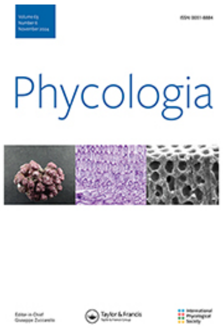

Phycologia

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ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/uphy20

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To cite this article: Nickolai Davidovich, Olga Davidovich, Vyacheslav Nikulin, Julijana Cvjetinovic & Dmitry A. Gorin (05 Mar 2025): Sexual reproduction of the ultrahalophilic diatom *Nitzschia siwaschica sp. nov*, Phycologia, DOI: 10.1080/00318884.2025.2471608

To link to this article: https://doi.org/10.1080/00318884.2025.2471608

PHYCOLOGIA
https://doi.org/10.1080/00318884.2025.2471608

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Sexual reproduction of the ultrahalophilic diatom *Nitzschia siwaschica sp. nov*

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ABSTRACT
We describe the sexual reproduction and breeding behaviour of a novel sigmoid pennate diatom *Nitzschia siwaschica sp. nov*. To date, sexual reproduction has been described in 21 representatives of the highly heterogeneous genus *Nitzschia*, including three sigmoid freshwater species. *Nitzschia siwaschica* originates from a hypersaline environment. The specific growth rate of *N. siwaschica* weakly depended on salinity levels, varying from 0.75 to 0.99 divisions per day, across salinity levels from 10 to 100 psu. This diatom exhibited heterothallic sexual reproduction. Compatible pairs of clones were able to reproduce sexually in the relatively broad range of salinities, 20–80 psu. According to Geitler's classification, the pattern of sexual process in this species (*cis*-anisogamy) corresponds to type IA2b. Furthermore, we determine the phylogenetic relationships of *N. siwaschica* with other *Nitzschia* species to provide a comprehensive understanding of the species' evolutionary position.

ARTICLE HISTORY
Received 27-03-2024
Accepted 20-02-2025
Version of Record 05-03-2025

KEYWORDS
Cis-anisogamy; Halophyte; Heterothally; Salinity tolerance; Sigmoid diatoms

INTRODUCTION

A minority of diatoms displays euryhaline characteristics, with a spectrum of salinity tolerance limits (Schultz & McCormick, 2013). Particularly, diatoms thriving in hypersaline environments exhibit remarkable halotolerance, enduring osmotic stress across a broad salinity spectrum. Conversely, certain diatom species exhibit a narrower habitat range, restricted to specific environments.

Sivash Bay, situated in the Sea of Azov, is the world's largest hypersaline lagoon, currently exhibiting salinity levels ranging from 30 to 120 psu (*Shadrin et al.*, 2019). There are man-made shallow salt ponds near the bay that were used for salt production. The salinity in these ponds is many times higher than in the bay and can reach levels that cause crystallization and precipitation of salts. In the region, the total solar radiation is approximately 4.9 $\mathrm{GJ{\cdot}m^{-2}{\cdot}year^{-1}}$ (Gorbunov *et al.*, 2020), contributing to water temperature exceeding 35°C during summer months, with heightened UV radiation exacerbating environmental harshness.

Despite the environmental distinctness of the area, data regarding microalgal diversity remain scarce. Analysis of microphytobenthos samples collected in the lagoon identified 35 diatom species, including *Nitzschia sigma* (Kützing) W.Smith (Shadrin *et al.*, 2021), previously observed in the Sea of Azov (Ryabushko & Bondarenko, 2017) and Crimean hypersaline lakes (Nevrova & Shadrin, 2005). Lange-Bertalot and Van de Vijver highlighted the taxonomic ambiguity surrounding *N. sigma*, indicating varied application of the name across distinct marine and freshwater habitats (Lange-Bertalot & Van de Vijver, 2022). We found a sigmoid diatom morphologically similar to *N. sigma* in samples taken in Sivash Bay and adjacent artificial salt ponds.

The long-term survival of diatom populations in certain ecological niches depends on a multitude of factors. Stenger-Kovács *et al.* (2023) comprehensively outlined the impacts of salinity on diatoms, emphasizing the intricate biochemical, physiological and morphological responses. Notably, the effects of high salinity (exceeding 50 psu) on crucial life cycle stages of diatoms remain inadequately explored. The ability of diatoms to grow vegetatively at higher salinity is insufficient for long-term population survival. While vegetative cell acclimation to elevated salinity fulfils short-term intra-generational adjustments, long-term adaptation leading to evolutionary fitness necessitates genotype alterations, through sexual reproduction, spanning multiple generations (Orr, 2005). The progressive reduction in cell size during cell division restricts a clone's longevity to mere months or a few years (Davidovich & Davidovich, 2022b; Mann, 2011; Mann *et al.*, 2017). Sexual reproduction and auxosporulation serve as mechanisms to reset the maximum cell size and produce new generations (Chepurnov *et al.*, 2004; Poulíčková & Mann, 2019; Round *et al.*, 1990), underscoring the pivotal role of sexual reproduction in ensuring the enduring survival of diatom populations.

This study endeavours to investigate different aspects of our novel diatom collected from Sivash Bay: its phylogenetic position in relation to other *Nitzschia* species, the possibility of sexual reproduction in extreme conditions, and the species' salinity thresholds and optima for growth and reproduction. The term 'extreme halophiles' is usually applied to species that

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/00318884.2025.2471608.

live in hypersaline waters. In reality, they may prefer significantly lower salinities compared to their natural habitat and are better described as 'halotolerant'. This study of halotolerant species holds pragmatic implications in light of the escalating global concern over freshwater salinization and its potentially deleterious impacts on biodiversity, ecosystems and societies (Cañedo-Argüelles *et al.*, 2016; Jeppesen *et al.*, 2023; Melles *et al.*, 2023; Zadereev *et al.*, 2020). Moreover, insights into the adaptive capacities of such unique diatoms hold promise for enhancing their cultivation efficiency for practical applications.

MATERIAL AND METHODS

Benthic samples were taken from Sivash Bay (45°19.62′N, 35° 23.76′E) and nearby hypersaline ponds (45°19.74′N, 35° 24.12′E). Original salinity in the samples was 40 and 200 psu, respectively. Salt deposition was observed at the latter site. At sampling sites, the chemical composition of the Sivash brines is close to that of waters of open seas; the sediment mainly contains sodium chloride and calcium sulphate, while the solution is dominated by sodium chloride and magnesium sulphate (Ponizovsky, 1965).

Single cells were isolated into clonal cultures by the micropipette method (Andersen & Kawachi, 2005). Clones were placed in 100 ml glass Erlenmeyer flasks filled with 50 ml of the slightly modified (Polyakova *et al.*, 2018) ESAW medium (Andersen *et al.*, 2005) of different salinities. Lower salinity levels were obtained by diluting the original medium with distilled water. Higher levels were obtained by adding sodium chloride. Salinity levels were measured by a refractometer RHS-10ATC (Zhangzhou Sino Science & Technology Co., China). For a year, the cultures were kept at salinities of 10, 20, 40, 60, 80, 100, 150 psu and dim natural light on the north facing window and semi-stable temperature (20 ± 2°C). Once a month, the cultures were examined under the microscope to detect possible intraclonal reproduction and re-inoculated into fresh medium.

Heterothallic sexual reproduction was initiated by pairwise combination of clones. Before experiments, clones were inoculated twice into fresh medium at shorter intervals (once per week) to maintain exponential growth, which is essential for initiation of the sexual process. The first pair of clones that revealed sexual compatibility was then used as a control in mating experiments. Before mating experiments, clonal cultures were re-inoculated twice (with intervals of 4 to 5 days) into fresh medium to enhance exponential growth, which is a necessary condition for successful sexual reproduction (Chepurnov *et al.*, 2004). Cells involved in the sexual process were observed and photographed under a Biolar PI microscope (PZO, Warszawa, Poland) equipped with a Canon A640 digital camera (Japan). Cells were counted and measured using inverted microscopes Nib-100 (Jinan Biobase Biotech Co., Jinan, Shandong, China) and Altami INVERT 3 (Altami, Moscow, Russia), equipped with a Moticam 1080 digital camera (Motic China Group Co., Xiamen, Fujian, China).

Cultures are deposited and available in the World Ocean Diatoms Collection, the Center of Collective Use at the Karadag Scientific Station (https://ibss-ras.ru/about-ibss/structure-ibss/tsentry-kollektivnogo-polzovaniya/collection-of-diatoms-of-world-ocean/).

The effect of salinity on vegetative cells was assessed by their growth rate (doubling rate), which is an integral measure of environment suitability. A sharp change in salinity was applied in one experiment, clones 22.0622-OD and 22.0622-OG maintained in ESAW medium (40 psu) were inoculated into media of the same composition in the range of salinity (0, 10, 20, 30, 40, 60, 80, 100, 200 psu). In another experiment, we used clones pre-adapted for a year to different salinities (10, 20, 40, 60, 80, 100 psu) by regular re-inoculation. After the first experiment, Petri dishes with a salinity of 100 psu were left and observed for another 50 days; the salinity in the dishes gradually increasing due to evaporation of water.

The number of cells in 15 fields of view of the microscope was counted on the third, fourth and fifth days after inoculation. The specific growth rate (r, day^{-1}) was determined based on the equation of exponential population growth

$$N_t = N_0 e^{r\Delta t} \tag{1}$$

where N_t and N_0 are the average number of cells at time t and the initial time, t_0; Δt is the time interval between t and t_0. The values of the coefficient r were calculated by the least squares method using data obtained for 3 days of cell growth. To obtain the doubling rate (K, division·day^{-1}), the r values were divided by ln2 (Wood *et al.*, 2005).

The influence of salinity on the sexual reproduction of *Nitzschia siwaschica* was studied in experiments with a reproductively compatible pair of clones (22.0622-OP + 22.0622-OR). Petri dishes (5 cm diameter) were half filled with modified ESAW medium of different salinity (10, 30, 40, 60, 80, 100 psu). A small amount (1.0 μl) of a mixture of sexually compatible clones maintained before experiments at 40 psu was then added to each dish. The results of sexual reproduction were assessed on the third and fourth days after crossing. The number of vegetative and generative cells was counted in 15 fields of the microscope view. Generative cells included gametes, zygotes, auxospores and developing initial cells. The relative number of cells that entered into the sexual process was determined as the ratio of the number of generative cells to the total number of cells.

Salinity values optimal for sexual reproduction were determined by the maximum of the function used for data approximation (Zaitsev, 1984):

$$A = aF^b e^{cF} \tag{2}$$

where A is the relative number of cells that have entered into the sexual process (frequency of auxosporulation) and F is a variable (salinity). The values of the coefficients a, b and c were adjusted to provide minimal dispersion of points in relation to the approximating curve.

The optimal salinity (F_{opt}, psu) corresponding to the maximum for equation 2 can be found as:

$$F_{opt} = -b/c \quad . \tag{3}$$

One-way analysis of variance (ANOVA) was performed in Excel using the Data Analysis add-in to obtain F statistics values. Means are presented with standard errors.

For scanning electron microscopy (SEM), cells were gently boiled in 30% hydrogen peroxide (H_2O_2) for 3 hours on two consecutive days, followed by centrifugation and washing of the precipitated cells with distilled water. The washing operation was repeated seven to eight times. A few drops of the resulting suspension were placed on aluminium stubs, air dried and coated with gold. Electron micrographs were taken on a Hitachi SU8020 (Japan) scanning electron microscope.

For DNA analysis, the unialgal cultures were harvested during the exponential growth phase and concentrated by centrifugation. Total genomic DNA was extracted according to (Abdullin *et al.*, 2021). PCR amplification of the plastid-encoded *rbc*L gene region and the nuclear-encoded small subunit ribosomal RNA gene (SSU) was carried out using primers DPrbcL1 and DPrbcL7 (Daugbjerg & Andersen, 1997), and 18SP2F and 18SP4R (Guillou *et al.*, 1999; Van Hannen *et al.*, 1999), respectively, with Encyclo Plus PCR kit (Evrogen, Moscow, Russia). The PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, California, USA) and sequenced in both directions and the same primers used for PCR. Sequences were assembled with the Staden Package v. 1.4 (Bonfield *et al.*, 1995).

A dataset was assembled using sequences retrieved from the NCBI and the dataset of Mann *et al.* (2021). The sequences (taxa and accession numbers are given in the tree) were aligned in SeaView (Galtier *et al.*, 1996). To clarify the phylogenetic position of the new clones, we used a concatenated dataset of 85 diatom sequences of *rbc*L and SSU (3043 bp). *Eunotia* sequences were used as an outgroup. In order to determine the most appropriate DNA substitution model for the datasets, the Akaike information criterion (AIC; Akaike, 1974) was applied with jModelTest 2.1.1 (Darriba *et al.*, 2012). The combination of GTR+I +G and TIM3 +I +G models (partition) were selected as the best fits for *rbc*L and SSU datasets respectively. Phylogenetic trees were constructed using maximum likelihood (ML) in RAxML-NG (https://github.com/amkozlov/raxml-ng; Kozlov *et al.*, 2019) and Bayesian inference (BI) in MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). In BI, four runs of four Markov chains were executed for 5 million generations, sampling every 100 generations for a total of 50,000 samples. Convergence of the chains was assessed, and stationarity was determined according to the 'sump' plot with the first 12500 samples (25%) discarded as burn-in; posterior probabilities were calculated from the remaining trees. The convergence of the stationary distribution was accessed by ESS values (> 200) using Tracer v.1.7.1 (Rambaut *et al.*, 2018). The robustness of the ML trees was estimated by bootstrap percentages (BP) following the rapid bootstrap algorithm (Stamatakis *et al.*, 2008) and posterior probabilities (PP) in BI. BP < 70% and PP < 0.95 were not considered.

RESULTS

Phylogenetic analyses

The partial sequences of the chloroplast *rbc*L gene obtained for the analysed strains revealed two closely related genotypes, differing by only one base substitution. The first genotype was found in the strain 22.0622-OR, the second in 22.0622-OA, 22.0622-OP and 22.0622-OQ. The SSU rRNA gene sequences of our clones were identical.

Phylogenetic analyses based on a concatenated dataset of 85 accessions representing eight major clades of Bacillariaceae (numbered 1–8 in Fig. 1) placed our strains into clade 6B (90/0.97 for BP/PP) following Mann *et al.* (2021). The relationships between accessions within the clade remained largely unresolved. In the phylogenetic tree, our strains were sister to *N. omanensis* Barkia & C.Li but without support (Fig. 1).

Phylogenetic analyses were consistent with the polyphyletic nature of the genus *Nitzschia*, in which *Nitzschia* species are interspersed among representatives of the genera *Pseudo-nitzschia, Denticula, Cylindrotheca, Psammodictyon, Tryblionella, Hantzschia* and *Bacillaria*.

Morphological difference from genetically related species

No single character can be used to distinguish our diatom from all other genetically related species. The key discriminant varies among the species being compared. Our diatom markedly differs from *N. capitellata* Hustedt, *N. filiformis* (W.Smith) Van Heurck and *N. sigmaformis* Hustedt by the absence of a central nodule (Table 1). The biometric differences between our diatom and *N. omanensis* are negligible. However, *N. omanensis* can be distinguished from the former by its general shape, wedge-shaped ends and the absence of a sigmoid bend in the frustules (see illustrations in Barkia *et al.*, 2019). Morphologically, our diatom is to some extent similar to *N. sigma* (clade 6A) but genetically distant.

The phylogenetic placement of our new strains and their morphological differences from closest relatives suggest a new species, which we now propose.

Nitzschia siwaschica N.Davidovich & O.Davidovich *sp. nov.*

Figs 2–4 (LM), Figs 5–9 (SEM).

DESCRIPTION: Valves sigmoid with a linear central part due to parallel margins, gradually tapering to finely subcapitate apices, 46–401 μm long (*n* = 1442), 5.4–9.2 μm width (*n* = 469; Fig. 2,3). Two chloroplasts with pyrenoids per cell, one towards each pole of the cell (Fig. 4). Fibulae somewhat irregular spaced, 7–12 in 10 μm (*n* = 24). The two middle fibulae not more distant than other fibulae (Fig. 5). Transapical striae are visible in LM, parallel throughout the entire valve, 19–34 in 10 μm (*n* = 23). Each stria is composed of one row of rounded or oblong poroids, 20–26 in 10 μm (*n* = 23). The girdle bands have singular row of poroids. The canal raphe strongly eccentric. At the apices, the terminal raphe endings are strongly hooked on the same side of the valve (Fig. 6). Internally, the raphe terminates at apices in small helictoglossae

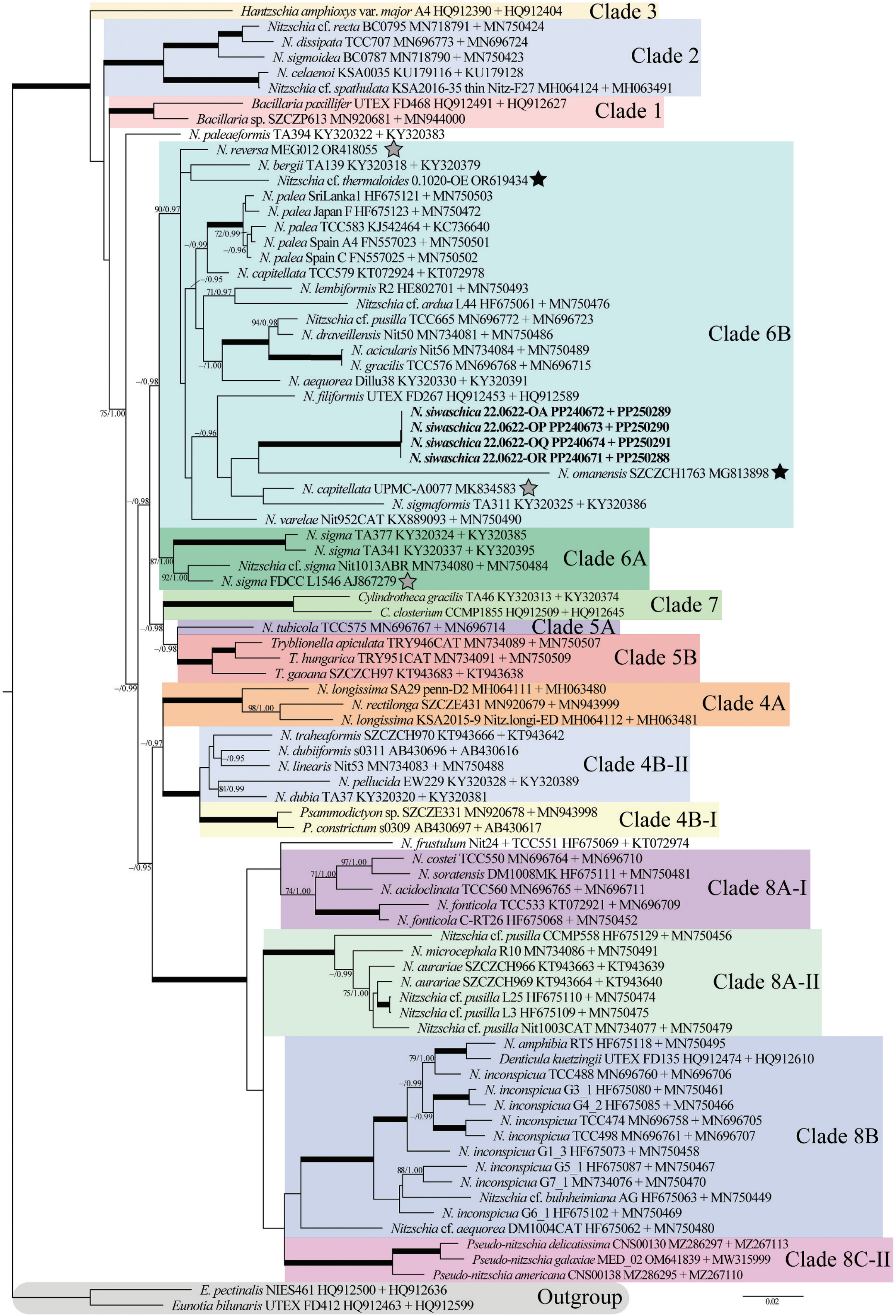


Fig. 1. ML tree showing the phylogenetic position of *Nitzschia siwaschica sp. nov*. clones based on plastid-encoded *rbc*L gene and nuclear-encoded SSU RNA gene sequence data. Supports [ML/BI above/below, (BP) ≥ 70% and (PP) ≥ 0.95] are given at the branches. New strains shown in bold, and branches with 100% BP and 1.00 PP with thick lines. The grey stars mark strains with only the SSU sequences; the black star marks the strain with only the *rbc*L sequence. Clade designations follow Mann *et al*. (2021). Scale bar = substitutions per site.

Table 1. Morphological characteristics of *Nitzschia siwaschica sp. nov.* compared to four genetically related *Nitzschia* species.

Character	Species				
	N. siwaschica sp. nov.	*N. capitellata*	*N. filiformis*	*N. omanensis*	*N. sigmaformis*
Length (μm)	46–401* (*n* = 1442)104–293**(*n* = 372)	45–65	27–150	31.0–46.0 (*n* = 15)	75–170
Width (μm)	5.4–9.2 (*n* = 469)	4–5	4–5	4.5–6.5 (*n* = 15)	4–5
Stria density (per 10 μm)	19–34(*n* = 23)	*ca.* 30	29–34	32–37	30
Fibula density (per 10 μm)	7–12(*n* = 24)	*ca.* 12	7–9	8–13	8–12
Poroid density in the stria (per 10 μm)	20–26(*n* = 23)	nd	nd	nd	nd
Central nodule	no	yes	yes	no	yes
Paired areolae in the raphe canal	yes/no	nd	no	no	nd
Source	This study	Hustedt, 1930; Barkia *et al.*, 2019	Barkia *et al.*, 2019	Barkia *et al.*, 2019	Hustedt, 1955

* In total for cells from the natural population and laboratory cultures
** For cells from the natural population
nd, no data
n, number of measurements

(Fig. 7). The central nodule and corresponding external proximal raphe endings absent (Figs 8, 9). Sexual reproduction *cis*-anisogamous and predominantly heterothallic, with two modes of gametogenesis (apical and transapical cell division in the two sexually compatible mating types correspondingly). The size range of initial cells 238–401 μm (*n* = 869).

HOLOTYPE: Permanent slide no. WODC.2023.10.30.*N*-1 (holotype here designated, Fig. 2) deposited in the Collection of the Komarov Botanical Institute of the Russian Academy of Sciences, (Saint Petersburg, Russia) under accession no. LE A0004253, comprised of cells of the two sexually compatible clones 22.0622-OP and 22.0622-OR from benthic samples and initial cells resulting from their mating. GenBank Accession numbers of *rbc*L (PP240673, PP240671) and SSU rRNA (PP250290, PP250288) genes of the clones 22.0622-OP and 22.0622-OR.

ISOTYPE: Permanent slide no. WODC.2023.10.30.*N*-2 and cleaned frustules deposited in the World Ocean Diatoms Collection at the Karadag scientific station, Crimea, Russia.

TYPE LOCALITY: 45°19.74′N, 35°24.12′E; a shallow (less than 0.5 m deep) hypersaline pond on a sandbar, the Arabat Spit, between the Sivash Bay and the Sea of Azov. Collected by N.A.Davidovich & O.I.Davidovich on 20 June 2022.

DISTRIBUTION: Currently known only from Sivash Bay and nearby hypersaline ponds located on the Arabat Spit.

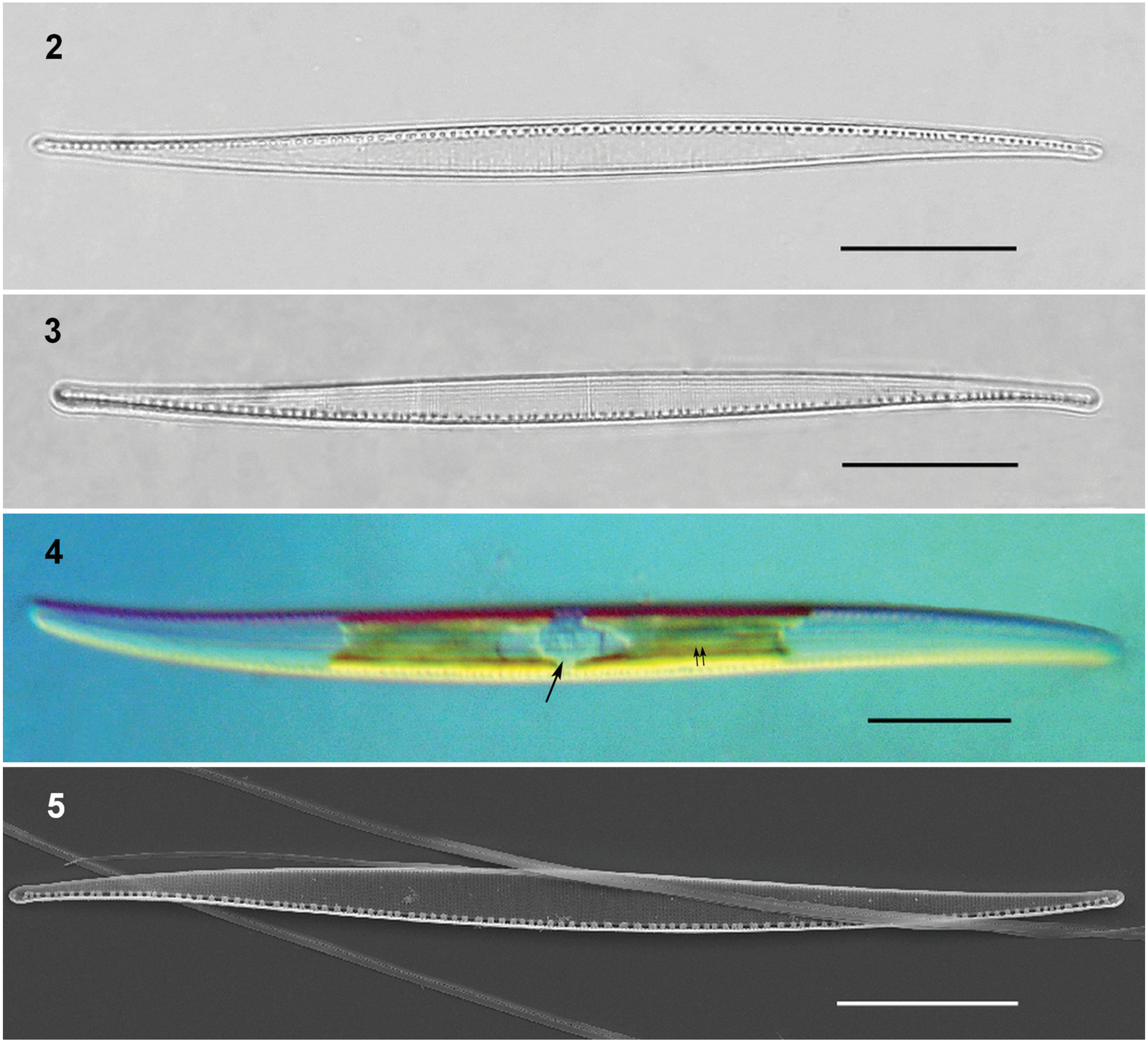
ETYMOLOGY: Named for the Sivash, a system of shallow water lagoons in the western part of the Sea of Azov.

REGISTRATION: Registration in PhycoBank at the link http://phycobank.org/105123.

Sexual reproduction

Nitzschia siwaschica sp. nov. exhibits heterothallic sexual reproduction. The sexual process can be initiated in mixtures of sexually compatible clones (Table 2), providing the cell sizes, light intensity, photoperiod, salinity, temperature *etc* are in tolerance ranges suitable for reproduction. The normal process involves pairing of two sexualized cells derived from parental clones. Sexualized cells join together and stop moving (Fig. 10). Instances of multiple gametangial cells clustering were also observed (Fig. 11). No noticeable mucilage was seen around the gametes, as well as no mucilage capsule was seen around the gametangial pairs. Each gametangial cell yields two gametes (Fig. 12). The mode of gametogenesis depends on the mating type. Cells of clones designated as mating type 1 (mt1) undergo protoplast contraction and division along the transapical plane (Fig. 13), giving rise to two rounded gametes liberated from the maternal valves in a few to tens of minutes (Fig. 14). Gametes of mating type 2 clones (mt2), generated via apical cell division, initially remain attached to parental valves (Fig. 15), undergoing a morphological transition from hemispherical to spherical (Fig. 16). The diameter of gametes formed by both mating types ranges from 15 to 18 μm. Several times we observed fusion of round gametes with gametes of the opposite mating type at a very early stage of their formation. Normally, a pair of parental cells gives rise to two zygotes (Fig. 17). Due to contraction of zygotes after syngamy, zygotes and fully formed gametes are more or less equal in size. At the same time, zygotes are easily distinguished from gametes by their double set of plastids. Spherical zygotes start bipolar expansion several minutes after syngamy (Fig. 18). Developing auxospores tend to align parallel to each other (Figs 19–23) and exhibit mostly synchronous growth. Position of auxospores in relation to parental frustules is somewhat random. The tips of auxospores are covered by caps (Fig. 24), which represent the halves of the zygote envelope (incunabula). While the caps are not always easily detectable, perizonium rings are clearly visible under the light microscope (Fig. 25). The transapical size of the initial cell is prominently smaller relative to the perizonium. Often, initial cells begin vegetative division inside the perizonium without leaving the latter, which suggests that the perizonium rings are either open or quite flexible, otherwise post-initial cells would be unable to leave the perizonium.

During a year of cultivation, no instances of intraclonal reproduction were observed, leading to the initial assumption that the heterothallic mode of reproduction prevailed in this species. However, later observations revealed cells exhibiting twice the size in monoclonal cultures 22.0622-OL and 22.0622-ON, indicative of homothallic reproduction. A single case of pedogamy was documented in the clone



Figs 2–5. *Nitzschia siwaschica sp. nov*., the whole valve view. Light microscopy (LM) and scanning electron microscopy (SEM).

Fig. 2. Holotype, the cell of the clone 22.0622-OP. The image was taken from the permanent slide WODC.2023.10.30.*N*-1, LM. Scale bar = 20 *μ*m.

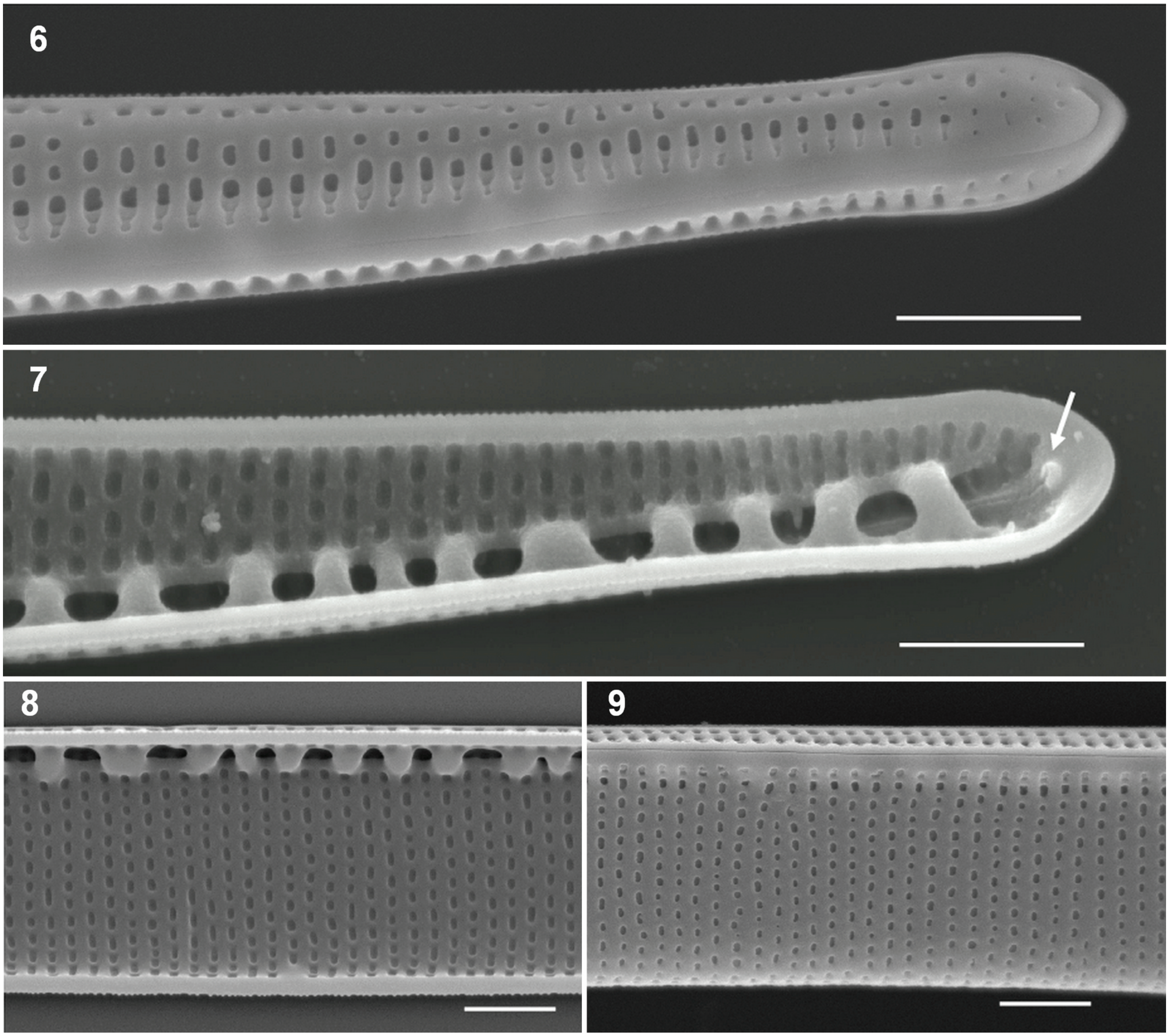
Fig. 3. Another cell from the same slide, LM. Scale bar = 20 *μ*m.

Fig. 4. Live cell with two chloroplast each containing a single pyrenoid (double arrow); the nucleus and nucleolus are clearly visible (arrow). Differential interference contrast image. Scale bar = 20 *μ*m.

Fig. 5. One theca of the frustule and girdle bands, SEM. Scale bar = 20 *μ*m.

22.0622-OP, where two gametes formed after transapical division of a single cell fused to form a zygote.

In most diatoms, the apical size of cells decreases during vegetative multiplication and restores in the process of sexual reproduction and auxosporulation in accordance with the McDonald-Pfitzer rule (MacDonald, 1869; Pfitzer, 1871; see also Chepurnov *et al*., 2004; Mann, 2011; Round *et al*., 1990; Fig. S1). This phenomenon gave rise to the size spectrum within the natural population of *N. siwaschica* (Fig. S2). At the time of sampling, over three quarters of cells in the natural population were within the reproductive (sexually inducible) phase, with no large cells resulting from recent sexual reproduction detected. The length of the largest cell measured in the population (*n* = 372) was 293 *μ*m, and the largest cell obtained as a result of sexual reproduction in mating experiments was 401 *μ*m (Table 3), which should be considered as the maximum species-specific size. According to the results of all crossing experiments, the 'cardinal point' (*sensu* Geitler, 1932), dividing the life cycle of *N. siwaschica* into pre-reproductive and reproductive phases

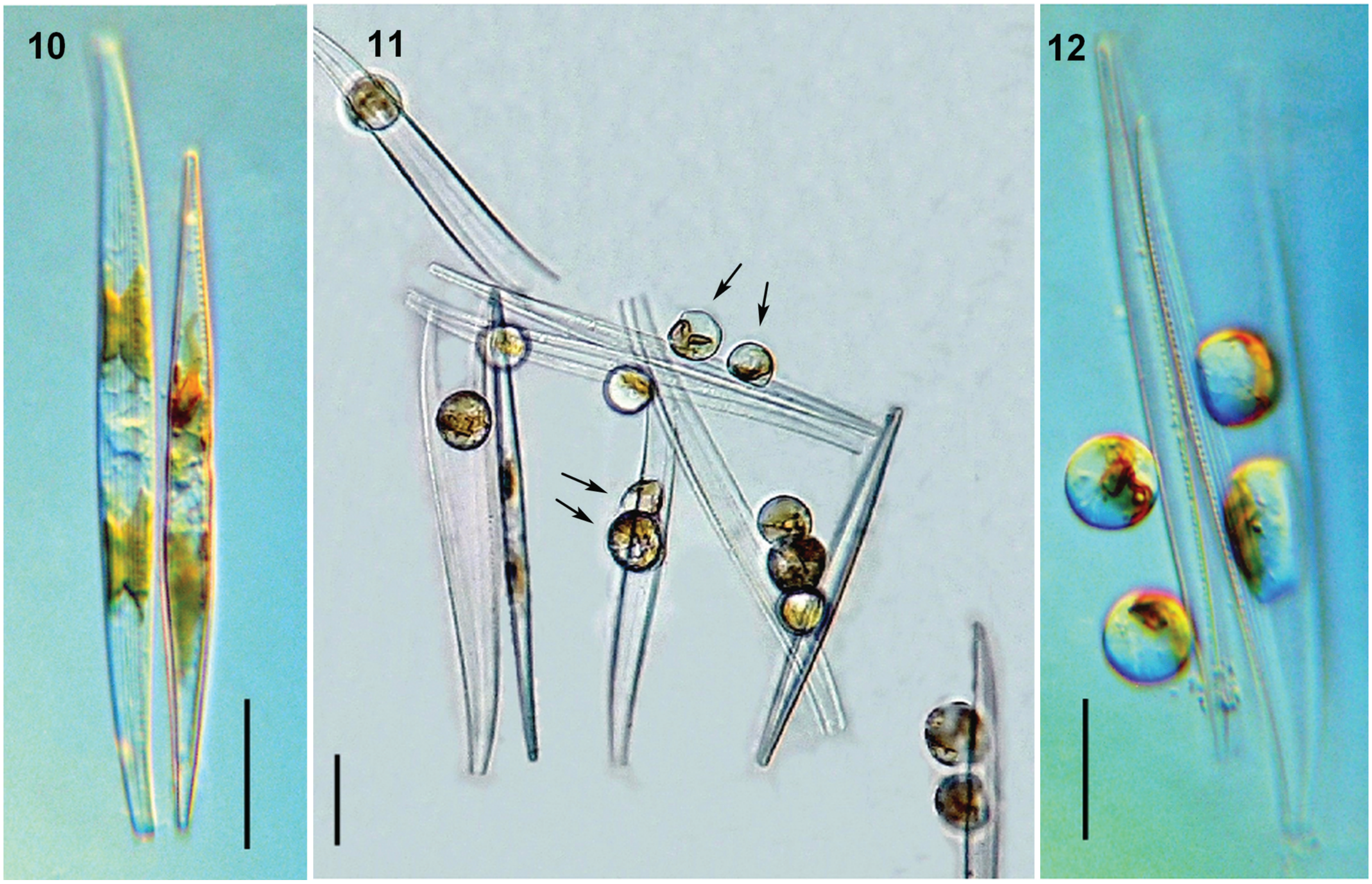


Figs 6–9. *Nitzschia siwaschica sp. nov*., the valve apices and central part.
Fig. 6. External view of the cell apex showing hooked raphe ending, SEM. Scale bar = 2 *μ*m.
Fig. 7. Internal view of the valve apex. Note helictoglossa (arrow) and irregular spaced fibulae, SEM. Scale bar = 2 *μ*m.
Fig. 8. Internal view of the central part of the valve. Note the absence of the central nodule, SEM. Scale bar = 2 *μ*m.
Fig. 9. External view of the central part of the valve, SEM. Scale bar = 2 *μ*m.

Table 2. Reproductive compatibility of *Nitzschia siwaschica sp. nov*. clones. Numbers indicate maximum score of relative abundance of auxospores (0 – absence, 1 – rare, 2 – frequent, 3 – abundant) in series of two to four crossing experiments or within clones (in square brackets).

		22.0622-OK	22.0622-OP	22.0210-OA	22.0622-OD	22.0622-OE	22.0622-OF	22.0622-OK	22.0622-OR	22.0622-OS
Clones	Mating type	mt1	mt1	mt2	mt2	mt2	mt2	mt2	mt2	mt2
22.0622-OK	mt1	[1]								
22.0622-OP	mt1	0	[1]							
22.0210-OA	mt2	nd	2	[0]						
22.0622-OD	mt2	nd	3	0	[0]					
22.0622-OE	mt2	nd	3	0	0	[0]				
22.0622-OF	mt2	nd	3	0	0	0	[0]			
22.0622-OK	mt2	nd	2	0	0	0	0	[1]		
22.0622-OR	mt2	1	3	0	0	0	0	0	[0]	
22.0622-OS	mt2	2	3	0	0	0	0	0	0	[0]

nd, no data; combinations of reproductively compatible clones are outlined by a dashed line



Figs 10–12. Early stages of the sexual reproduction in *Nitzschia siwaschica sp. nov.*, LM. Scale bar = 20 μm.

Fig. 10. Pairing of two gametangial cells in the mixture of clones 22.0622-OP and 22.0622-OR. The chloroplasts starting to divide before meiosis and gamete formation. Note the difference in the length of cells in the pair, the feature allowing us to distinguish between cells of two sexually compatible clones.

Fig. 11. Sometimes gametangial cells form groups producing many gametes (arrows).

Fig. 12. Normally, each gametangial cell in a pair produces two gametes.

corresponds to the size of the largest cell capable of sexual reproduction (189 μm).

Tolerance to salinity

The specific growth rate of *N. siwaschica* (clone 22.0622-OD) exhibited no salinity dependence varying from 0.70 to 0.83 divisions per day across the 10–100 psu range (Fig. 26). No significant difference exists between the tested salinity levels (F-statistics was 0.60 which is less than the critical value 2.53; *p*-value = 0.703).

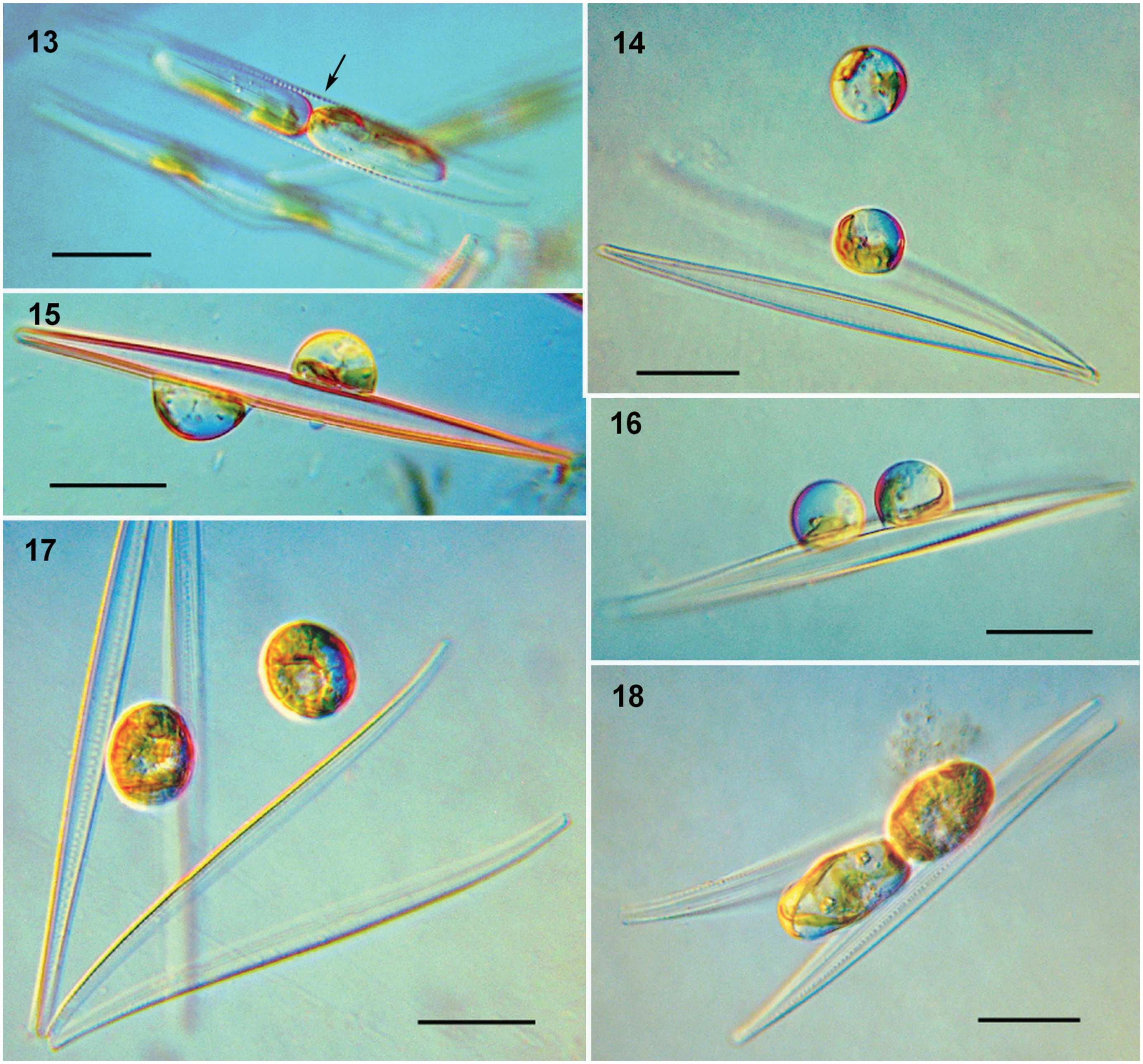
Control pairs of clones (22.0622-OK+22.0622-OP and 22.0622-OP+22.0622-OS) were able to reproduce sexually within a relatively broad salinity range from 20 to 80 psu. Maximum abundance of gametes, zygotes and auxospores resulting from sexual reproduction, of clones acclimated to 40 psu ESAW medium and subsequently exposed to varied salinities, peaked at 40 psu (Fig. 27). Notably, larger initial cells were seen at higher salinity levels (Fig. 28). Statistically significant differences between the mean length of initial cells arose at different salinities (F = 209.46 for the pair of clones 22.0622-OK+22.0622-OP and F = 92.89 for the pair of clones 22.0622-OP+22.0622-OS, which is much higher than the critical values at very high probability, $p < 0.01$).

In addition to its high halotolerance, *N. siwaschica* exhibited resistance to desiccation. In one of our experiments, cells resumed vegetative division upon rehydration of a completely evaporated Petri dish.

DISCUSSION

A number of diatom species have been identified in hypersaline environment (Sapozhnikov & Kalinina, 2018; Shadrin *et al.*, 2021). Permanent presence of these diatoms in the biotopes suggests that they live in these extreme habitats over numerous generations periodically entering sexual reproduction. Although these hypersaline habitats are potentially suitable for sexual reproduction, empirical evidence to support this assumption other than cell size changes is currently lacking.

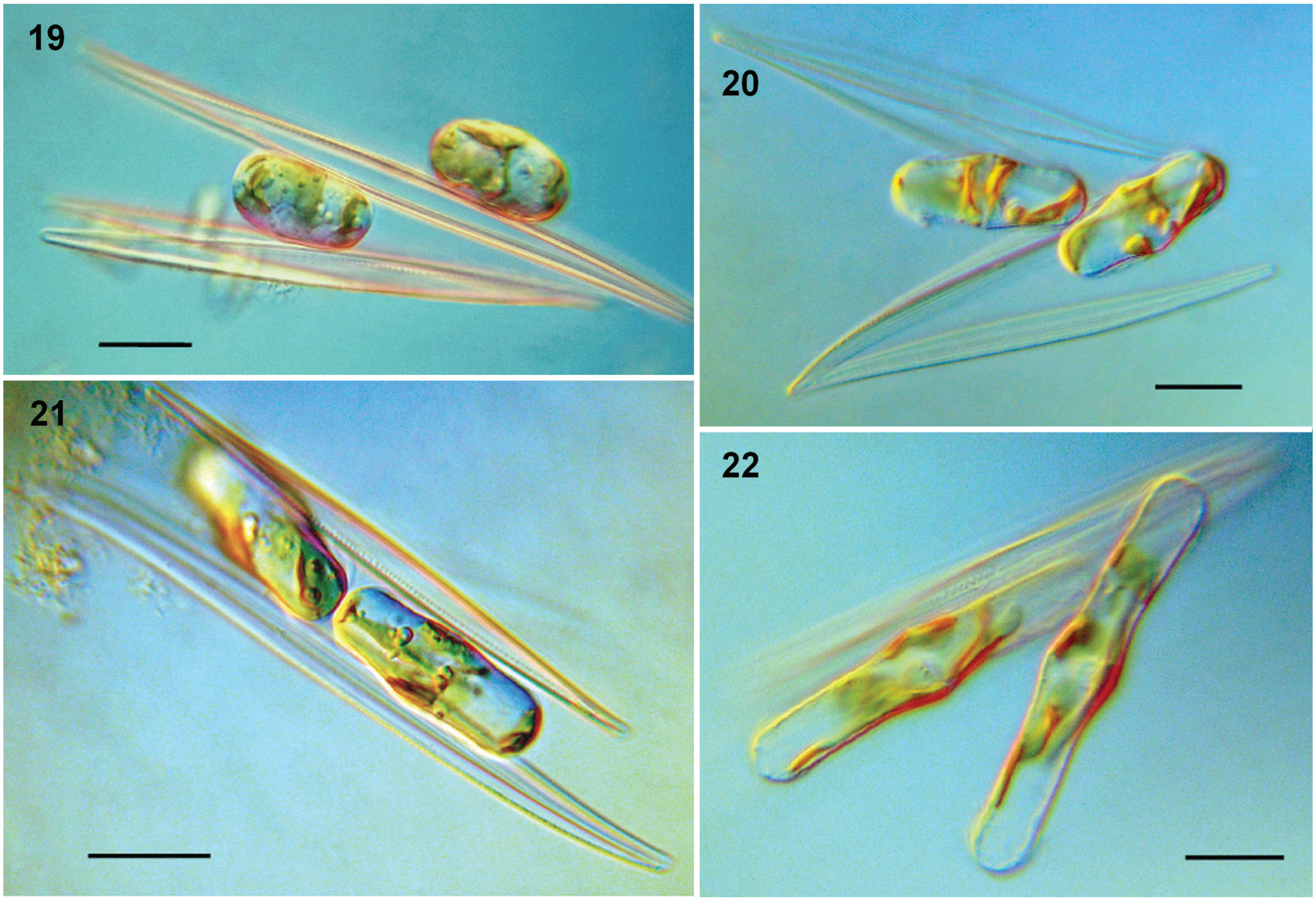
Physiological aspects of salt resistance in diatoms are poorly studied. Salt stress can significantly affect their photosynthetic activities, pigment contents, growth rate, metabolism, toxin synthesis, *etc.* (Stenger-Kovács *et al.*, 2023). The persistence of natural populations of diatoms hinges on the



Figs 13–18. Gametogenesis and zygote formation in *Nitzschia siwaschica sp. nov*., LM. Scale bar = 20 μm.
Fig. 13. Protoplast of the mt1 gametangium divides in the transapical plane (arrow).
Fig. 14. Gametes produced by mt1 clones usually lose contact with parental valves.
Fig. 15. Hemispherical gametes of mt2 clones remain attached to parental valves for a longer time.
Fig. 16. The shape of mt2 gametes gradually changes to round.
Fig. 17. Pair of parental cells gives rise to two zygotes. Zygotes as well as growing auxospores (see Figs 18–23) bear four chloroplasts.
Fig. 18. Zygotes beginning bipolar expansion.

environmental fitness for both vegetative growth and sexual reproduction, a vital stage in the life cycle of diatoms. In the case of *Nitzschia siwaschica sp. nov*., a notable tolerance to extreme salinity has been exhibited, with a consistent high growth rate observed across 10–100 psu salinity spectrum. However, this resilience alone does not ensure population stability. Sexual reproduction, being more susceptible to environmental influences, emerges as a fundamental requirement for sustainable population survival across generations. It is well known that sexual reproduction cannot be initiated under unfavourable culture conditions (Chepurnov *et al.*, 2004; Drebes, 1977). The ability of *N. siwaschica* to reproduce sexually across a salinity range from 20 to 80 psu, the widest known for diatoms, highlights the uniqueness of this species.

Further investigations are imperative to delineate the optimal salinity range conducive to sexual reproduction, especially considering that the cultures in our acute experiments were not acclimated to varying salinities. Studies have shown



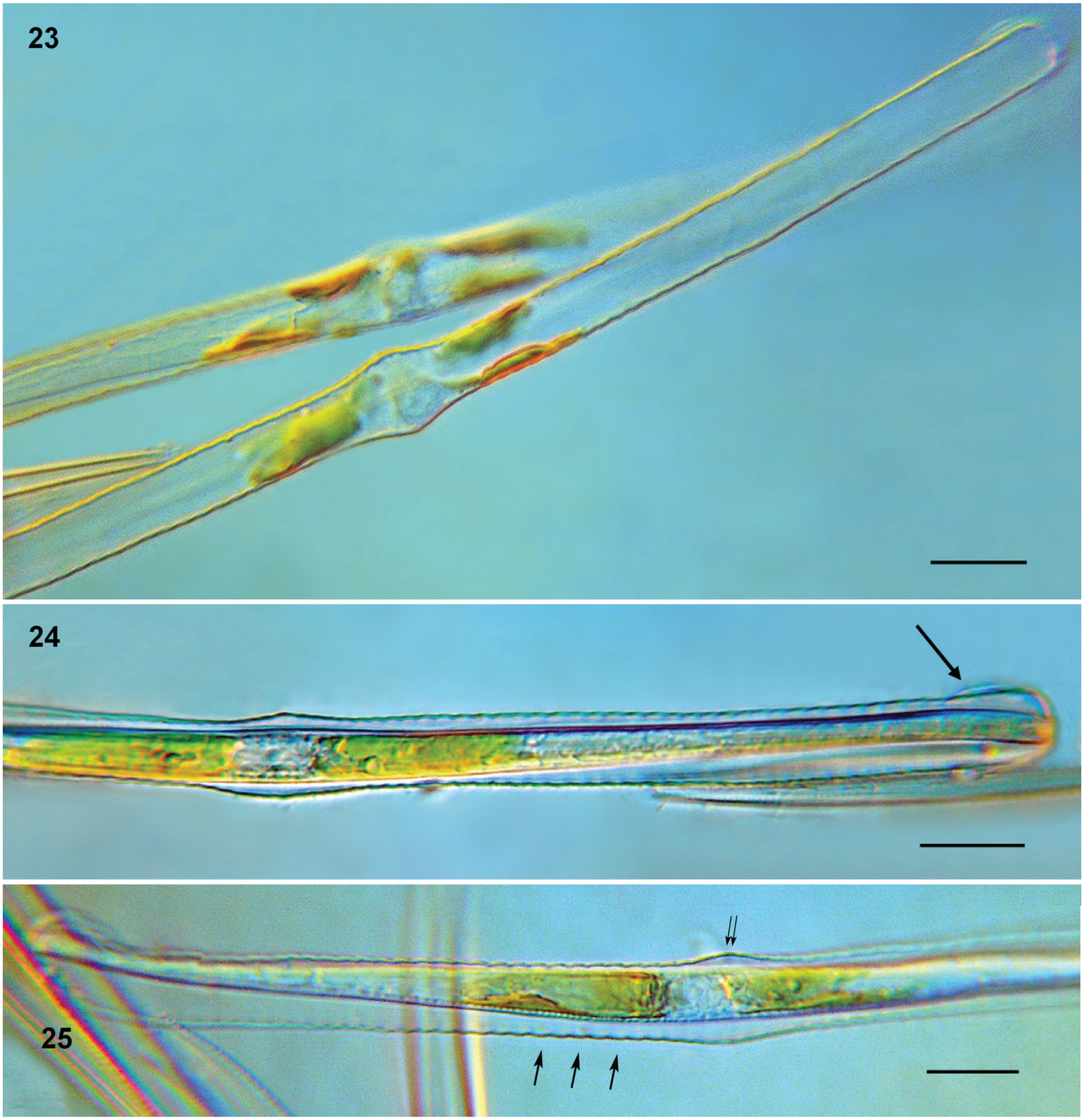
Figs 19–22. Expanding auxospores in *Nitzschia siwaschica sp. nov.*, clones 22.0622-OP and 22.0622-OR, LM. Scale bar = 20 μm.
Fig. 19-22. Growing auxospores lie in most cases more or less parallel to each other and elongate synchronously.

that slight salinity increments favour sexual reproduction in some diatoms (Davidovich *et al.*, 2012; Davidovich & Davidovich, 2022b).

Interestingly, the largest initial cells in *N. siwaschica* occurred under elevated salinity conditions. Euryhaline taxa that invaded freshwaters were noted to have smaller cell sizes compared to their marine populations (Edlund *et al.*, 2000). On the contrary, Nagai & Imai (1999) reported that initial cells formed through vegetative cell enlargement in *Coscinodiscus wailesii* Gran & Angst were smaller at higher salinities. The inverse correlation between the length of initial cells and medium salinity was confirmed in *Entomoneis sp.* (O. I. Davidovich *et al.*, 2022). Auxospores within a hypertonic environment have shown susceptibility to plasmolysis (Mann, 1994). At the same time, in the case of *Tabularia tabulata* (C.Agardh) Snoeijs, the size of initial cells did not depend on the salinity of the medium (Davidovich & Davidovich, 2022b). Regardless of the mechanism controlling the size limits of the initial cells, it is axiomatic that the duration of the life cycle in diatoms is determined by the initial cell size and the rate of cell size reduction (Chepurnov *et al.*, 2004; Mann, 2011; Round *et al.*, 1990). Therefore, clones of *N. siwaschica* resulting from sexual reproduction under higher salinity conditions potentially have longer life spans.

In the genus *Nitzschia*, known for its considerable heterogeneity (Mann *et al.*, 2021), a variety of sexual reproduction modes have been documented (Mann, 1986, 1993). While some species produce a single gamete per gametangium, the majority are digametic. In some representatives of the genus, gametangial pair forms specific copulation papillae. The gametes are often morphologically alike, but some *Nitzschia* exhibit physiological anisogamy (Chepurnov *et al.*, 2004; Mann, 1986). Several species have adopted automixis (Mann *et al.*, 2013).

To date, sexual reproduction has been discovered and described with varying degrees of detail in 21 representatives of the genus, namely *N. amphibia* Grunow, *N. dicrogramma* D.G.Mann & Trobajo, *N. dissipata* (Kützing) Rabenhorst, *N. flexoides* Geitler, *N. fonticola* (Grunow) Grunow, *N. frustulum* var. *perpusilla* (Rabenhorst) Grunow *ex* Van Heurck, *N. lanceolata* W.Smith, *N. linearis* W.Smith, *N. longissima* (Brébisson *ex* Kützing) Grunow, *N. palea* (Kützing) W.Smith, *N. recta* Hantzsch *ex* Rabenhorst,



Figs 23–25. Finalization of auxospore growth and formation of initial cells in *Nitzschia siwaschica sp. nov.*, LM. Scale bar = 20 μm.

Fig. 23. Fully expanded auxospores contain four chloroplasts located close to the centre of the cell.

Fig. 24. The initial cell inside the perizonium. The tips of auxospores are covered by caps (arrow) representing the halves of the zygote envelope. Only two chloroplasts are observed in the initial cell.

Fig. 25. An initial cell inside the perizonium, with rings (arrows) clearly visible. In the transverse perizonium, the central ring (primary band following terminology proposed by Kaczmarska *et al.*, 2013; double arrow) has larger diameter. Only two chloroplasts are observed in the initial cell.

N. rectilonga Takano, *N. reversa* W. Smith, *N. sigmoidea* (Nitzsch) W.Smith, *N. spathulata* Brébisson *ex* W.Smith, *N. subtilis* (Kützing) Grunow (reviewed in Davidovich & Davidovich, 2022a), *N. acidoclinata* Lange-Bertalot (Bagmet *et al.*, 2022), *N. inconspicua* Grunow (Mann *et al.*, 2013, 2017), *N. hybrida* Grunow (Karsten von, 1899), *Nitzschia* cf. *pusilla* Grunow [as *Nitzschia* cf. *kützingiana* Hilse] (Geitler, 1968) and *Nitzschia* cf. thermaloides Hustedt (Davidovich *et al.*, 2023). They reveal diverse patterns of sexual reproduction (Table 4).

Table 3. The apical size of cells at different stages of the life cycle in *Nitzschia siwaschica sp. nov.*

Life cycle stage	Number of cells measured	Cell length, *μm*	
		min	max
Vegetative cells	1289	104	401
Gametangia	48	117	189
Initial cells	869	238	401

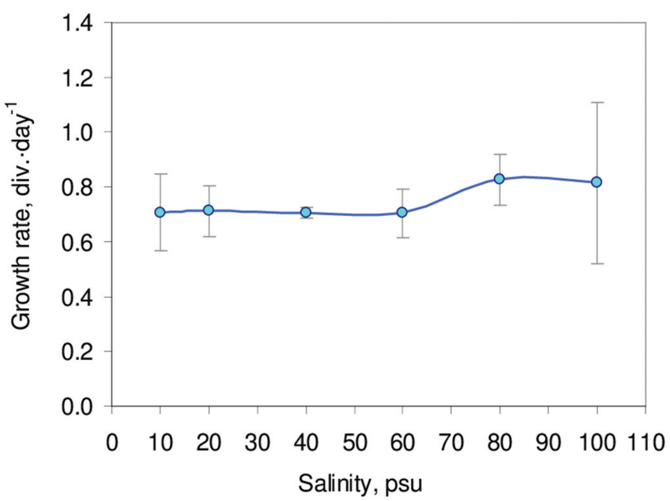


Fig. 26. *Nitzschia siwaschica sp. nov.* (clone 22.0622-OD), growth rate in various salinities. Each mean represents the average of six replicate experiments. Standard error bars are shown for the means.

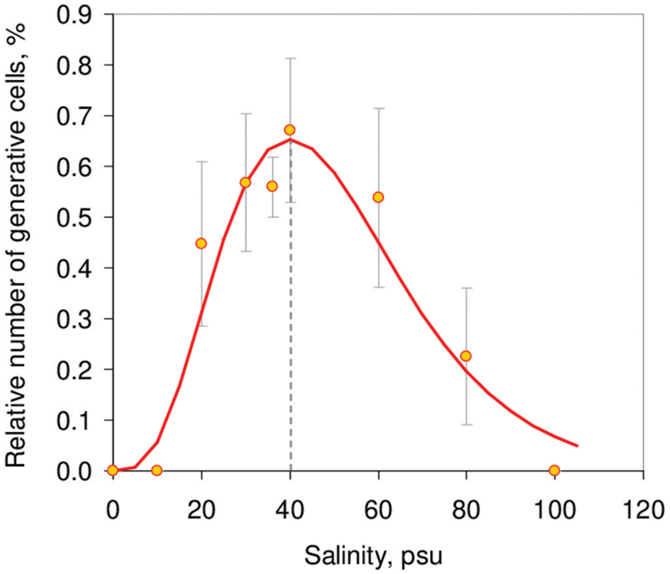


Fig. 27. Relative number of generative cells (sum of gametes, zygotes and auxospores in relation to the total number of cells) in *Nitzschia siwaschica sp. nov.* in mating experiments at different salinity levels. Before mating cultures maintained in 40 ‰ ESAW medium and natural lighting. Mean values and standard errors are shown.

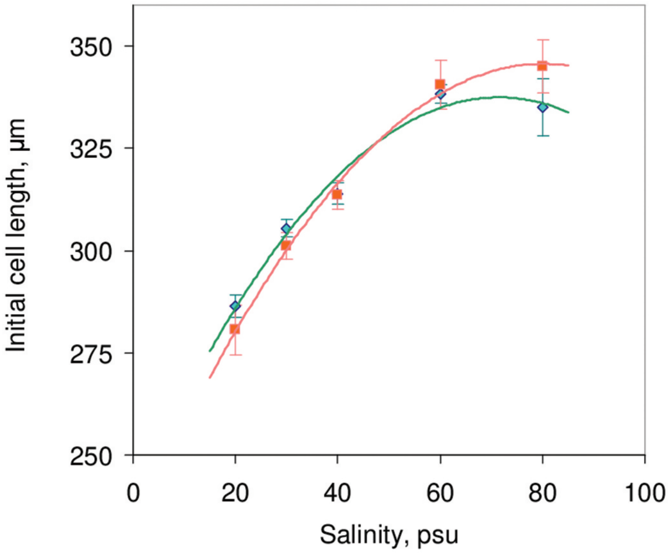


Fig. 28. Length of initial cells in reproductive cultures at different salinity levels in two pairs of sexually compatible clones of *Nitzschia siwaschica*: (*square*) 22.0622-OP + 22.0622-OS and (*diamond*) 22.0622-OK + 22.0622-OP.

The type species of the genus *Nitzschia, N. sigmoidea* demonstrates *trans*-behavioural anisogamy (Mann, 1986). In this case, one of two gametes formed in each gametangium is actively mobile. In a gametangial pair, active gametes reciprocally migrate through the copulation tube. By definition, this mode of sexual reproduction can be regarded as typical for the genus, at least for the 'subgenus *Nitzschia*' (*sensu* Mann, 1986). The mode of reproduction in *N. siwaschica* is evidently different. *Cis*-anisogamy (category IA2b; Amato, 2010; Geitler, 1973) is the main type of sexual process in *N. siwaschica* following Geitler's system. This pattern of sexual reproduction we can observe in diatoms belonging to several phylogenetic clades. *Cis*-anisogamy is typical for *N. longissima* (Shorenko *et al.*, 2014) and *N. rectilonga* Takano (our unpublished observation). Interestingly, *N. siwaschica* shares the trait of *cis*-anisogamy with the marine planktonic genus *Pseudo-nitzschia*, a relationship that correlates with the earlier recognized affinity between the Nitzschiella and *Pseudo-nitzschia* groups (Mann, 1993; Trobajo *et al.*, 2006). The above species are sufficiently distant genetically (see Fig. 9), and *cis*-anisogamous reproduction can be regarded as an example of homoplasy, which was noted to be common in both light microscopical and ultrastructural characters, *e.g.* sigmoid shape is also considered homoplasious (Mann *et al.*, 2021).

The sigmoid *Nitzschia* species segregate into three distinct clades (Mann *et al.*, 2021). Presently, sexual reproduction was elucidated for only four sigmoid diatoms. Among them, three (including freshwater *N. sigmoidea, N. dissipata* and *N. flexoides*) form a cluster exhibiting a specific sexual reproduction type aligning with Geitler's (1973) category IAla(gamma). *Nitzschia sigmoidea* and *N. dissipata* are affiliated with clade 2 as per Mann *et al.* (2021). Our results place *N. siwaschica* in clade 6B, along with several other sigmoid diatoms, which await further study of their sexual reproduction in terms of homoplasy, evolutionary relationship and diversity.

Table 4. Patterns of sexual reproduction characteristic of representatives of the genus *Nitzschia*.

Species	Dominant mode of reproduction	Number of gametes per gametangium	Copulation papillar/ tube	Gamete rearrangement	Morphological isogamy/ anisogamy	Behavioural isogamy/ anisogamy (type *cis-* or *trans-*)	Position of auxospores and gametangial frustules	Geitlers's type (Geitler, 1973)	Source
N. acidoclinata	homothallic (automictic, pedogamous)	2	no	?	isogamy	isogamy	more or less parallel	IIIA	Bagmet *et al.*, 2022
N. amphibia	heterothallic	1	present (on the cell poles)	no	isogamy	isogamy	chaotic	IIA	Geitler, 1969
N. dicrogramma	heterothallic	2	no	yes	isogamy	*trans*-anisogamy	parallel	IA1a (betta)	Mann & Trobajo, 2014
N. dissipata	heterothallic	2	present (on the cell equator)	yes	isogamy	*trans*-anisogamy	parallel	IA1a (gamma)	Geitler, 1958, Geitler, 1973; Mann, 1986
N. flexoides	heterothallic	2	present (on the cell equator)	yes	isogamy	*trans*-anisogamy	parallel	IA1a (gamma)	Geitler, 1968; Mann, 1986
N. fonticola	heterothallic	2	no	?	isogamy	isogamy	chaotic	IC	Geitler, 1932
	homothallic (automictic, pedogamous)	2	no	no	isogamy	isogamy	chaotic	IIIA	Trobajo *et al.*, 2006
N. frustulum var. *perpusilla**	homothallic (automictic, pedogamous)	2	no	yes	isogamy	isogamy	more or less parallel	IIIA	Geitler, 1970
N. hybrida	heterothallic	2	no	?	isogamy	isogamy	parallel	IB2a	Karsten von, 1899
*N. inconspicua**	homothallic (automictic, pedogamous)	2	no	yes	isogamy	isogamy	more or less parallel	IIIA	Mann *et al.*, 2013
N. longissima	heterothallic	2	no	no	anisogamy in the beginning	*cis*-anisogamy	more or less parallel	IA2b	Karsten, 1897; Chepurnov in Roshchin, 1994
*N. palea***	heterothallic	2	present (on the cell equator)	no	isogamy	*trans*-anisogamy	chaotic	IA1b	Geitler, 1968; Trobajo *et al.*, 2009
Nitzschia cf. *pusilla* (=*Nitzschia* cf. *kützingiana*)	heterothallic	2	present	no (?)	isogamy	*trans*-anisogamy	(?)	IA1b	Geitler, 1968; Mann, 1986
N. recta	heterothallic	2	present (on the cell equator)	yes	isogamy	*trans*-anisogamy	parallel	IA1a (gamma)	Mann, 1986
N. rectilonga	heterothallic	2	no	no	anisogamy in the beginning	*cis*-anisogamy	more or less parallel	IA2b	Shorenko *et al.*, 2016
N. reversa	heterothallic	2	no	no	isogamy	isogamy	chaotic	IC	Mann, 1993
N. sigmoidea	heterothallic	2	present (on the cell equator)	yes	isogamy	*trans*-anisogamy	parallel	IA1a (gamma)	Geitler, 1973; Mann, 1986
N. siwaschica	heterothallic	2	no	no	anisogamy in the beginning	*cis*-anisogamy	chaotic	IA2b	present paper
N. spathulata	heterothallic	2	present	yes	isogamy	*trans*-anisogamy	parallel	IA1a (gamma)	Mann & Trobajo, 2014
*N. subtilis***	heterothallic	2	present	no	isogamy	*trans*-anisogamy	chaotic	IA1b	Geitler, 1928, Geitler, 1958; Mann, 1986
Nitzschia cf. *thermaloides*	heterothallic	2	no	no	isogamy	isogamy	more or less parallel	IB2a	Davidovich, *et al.*, 2023

* *N. inconspicua* was thought to be synonymous with, and referred to as, *N. frustulum* var. *inconspicua* (Grunow) Grunow *(see* Rovira *et al.*, 2015).

** Geitler originally described the auxosporulation in *N. palea*, considering it *N. subtilis* (see Geitler, 1968)

Details of the reproduction of *N. lanceolata* (Roshchin, 1990) and *N. linearis* W.Smith (Chepurnov *et al.*, 2005) are not presented, since they are extremely scarce.

ACKNOWLEDGEMENTS

We thank reviewers for their valuable comments and improving the manuscript.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

FUNDING

This work was supported by Russian Science Foundation (RSF) grant No. 22-14-00209. The investigated marine diatom strains were isolated and introduced in a culture thanks to the project number 124030100100-0 funded by the Ministry of Science and Higher Education of the Russian Federation; 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.

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