ISSN 1063-0740, Russian Journal of Marine Biology, 2018, Vol. 44, No. 3, pp. 192–201. © Pleiades Publishing, Ltd., 2018.
Original Russian Text © I.V. Stonik, M.P. Isaeva, N.A. Aizdaicher, E.S. Balakirev, F.J. Ayala, 2018, published in Biologiya Morya.

ORIGINAL PAPERS

Morphological and Genetic Identification of *Pseudo-nitzschia* H. Peragallo, 1900 (Bacillariophyta) from the Sea of Japan

I. V. Stonik[a], *, M. P. Isaeva[b], N. A. Aizdaicher[a], E. S. Balakirev[a], and F. J. Ayala[c]

[a] *National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, 690041 Russia*
[b] *Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, 690022 Russia*
[c] *Department of Ecology and Evolutionary Biology, University of California, California, Irvine, 92697-2525 United States*
**e-mail: innast2004@mail.ru*

Received November 23, 2017

Abstract—A total of 15 *Pseudo-nitzschia* strains isolated from Russian coastal waters of the Sea of Japan were identified using light and electron microscopy and phylogenetic analysis of ribosomal genes. *Pseudo-nitzschia* species are known to be potential producers of neurotoxic domoic acid and cause blooms. Phylogenetic analysis of LSU and ITS1-5,8SrDNA-ITS2 sequences revealed that the examined strains belong to *P. calliantha*, *P. hasleana*, *P. multistriata*, *P. pungens* var. *pungens*, and *P. delicatissima*. *P. hasleana* is a new record for the Sea of Japan. Some morphological distinctions of the strains of *P. calliantha* and *P. pungens* var. *pungens* from the original descriptions of the species were detected. The ITS1-5,8SrDNA-ITS2 topologies showed that the *P. delicatissima* clade A is formed from three subclades, including Pacific strains, as well as isolates from Scotland and Spain. The high values of genetic distances between subclades (3.7–5%) and the morphological similarity between strains are indicative of cryptic genetic diversity within the *P. delicatissima* clade A. Different ITS types, including a possibly recombinant type, occur in the same monoclonal culture of *P. multistriata,* suggesting the hybridization of the local populations in the studied area.

Keywords: diatoms, *Pseudo-nitzschia*, morphology, rDNA
DOI: 10.1134/S1063074018030100

INTRODUCTION

To date, 37 species of marine diatoms of the genus *Pseudo-nitzschia* H. Peragallo are known, of which 14 are able to produce the neurotoxin domoic acid [11]. The systematics of *Pseudo-nitzschia* has been inadequately studied due to high intraspecific morphological and genetic variability and the presence of sympatric cryptic species. The detection of such species is necessary for accurate assessment of marine diatom biodiversity [17]. Previous studies have shown that *P. pungens*, *P. pseudodelicatissima/cuspidata,* and *P. delicatissima* are species complexes with high morphological and genetic diversity [4, 15, 16, 22 and others]. Representatives of the complexes cause blooms at the coasts of Primorye (the Sea of Japan) almost every year, reaching densities of more than 10^6 cells/L [2]. However, no data are available on the morphological and genetic variability of *Pseudo-nitzschia* from Russian waters of the Sea of Japan.

The present research is aimed at a morphological and genetic identification of 15 strains of the diatom genus *Pseudo-nitzschia* from the northwestern Sea of Japan.

MATERIALS AND METHODS

A morphological and molecular-genetic study was carried out on 15 *Pseudo-nitzschia* strains isolated from the northwestern Sea of Japan during the period from 2006 to 2013 (Table 1) according to the previously described procedure [1]. The strains are maintained in the Marine Biobank Resource Collection (http://marbank.dvo.ru/index.php/en/). The morphology of diatoms was examined using an Olympus BX 41 (Tokyo, Japan) light microscope (LM) and a Carl Zeiss LIBRA-120 (Carl Zeiss Group, Germany) transmission electron microscope (TEM) at the Far East Center for Electron Microscopy of the National Scientific Center of Marine Biology (Far East Branch of the Russian Academy of Sciences). For morphological study by TEM, the diatom frustules were cleaned by treatment with sulfuric acid and saturated aqueous solutions of potassium permanganate and oxalic acid followed by centrifugation with distilled water [14]. For TEM observation, a drop of the washed sample was placed on formvar-coated grids and dried in the air.

The morphometric characteristics of the frustules were determined for ten specimens from each clonal culture. The data were tested for normality of distribution (Shapiro–Wilk test) and for the homogeneity of

Table 1. *Pseudo-nitzschia* strains used in this study

Name of strain	Coordinates of isolates	Date of isolation	GenBank access number
MBRU_PC-06	43°11′21″ N, 132°06′50″ E	November 30, 2006	KT247437
MBRU_PC-081	42°5337″ N, 132°43′55″ E	September 23, 2008	KT247438
MBRU_PC-101	43°06′44″ N, 131°53′28″ E	November 24, 2010	KT247439
MBRU_PC-12	43°05′13″ N, 131°58′35″ E	November 14, 2012	KT247436
MBRU_PC-13	43°04′14″ N, 131°57′37″ E	December 5, 2013	KT247434
MBRU_PC-124	43°04′19″ N, 131°57′59″ E	October 29, 2012	KT247435
MBRU_PH-07	43°12′00″ N, 131°54′50″ E	July 23, 2007	KT247440
MBRU_PD-101	43°06′44″ N, 131°53′28″ E	August 3, 2010	KT247429
MBRU_PD-102	43°06′44″ N, 131°53′28″ E	August 31, 2010	KT247427
MBRU_PD-111	43°06′44″ N, 131°53′28″ E	July 25, 2011	KT247428
MBRU_PP-071	42°53′37″ N, 132°43′55″ E	December 20, 2007	KT247433
MBRU_PPR-12	43°01′26″ N, 131°47′49″ E	July 24, 2012	KT247432
MBRU_PP-13	43°12′00″ N, 131°54′50″ E	February 20, 2013	KT247431
MBRU_PP-131	43°04′136 N, 131°59′72″ E	June 18, 2013	KT247430
MBRU_PMS-11	43°12′00″ N, 131°54′50″ E	July 31, 2011	KT247441-44

the variance (Levene's test) and then subjected to a one-factor ANOVA. In the case of the statistical significance of a result of the analysis of the variance, Tukey's multiple comparison test was used. All calculations were carried out with the Statistica (StatSoft, United States) program.

The genomic DNA was separated using a DNeasy Plant Mini Kit (Qiagen, United States). For all *Pseudo-nitzschia* strains, PCR amplification of the region that includes the ITS1-5,8SrDNA-ITS2 and LSU (D1-D3 domains) was carried out using the 1380-F and ITS055-R primers [15, 18]. For the MBRU_PH-07 strain, a PCR-specific fragment containing 5,8SrDNA-ITS2 and LSU was amplified using the ITS03-F and ITS055-R primers [15, 18]. Amplification was carried out according to the generally accepted methods [15]. PCR products were purified and sequenced with fluorescently labeled terminators ((2'-3'-dideoxynucleoside triphosphates, BigDye™, Applied Biosystems) on ABI PRISM 377 (Perkin Elmer, United States) or 3130×L (Hitachi, Japan) DNA analyzers using the above primers. The sequences of both DNA chains obtained from two independent amplifications were determined in order to avoid possible error.

For the isolate of *P. multistriata*, PCR fragments that were unreadable in direct sequencing were cloned into the pTZ57R/T vector (Fermentas, Lithuania) and transformed into cells of *Escherichia coli* strain Top10 (Invitrogen, United States). Positive PCR fragments obtained from ampicillin-resistant colonies were purified and sequenced using M13 primers (Sibenzim, Russia).

A search for homologous sequences was performed at the NCBI and (http://blast.ncbi.nlm.nih.gov) and EMBL-EBI (http://www.ebi.ac.uk/ena). Ribosomal RNA gene sequences were assembled using the SeqMan program (Lasergene, DNASTAR). Multiple alignments were carried out using the MUSCLE program [7]. Kimura two-parameter distances (K2) [10] were calculated using the MEGA v.6 program [21]. Optimal evolutionary models were selected using the Bayesian information criterion (BIC) and the Akaike information criterion (AIC). On this basis, phylogenetic trees were constructed by the neighbour-joining (NJ) method. The significance of phylogenetic trees was assessed by bootstrap analysis (500–1000 replicates). A search for recombinant events was carried out using a number of methods in the RDP3 [19] and DnaSP [12] programs.

RESULTS AND DISCUSSION

Morphology

According to their cell morphology, the MBRU_PC-06, MBRU_PC-081, MBRU_PC-101, MBRU_PC-12, MBRU_PC-124, MBRU_PC-13, and MBRU_PH-07 strains (Fig. 1) were identified as belonging to the *Pseudo-nitzschia pseudodelicatissima/cuspidata* species complex [15]. The frustule morphology of the first of the 15 strains (Table 2, Figs. 1a–1e) generally conformed to the known descriptions of *P. calliantha* Lundholm, Moestrup et Hasle [13, 15]. However, the average values of fibulae density in cells from the MBRU_PC-081, MBRU_PC-12, and MBRU_PC-124 strains (Table 2) did not match the range of values (18–24) reported by Lundholm et al. [13]. Morphological examination revealed a high average density of fibulae in MBRU_PC-081, MBRU_PC-124, and MBRU_PC-12,

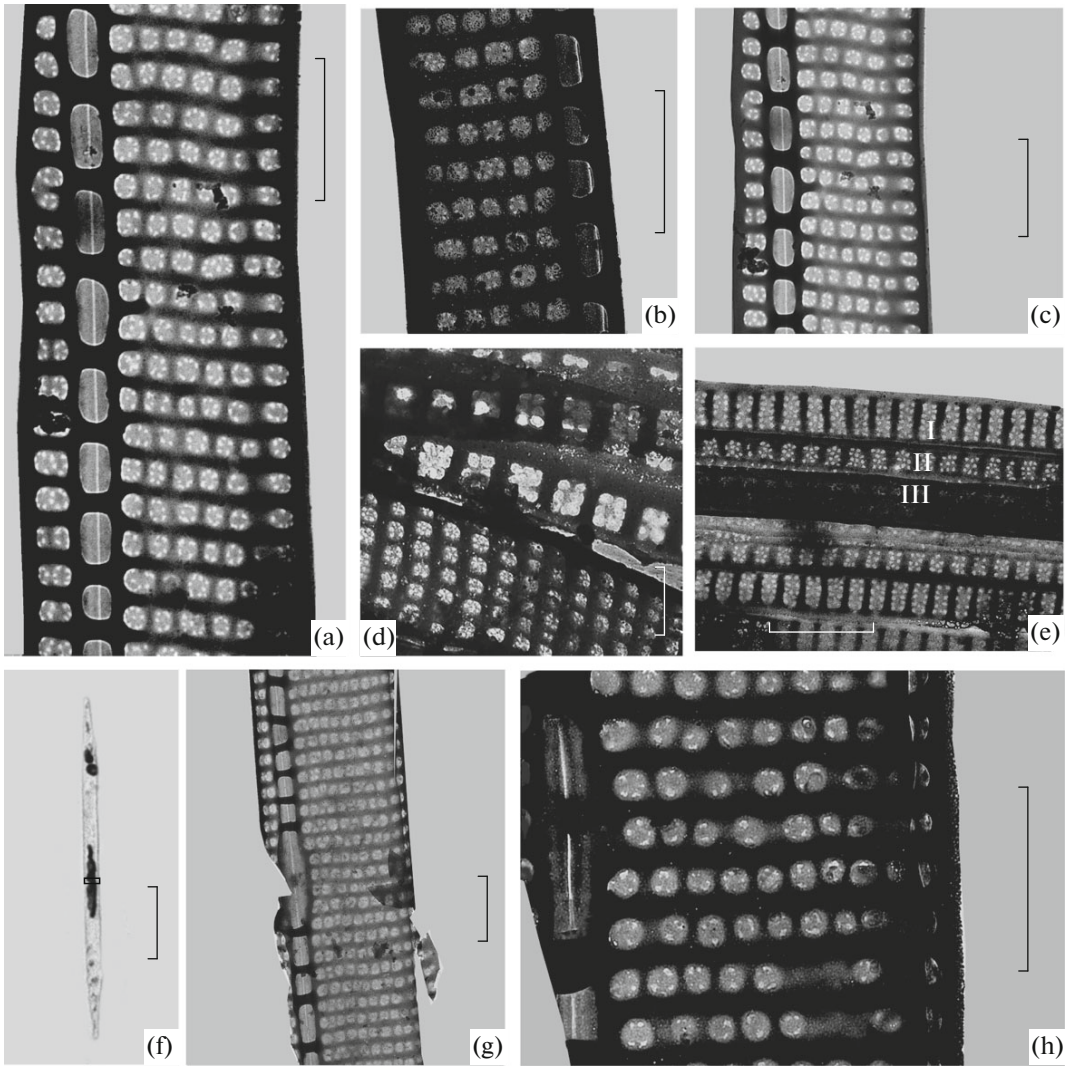

Fig. 1. The morphology of strains MBRU_PC-12 (a, b), MBRU_PC-081 (c), MBRU_PC-124 (d, e), and MBRU_PH-07 (f–h). (a) fibulae and striae on the valve; (b, c) structure of poroids; (d) fragment of valve and girdle bands; (e) structure of valvocopula (I), second (II), and third (III) girdle bands; (f) general view of cell; (g) fragment of valve showing fibulae and striae; (h) structure of valve poroids. (a–e, g, h) TEM; (f) LM. Scale: (a, f) 10 μm; (b–e, g, h) 1 μm.

compared to that in other strains from the northwestern Sea of Japan (Turkey test, $p < 0.001$; Table 2).

The morphometric features of the strain MBRU_PH-07 (Table 2, Figs. 1f–1h) coincided with the data from the original description of the species *P. hasleana* Lundholm [13]. The morphology of the MBRU_PMS-11 strain (Table 2, Figs. 2a–2c) completely corresponded to the descriptions of *P. multistriata* (Takano) previously given by Takano [20].

The TEM examination revealed that the strains MBRU_PD-101, MBRU_PD-102, MBRU_PD-111 belong to the *P. delicatissima* species complex [16] (Figs. 2d, 2e). The average values of the main morphometric characteristics of cells of the studied strains (Table 2) corresponded to the data provided in the original description of *P. delicatissima* (Cleve) Heiden, including the epitype (strain Læsø 5) from Danish waters and Pacific strains OFPd972 and Tasm10 [16]. The mean values of the morphometric characteristics of the studied strains corresponded to the range of these characters in strains from coastal waters of Scotland and Spain [22].

The TEM investigation of cells of strains MBRU_PP-071, MBRU_PPR-12, MBRU_PP-13, and MBRU_PP-131 identified them as belonging to the species *P. pungens* (Cleve) Hasle (Figs. 2f–2m). However, the identification of varieties of the species was hindered in some cases. The average density of striae and fibulae on the valve of our strains (Table 2) did not correspond to their ranges for the type variety (8.6–11.6, cited after Churro et al. [5]). The average density of striae on the valve of MBRU_PP-071 (Table 2) corresponded to its variation range in *P. pun-*

Table 2. The morphometric characteristics of *Pseudo-nitzschia* strains

Name of strain	Width of valve, μm	Fibulae density in 10 μm	Striae density in 10 μm	Poroids density in 10 μm	Striae density on valvocopula in 10 μm
		P. calliantha			
MBRU_PC-06	1.5–1.6 1.5 ± 0.05	19–22 **20.4 ± 0.9^{A}**	33–44 37.1 ± 3.2	4–5 4.7 ± 0.5	47–48 47.5± 0.5
MBRU_PC-081	1.5–2.0 1.7 ± 0.2	26–28 **27.1 ± 0.8^{B}**	34–38 35.3 ± 1.6	4–6 5.2 ± 0.7	46–48 46.6 ± 0.3
MBRU_PC-101	1.4–2 1.7 ± 0.2	19–28 **22.8 ± 2.5^{C}**	35–44 39.2 ± 2.9	3–6 4.9 ± 1.0	38–50 44.8 ± 4.3
MBRU_PC-12	1.4–2 1.6 ± 0.2	25–27 **25.9 ± 0.8^{D}**	39–45 42 ± 1.9	5–6 5.4 ± 0.5	42–43 **42.4 ± 0.5^{G}**
MBRU_PC-124	1.6–2 1.7 ± 0.2	26–28 **26.9 ± 0.9^{E}**	40–42 41 ± 0.9	4–6 4.9 ± 0.9	–
MBRU_PC-13	1.5–1.9 1.7 ± 0.1	16–27 **20.3 ± 3.3^{F}**	39–49 42.3 ± 3.8	5–6 5.4 ± 0.5	47–52 **49.3 ± 2.6^{H}**
		P. hasleana			
MBRU_PH-07	1.7–2.6 2.1 ± 0.4	19–20 19.6 ± 0.6	36–39 37.7 ± 1.5	5–7 6 ± 0.6	48
		P. delicatissima			
MBRU_PD101	1.4–2.1 1.8 ± 0.2	18–26 21.4 ± 1.9	34–41 36.8 ± 1.5	8–12 10.1 ± 1.0	43–48 44.8 ± 1.9
MBRU_PD102	1.4–1.9 1.6 ± 0.1	22–27 24.3 ± 1.3	40–44 40.9 ± 1.3	10–12 11.1 ± 0.8	40–50 45.8 ± 3.8
MBRU_PD111	1.5–2.1 1.8 ± 0.1	22–26 23 ± 1.4	36–42 39.3 ± 1.6	11–14 12.4 ± 0.9	48–50 48.2 ± 0.5
		P. pungens			
MBRU_PP-071	2.8–4 3.3 ± 0.4	10–13 **12 ± 0.9^{I}**	9–13 12 ± 0.9	3–4 3.5 ± 0.5	16–19 **17.2 ± 0.4^{M}**
MBRU_PPR-12	3.1–3.4 3.3 ± 0.1	13–15 **13.8 ± 1^{J}**	13–15 13.8 ± 1	3–4 3.5 ± 0.5	15–17 **16 ± 0.8^{O}**
MBRU_PP-13	3–3.7 3.3 ± 0.2	14–16 **15.2 ± 0.6^{K}**	14–16 15.2 ± 0.6	3–4 3.5 ± 0.5	19–26 **21.7 ± 2.8^{N}**
MBRU_PP-131	3.4–3.8 3.6 ± 0.1	14–15 **14.5 ± 0.5^{L}**	14–15 14.5 ± 0.5	3–4 3.5 ± 0.5	18–24 **20.3 ± 1.9^{P}**
		P. multistriata			
MBRU_PMS-11	2.5–3.8 3.3 ± 0.5	22–27 24.5 ± 2.1	37–44 39 ± 3.4	10–12 10.7 ± 0.9	–

The first line, the variation range of the character; the second line, the mean value ± standard deviation; "–" means no data. Bold values are statistically significantly different mean values of A and B, A and D, A and E, B and C, B and F, C and E, D and F, E and F, G and H, I and J, I and K, I and L (Tukey test, $p < 0.001$).

gens var. *cingulata* Villac (10–13), while the average density of striae and fibulae on the valve in MBRU_PPR-12, MBRU_PP-13, and MBRU_PP-131 (Table 2) were consistent with those in *P. pungens* var. *aveirensis* Lundholm, Churro, Carreira et Calado (13–16 in 10 μm) [5]. In most of the studied specimens, one transverse row of large oval poroids was observed on the valvocopula (Figs. 2i and 2j), which is characteristic of the type variety of *P. pungens* var. *pungens* [5]. Thus, the strains PP-07, PPR-12, and PP-13 were characterized by the presence of one row of large oval poroids on the valvocopula (Figs. 2i, 2j). However, cells of MBRU_PP-131 had one row of rectangular or square poroids on the valvocopula; each poroid was split in two or more parts in some specimens (Figs. 2k–2m). In cells of MBRU_PP-13 and

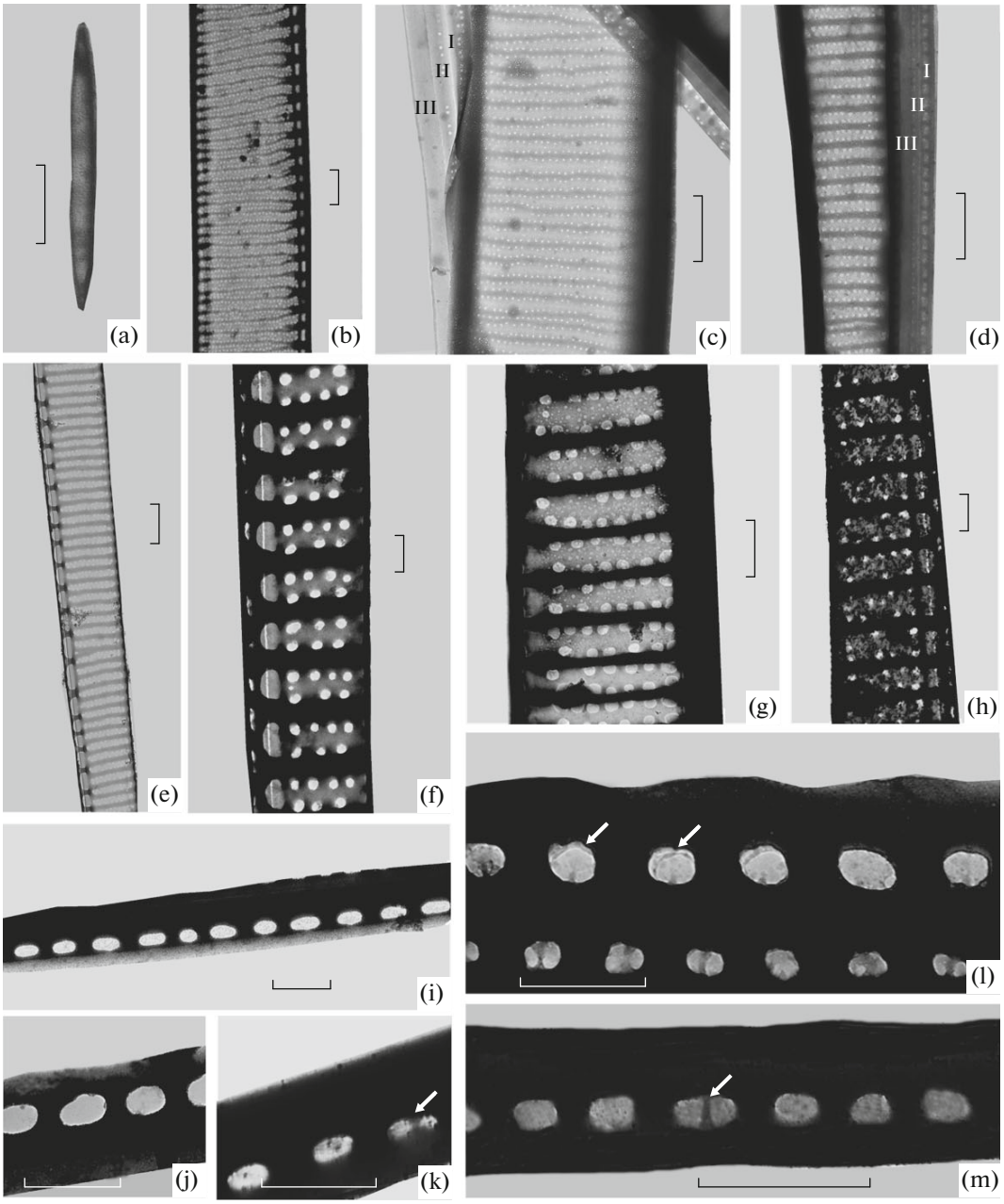

Fig. 2. The morphology of strains MBRU_PMS-11 (a–c), MBRU_PD-111 (d), MBRU_PD101 (e), MBRU_PPR-12 (f), MBRU_PP-13 (g), MBRU_PP-071 (h, i), MBRU_PP-13 (j), and MBRU_PP-131 (k–m). (a) General view of the cell; (b) fibulae and striae on valve; (c) structure of striae on valve; (d) structure of a stria on valve and fragment of valvocopula (I), second (II), and third (III) girdle bands; (e) fibulae and striae of valve; (f–h) fragments of valves showing fibulae and striae; (i, j) one row of large oval poroids on valvocopula; (k–m) one row of rounded-rectangular poroids on valvocopula, valvocopula poroids are split into two and more parts (arrows). (a–m) TEM. Scale: (a) 10 μm, (b–m) 1 μm.

MBRU_PP-13 the average density of striae on the valvocopula corresponded to the measurements reported in the original description of *P. pungens* var. *cingulata* Villac (20–24) [23]. Thus, the studied specimens of the above strains differed from *P. pungens* var. *pungens* in a number of features (the density of striae and fibulae on the valve, the density of striae of the valvocopula, and the structure of poroids). We found a high average density of striae on the valve in MBRU_PP-13, MBRU_PP-131, and MBRU_PPR-12, as well as a high density of striae of the valvocopula in PP-13 and PP-131, compared to the data for other isolates (Tukey test, $p < 0.001$; Table 2).

Analysis of LSU Nucleotide Sequences

For phylogenetic reconstructions, 35 LSU sequences of different species of *Pseudo-nitzschia* that are closest to our sequences were additionally derived from the GenBank and EMBL-EBI. The length of the analyzed sequence consisting of the first three conserved domains D1–D3 28SrDNA was 506–508 b.p. The optimal evolutionary model with the minimum value of BIC was the Kimura 2-parameter model with gamma distribution (K2 + G). In contrast to other models that are closest in values of BIC (K2 + G + I, HKY + G, and GTR + G + I), only this model separated *P. mannii* Amato et Montresor and *P. calliantha* in the NJ-tree (75% node bootstrap support). LSU analysis showed a high level of intraspecific similarity of sequences of the Sea of Japan strains *P. calliantha*, *P. pungens,* and *P. multistriata* with the type strains of the respective species (Fig. 3). The strain MBRU_PH-07 was identified as *P. hasleana* according to its clustering with the available strains of this species (99% bootstrap support).

Analysis of the LSU fragment revealed no sufficient resolution for differentiating *P. delicatissima*, *P. decipiens* Lundholm et Moestrup, *P. dolorosa* Lundholm et Moestrup, *P. galaxiae* Lundholm et Moestrup, and *P. micropora* Priisholm, Moestrup et Lundholm (Fig. 3). The strains MBRU_PD-101, MBRU_PD-102, and MBRU_PD-111, which were identified as *P. delicatissima* according to their morphological features, were taxonomically unresolved.

Analysis of ITS1-5,8SrDNA-ITS2 Nucleotide Sequences

For taxonomic identification of *Pseudo-nitzschia* strains from the Sea of Japan, we constructed phylogenetic trees based on ITS1-5,8SrDNA-ITS2 sequences. The multiple alignment also included 48 closest sequences that were derived from the NCBI and EMBL-EBI databases. The analyzed sequence was 870 b.p. long. No outgroup was used for the construction of ITS-tree of *Pseudo-nitzschia*, because with its inclusion the number of informative sites was decreased, which markedly reduced the tree resolution. The strains that were identified as *P. calliantha*, *P. pungens,* and *P. multistriata* based on the morphology and LSU analysis were grouped with the respective taxa (Fig. 4).

All the phylogenetic ITS-trees clearly differentiated the three clades of *P. pungens* described previously [4]. Four strains of *P. pungens* (PP-071, PPR-12, PP-13, and PP-131) fell within clade I (*P.* var. *pungens*).

In the ITS1-5,8SrDNA-ITS2-based tree, the studied *P. calliantha* strains (MBRU_PC-06, MBRU_PC-081, MBRU_PC-101, MBRU_PC-12, MBRU_PC-124, and MBRU_PC-13) were clustered with Vietnamese strains PC DS2, PC TA1, and PC HA-D4, as well with the Atlantic strain PC Pn-2 (Fig. 4). *P. calliantha* strains (MBRU_PC-081, MBRU_PC-12, and MBRU_PC-13) were 99–100% identical to each other in LSU and 5,8SrDNA sequences but were polymorphic in ITS1 and ITS2 regions at positions 187, 234, 238–239, and 252.

The unresolved LSU-tree topology of the three strains of *P. delicatissima* (MBRU_PD-101, MBRU_PD-102, and MBRU_PD-111) is clearly resolved using the ITS1-5,8SrDNA-ITS2 based tree. These strains were clustered with strains of *P. delicatissima sensu stricto* (clade A); three subclades were clearly differentiated within this clade (Fig. 4), which combined our strains, strains PD OFPd972 from Japan, and strains PD Tasm10 from Tasmania (subclade I), as well as the isolates from Scotland and Denmark (subclade II) [8] and Spain (subclade III) [22]. The values of distances between subclades were within the range of 3.74–5.03%, which corresponds to the level of interspecific divergence in the diatom genus *Pseudo-nitzschia* [13]. The main differences between Pacific strains were found in the ITS1 fragment. *P. delicatissima* strains from the Sea of Japan that were isolated in different years were identical at ITS1, 5,8S rRNA, ITS2, and LSU (D1–D3). Data on the *P. hasleana* strain from the Sea of Japan are not shown in the ITS1-5,8SrDNA-ITS2 tree (Fig. 4), as satisfactory amplification of PCR-fragment was only accomplished with the direct primer for the beginning of 5,8SrDNA. The *P. hasleana* strain MBRU_PH-07 in the 5,8SrDNA-ITS2 tree was grouped with the known strains of this species (NJ, 100%), which fully agrees with the LSU data (Fig. 3).

Intragenomic Variations in the ITS Region in P. multistriata

Direct sequencing of the ITS region in the strain MBRU_PMS-11 yielded unsatisfactory chromatograms with displacement and overlapping of the peaks. For determination of intragenomic polymorphism, the PCR product of the ITS fragment was cloned and positive colonies were subsequently sequenced. This revealed four different ITS subtypes: B1, B2, B3, and A/B2 (presumably recombinant) (Table 3), which were previously determined by D'Alelio et al. [6]. The ITS subtypes were characterized by specific substitutions and insertions at certain positions in the ITS1 and ITS2 regions. At position 36, an insertion of one nucleotide, A, occurred, which is also characteristic of other *P. multistriata* strains from the Mediterranean Sea and coastal waters of Australia and Korea (GenBank access numbers DQ990368, DQ990369, DQ990367, KC017470, and AY257843). At position 798, a unique substitution G>C was found in the subtype A/B2 in the PSM-11-A/B2 sequences and in the Korean strain A (AY257843).

We investigated the recombination signal using a number of methods in the RDP3 program [19], as well as the method of the minimum number of recombina-

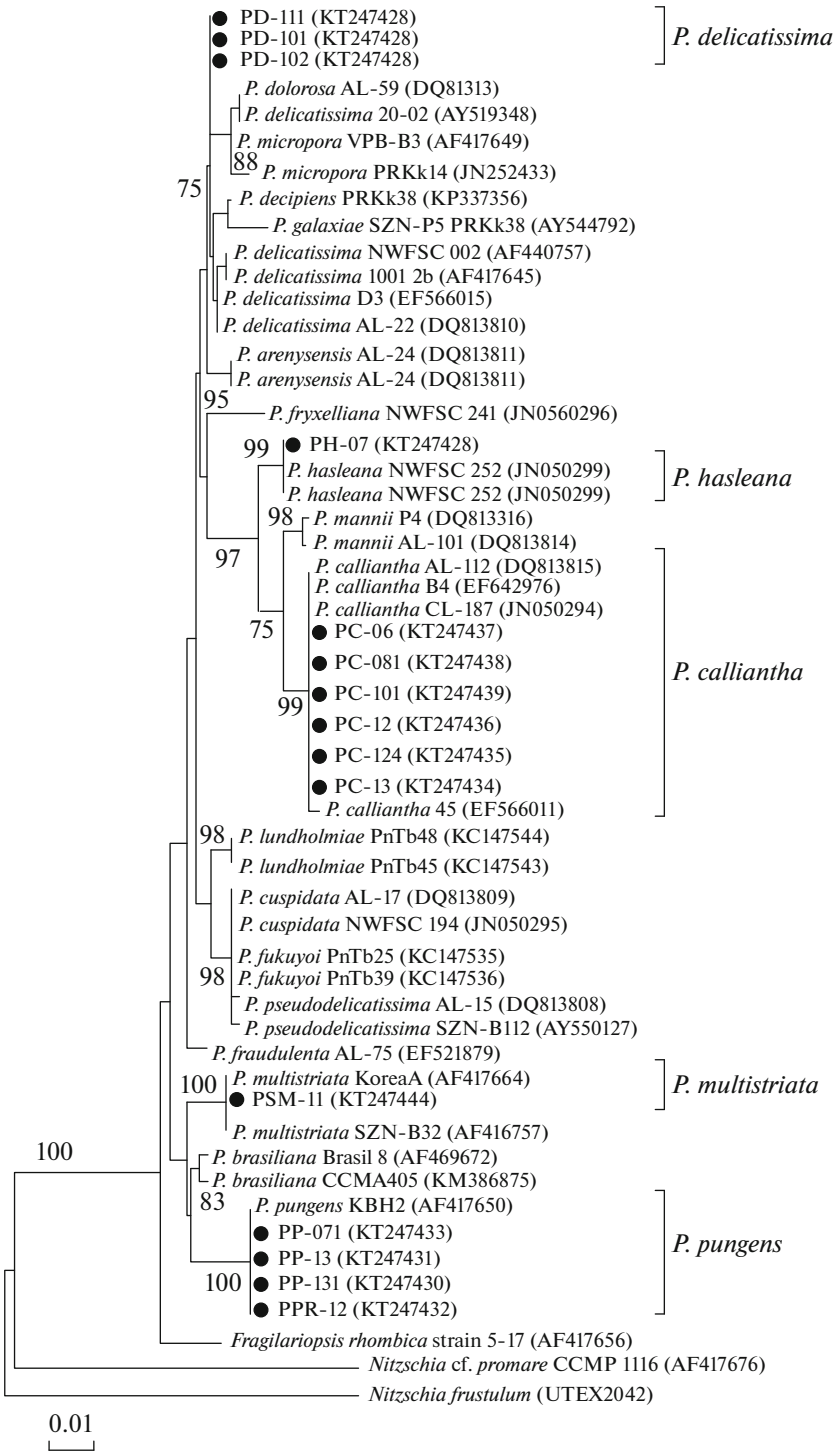

Fig. 3. A D1–D3-28S-rRNA phylogenetic tree of the genus *Pseudo-nitzschia* constructed by the NJ method using K2 + G as the optimal evolutionary model. The scale shows the number of nucleotide substitutions per nucleotide site. Values of bootstrap support (1000 replicates) greater than 75% are shown in tree nodes. The number in parentheses is the GenBank access number. The outgroups are *Fragilariopsis rhombica* (O'Meara) Hustedt, *Nitzschia* cf. *promare* Medlin, and *Nitzschia frustulum* (Kützing) Grunow.

tion events [9] in the DnaSP software [12]. With five polymorphic sites in the alignment of 5,8SrDNA-ITS2 sequences, the methods in the RDP3 program failed to reveal recombination, evidently because of the low variability of the studied nucleotide region. However, the method of Hudson and Kaplan [9] revealed one, which was probably recombinant even in

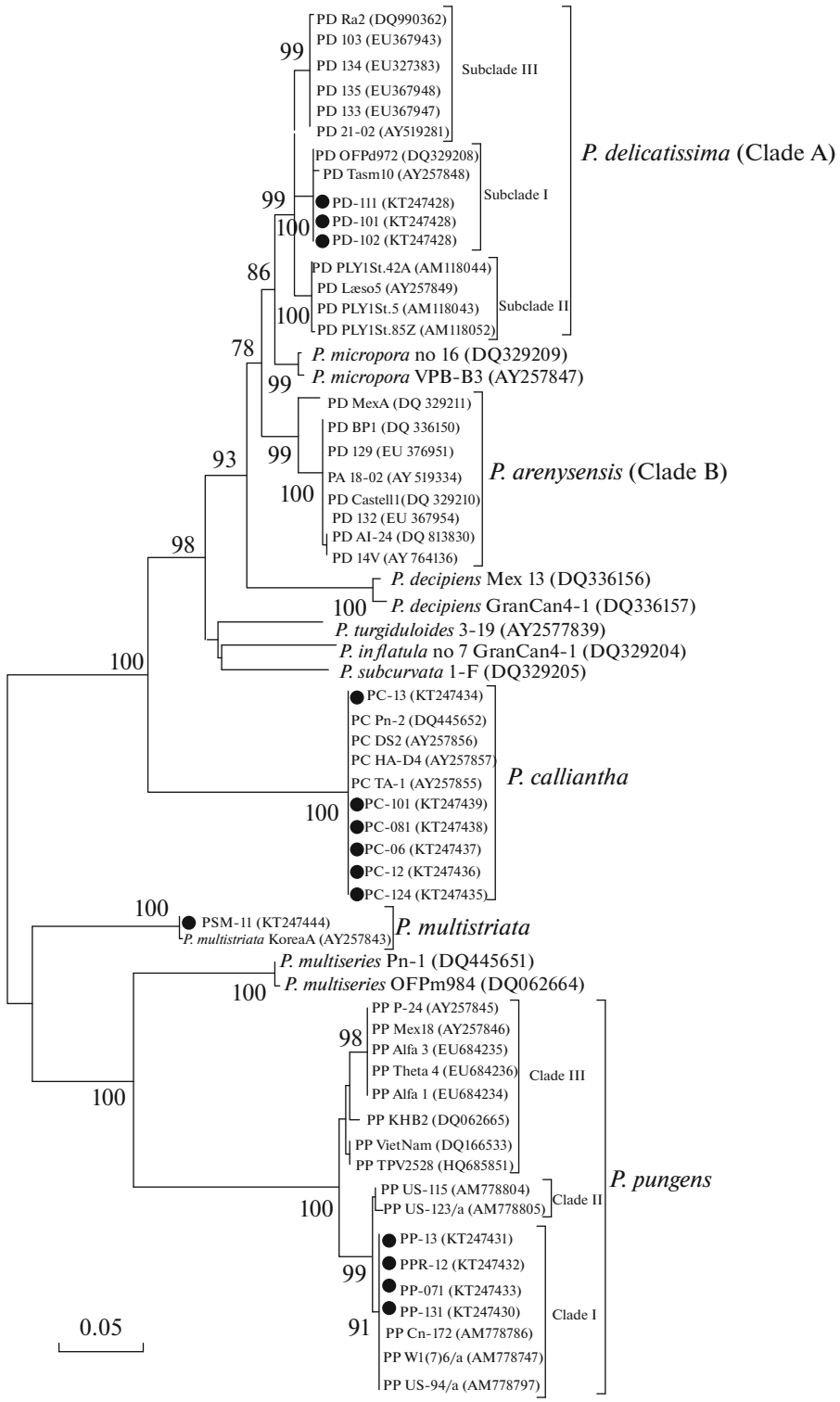

Fig. 4. An ITS1-5,8SrDNA-ITS2 phylogenetic tree of the genus *Pseudo-nitzschia* constructed by the NJ method using T92 + G as the optimal evolutionary model. Other designations are as in Fig. 3.

the coordinates of 301–797, confirming the possible hybridization of the local populations of *P. multistriata*.

Thus, for the first time, the present work presents the results of a complex study of cultures of *Pseudo-nitzschia* species from the Sea of Japan using molecular-genetic and morphological methods. *P. hasleana* was found in the Sea of Japan for the first time, supplementing the few records of the species from coasts of Japan and Australia [3, 13].

Table 3. ITS-polymorphism in *Pseudo-nitzschia multistriata* strain MBRU_PMS-11 from the Sea of Japan

ITS-subtype (GenBank access number)	Insertions			Single nucleotide polymorphism			Subtypes	Unique nucleotide polymorphism		
	141–150	579–591	793–796	69	170	301	151–154	36	42	798
A	–	–	+	T	C	T	$(TA)_2$	–	T	G
B	+	+	–	C	T	C	$(TA)_2$	–	T	G
B1 (KT247442)	**+**	**+**	**–**	**C**	**T**	**C**	**$+(TA)_2$**	**A**	**T**	**G**
B2 (KT247444)	**+**	**+**	**–**	**C**	**T**	**C**	**$+(TA)_4$**	**A**	**T**	**G**
B3 (KT247443)	**+**	**+**	**–**	**C**	**T**	**C**	**$+(TA)_5$**	**A**	**T**	**G**
A/B2 (KT247441)	**–**	**+**	**–**	**C**	**C**	**T**	**$(TA)_2$**	**A**	**C**	**C**

For ITS-subtypes A and B, the pattern of differences in ITS1-2 sequences is given according to [6]. Numerals indicate the position of nucleotides. Bold font indicates ITS-subtypes identified for the strain MBRU_PMS-11, including a probably recombinant subtype A/B2.

The phylogenetic and morphological study allowed us to identify the strains MBRU_PC-06, MBRU_PC-081, MBRU_PC-101, MBRU_PC-124, MBRU_PC-12, and MBRU_PC-13 as *P. calliantha*. An earlier phylogenetic study of strains of the *Pseudo-nitzschia delicatissima* complex revealed two genotypes (clades): *P. delicatissima* (clade A) and *P. arenysensis* Quijano-Scheggia, Garcés et Lundholm (clade B) [16, 22]. This phylogenetic study identified strains from the northwestern Sea of Japan as *P. delicatissima* sensu stricto (clade A according to [16]). Despite the absence of morphological differences between Pacific strains and strains from other places, the molecular-genetic data indicated cryptic diversity within clade A: the presence of three subclades, with subclade I combining Pacific strains from Russia, Japan, and Tasmania. The values of the genetic distances that we found between the three subclades (3.74–5.03%) were comparable to the distances between *P. micropora* and *P. delicatissima* (3.8–4.3%), but were lower than the distances between *P. delicatissima* and *P. arenysensis* (6.2–7.8%), as well as between *P. micropora* and *P. arenysensis* (5.6–6.9%) [13]. The relatively high values of divergence between the three subclades indicated the probable presence of cryptic taxa within clade A.

Three clades were previously distinguished for *P. pungens* from different areas of the World Ocean that correspond to *P. pungens* var. *pungens*, *P. pungens* var. *cingulata*, and *P. pungens* var. *aveirensis* [4, 5]. The present phylogenetic study allowed us to assign our *P. pungens* strains to clade I [4]. These results, as well as the split poroid structure in valvocopulae in the strain MBRU_PP-131, which is characteristic of clades II and III, were indicative of high intraspecific morphological variability of genetically identical *P. pungens* strains from the Sea of Japan.

Intragenomic variations in the ITS sequences of *P. multistriata* and the presence of a probably recombinant subtype A/B pointed to the possible hybridization of the local populations of *P. multistriata* in the studied area. The GenBank-derived sequence of *P. multistriata* strain from coastal waters of Korea (AY257843) was genetically close to the possible recombinant subtype A/B identified for MBRU_PMS-11 (average genetic distance, 0.25 ± 0.17%). This suggests that genetically similar local populations that are close in geographical origin co-occur in the northwestern Sea of Japan.

The results of this research showed that the studied strains belong to *P. calliantha*, *P. hasleana*, *P. multistriata*, *P. pugens* var. *pungens*, and *P. delicatissima*. Intragenomic variations in the *P. multistriata* strain, including the probable presence of a hybrid ITS subtype, were detected for the first time. Clade A (*P. delicatissima sensu stricto*) was formed by three subclades, including Pacific clades, as well as isolates from Scotland and Spain. The high values of the genetic distances between subclades and the morphological similarity between strains were indicative of cryptic genetic diversity within clade A. The geographic divergence of the strains may suggest intraspecific genetic differentiation of the *P. delicatissima* isolates from the northwestern Pacific, which requires further investigation.

ACKNOWLEDGMENTS

The morphological analysis of diatom strains was supported by the Dal'niy Vostok Program no. 18-5-074/0268-2018-0021. The analysis of nucleotide sequences of *Pseudo-nitzschia pungens* and *P. delicatissima* was performed with the financial support from the Bren Professor Funds at the University of California, Irvine, United States.

REFERENCES

1. Orlova, T.Yu., Aizdaicher, N.A., and Stonik, I.V., *Laboratornoe kul'tivirovanie morskikh mikrovodoroslei, vklyuchaya produtsentov fititoksinov: nauchno-metodicheskoe posobie* (Laboratory Cultivation of Marine Microalgae, Including Producers of Phytotoxins: A

Scientific-Methodical Manual), Vladivostok: Dal'nauka. 2011.

2. Stonik, I.V. and Orlova, T.Yu., The species composition and quantitative distribution of the diatom genus *Pseudo-nitzschia* H. Peragallo, 1900 in Russian waters of the Sea of Japan and the Sea of Okhotsk, *Russ. J. Mar. Biol.*, 2013, vol. 39, no. 4, pp. 238–245.
3. Ajani, P., Murray, S., Hallegraeff, G., et al., First reports of *Pseudo-nitzschia micropora and P. hasleana* (Bacillariaceae) from the Southern Hemisphere: morphological, molecular and toxicological characterization, *Phycol. Res.*, 2013, vol. 61, pp. 237–248.
4. Casteleyn, G., Chepurnov, V.A., Leliaert F., et al., *Pseudo-nitzschia pungens* (Bacillariophyceae): a cosmopolitan diatom species, *Harmful Algae*, 2008, vol. 7, pp. 241–257.
5. Churro, C.I., Carreira, C., Rodrigues F.J., et al., Diversity and abundance of potentially toxic *Pseudo-nitzschia* Peragallo in Aveiro coastal lagoon, Portugal and description of a new variety, *P. pungens* var. *aveirensis* var. nov., *Diatom Res.*, 2009, vol. 24, no. 1, pp. 35–62.
6. D'Alelio, D., Amato, A., Kooistra, W.H.C.F., et al., Internal transcribed spacer polymorphism in *Pseudo-nitzschia multistriata* (Bacillariophyceae) in the Gulf of Naples: recent divergence or intraspecific hybridization?, *Protist*, 2009, vol. 160, pp. 9–20.
7. Edgar, R.C., MUSCLE: Multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Res.*, 2004, vol. 32, no. 5, pp. 1792–1797.
8. Fehling, J., Davidson, K., Bolch, C., and Tett, P., Seasonality of *Pseudo-nitzschia* spp. (Bacillariophyceae) in western Scottish waters, *Mar. Ecol.: Prog. Ser.,* 2006, vol. 323, pp. 91–105.
9. Hudson, R.R. and Kaplan, N.L., Statistical properties of the number of recombination events in the history of a sample of DNA sequences, *Genetics*, 1985, vol. 111, pp. 147–164.
10. Kimura, M., A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences, *J. Mol. Evol.*, 1980, vol. 16, pp. 111–120.
11. Lelong, A., Hégaret, H., Soudant, P., and Bates, S.S., *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: Revisiting previous paradigms, *Phycologia*, 2012, vol. 51, pp. 168–216.
12. Librado, P. and Rozas, J., DnaSP v.5: A software for comprehensive analysis of DNA polymorphism data, *Bioinformatics*, 2009, vol. 25, pp. 1451–1452.
13. Lundholm, N., Bates, S.S., Baugh, K.A., et al., Cryptic and pseudo-cryptic diversity in diatoms – with descriptions of *Pseudo-nitzschia hasleana* sp. nov. and *P. fryxelliana* sp. nov., *J. Phycol.*, 2012, vol. 48, no. 2, pp. 436–454.
14. Lundholm, N., Daugbjerg, N., and Moestrup, Ø., Phylogeny of the Bacillariaceae with emphasis on the genus *Pseudo-nitzschia* (Bacillariophyceae) based on partial LSU rDNA, *Eur. J. Phycol.*, 2002, vol. 37, pp. 115–134.
15. Lundholm, N., Moestrup, Ø., Hasle G.R., and Hoef-Emden, K.A., A study of the *Pseudo-nitzschia pseudodelicatissima/P. cuspidata* complex (Bacillariophyceae): What is *P. pseudodelicatissima*?, *J. Phycol.*, 2003, vol. 39, pp. 797–813.
16. Lundholm, N., Moestrup, Ø., Kotaki, Y., et al., Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses, *J. Phycol.*, 2006, vol. 42, pp. 464–481.
17. Mann, D., The species concept in diatoms, *Phycologia*, 1999, vol. 38, pp. 437–495.
18. Marin, B., Klingberg, M., and Melkonian, M., Phylogenetic relationships among the Cryptophyta: analyses of nuclear-encoded SSU rRNA sequences support the monophyly of extant plastid-containing lineages, *Protist*, 1998, vol. 149, pp. 265–276.
19. Martin, D.P., Lemey, P., Lott, M., et al., RDP3: A flexible and fast computer program for analyzing recombination, *Bioinformatics*, 2010, vol. 26, pp. 2462–2463.
20. Moschandreou, K.K., Baxevanis, A.D., Katikou, P., et al., Inter- and intra-specific diversity of *Pseudo-nitzschia* (Bacillariophyceae) in the northeastern Mediterranean, *Eur. J. Phycol.*, 2012, vol. 47, pp. 321–339.
21. Tamura, K., Stecher, G., Peterson, D., et al., MEGA6: Molecular evolutionary genetics analysis version 6.0, *Mol. Biol. Evol.*, 2013, vol. 30, pp. 2725–2729.
22. Quijano-Scheggia, S.I., Garcés, E., Lundholm, N., et al., Morphology, physiology, molecular phylogeny and sexual compatibility of the cryptic *Pseudo-nitzschia delicatissima* complex (Bacillariophyta), including the description of *P. arenysensis* sp. nov., *Phycologia*, 2009, vol. 48, pp. 492–509.
23. Villac, M.C. and Fryxell, G.A., *Pseudo-nitzschia pungens* var. *cingulata* var. nov. (Bacillariophyceae) based on field and culture observations, *Phycologia*, 1998, vol. 37, pp. 269–274.

Translated by T. Koznova