Marine Biology (2027) 174:5
https://doi.org/10.1007/s00227-026-04986-4

ORIGINAL PAPER

Life-stage-dependent toxin production and reproductive isolation in geographically separated populations of *Pseudo-nitzschia delicatissima*

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Received: 29 June 2026 / Accepted: 23 October 2026
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Abstract

Pseudo-nitzschia cells for clonal cultures were isolated from geographically distant populations (approximately ten thousand kilometers apart) in the Black Sea and the Sea of Japan. Reproductive compatibility with a previously identified strain from the Marine Biobank collection, together with molecular genetic analysis, confirmed that the isolated cultures belong to the toxigenic species *Pseudo-nitzschia delicatissima*. Using reproductive biology methods, successful intrapopulation sexual reproduction was achieved, whereas postzygotic isolation was observed during interpopulation sexual reproduction. Molecular genetic analysis revealed the divergence of the strains into two genotypes, Pacific and Mediterranean. In two strains of *P. delicatissima*, post-initial cells (at the beginning of their life cycle) were found, indicating cases of intraclonal reproduction. Cell length ranged from 106 µm in the largest initial cells to 16 µm in extremely small ones, which allowed us to expand the previously established upper size limit in the life cycle of *P. delicatissima*. Enzyme-linked immunosorbent assay revealed a relatively high concentration of the neurotoxic domoic acid – a compound that causes amnesic shellfish poisoning (ASP) in humans – in clonal cultures of *P. delicatissima* from the Sea of Japan and the Black Sea at the reproductive stage of the life cycle. The toxin was not detected in cells at the prereproductive stage.

Keywords *Pseudo-nitzschia delicatissima* · Sexual reproduction · Life cycle · Homothallism · Toxigenic species

Introduction

Species of the genus *Pseudo-nitzschia* H. Peragallo, 1900 are widely distributed in the world's oceans (Hasle 2002). To date, 62 validly described species of this genus have been reported in the literature (Guiry and Guiry 2021), and 26 of them have been found to contain domoic acid (DA) (Bates et al. 2018). The DA neurotoxin can accumulate in significant amounts in filter-feeding organisms and be further transmitted through food chains, posing a danger to the health and life of upper consumers (Davidovich and Bates 1998).

Despite considerable interest of researchers in toxigenic species, the life cycle and reproductive systems of representatives of the genus *Pseudo-nitzschia* remain insufficiently studied. The cell size characteristic of the pre-reproductive and reproductive phases have been described only for eleven species, and sexual reproduction has been described in some detail for fifteen species and intraspecific taxa of

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Published online: 20 November 2026

the genus *Pseudo-nitzschia* (Davidovich and Davidovich 2022). Many researchers consider heterothallic reproduction to be predominant in species of this genus (Davidovich and Bates 1998; Chepurnov et al. 2004; Amato et al. 2005; D'Alelio et al. 2008). To date, the only confirmed case of homothallism has been observed in *P. brasiliana* Lundholm, Hasle & Fryxell (Quijano-Scheggia et al. 2009a).

In heterothallic reproduction, meiosis results in the formation of two types of gametes (mt + and mt-), their fusion, zygote formation, auxospore development, and the production of initial cells that give rise to a new generation. Initial cells are typically several times larger than gametangial cells (Mann 1993, 2002; Davidovich and Davidovich 2022). A small group of planktonic pennate diatoms, including the genus *Pseudo-nitzschia*, faces challenges in delivering gametes to the site of syngamy. Unlike centric diatoms, which produce motile flagellated male gametes, pennate diatoms must synchronize meiosis with their mass development to ensure that sexual partners encounter each other in three-dimensional space. Such synchronization has been described in *P. calliantha* Lundholm, Moestrup and Hasle

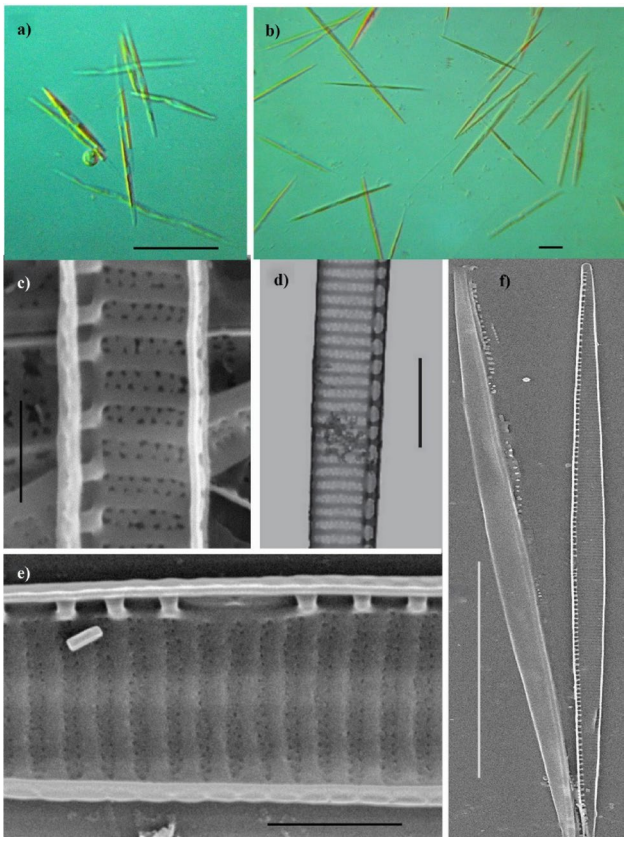

Fig. 1 *Pseudo-nitzschia delicatissima* (Cleve) Heiden live cells: (**a**) in mixture of two clones, (**b**) in monoclonal culture, light microscopy, LM. (**c**, **d**) fragment of the valve, structure of the strokes, SEM, TEM. (**e**) central part of valve showing central nodule, mantle and stria structure. **(f)** strain 25.0603-SK, SEM. Scale bar: (**a**, **f**) 20 µm, (**b**) 10 µm, (**c**, **d**, **e**) 1 µm

(Polyakova et al. 2025) and is likely to be widespread in other members of this genus.

In the *P. delicatissima* (Cleve) Heiden group, Lundholm et al. (2006) described two new species and two clades based on morphology, molecular probes, and phylogenetic analysis of the internal transcribed spacers ITS1, 5.8 S, and ITS2. The authors found significant genetic diversity among *P. delicatissima* strains and concluded that this species should be divided into two main clades representing cryptic species. Intraspecific cryptic diversity was noted during a *P. delicatissima* bloom in the Gulf of Naples (Mediterranean Sea) (Orsini et al. 2004) and also during the study of strains isolated from Russian coastal waters of the Sea of Japan (Stonik et al. 2018). Reproductive isolation as a method of establishing interspecies boundaries was not examined in these cases, although it was noted that the presence of different ITS types (Orsini et al. 2004) may underlie reproductive isolation, despite the similarity of morphological characteristics (Amato et al. 2005). A clear example where reproductive biology was successfully applied comes from the work of Quijano-Scheggia et al. (2009b). Their study of 26 Catalan strains of *P. delicatissima* not only identified two genotypes but also used evidence of reproductive isolation, combined with molecular and physiological differences, to establish one genotype as the new species *Pseudo-nitzschia arenysensis* sp. nov.

The aim of our study was to investigate the life cycle and reproductive compatibility of *P. delicatissima* strains from geographically distant populations (the Sea of Japan and the Black Sea), to identify possible phylogenetic differences, and to determine the toxigenic activity of the studied *P. delicatissima* strains during the pre-reproductive and reproductive phases of the life cycle.

Materials and methods

Study organism

The organism studied was the planktonic pennate diatom *Pseudo-nitzschia delicatissima* (Cleve) Heiden (Fig. 1a – f). Individual cells that gave rise to clonal cultures were isolated from samples collected in the Sea of Japan and the Black Sea (Table 1) using a micropipette method (Andersen and Kawachi 2005). The PD_191 strain was obtained from the Marine Biobank Center of the A. V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences (Vladivostok, Russia).

Table 1 The studied strains of *Pseudo-nitzschia delicatissima*

Strain	Salinity (‰)	Date of sampling	Strain origin
25.06.29-SL intraclonal [19.10.10-SD]*	36	June 2025	Laboratory culture [Sea of Japan, Sobol Bay]*
25.06.03-SK [24.03.15-SL+21.03.16-SC]*	36	June 2025	Laboratory culture Black Sea [44.26883 N, 34.15818 E+44.93192 N, 35.24803 E]*
25.03.13-SA [PD_19_1+22.12.20-SL]*	36	March 2025	Laboratory culture Sea of Japan [Sobol Bay+Amur Bay 43.191113 N, 131.913660 E]*
25.03.13-SE [PD_19_1+22.12.20-SL]*	36	March 2025	Laboratory culture Sea of Japan [Sobol Bay+Amur Bay 43.191113 N, 131.913660 E]*
PD_19_1	36	October 2019	Sea of Japan, Sobol Bay
22.12.20-SL	36	November 2022	Sea of Japan, Sobol Bay 43.191113 N, 131.913660 E
22.01.08-SG	18	October 2022	Black Sea, Karadag 44.93192 N, 35.24803 E
21.03.16-SC	18	March 2021	Black Sea, Karadag 44.93192 N, 35.24803 E
22.02.12-SCC	18	February 2022	Black Sea, Karadag 44.93192 N, 35.24803 E
22.10.26-ST	18	October 2022	Black Sea, Karadag 44.93192 N, 35.24803 E
22.02.16-SL	18	February 2022	Black Sea, Karadag 44.93192 N, 35.24803 E
24.03.15-SL	18	March 2024	Black Sea, Semeis 44.26883 N, 34.15818 E

*, Parental strain

Sampling and cultivation

In the laboratory, the cultures were maintained in 100 ml Erlenmeyer flasks containing modified ESAW artificial seawater medium (Polyakova et al. 2018), with salinity adjusted to match the sampling site. Cultures were kept in a temperature-controlled room at 20 ± 2 °C under natural light from a north-facing window. Crossing experiments were conducted at a oceanic salinity of 35–36‰, without acclimating the Black Sea strains to higher salinity, since their ability to reproduce within a population at elevated salinity had been previously demonstrated. For mating, strains were inoculated in pairs into glass Petri dishes (diameter 4.5 cm). Strains intended for enzyme-linked immunosorbent assay (ELISA) and phylogenetic analysis were grown in 500 mL Erlenmeyer flasks.

Weekly monitoring of *Pseudo-nitzschia* species in the natural population was conducted by sampling from a pier (100 m long) near the Karadag Biological Station. In the laboratory, samples were dispensed in 10 mL aliquots into Petri dishes (9.5 cm diameter) in triplicate. Cells were counted across 15 fields of view (at 200× magnification) using an Altami inverted optical microscope (China), and measured with an eyepiece micrometer (at 400× magnification) in each dish.

Species identification was based on morphological and biometric data obtained by light microscopy (LM), scanning electron microscopy (SEM, Hitachi SU3500, Japan), and transmission electron microscopy (TEM, Carl Zeiss LIBRA-120). Sample preparation for SEM and TEM followed the protocols described by Polyakova et al. (2023) and Stonik et al. (2018), respectively.

The relationship between cell length and cultivation time, as well as the relationship between initial and parental cell size, were approximated by linear regression. In tables and text, mean values are presented as the standard error of the mean and the number of measurements (n).

Molecular Genetic Method

Total genomic DNA was extracted following the protocol of Abdullin et al. (2021). The ITS rDNA region was amplified by PCR using primers ITS03-F and ITS055-R (Marin et al. 1998; Lundholm et al. 2003) with the Encyclo Plus PCR kit (Evrogen, Moscow, Russia). PCR products were purified with the ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix, Santa Clara, CA, USA). Sequencing in both directions was performed on an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA) at the Instrumental Center of Biotechnology and Gene Engineering of FSCEATB FEB RAS, using the BigDye Terminator v.3.1 sequencing kit (Life Technologies Corporation, Austin, TX, USA) and the same primers used for PCR. Sequences were assembled with the Staden Package v.1.4 (Bonfield et al. 1995). The obtained sequences were deposited in Genbank (taxa and accession numbers are provided in the phylogenetic tree) and aligned using SeaView program (Galtier et al. 1996).

The dataset was used to clarify the phylogenetic position of the novel clones. The alignment, initially based on the dataset of Stonik et al. (2018), was extended to include all new members of three previously identified major subclades of the genus. The final alignment comprised 54 sequences (1317 bp), with sequences of *Pseudo-nitzschia*

turgiduloides G.R. Hasle, *P. subcurvata* (Hasle) G.A. Fryxell, and *P. inflatula* (Hasle) Hasle used as the outgroup.

Phylogenetic analysis was performed using the IQ-TREE web server (Trifinopoulos et al. 2016) to determine the optimal DNA substitution model based on the Bayesian information criterion (BIC) and to infer maximum likelihood (ML) phylogenies. For the ITS dataset, the selected model was TPM2 + F+G4. The reliability of the tree topology was assessed using ultrafast bootstrap analysis (10,000 replicates) (Minh et al. 2013). Bayesian inference (BI) was conducted using MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001) with four parallel Markov chain Monte Carlo (MCMC) runs for 2 million generations, sampling every 100 generations. Chain convergence was evaluated using Tracer v.1.7.1 (Rambaut et al. 2018) to ensure that the analysis yielded a sufficient number of effective independent samples from the posterior distribution. Stationarity was determined from the "sump" plot, with the first 5000 sampled trees (25%) discarded as burn-in. Posterior probabilities (PP) were calculated from the trees sampled during the stationary phase. The robustness of the phylogenetic trees was assessed using ultrafast bootstrap percentages (UFBP) from the ML analysis and posterior probabilities from the BI analysis. Clades with UFBPs $\geq$ 0.95 and PPs $\geq$ 0.95 were considered strongly supported.

Competitive enzyme-linked immunosorbent assay

The total concentration of domoic acid (DA) in the cultures (cells plus medium) was measured using a competitive enzyme-linked immunosorbent assay (ELISA) with a commercial test system (EuroProxima, Netherlands). Because DA production in *Pseudo-nitzschia* is known to peak during the stationary phase (Lelong et al. 2013; Fernandes et al. 2014), 50 mL culture samples were collected on day 30, when cultures had reached this stage. Sample preparation followed the method described by Fehling et al. (2004). The ELISA was performed according to the manufacturer's protocol (https://food.r-biopharm.com/products/europroxima-domoic-acid/) and in accordance with the certified methodological guideline for measuring domoic acid in seafood using ELISA (MUK K003-2023, developed by Komprodservice LLC). This guideline is certified under Order No. 4091 of the Ministry of Industry and Trade of the Russian Federation (dated December 15, 2015) and complies with GOST R 8.563–2009 (https://komprod.com/wp-content/uploads/2021/03/muk-k003-2023-fikotoksiny.pdf). Toxin concentrations, expressed as DA pg cell^{-1}, were measured as total DA in the whole culture divided by the number of cells present at culture harvesting.

The buffer-diluted sample was analyzed in duplicate. The procedure included the following steps: (1) addition of standards and samples to the microplate wells; (2) addition of conjugate and antibody solutions followed by incubation; (3) plate washing; (4) substrate addition and incubation; (5) addition of a stop solution; (6) measurement of optical density. Optical density was measured at 450 nm using a BioTek ELx800 microplate photometer (BioTek, USA). Data were processed using RIDASOFT® Win. software NET.

Results

Morphology

The biometric characteristics of our *Pseudo-nitzschia delicatissima* strains were compared with the literature data (Table 1). Most morphometric parameters corresponded to those previously reported, except for cell length, which was higher in our study (106 µm) compared to the previously noted maximum of 94 µm (Amato et al. 2005). The degree of cell overlap was constant (regardless of the cell length) both in the natural population and in the culture (Table 2).

Sexual process and reproductive compatibility

Within each population, strains readily intercrossed when reproductively compatible. In interpopulation crosses between Black Sea strains and strains isolated from the Ussurii and Peter the Great bays (Sea of Japan), prezygotic isolation was absent; active gametogenesis was observed, followed by zygotes and auxospore formation. However, postzygotic isolation occurred, since no initial cells were formed (Table 3).

The cis-anisogamous sexual process, characteristic of the genus *Pseudo-nitzschia*, involves the formation of two active gametes in the male gametangium (mating type mt1) and two passive gametes in the female gametangium (mt2). In male cells, the protoplast divides apically, whereas in female cells, division is transapical. Gamete reorganization occurs in the first gametangium but not in the second. Following gamete fusion, two zygotes are formed. Zygotes and forming auxospores are attached perpendicularly to the female gametangium and remain parallel to each other (Fig. 2a–f). This type of sexual reproduction corresponds to category IA2 in Geitler's classification (Geitler 1973).

The size range in the life cycle

A correlation was found between the size of initial and parental cells in *P. delicatissima* (Fig. 3). The apical size of parental cells (gametangia) ranged from 20 to 45 µm, while initial cells measured between 63 and 106 µm. During three years of observation (2022–2025), Black Sea strains of *P.*

Table 2 Comparison of biometric characteristics of *Pseudo-nitzschia delicatissima* (Cleve) Heiden, according to literature data

Valve length (µm)	Valve width (µm)	Stria, per 10 (µm)	Fibulae, per 10 (µm)	Poroids, per 1 (µm)	Poroid, series	Cell overlap (µm)	Sources
16–106	**2.0–2.5**	**35–39**	**19–24**	**12–13**	**2**	**1/3 − 1/18 (6 µm)** (n = 300)	**Our data**
19–76	1.5–2.0	35–40	19–26	8–12	2	–	Lundholm et al. (2006)
40–76	1.0–2.0	36–40	19–25	10–12	2	–	Hasle (1965)
8–94	2.65–2.78	33–43	22–29	6–11	–	–	Amato et al. (2005)
42–66	1.0–1.5	40	20–23	10–12	2	1/9	Hasle et al. (1996)
37.5 − 77.5	0.9–1.8	37–43	20–26	8–12	–	1/5 − 1/10	Skov et al. (1999)
27–88	1.0–2.2	36–40	20–23	10–12	–	1/9	Ferrario et al. (2004)
50–70	0.6–1.8	–	18–26	8–9	2	–	Caroppo et al. (2005)
24.2–40.0	1.5–2.3	36–42	21–24	7–12	2	–	Rivera-Vilarelle et al. (2013)
39–71	1.3–1.7	37–42	20–30	9.0 − 12.5	2	–	Kaczmarska et al. (2005)
19–78	1.0–2.4	35–42	19–28	8–12	2	–	Quijano-Scheggia et al. (2008)

Table 3 Reproductive compatibility of *Pseudo-nitzschia delicatissima* strains isolated from the Black Sea and the Sea of Japan populations

Location	Clone	mating type	PD_19_1	22.12.20-SL	22.12.13-SB	23.05.02-SB	23.05.02-SC	23.03.17-SG	22.02.12-SCC	22.01.08-SG	22.02.25-SP	21.03.01-SZ	21.03.16-SC	22.02.16-SL	22.10.26-ST	24.03.15-SL
			mt1	mt2	mt2	mt2	mt2	mt1	mt1	mt1	mt1	mt1	mt1	mt1	mt2	mt2
Japan Sea, Sobol Bay	PD _ 19_1	mt1														
Japan Sea, Sobol Bay	22.12.20-SL	mt2	+													
Japan Sea, Sobol Bay	22.12.13-SB	mt2	+	0												
Japan Sea, Peter the Great	23.05.02-SB	mt2	+	-	-											
Japan Sea, Peter the Great	23.05.02-SC	mt2	+	-	-	-										
Black Sea, Karadag	23.03.17-SG	mt1	0	x	0	-	-									
Black Sea, Karadag	22.02.12-SCC	mt1	0	x	0	-	-	-								
Black Sea, Karadag	22.01.08-SG	mt1	0	x	0	-	-	-	-							
Black Sea, Karadag	22.02.25-SP	mt1	0	x	0	-	-	-	-	-						
Black Sea, Karadag	21.03.01-SZ	mt1	0	x	0	-	-	-	-	-	-					
Black Sea, Karadag	21.03.16-SC	mt1	0	x	-	-	-	-	-	-	-	-				
Black Sea, Karadag	22.02.16-SL	mt1	0	x	-	-	-	-	-	-	-	-	-			
Black Sea, Karadag	22.10.26-ST	mt2	x	0	-	-	-	0	-	-	-	-	-	0		
Black Sea, Semeis	24.03.15-SL	mt2	x	0	-	-	-	+	+	+	+	+	+	+	0	

+, Successful sexual reproduction observed; **0**, sexual reproduction not observed; **x**, gametes, zygotes, and auxospores observed without formation of initial cells; assessment of auxosporulation in mixtures; -, no data available; dash and dot outlines indicate zones of inter- and intrapopulations mating accordantly

delicatissima exhibited a gradual and proportional decrease in apical length, at an average 0.84 ± 0.3 µm per month (n = 6). Cells of strain PD_19_1, isolated from the Sea of Japan (Ussurii Bay), reproduced intraclonally after decreasing to 17.7 µm by the end of 2022. Although this event was not directly observed, intraclonal reproduction is evidenced by the appearance of larger cells (40.5 µm; Fig. 4) in the culture in August 2023. This suggests that auxosporulation had occurred earlier, producing larger initial cells at that time. Over time, small cells disappeared from the regularly re-inoculated culture, and no further intraclonal sexual reproduction was observed. When cell size in the culture decreased to about 32 µm, cells became capable of reproduction in mixtures with sexually compatible clones. However, no sexual reproduction was observed in the culture itself. In May 2025, a case of homothallism was reported in strain 19.10.10-SD from the Sea of Japan, which had been in culture since 2019 and had diminished to 16 µm. As a result of reproduction, initial cells with a length of 63 µm appeared (Fig. 2g, h). Cells that did not participate in sexual reproduction died, thereby determining the lower limit of the life cycle for this strain.

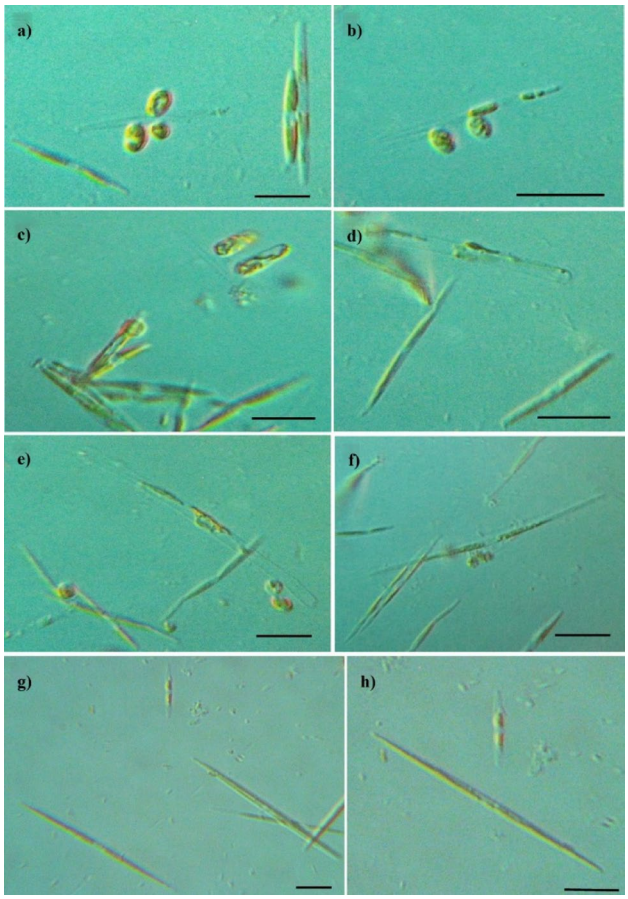

Fig. 2 Sexual process occurring in a mixture of *Pseudo-nitzschia delicatissima* strains. (**a**, **b**) gametes, zygotes, (**c**, **d**, **e**) growing auxospores, (**f**) initial cell, (**g**, **h**) the result of homothallic sexual reproduction of the *P. delicatissima* 19.10.10-SD strain, gametangial and post-initial cells. LM. Scale bar: (**a - f**) 20 µm, (**g**, **h**) 10 µm

By initiating the sexual process, we obtained large cells that extended the previously known upper size limit for *P. delicatissima* from 94 µm to 106 µm (Table 2). The size range of gametangial cells constitutes only 20% of the total size range in the life cycle (Fig. 5).

Population dynamics

Weekly monitoring of the natural phytoplankton community in the waters of the Karadag Nature Reserve (Black Sea) during 2025 (from January to June inclusive) revealed relatively stable cell numbers of *Pseudo-nitzschia*. Minor peaks in abundance (20–60 cells mL^{-1}) were recorded in the spring, from March to May. A sharp increase in the abundance of *P. delicatissima* (up to 160 cells mL^{-1}) was observed in early June (Fig. 6a). Cells of other *Pseudo-nitzschia* species occurred only sporadically.

Cell size within the population was measured before and during this surge (04.06.2025; 10.06.2025; 16.06.2025). Analysis showed an inverse relationship between the proportion of sexually induced cells and large cells approaching the species-specific maximum size, indicating ongoing sexual reproduction (Fig. 6b, arrows).

Phylogenetic analyses

The ITS rDNA sequences were successfully amplified and sequenced only for five *Pseudo-nitzschia delicatissima* strains: three from the Sea of Japan (strains 25.03.13-SA, 25.03.13-SE, and 22.12.20-SL) and two from the Black Sea (strains 22.01.08-SG and 21.03.16-SC), yielding an amplification success rate of approximately 42% for the newly isolated strains. Phylogenetic relationships within the *Pseudo-nitzschia delicatissima* complex were inferred from

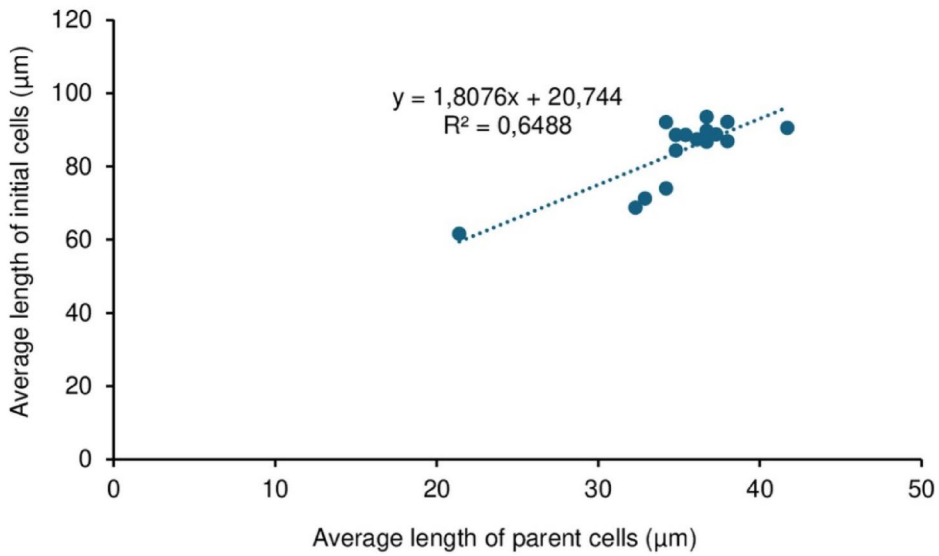

Fig. 3 Dependence of the size of initial cells on the parental cell sizes in *Pseudo-nitzschia delicatissima*

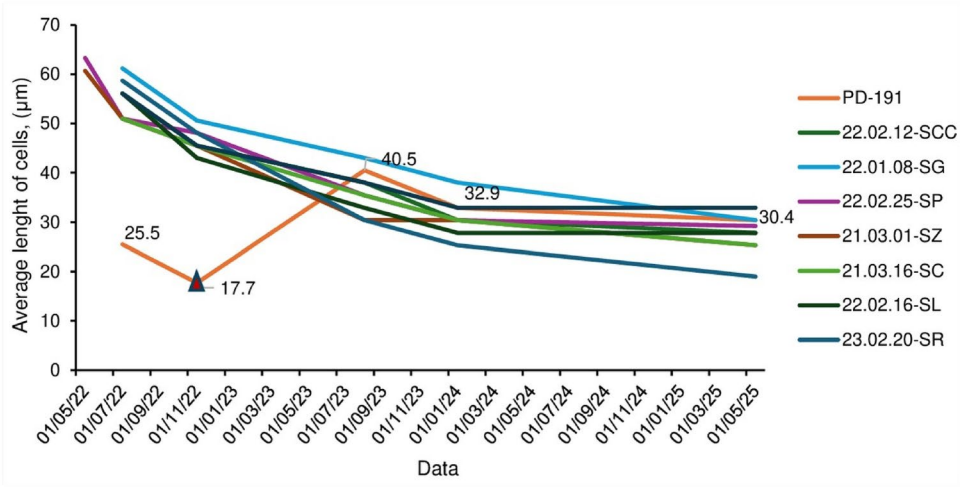

Fig. 4 Changes in the size of seven strains of *Pseudo-nitzschia delicatissima* maintained in the exponential growth phase (ESAW medium) at a constant temperature of 20±2 °C under natural light from a north-facing window. ▲ – at this size, homothallic reproduction was observed in strain PD_191_1

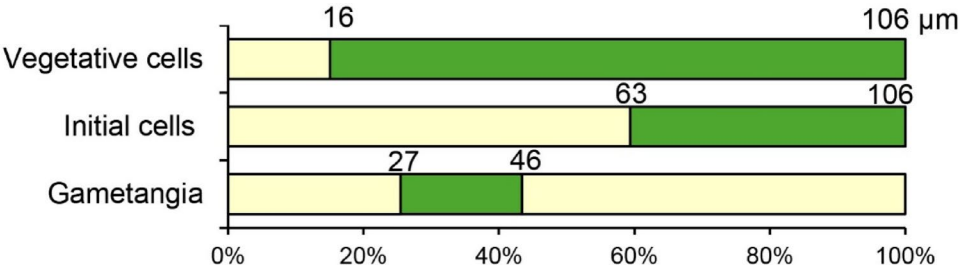

Fig. 5 Diagram showing the cell sizes of the diatom *Pseudo-nitzschia delicatissima* at different phases of the life cycle. Pre-reproductive phase (Initial cells), reproductive phase (Gametangia), the total size range of cells (Vegetative cells). The x-axis shows the cell size in micrometers and percentages

an ITS rDNA dataset of 54 sequences, with a total alignment length of 1317 bp. Representatives of *P. turgiduloides*, *P. subcurvata*, and *P. inflatula* served as outgroups. Phylogenetic reconstructions using Maximum Likelihood (ML) and Bayesian Inference (BI) produced congruent topologies. The analysis confirmed the identity of all investigated strains as *P. delicatissima* (Fig. 7). Within this well-supported clade (96/1.00), three subclades were resolved only topologically (Fig. 7). Most basal nodes of the tree received high statistical support.

Strains from the Sea of Japan (25.03.13-SA, 25.03.13-SE, and 22.12.20-SL) formed a distinct and well-supported lineage within Subclade I. This subclade predominantly comprises sequences from the Pacific region (Japan, China, Tasmania, USA, and New Zealand), along with a single isolate from Namibia (southern Atlantic Ocean). The clustering of these strains reflects a clear phylogeographic signal associated with the Pacific basin. Black Sea strains (22.01.08-SG, 21.03.16-SC) clustered together with isolates from the Mediterranean region (France, Spain, Italy, and one accession of unknown origin), forming Subclade III. This subclade was clearly separated from the Pacific lineage, suggesting genetic differentiation between Atlantic–Mediterranean and Pacific populations. Subclade II comprised sequences from predominantly Atlantic regions and occupied a sister position to the other subclades within *P. delicatissima*.

Overall, the ITS phylogeny reveals pronounced genetic structuring within *P. delicatissima*, with geographically coherent subclades corresponding to major oceanic regions. Although internal resolution within the subclades was relatively low, their divergence was consistently supported by both ML and BI analyses.

Enzyme immunoassay

An enzyme-linked immunosorbent assay (ELISA) revealed a relatively high concentration of domoic acid (DA) in gametangial cells of *Pseudo-nitzschia delicatissima* (Table 4). The highest concentrations of DA were observed in the Black Sea strain 22.10.26-ST, 22.02.12-SCC and 22.02.16-SL, which were in the reproductive phase of their life cycle. In contrast, DA was not detected in the post-initial cells resulting from sexual reproduction.

Discussion

Changing the size in the life cycle

Our study showed that the initial cells obtained as a result of successful reproduction cover almost half of the entire size range in the life cycle (Fig. 5). This wide range shortens the period of vegetative growth, allowing relatively small

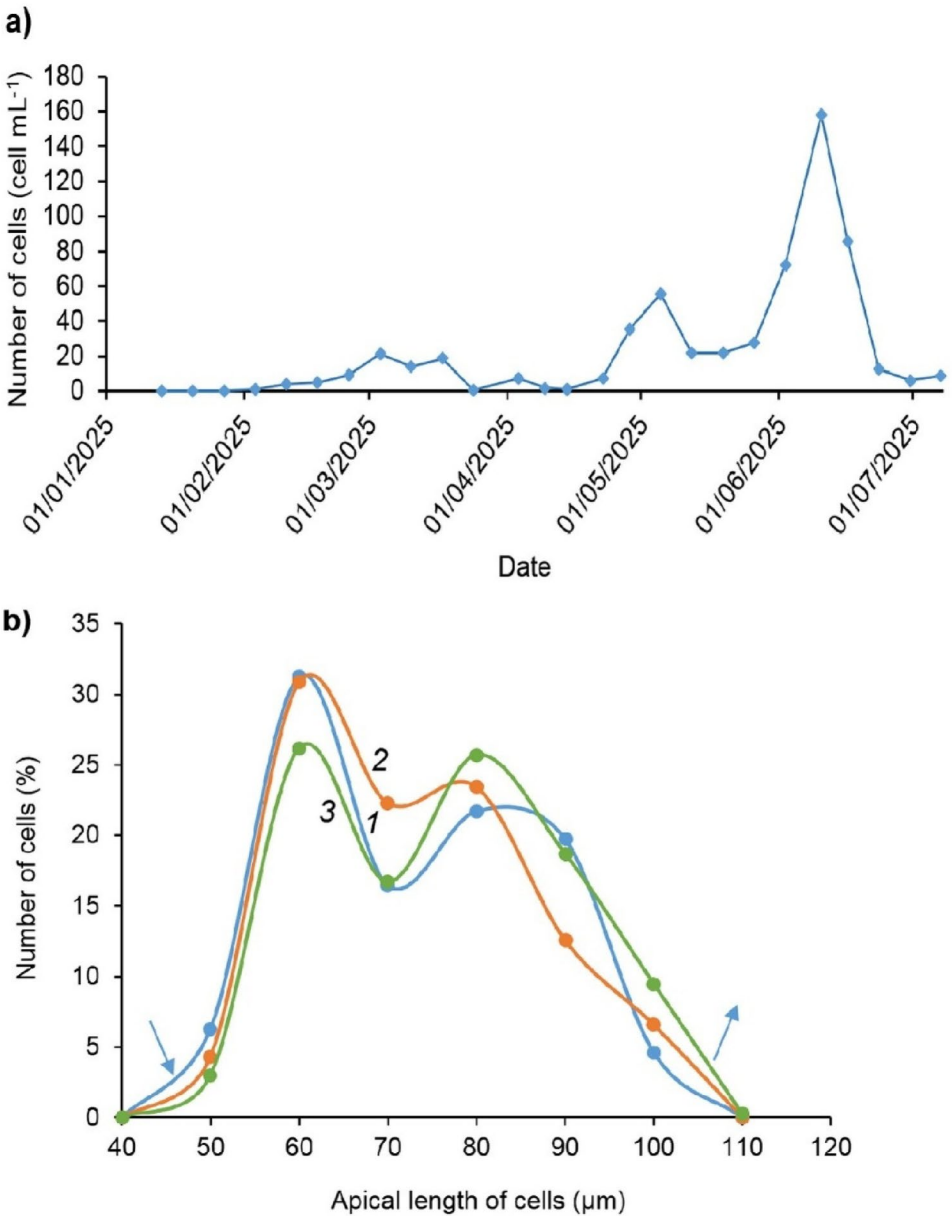

Fig. 6 (**a**) Seasonal variability of the number of cells of the genus *Pseudo-nitzschia* H. Peragallo, 1900 in the coastal waters near Karadag biostation in 2025. (**b**) Cell size distributions in plankton samples, showing three time points from the beginning of the increase in number of cells to its decrease. 04.06.2025 (*1*), 10.06.2025 (*2*), 16.06.2025 (*3*)

post-initial cells to enter the sexual process, if not immediately, then after a very short period of time. As a result, a two-stage formation of auxospores is possible (Roshchin 1994; Chepurnov et al. 2004). In our case, gametangial cells, unlike initial cells, cover only about 20% of the entire size range of the life cycle. Typically, gametangial cells occupy a wider size range, from 30% to half of the total range (Davidovich and Davidovich 2022).

For *Pseudo-nitzschia delicatissima*, as for most diatoms, size decreases throughout the life cycle. Amato and co-authors (2005) indicated that *P. delicatissima* cells can decrease to 8 µm in length. However, they note that such small cells (less than 30 µm) were not observed in natural populations, which raises the question of why these small cells are not preserved in the population. Monitoring of the cell sizes in culture allowed us to observe cases of homothallic reproduction in two strains when they reached 17.7 and 16 µm. It is worth noting that, despite the reduction in the size of *P. delicatissima* cells to 8 µm, Amato and colleagues did not observe intraclonal reproduction.

Previously, a relatively high rate of cell length reduction was reported for *P. delicatissima*, averaging from 1.6 to 3.5 µm per week, and reaching up to 8.6 µm per week in the largest cells (Amato et al. 2005; Quijano-Scheggia et al. 2009b; Amato and Orsini 2015). According to our data, the rate of cell size reduction in *P. delicatissima* cultures is low (on average 0.84 ± 0.3 µm per month). This difference is possibly due to differences in cultivation conditions.

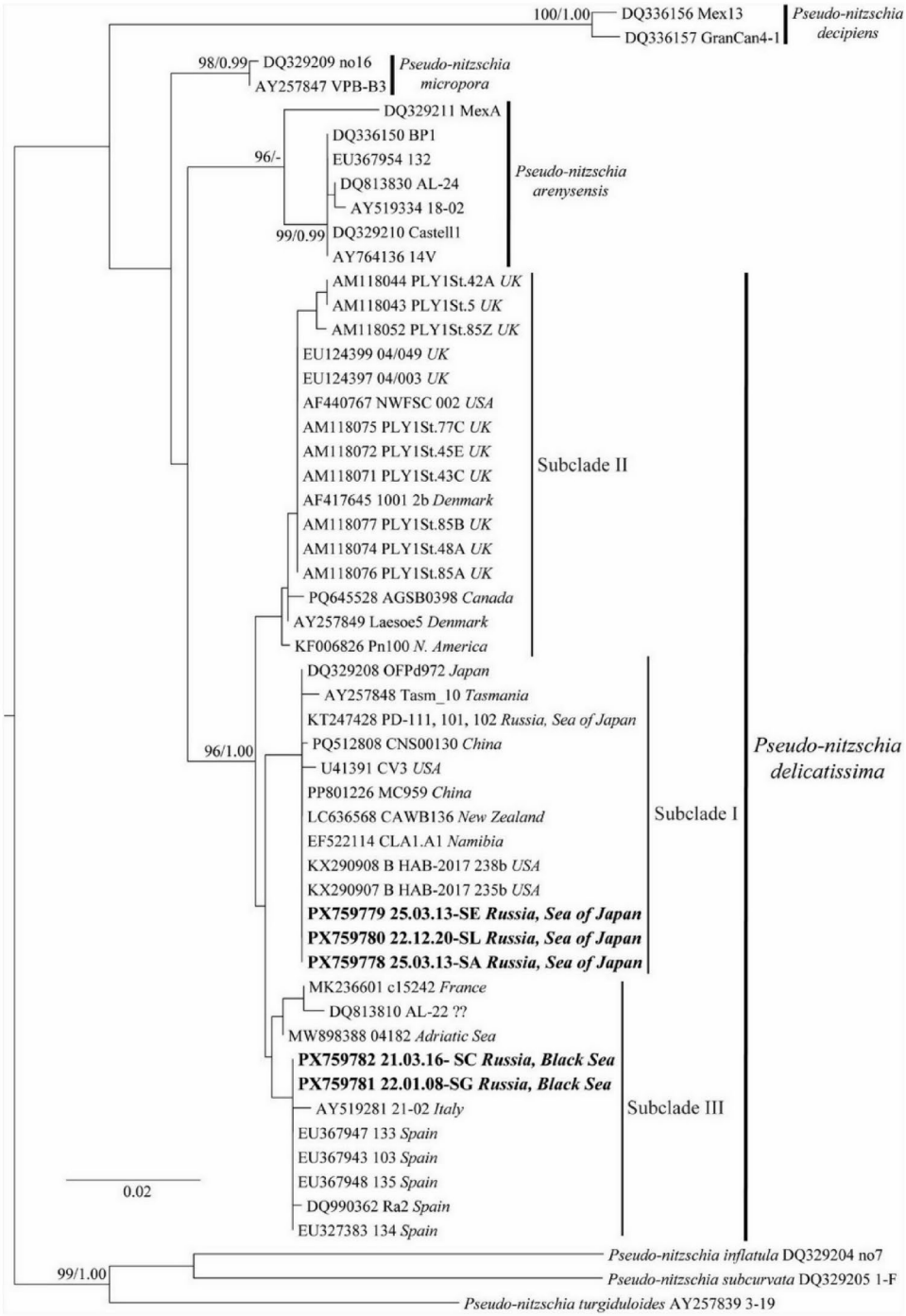

Fig. 7 ML phylogenetic tree showing position of novel strains (bold) based on the ITS rDNA sequence data (54 accessions). Support values (UFBP)≥95%/(PP)≥0.95 are given on branches. The sequences have GenBank accession numbers, strain/voucher names, and geographic data for *Pseudo-nitzschia delicatissima* samples. Scale bar is substitutions per nucleotide position

Genetic differentiation and phylogenetic implications

The ITS rDNA phylogeny obtained in this study confirms the assignment of all newly analyzed strains to *Pseudo-nitzschia delicatissima* and reveals a structured pattern of genetic variation within the species. Both Maximum Likelihood and Bayesian analyses recovered congruent overall topologies, indicating a stable phylogenetic signal in the dataset. However, the relatively low success rate of amplification and sequencing of the rDNA ITS region in this study (~42%) represents a significant limitation of the molecular analysis. Only five newly isolated strains yielded reliable ITS sequences, and we cannot exclude the possibility that the unsuccessfully amplified strains harboured additional genetic variants that are not represented in the phylogeny. The specific causes of PCR failure were not investigated and may include technical factors such as primer-template

Table 4 Domoic acid concentration determined by ELISA/ELISA in *Pseudo-nitzschia delicatissima* cultures from the Black Sea and the Sea of Japan

Strain	Cell length (µm) ($n=30$)	Cell density (cells mL^{-1})	Total concentration of DA in culture	
			ng mL^{-1}	pg $cell^{-1}$
25.06.29-SL (intraclonal [19.10.10-SD])	55.6*± 0.1	228.571±68.570	ND	ND
25.06.03-SK [24.03.15-SL+21.03.16-SC]	96.3*± 0.1	78.400±23.520	ND	ND
25.03.13-SA [PD_19_1+22.12.20-SL]	73.0*± 0.3	466.285±139.885	ND	ND
25.03.13-SE [PD_19_1+22.12.20-SL]	63.3*± 0.2	461.714±138.514	ND	ND
PD_19_1	30.4±0.5	251.657±75.497	2.32±0.69	0.009±0.003
22.12.20-SL	32.9±0.9	791.771±237.531	8.32±2.50	0.010±0.003
22.01.08-SG	33.9±0.2	220.114±66.034	6.05±1.82	0.027±0.083
21.03.16-SC	25.3±0.8	165.257±49.577	7.69±2.31	0.046±0.014
22.02.12-SCC	27.8±0.2	53.257±15.977	9.42±2.83	0.177±0.053
22.10.26-ST	40.5±0.7	189.714±56.914	14.86±4.46	0.078±0.024
22.02.16-SL	25.3±1.6	158.171±47.451	13.03±3.91	0.082±0.025
24.03.15-SL	43.0±2.9	167.543±50.263	7.48±2.24	0.045±0.0137

*, Cells in the beginning of the life cycle; ND – not detected (the limit of measurement sensitivity (<0. 15 ng mL^{-1}))

mismatches, template quality or quantity, and variation among multiple rDNA copies. Such intragenomic variation is particularly relevant for *Pseudo-nitzschia*: multiple ITS types within individual strains of *P. delicatissima* have been documented, demonstrating substantial intragenomic variation in this region (Orsini et al. 2004). Although this study concerns the 18 S rDNA V4 region rather than ITS, extensive intragenomic variation has also been demonstrated in *P. multistriata*, indicating that rDNA heterogeneity may be a broader feature of the genus (Wang et al. 2024). Therefore, the present ITS dataset should be considered representative of the successfully sequenced isolates rather than exhaustive of the genetic diversity present in the sampled populations.

Within the *P. delicatissima* clade, several geographically associated lineages can be distinguished. Strains from the Sea of Japan grouped together within a predominantly Pacific assemblage, whereas the Black Sea strains clustered with isolates from the Mediterranean region. This pattern is consistent with previously reported phylogeographic structuring in the *P. delicatissima* complex (Orsini et al. 2004; Amato et al. 2005; Lundholm et al. 2006; Quijano-Scheggia et al. 2009b; Stonik et al. 2018) and supports the view that genetic differentiation within this taxon is not randomly distributed, but is at least partly associated with major oceanic basins.

Although internal relationships among some sublineages remain only moderately resolved, the recurrent recovery of geographically coherent groups across analytical approaches suggests that the observed subdivision reflects biologically meaningful structuring rather than analytical artefacts. Such a pattern is typical of species complexes with extensive dispersal potential but constrained long-term gene flow between oceanographic regions.

Importantly, the molecular structuring detected here corresponds to the results of crossing experiments. In mixed cultures involving Black Sea and Sea of Japan strains, postzygotic reproductive isolation was observed. Although gametogenesis, syngamy, and auxospore formation occurred in mixed cultures, no viable offspring were produced, indicating the presence of intrinsic postzygotic barriers. The concordance between genetic clustering and reduced reproductive compatibility suggests that the detected lineages represent partially differentiated evolutionary units within *P. delicatissima* sensu lato.

At the same time, the overall level of ITS divergence among the identified lineages falls within the range generally reported for intraspecific variability in *Pseudo-nitzschia*. As emphasized by several authors, ITS-based lineages alone cannot be unequivocally treated as distinct species without consistent evidence of complete reproductive isolation and/or stable diagnostic morphological characters (Amato et al. 2007; Vanormelingen et al. 2008). Rather, our data support the interpretation of *P. delicatissima* as a genetically structured species complex undergoing ongoing divergence.

The association of Black Sea strains with Mediterranean isolates may reflect historical connectivity within the Atlantic–Mediterranean system, whereas the distinct position of Pacific strains is compatible with more prolonged or recurrent geographic separation. Whether the observed postzygotic barriers represent an early stage of speciation or a stable form of intraspecific incompatibility requires further investigation using additional genetic markers and broader sampling.

Overall, the integration of molecular phylogeny and reproductive biology provides a coherent framework for interpreting genetic diversity within *P. delicatissima*. Our results indicate that *P. delicatissima* represents a species

complex characterized by cryptic genetic diversity, partial reproductive isolation, and ongoing evolutionary differentiation across oceanic regions, while remaining consistent with a conservative taxonomic treatment of the species at the present stage of knowledge.

DA production

Pseudo-nitzschia delicatissima is a cosmopolitan species and is one of the 26 species within the genus that have a confirmed ability to produce DA toxin (Bates et al. 2018). Numerous hypotheses regarding DA production have been proposed in the literature. Many researchers associate DA production with nutrient availability (Lelong et al. 2012; Trainer et al. 2012; Bates et al. 2018). Numerous studies demonstrate that a deficiency or, conversely, an excess of phosphorus and silicon is not a "trigger" for increased DA production in *P. australis* (Garrison et al. 1992; Cusack et al. 2002; Howard et al. 2007; Cochlan et al. 2008; Thorel et al. 2014; Radan and Cochlan 2018; Ayache et al. 2019; Kudela et al. 2020; Wingert and Cochlan 2021). *P. multiseries* increases DA production during the transition to the stationary phase, a condition in which cell division slows due to limitation by macronutrients such as phosphorus (P) or phosphate (PO_4^{3-}), or silicon in the form of silicic acid [$Si(OH)_4$] (Bates 1998; Bates and Trainer 2006). However, no relationship has been found between DA concentrations and the concentrations of silicon or phosphorus (Trainer et al. 2009a, b; Trick et al. 2018; Cochlan et al. 2023).

Extracellular bacteria isolated from cultures of *P. multiseries* were unable to produce DA autonomously, even in the presence of *P. multiseries* exudates (Bates et al. 2004). However, the SEM used in that study revealed the presence of epiphytic bacteria on *P. multiseries* cells, which does not rule out their potential role in DA production. It has been noted that, in the presence of other organisms, toxigenic *Pseudo-nitzschia* species increase DA synthesis (Lelong et al. 2014; Sison-Mangus et al. 2014; Sauvey et al. 2021). This may be a stress response triggered by the presence of other organisms. In our study, we did not examine the bacterial composition of the cultured strains. The initial cells used to establish the strain cultures were isolated from a mixture of parental cells, and all cultures (Table 4) were maintained under identical growth conditions. Therefore, although a bacterial contribution cannot be formally excluded, any such effect would have been identical across all cultures and thus cannot account for the differences in DA concentrations observed between strains. In their study, Lelong et al. (2014) reported species-specific responses to bacteria: DA production was unaffected in *P. delicatissima*, but increased in *P. multiseries* in the presence of bacteria. Notably, they do not provide dimensional characteristics of the studied species – information that is also essential when measuring DA, as our study illustrates.

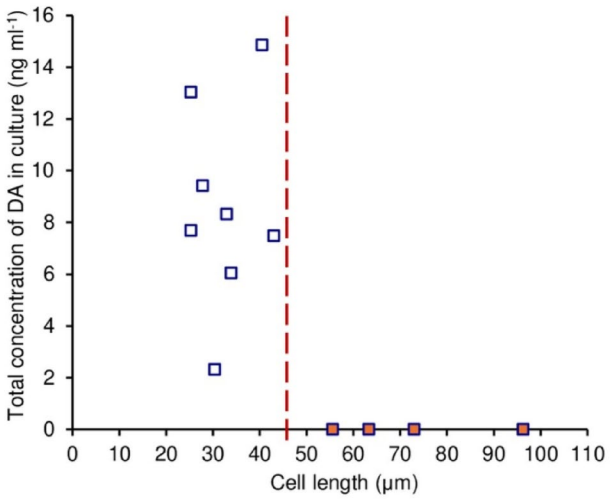

Fig. 8 Total DA concentration (ng mL^{-1}) in cultures of different cell sizes (µm). Cells in the pre-reproductive phase are shown by colored squares; cells in the reproductive phase of the life cycle are shown as squares without shading. The red dashed line indicates the border between the pre-reproductive and the reproductive phase of the life cycle (see Fig. 5)

The data from the ELISA on the DA toxin content in our *P. delicatissima* strains revealed differentiation between cells in the reproductive phase of the life cycle and post-initial cells at the beginning of the life cycle. Analysis of these data shows that the toxin content depends on the life cycle phase. In post-initial cells with a size range of 55.6–96.3 µm that have not reached the critical threshold for transition from the pre-reproductive phase of the life cycle to the reproductive phase, DA was not detected (Fig. 8). In all studied strains, cells in the reproductive phase of the life cycle (cells with a size range of 25.3–43.0 µm) were capable of producing DA. The values of cellular DA concentration in gametangial cells for studied strains ranged from 0.009 to 0.177 pg $cell^{-1}$, which is close to the concentrations previously reported for other isolates of this species (0.005–0.12 pg $cell^{-1}$) (Trainer et al. 2012). The highest concentrations of DA were recorded in the Black Sea strains: 22.10.26-ST, 22.02.16-SL, and 22.02.12-SCC with apical lengths of 40.5, 25.3, and 27.8 µm respectively. The apical length of these cells did not correlate with the total and cellular concentration of DA (Table 4).

It has been supposed that DA production in *Pseudo-nitzschia* is related to culture age and cell size (Sauvey et al. 2019). However, among the three toxigenic species studied (*P. australis*, *P. fraudulenta* and *P. pungens*), this was confirmed only for *P. australis*, suggesting a link between toxin production and the life cycle. For large cells of *P. australis*, the DA concentrations were low but not zero. Our

data showed an undetectable level of DA in cells that had not reached sexual maturity, while sexually inducible cells were capable of producing it. We also cannot completely rule out the possibility that the post-initial cells can produce extremely low concentrations of DA. However, we cannot estimate these concentrations because they are below a limit of measurement sensitivity (<0.15 ng mL^{-1}) of the ELISA method we used.

An increase in DA content in exponentially growing *P. australis* and *P. fraudulenta* (without nutrient restriction) was induced by factors of 14 and 58 in the presence of the oyster *Crassostrea gigas* (Thunberg 1793) and by factors 30 and 24 times in the presence of the scallop *Pecten maximus* (Linnaeus 1758), respectively (Sauvey et al. 2021). Whether this induced DA production deters grazers is unknown; the toxin might not inhibit feeding at all. Nevertheless, considering the density-dependent reproduction of *P. delicatissima* (Polyakova et al. 2025), we speculatively suggest that if DA has any defensive property, its secretion could be an adaptive trait to mitigate predation pressure and sustain the population size necessary for successful mating.

Conclusion

As in most other diatoms, the apical cell size of *Pseudo-nitzschia delicatissima* decreases throughout the life cycle. Cells that had decreased to about 46 µm became capable of reproduction in cross-cultures, producing initial cells longer than 100 µm. The longest initial cell (106 µm) was observed in a mixture of large, sexually competent cells. Intraclonal reproduction occurred in strains where cells had decreased to 18 and 16 µm, yielding initial cells of 63 and 56 µm, respectively. Our study revealed a correlation between the size of the initial and their parental cells in *P. delicatissima*. These are the first recorded cases of homothallism in this species. Using reproductive biology methods, we achieved successful intrapopulation sexual reproduction in *P. delicatissima* strains, while noting postzygotic isolation during interpopulation crosses. Phylogenetic analysis confirmed that all the studied strains belong to *P. delicatissima*. The Black Sea strains formed a distinct subclade together with isolates from the Mediterranean region. This subclade was topologically separated from the Pacific lineage, indicating genetic differentiation between the Atlantic-Mediterranean and Pacific populations. Although the internal resolution of some relationships was limited, the recurrent recovery of geographically associated groups suggests structured intraspecific variability rather than random genetic heterogeneity. Therefore, we regard *P. delicatissima* as a genetically structured species exhibiting population-level differentiation across distant marine regions, while remaining within the limits of a single, morphologically coherent taxon. Enzyme-linked immunosorbent assay (ELISA) data on domoic acid (DA) content showed relatively high toxin concentrations in gametangial cells of *P. delicatissima*. In contrast, DA was not detected in post-initial cells of the new generation resulting from sexual reproduction. Thus, as cells decrease in size and enter the reproductive phase, they acquire the ability to reproduce sexually and to produce the toxin.

Acknowledgements The authors express their gratitude to the head of the microscopy laboratory Vyacheslav Nikolaevich Lishaev for assistance in obtaining SEM images.

Author contributions SLP: Conceptualization, methodology, investigation, formal analysis, data curation, visualization, writing – original draft. NAD: Methodology, formal analysis, visualization, writing – review & editing. IVS: Methodology, investigation, formal analysis, data curation, visualization, editing. AYuN: Methodology, investigation, formal analysis, data curation, visualization, editing. VYuN: Methodology, investigation, formal analysis, data curation, visualization, editing. TYuO: methodology, investigation, formal analysis, editing.

Funding This work was supported by the Ministry of Science and Higher Education of the Russian Federation, project number 124030100100-0. Immunoenzymatic analysis of the toxin content in cultures was carried out as part of the "Dynamics of Marine Ecosystems, Adaptation of Marine Organisms and Communities to Habitat Changes," state registration number 121082600038-3. Molecular genetic analysis was carried out within the state assignment of the Ministry of Science and Higher Education of the Russian Federation, project number 124012400285-7.

Data availability The obtained sequences are deposed in Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Other data are presented in this article.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval No approval of research ethics committees was required.

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