).

In the phylogenetic tree constructed based on the combined DNA sequences (Fig. 1), *S. grigorievi* and *S. nozawae* form separate clusters. The central branch nodes of these cluster are supported by significant values of *a posteriori* probability (0.95 and 0.97), and thus, their formation is reliable.

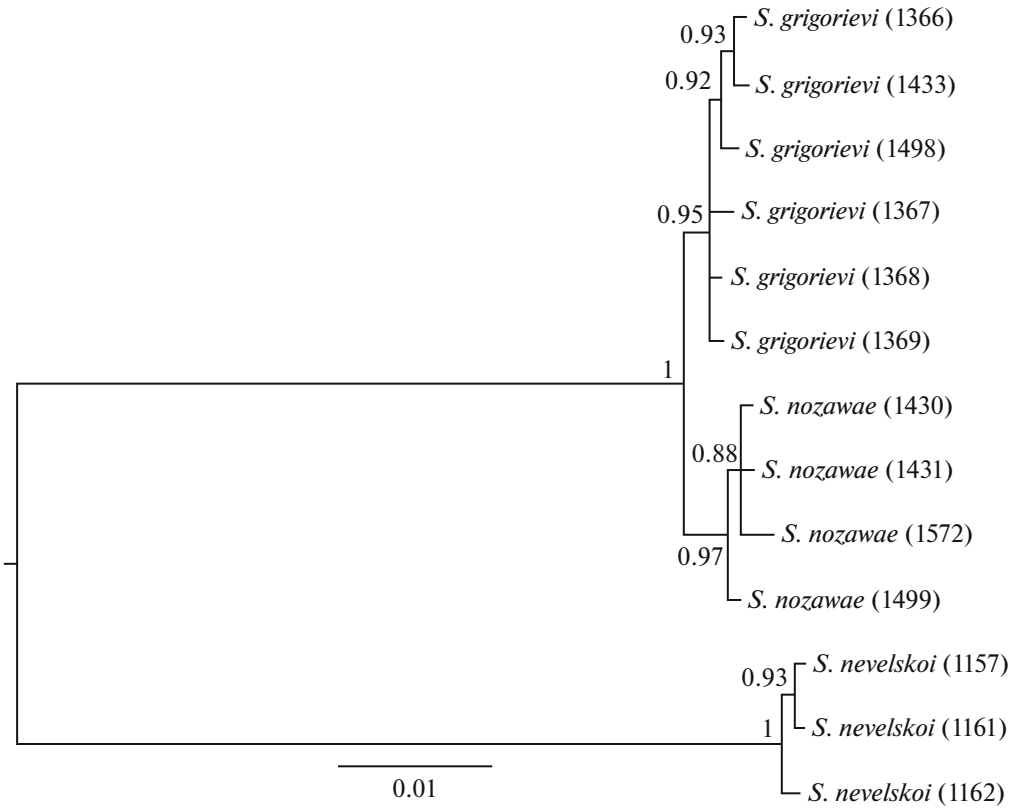

Fig. 1. The phylogenetic pattern of the relationships between *Stichaeus nozawae* and *S. grigorievi* based on the data of the combined nucleotide sequences of genes for *COI*, cytochrome *b*, 16S rRNA of mtDNA, RNF213, and rhodopsin of nDNA. Estimates of *a posteriori* probability are shown on the branches.

Karyological Analysis

The analysis of 100 and 85 stained metaphase plates from *S. nozawae* and *S. grigorievi* indicates that their karyotypes are stable and contain 48 chromosomes (2n); the number of chromosome arms (NF) is 70 (Fig. 2). Bi-armed chromosomes in *S. nozawae* and *S. grigorievi* are represented by four M chromosomes. M chromosomes of the first pair in the karyotype of *S. nozawae* are large (Fig. 2a); their relative sizes are similar to sizes of large SM chromosomes (Figs. 2a and 2b, pair *4*). The second pair of M chromosomes of *S. nozawae* is identical to the first pair of M chromosomes of *S. grigorievi* (Fig. 2a, pair *2*; Fig. 2b, pair *1*).

The M chromosomes of the second pair in karyotype of *S. grigorievi* (Fig. 2b) are small, and according to their size correspond to the smallest SM chromosomes (Figs. 2a and 2b, pair *11*). The eighteen SM chromosomes, of which the first two pairs are the largest (Figs. 2a and 2b, pairs *3, 4*), are grouped into a separate row and arranged in the order of their decrease (Figs. 2a and 2b, pairs *3–11*).

Uni-armed chromosomes in *S. nozawae* are represented by 20 ST and 6 A chromosomes (Fig. 2a, pairs *12–21* and *22–24*); in *S. grigorievi*, 22 ST and 4 A chromosomes (Fig. 2b, pairs *12–22* and *23, 24*). The short arms of ST chromosomes in the karyotypes of both species are expressed well enough to be selected into a separate row with gradually decreasing sizes.

A total of 73 Ag-NOR banded metaphase plates from *S. nozawae* and 40 metaphase plates from *S. grigorievi* were examined. In *S. nozawae*, active NORs in 29% of cells are stained in the near-centromeric regions of the long arms of two homologous ST chromosomes; in 71% of the cells are stained in one of the homologues (Table 4; Fig. 2a, inset). In *S. grigorievi*, NORs are located in the near-centromeric region of the long arm of one ST chromosome (Fig. 2b, inset). The sizes of the long arms of nucleolus-forming ST chromosomes (NOR-chromosomes) in the karyotypes of *S. nozawae* and *S. grigorievi* correspond to the sizes of long arms of medium ST chromosomes (Figs. 2a and 2b, pair *14*, insets). In interphase nuclei of the studied species, one and/or two nucleoli are stained.

Ultrastructural Analysis of Spermatozoa

Spermatozoa of both fish species have a flattened bullet-shaped head, distinguished by a pronounced pit on the surface in *S. nozawae* and a less-pronounced one in *S. grigorievi* and a long flagellum (Figs. 3 and 4).

The size of the head and the length of the flagellum vary significantly (Table 5).

In the spermatozoa of both fish species, the nuclear chromatin has a similar electron density; there are only small lacunae filled with the electron-lucent matrix (Figs. 4a, 4b, 4d, and 4g). In the basal part of nucleus, there are centriolar pits, in which the centriolar apparatus, consisting of two centrioles arranged parallel to each other, is located (Figs. 4b and 4g). The proximal centriole has a striated centriolar rootlet, which contacts with the nucleus (Figs. 4b, 4c, 4g, and 4f). The distal centriole is the basal body of the flagellum, which has a 9 + 2 structure. In both species, the middle part contains the annular mitochondrion (Figs. 4e and 4h).

DISCUSSION

Morphologically, *Stichaeus nozawae* and *S. grigorievi* are well distinguished by such non-overlapping characters as the number of rays in the dorsal and anal fins, the number of vertebrae, the size and position of the eyes and mouth, the length of the upper jaw, and the number of pores of the seismosensory system on the head [5, 6, 8]. According to Makushok [6], *S. grigorievi* is so significantly specialized and isolated that it can be placed in a separate subgenus.

The pricklebacks *S. grigorievi* and *S. nozawae* have a similar benthic mode of life, but the former species is found at a depth of 288 m, whereas the latter lives at a depth of up to 520 m. They breed in shallow waters, at a depth of less than 10 m, in algal beds on a boulder/pebbly bottom. These species differ quite significantly in their biological characteristics: Grigorev's prickleback is larger and has a higher growth rate; the absolute and relative fecundity is higher in Nozawa's prickleback; according to its type of feeding Grigorev's prickleback is ichthyobenthophagous, while Nozawa's prickleback is nektobenthophagous [3].

The conclusion of the species independence of *S. nozawae* and *S. grigorievi* according to morphological and biological characteristics is not consistent with the results of genetic studies. Based on the data of polymorphism of the mitochondrial genes for *COI*, cytochrome *b*, and 16S rRNA, as well as the nuclear RNF213 and RAG2 genes, these species were shown to have minor inter-species differences [26, 29, 41]; it has even been suggested to consider these taxa as a single species [26].

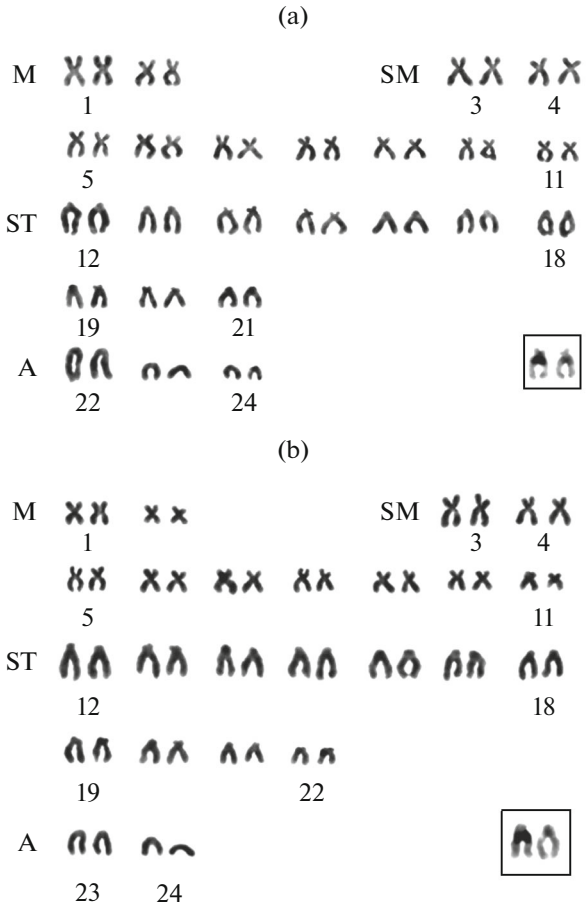

Fig. 2. Karyograms and NOR chromosomes of *Stichaeus nozawae* (a) and *S. grigorievi* (b), $2n = 48$, NF = 70. Chromosomes are as follows: M, metacentric; SM, submetacentric; ST, subtelocentric; A, acrocentric; NOR chromosomes are highlighted by frame. Magnification: 10 × 100.

According to our data on the variability of the mtDNA and nDNA genes, a low level of genetic differences was also revealed between the species *S. noza-*

Table 4. NOR chromosomes in the cells of the anterior kidney in *Stichaeus nozawae*

No. of specimen, sex	Number of cells with NOR in		Number of		
	ST 1	ST 2	NOR chromosomes in cell	nucleoli	metaphase plates
1430, female	18	7	1–2	1–2	25
1431, female	18	5	1–2	1	23
1499, male	11	1	1–2	1–2	12
1572, male	5	8	1–2	1–2	13
Proportion, %	52/71	21/29	1–2	1–2	73/100

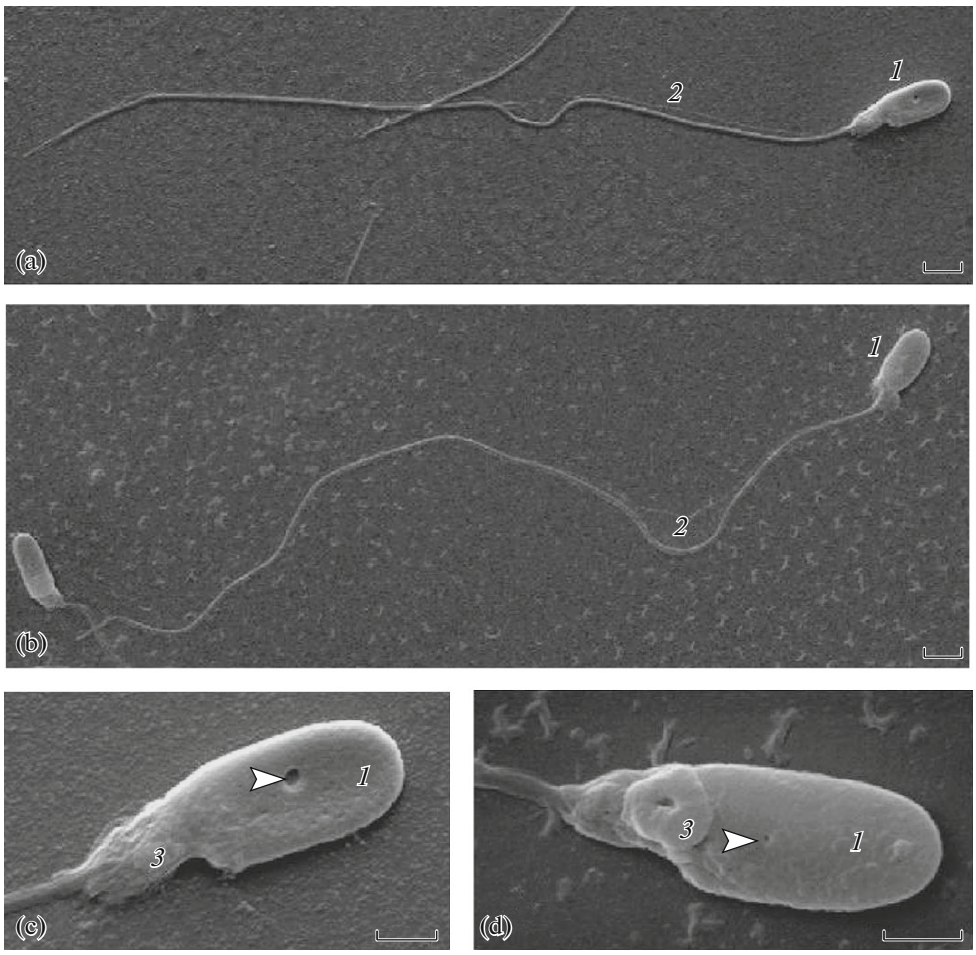

Fig. 3. The external form of the spermatozoon and head in *Stichaeus nozawae* (a, c) and *S. grigorievi* (b, d) (SEM).

wae and *S. grigorievi*, but in the phylogenetic tree (Fig. 1) they were located in their own "specific" clusters. Low levels of mtDNA differentiation were also found for other valid species of the suborder Zoarcoidei: 0.95% for *Zoarces fedorovi* and *Z. viviparus* [10] and 0.74% for *Lycodes knipowitschi* and *L. brevicaudus* [12]. These are probably adolescent species, well isolated morphologically and biologically, that have not yet accumulated a sufficient amount of DNA changes for genetic species identification.

A comparison of karyotypes of *S. nozawae* and *S. grigorievi*, $2n = 48$, NF = 70 (Figs. 2a and 2b), showed their similarity in the set of chromosomes. A pair of identical M chromosomes (Fig. 2a, pair *2*; Fig. 2b, pair *1*) and nine pairs of identical in size SM chromosomes (Figs. 2a and 2b, pairs *3–11*) were selected in both species. Nozawa's and Grigorev's pricklebacks are similar in the number of uni-armed chromosomes (26 ST and A chromosomes) (Figs. 2a and 2b, pairs *12–24*) but differ in their quantitative proportion (Fig. 2a: ST, pairs *12–21*; A, pairs *22–24*; Fig. 2b: ST, pairs *12–22*; A, pairs *23*, *24*). The size of the arms of the smallest A chromosomes in the karyotype of *S. nozawae* (Fig. 2a, pair *24*) corresponded to the size

Table 5. The sizes (in μm) of spermatozoa in *Stichaeus nozawae* and *S. grigorievi* ($n = 43$)

Species	Head length	Head width	Length of middle part	Width of middle part	Flagellum length
Stichaeus nozawae	2.42–3.94 3.31 ± 0.05	1.47–1.85* 1.67 ± 0.01	0.77–2.38 1.32 ± 0.04	0.7–1.36 1.03 ± 0.02	27.99–46.33* 40.37 ± 05.
S. grigorievi	2.73–4.61 3.24 ± 0.04	1.39–1.90* 1.59 ± 0.02	0.57–2.14 1.1 ± 0.05	0.7–1.79 1.12 ± 0.03	40.34–54.52* 46.04 ± 0.4

* Significant differences between species at $p < 0.05$.

The numerator is the range of variations; the denominator is the mean and the error of the mean.

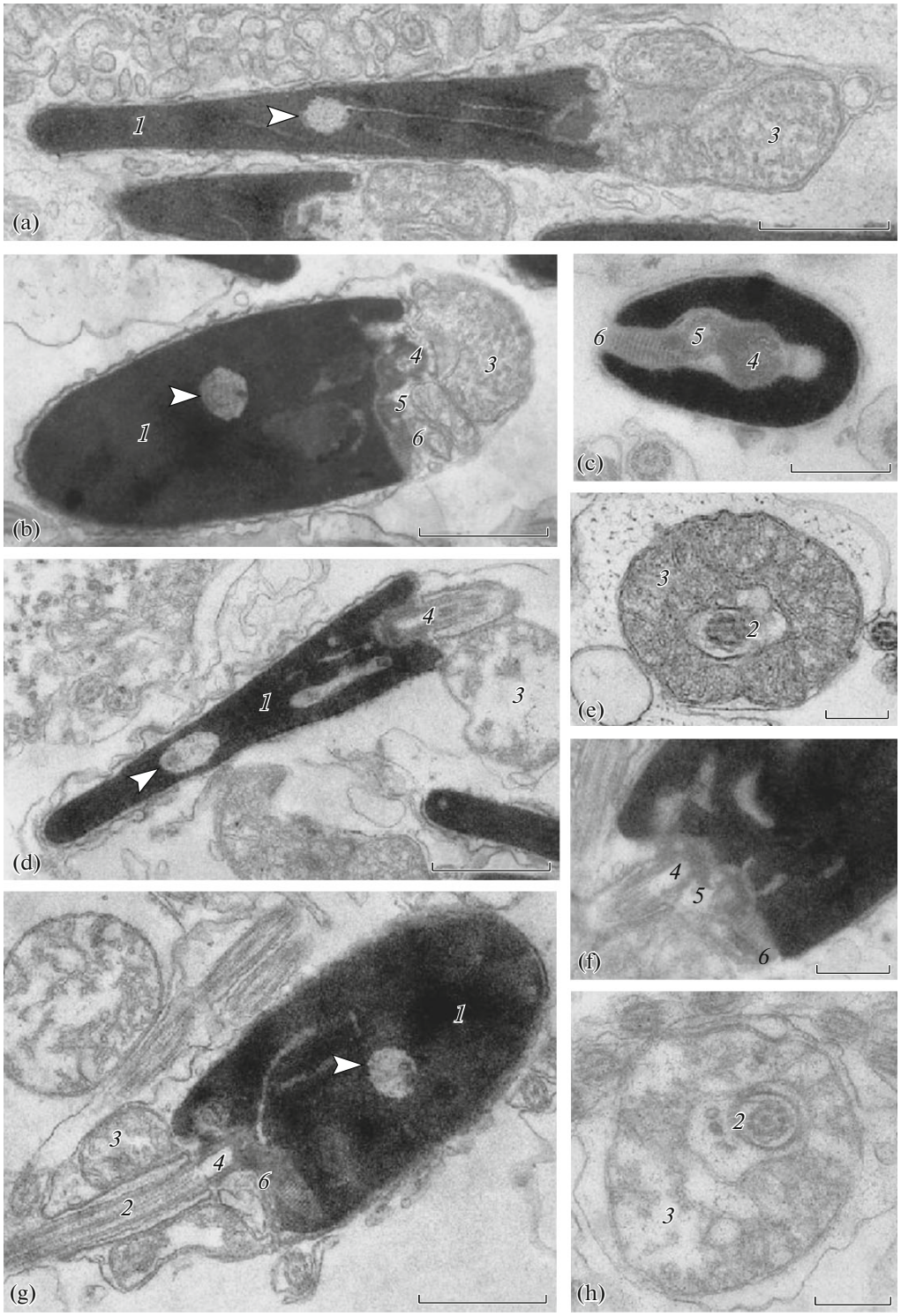

Fig. 4. Spermatozoon of *Stichaeus nozawae* (a, b, c, e) and *S. grigorievi* (d, g, f, h) (TEM): (a, d) sagittal section; (b, g) frontal section; (c, f) centriolar complex; (e, h) cross section on the level of mitochondria. (*1*) Nucleus; (*2*) flagellum; (*3*) mitochondrion; (*4*) distal centriole; (*5*) proximal centriole; (*6*) striated centriolar rootlet. Lacunae with the electron-lucent matrix are indicated by arrows. Scale bar: a, b, d, g, 1 μm; c, e, f, h, 0.5 μm.

of the long arms of the small ST chromosomes in *S. grigorievi* (Fig. 2b, pair *22*). However, the short arms of these ST chromosomes in *S. grigorievi* were not always clearly pronounced, which may be associated with the varying degree of chromosome spiralization in the studied mounts. A pairwise comparison of routinely

stained chromosomes showed that the karyotypes of the pricklebacks differ in the size of the M chromosomes (Fig. 2a, pair *1*; Fig. 2b, pair *2*). By drawing an analogy with the most studied groups of fish, in which the main mechanisms of karyotype changes in the course of evolution are Robertsonian translocations [15, 18, 35], we can assume that different uni-armed chromosomes participated in the formation of M chromosomes in *S. nozawae* (Fig. 2a, pair *1*) and *S. grigorievi* (Fig. 2b, pair *2*). A pair of large M chromosome in *S. nozawae* and a pair of small M chromosomes in *S. grigorievi* allow discrimination between the karyotypes of the studied species and can be considered marker ones for them.

The earlier studies on the species of the genus *Zoarces* (suborder Zoarcoidei), whose chromosome sets are stable within the genus ($2n = 48$, NF = 58), like those in *S. nozawae* and *S. grigorievi* ($2n = 48$, NF = 70), showed that the number and localization of active NORs in the chromosomes are good diagnostic characters [15, 28]. However it has been found that the localization of active NORs does not differentiate the karyotypes of *S. nozawae* and *S. grigorievi* (Figs. 2a and 2b, insets). The position of active NORs in the chromosomes is also not a determinative character for species in the family Nototheniidae, whose taxonomic status of undoubted: *Pseudotrematomus hansoni* (Boulenger, 1902), *Pagothenia borchgrevinki* (Boulenger, 1902), and *Trematomus newnesi* (Boulenger, 1902). Their karyotypes are "synonimic $2n = 46/45$, NF = 52/51" [34]; NORs in these species are located in the third pair of bi-armed chromosomes [9]. Cases where the number and localization of active NORs do not allow diagnostics of species are known for mammals, particularly for the small ground squirrel *Spermophilus pygmaeus* (Pallas, 1778) and the Caucasian mountain ground squirrel *S. musicus* (Menetries, 1832). However, genetic differentiation of these species, confirming their taxonomic status, was based on C- and G-banding of karyotypes [19].

The studied prickleback species differ in the number of NOR chromosomes in their karyotypes. The observed difference can be explained by an insufficient number of metaphases examined by this method in *S. grigorievi* or by different genetic activity of NORs in homologous chromosomes caused by the functional status of fish at the time of collection of the material. According to the published data, spawning in *S. grigorievi* occurs earlier than that in *S. nozawae* [3]. The presented data on karyotypes of *S. nozawae* and *S. grigorievi* (the similarity in the number and morphology of chromosomes, number of chromosome arms, and localization of active NORs) show their close relationship. The only character that marks the prikleback species is the size of M chromosomes. These cytogenetic data indicate the necessity of further investigation and complex analysis of the karyotypes of *S. nozawae* and *S. grigorievi* using other techniques of differential staining (C-banding, with chromomycin A_3), as well as a study of the karyotypes of other members of the genus *Stichaeus* for their comparative analysis.

A comparative analysis of spermatozoa from *S. nozawae* and *S. grigorievi* showed the structural and morphological similarity of their heads and sizes (Fig. 3, Table 5). The nuclear chromatin in the spermatozoa of these species is of the same electron density; only small lacunae filled with the electron-lucent matrix were found (Figs. 4a, 4b, 4d, and 4g). A similar structure of chromatin was described as well from cyprinid fishes [2, 7, 22]. Sperm cells of *S. nozawae* and *S. grigorievi* are typical non-acrosome primitive spermatozoa, which are characteristic for many members of bony fishes with external fertilization [25]. The striated centriolar rootlet contacting with the nucleus (Figs. 4b, 4c, 4g, and 4f), found in spermatozoa of *S. nozawae* and *S. grigorievi* is also typical for spermatozoa of fish in the family Anguillidae (freshwater eels). Unlike pricklebacks, the striated centriolar rootlet in freshwater eels is connected with the distal centriole [23, 36, 40]. This process is also found in rainbow trout at the spermatid stage, but this structure is absent from mature sperm cells [20].

According to the data from the statistical analysis (ANOVA procedure), the spermatozoa of *S. nozawae* and *S. grigorievi* significantly differ in the head width and flagellum length, which allows reliable differentiation of each of the samples (Table 5). These differences confirm the species specificity of the studied gametes. The comparative cytological analysis of the ultrastructure of spermatozoa from *S. nozawae* and *S. grigorievi* made it possible to establish the significant structural and morphological similarity of their heads and sizes. However, the observed differences between spermatozoa in head width and flagellum length are species-specific for the prickleback species under study.

Thus, the analysis of ultrastructural organization of sex cells in *S. nozawae* and *S. grigorievi* indicated characters that confirm the validity of these species. However, the data obtained as a result of the study of *S. nozawae* and *S. grigorievi* using genetic and karyological analyses are not consistent with the conclusions about their taxonomic independence according to morphological and biological characters. The Nozawa's and Grigorev's pricklebacks are assumed to be adolescent species. The lack of changes that are suitable for species differentiation in the DNA and chromosome sets of these species may be caused by different rates of evolution of morphological, genetic, and karyological characters.

ACKNOWLEDGMENTS

We are grateful to D.V. Fomin (Zhirmunsky Institute of Marine Biology, Far Eastern Branch, Russian Academy of Sciences) for his assistance in the work

with the electron and scanning microscopes. The study was financially supported by the Far Eastern Branch of the Russian Academy of Sciences, project nos. 15-I-6-081 and 15-I-6-014o, and the Russian Foundation for Basic Research, project no. 15-29-02416 ofi_m.

REFERENCES

1. Borovikov, V.P., *Populyarnoye vvedeniye v programmu Statistica* (Popular Introduction into the Statistica Software), Moscow: Komp'yuter-Press, 1998.
2. Emelyanova, N.G. and Makeeva, A.P., Ultrastructure of spermatozoa in some of cyprinid fishes (Cyprinidae), *Vopr. Ikhtiol.*, 1985, vol. 25, no. 3, pp. 459–468.
3. Kolpakov, N.V. and Klimkin, A.F., Specific features of biology of shannies *Stichaeus grigorjewi* and *S. nozawae* (Stichaeidae) in Waters of Northern Primorye, *J. Ichthyol.*, 2004, vol. 44, no. 7, pp. 592–599.
4. Lavrova, T.V., Preliminary list and distribution of fishes of the family Stichaeidae in the Sea of Okhotsk, *Tr. Zool. Inst., Akad. Nauk SSSR*, 1990, vol. 213, pp. 46–54.
5. Lindberg, G.U. and Krasyukova, Z.V., *Ryby Yaponskogo morya i sopredel'nykh chastei Okhotskogo i Zheltogo morei* (Fishes in the Sea of Japan and Adjacent Parts of the Sea of Okhotsk and Yellow Sea), Leningrad: Nauka, 1975.
6. Makushok, V.M., Morphological bases of stichaeids and similar fish families (Stichaeoidae, Blennioidei, Pisces), *Tr. Zool. Inst., Akad. Nauk SSSR*, 1958, vol. 25, pp. 3–129.
7. Neznanova, S.Yu., Ultrastructural study of spermiogenesis in Far-Eastern redfins of the genus *Tribolodon* (Pisces: Cyprinidae), *Russ. J. Mar. Biol.*, 2012, vol. 38, no. 2, pp. 156–165.
8. Pitruk, D.L., Lavrova, T.V., and Zemnukhov, V.V., A morphological description of the brown shanny *Stichaeus fuscus* Miki et Maruyama, 1986 (Perciformes: Stichaeidae), *Russ. J. Mar. Biol.*, 2011, vol. 37, no. 6, pp. 458–463.
9. Prirodina, V.P., Karyotypic and taxonomic diversity of nototenioidnyh fish of the suborder Notothenioidae (Perciformes) from the Southern Ocean, *Tr. Zool. Inst., Ross. Akad. Nauk*, 2010, vol. 314, no. 4, pp. 411–432.
10. Radchenko, O.A., Petrovskaya, A.V., Nazarkin, M.V., et al., Relationships of eelpouts of the genus *Zoarces* (Zoarcidae, Pisces) inferred from molecular genetic and morphological data, *Russ. J. Genet.*, 2010, vol. 46, no. 11, pp. 1344–1351.
11. Radchenko, O.A., Chereshnev, I.A., and Petrovskaya, A.V., New data on the phylogenetic relationships of taxa in the subfamily Gymnelinae (Zoarcidae) based on the results of analysis of mitochondrial and nuclear DNA, *Vestn. Sev.-Vost. Nauchn. Tsentra Dal'nevost. Otd. Ross. Akad. Nauk*, 2010, no. 4, pp. 80–85.
12. Radchenko, O.A., Chereshnev, I.A., and Petrovskaya, A.V., Relationships and divergence of some taxa of the subfamily Lycodinae (Zoarcidae, Pisces) based on molecular-genetic and morphological data, *J. Ichthyol.*, 2009, vol. 49, no. 5, pp. 585–598.
13. Radchenko, O.A., Chereshnev, I.A., and Petrovskaya, A.V., Genetic differentiation of species and taxonomic structure of the superfamily Stichaeoidea (Perciformes: Zoarcoidei), *Russ. J. Mar. Biol.*, 2014, vol. 40, no. 6, pp. 473–485.
14. Ryazanova, I.N., Evolution of sculpins (*Myoxocephalus* and *Megalocottus*) in the seas of Japan and Okhotsk based on karyological data, *Extended Abstract of Cand. Sci. (Biol.) Dissertation*, Vladivostok, 2008.
15. Ryazanova, I.N. and Frolov, S.V., Karyotype of the Fedorov's eelpout *Zoarces fedorovi* (Zoarcidae, Pisces) from Taui Bay, Sea of Okhotsk, *Vestn. Sev.-Vost. Nauchn. Tsentra Dal'nevost. Otd. Ross. Akad. Nauk*, 2008, no. 1, pp. 86–90.
16. Sokolovsky, A.S., Dudarev, V.A., Sokolovskaya, T.G., and Solomatov, S.F., *Ryby rossiiskikh vod Yaponskogo morya* (Fishes of the Russian Waters of the Sea of Japan), Vladivostok: Dal'nauka, 2007.
17. Taranets, A.Ya., Some changes in the taxonomy of fishes of the Soviet Far East, with notes on their distribution, *Vestn. Dal'nevost. Fil. Akad. Nauk SSSR*, 1935, no. 13, pp. 89–101.
18. Frolov, S.V., *Izmenchivost' i evolyutsiya kariotipov lososevykh ryb* (Variability and Evolution of Karyotypes of Salmonids), Vladivostok: Dal'nauka, 2000.
19. Tsvirka, M.V. and Korablev, V.P., On the issue of chromosomal speciation exemplified by the little (*Spermophilus pygmaeus* (Pallas, 1778)) and the Caucasian mountain (*Spermophilus musicus* (Menetries, 1832)) ground squirrels (Rodentia, Sciuridae), *Zool. Zh.*, 2014, vol. 93, no. 7, pp. 917–925.
20. Billard, R., Spermiogenesis in the rainbow trout (*Salmo gairdneri*), *Cell Tissue Res.*, 1983, vol. 233, pp. 265–284.
21. *Catalog of Fishes*, Eschmeyer, W.N., Ed. http://researcharchive.calacademy.org/research/ichthyology/catalog/. Accessed March 10, 2014.
22. Fürböck, S., Patzner, R.A., and Lahnsteiner, F., Fine structure of spermatozoa of *Chondrostoma nasus* and *Rutilius meidingerri* (Teleostei, Cyprinidae), as revealed by scanning and transmission electron microscopy, *Acta Zool.* (Stockholm), 2010, vol. 91, pp. 88–95.
23. Gibbons, B.H., Gibbons, I.R., and Baccetti, B., Structure and motility of the 9+0 flagellum of eel spermatozoa, *J. Submicrosc. Cytol.*, 1983, vol. 15, no. 1, pp. 15–20.
24. Howell, W.M. and Black, D.A., Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method, *Experientia*, 1980, vol. 36, pp. 1014–1015.
25. Jamieson, B.G.M., *Fish Evolution and Systematics: Evidence from Spermatozoa*, New York: Cambridge Univ., 1991.
26. Kartavtsev, Y.P., Sharina, S.N., Goto, T., et al., Sequence diversity at cytochrome oxidase 1 (Co-1) gene among sculpins (Scorpaeniformes, Cottidae) and some other scorpionfish of Russia Far East with phylogenetic and taxonomic insights, *Genes Genomics*, 2009, vol. 31, no. 2, pp. 183–197.
27. Kligerman, A.D. and Bloom, S.E., Rapid chromosome preparations from solid tissues of fishes, *J. Fish. Res. Board Can.*, 1977, vol. 34, pp. 266–269.
28. Klinkhard, M.B., Comparison of the karyology (C-banding, Ag-NOR) of eelpout (*Zoarces viviparus*) from

the White Sea and Baltic Sea, *Cytobios*, 1991, no. 68, pp. 29–36.

29. Kwun, H.J. and Kim, J.-K., Molecular phylogeny and new classification of the genera *Eulophias* and *Zoarchias* (PISCES, Zoarcoidei), *Mol. Phylogenet. Evol.*, 2013, vol. 69, no. 3, pp. 787–795.
30. Maniatis, T., Fritsch, E.F., and Sambrook, J., *Molecular Cloning, a Laboratory Manual*, New York: Cold Spring Harbor Lab., 1982.
31. Mecklenburg, C.W. and Møller, P.R., Biodiversity of arctic marine fishes: taxonomy and zoogeography, *Mar. Biodiversity*, 2011, vol. 41, pp. 109–140.
32. Mecklenburg, C.W. and Sheiko, B.A., Family Stichaeidae Gill, 1864 pricklebacks, *Calif. Acad. Sci. Annot. Checklist Fishes*, 2004, no. 35, pp. 1–36.
33. Miki, T. and Maruyama, Sh., New and rare stichaeid fishes from Okhotsk Sea, *Jpn. J. Ichthyol.*, 1986, vol. 32, no. 4, pp. 400–408.
34. Morescalchi, A., Pisano, E., Stanyon, R., and Morescalchi, M.A., Cytotaxonomy of Antarctic teleost of the *Pagothenia/Trematomus* complex (Nototheniidae, Perciformes), *Polar Biol.*, 1992, no. 12, pp. 553–558.
35. Ohno, S., Stenius, C., Faisst, E., and Zenzes, M.T., Post-zygotic chromosomal rearrangements in rainbow trout (*Salmo irideus* Gibbons), *Cytogenetics*, 1965, vol. 4, no. 2, pp. 117–129.
36. Okamura, A. and Motonobu, T., Spermatozoa of *Conger myriaster* observed by electron microscopy, *Zool. Sci.*, 1999, vol. 16, pp. 927–933.
37. Posada, D. and Crandall, K.A., Modeltest: Testing the model of DNA substitution, *Bioinformatics*, 1998, vol. 14, pp. 817–818.
38. Ronquist, F. and Huelsenbeck, J.P., MrBayes 3: Bayesian phylogenetic inference under mixed models, *Bioinformatics*, 2003, vol. 19, pp. 1572–1574.
39. Swofford, D.L., *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4*, Sunderland: Sinauer, 2002.
40. Todd, P.R., Ultrastructure of spermatozoa and spermiogenesis in New Zealand freshwater eels (Anguillidae), *Cell. Tissue Res.*, 1976, vol. 171, pp. 221–232.
41. Turanov, S.V., Kartavtsev, Y.P., Lipinsky, V.V., et al., DNA-barcoding of perch-like fishes (Actinopterygii: Perciformes) from far-eastern seas of Russia with taxonomic remarks for some groups, *Mitochondrial DNA*, 2014, vol. 27, no. 2, pp. 1–22. doi 10.3109/19401736.2014.945525

Translated by E. Shvetsov