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Original

Nitzschia gobbii sp. nov. (Bacillariophyceae): a common but overlooked planktonic diatom species from the northwestern Adriatic Sea / Giulietti, S.; Totti, C.; Romagnoli, T.; Siracusa, M.; Bacchiocchi, S.; Accoroni, S.. - In: PHYCOLOGIA. - ISSN 0031-8884. - 60:6(2021), pp. 558-571. [10.1080/00318884.2021.1952513]

Availability:

This version is available at: 11566/293651 since: 2024-03-26T16:43:37Z

Publisher:

Published

DOI:10.1080/00318884.2021.1952513

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Nitzschia gobbii sp. nov. (Bacillariophyceae): a common but overlooked
planktonic diatom species from the northwestern Adriatic Sea

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RUNNING TITLE

Nitzschia gobbii sp. nov. from the NW Adriatic Sea

ACKNOWLEDGEMENTS

The long-term monitoring has been carried in the framework of LTER-ITALY (Italian Long-Term Ecological Research Network) activities. Thanks to the Centro di Ricerca e Servizio di Microscopia delle Nanostrutture (CISMiN) of the Università Politecnica delle Marche for the Electron Microscopy service.

FUNDING

This research was partially supported by the Italian Ministry of Health (Ricerca Finalizzata 2016) under grant number GR-2016-02363211 and by the EU 2014–2020 Interreg V-A Italy-

Croatia CBC project ECOSS (Observing System in the Adriatic Sea: oceanographic
observations for biodiversity) ID: 10042301. The cruises carried out with the M/N Actea
were entirely funded by the Department of Life and Environmental Sciences (Università
Politecnica delle Marche).

ABSTRACT

Planktonic diatoms of the family Bacillariaceae, including *Nitzschia* spp, *Pseudo-nitzschia* spp and *Cylindrotheca closterium*, are common and often highly abundant in the Adriatic Sea. During the 30 years of phytoplankton monitoring at the coastal site of the LTER Senigallia-Susak transect, a nitzschioid diatom with morphology matching that of *Nitzschia* section *Nitzschiella* (dilated central portion and two long rostra) was frequently observed. In this study we describe this new species as *Nitzschia gobbii* sp. nov., combining morphological (TEM and SEM) and molecular (LSU and ITS rDNA) data. The morphology of *Nitzschia gobbii* makes it easy to confuse with other common species in the Adriatic Sea (*Cylindrotheca closterium* and *Pseudo-nitzschia galaxiae*). The partial LSU rDNA sequences of strains of *Nitzschia gobbii* sp. nov. were resolved in a well-supported clade. In this area, this species has been mainly recorded in spring-summer with a maximum abundance of 3.2×10^6 cells l⁻¹. None of the tested *Nitzschia gobbii* strains produced domoic acid in detectable amounts.

KEYWORDS

Diatom systematics; ITS; LSU; LTER-site; Phytoplankton; Time series.

INTRODUCTION

Species of the genus *Nitzschia* Hassall are widely distributed, have about 829 taxonomically accepted species (Guiry & Guiry 2021) that are found in a variety of habitats (freshwater, brackish and marine waters, and soil), and include both pelagic and benthic species (Hasle 1964; Hasle & Medlin 1990; Witkowski *et al.* 2000; Rovira *et al.* 2012; Trobajo *et al.* 2013). Recently, two species of *Nitzschia* were reported as producers of domoic acid, a neurotoxin responsible for Amnesic Shellfish Poisoning (ASP; Lundholm & Moestrup 2000; Smida *et al.* 2014). The identification of species of *Nitzschia* is highly problematic, particularly because the genus is not a natural taxonomic group, as suggested by differences in sexual

behaviour (e.g. copulation papillae are formed in some species but not in others; Mann 1993) and by molecular evidence showing that the genus is paraphyletic (Lundholm *et al.* 2002; Trobajo *et al.* 2009; Carballeira *et al.* 2017; Mann *et al.* 2021).

The genus was erected in 1845 by Hassall, accommodating all single-celled and colonial pennate diatoms with a linear to lanceolate (also sigmoid) shape of frustules, and with a predominantly eccentric (sometimes centric) raphe subtended by siliceous bridges (Mann 1986). Later, Cleve & Grunow (1880) subdivided the genus *Nitzschia* in 24 sections that, with some modifications, particularly following Hustedt (1930, 1955), Lange-Bertalot and Simonsen (1978), Mann (1986) and Round *et al.* (1990), are still in use. Species with a more or less lanceolate central part and fine projections (i.e. rostrate ends, designated as ‘terminal horns’ in Hasle 1964) are gathered in the section *Nitzschiella* (Hasle 1964). *Nitzschiella* was originally proposed as a separate genus (Rabenhorst 1864) and has been reduced afterwards to a section of the genus *Nitzschia* (Cleve & Grunow 1880).

The classical approach to the study of this genus has been based mainly on morphological analysis. Still numerous papers describe new species of *Nitzschia* without molecular information (Alakananda *et al.* 2012; Grady *et al.* 2020; Morales *et al.* 2020), making arduous the construction of a coherent phylogenetic classification. Portions of rDNA are widely used as genetic markers for many organisms, and in the last 10 years they have proved to be a useful tool also for diatoms (Alverson 2008; Amato *et al.* 2018), because they have numerous copies in the genome, they are highly conserved and the database of rDNA sequences for diatoms is growing rapidly. In particular, LSU (28S) rRNA genes have often been used for phylogenetic analysis at higher taxonomic levels (Lundholm *et al.* 2002; Lim *et al.* 2018), whereas ITS can resolve species and even population relationships (Beszteri *et al.* 2005).

The Adriatic Sea is a continental basin located in the northernmost part of Mediterranean Sea. The northern Adriatic basin is mainly characterized by shallow waters and high freshwater inputs, mainly due to the Po River outflow (Cozzi & Giani 2011). There is an extensive bibliography concerning the distribution and composition of phytoplankton in the northern Adriatic Sea (NAS), revealing a dominance of diatoms, which peak in abundance in late winter and spring, and of phytoflagellates (Bernardi Aubry *et al.* 2004, 2006, 2018; Cabrini *et al.* 2012; Mozetič *et al.* 2012; Totti *et al.* 2019).

In the phytoplankton communities of the NAS, the occurrence of diatom species belonging to the family Bacillariaceae, including *Nitzschia* spp, *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C. Lewin and *Pseudo-nitzschia* spp is common, and they sometimes reach high abundances (Bernardi Aubry *et al.* 2012, 2018; Cabrini *et al.* 2012; Cerino *et al.* 2019; Mozetič *et al.* 2019; Totti *et al.* 2019; Turk Dermastia *et al.* 2020). Among these diatoms, we have commonly observed a morphotype that matches that of *Nitzschia* sect. *Nitzschiella* and resembles both *Cylindrotheca closterium* and *Pseudo-nitzschia galaxiae* Lundholm & Moestrup (long morphotype *sensu* Cerino *et al.* 2005).

The aim of this work was to perform a characterization of the above morphotype, which we named *Nitzschia gobbii* sp. nov., combining a morphological and molecular approach, and analysing the production of domoic acid (DA) by liquid chromatography coupled to mass spectrometry (LC-MS/MS). Furthermore, we describe its seasonal and interannual variability, based on over 30 years of phytoplankton data from a coastal site of the NAS.

MATERIAL AND METHODS

Study area and sampling

The study area is the coastal station SG01 (43°45.86'N, 13°13.00'E) of the Senigallia-Susak transect, located in the southern part of the northern Adriatic sub-basin, at 1.2 nM from the Italian coastline (bottom depth = 12 m) and included in the Long-Term Ecological Research (LTER) Italian sites, where since 1988 we have carried out monitoring activity for physical and chemical parameters, and phytoplankton.

Sampling was carried out with a nearly monthly frequency from 1988 to 2020 in the framework of several research projects and on board of several research vessels (S. Lo Bianco, Tecnopeca 2, G. Dallaporta, Tethis, Copernaut Franca, Urania, Alliance, Minerva, Bannock, D'Ancona, Actea).

Water samples for phytoplankton analysis have been collected at the surface by Niskin bottles, transferred to 250 ml dark glass bottles, preserved by adding 0.8% prefiltered formaldehyde and neutralized with hexamethylenetetramine (Thronsen 1978), and stored at 4°C until analysis.

From January 2018 to December 2019, net samples (20-µm mesh net) have been collected to isolate cells and set up monoclonal cultures of strains of pennate diatoms belonging to Bacillariaceae for molecular and ultrastructural analyses.

Phytoplankton analysis

Phytoplankton analysis was carried out using an inverted microscope (ZEISS Axiovert 135) equipped with phase contrast, following the Utermöhl method (Edler & Elbrächter 2010). Identification and counting were carried out at 400× magnification along transects or in random visual fields, depending on cell abundance, to count a minimum of 200 cells.

Pennate diatoms with an expanded central part and two long rostra, for which no identity could be assigned, have been provisionally named '*Nitzschiella*', although sometimes they may have been misidentified as *Cylindrotheca closterium* or *Pseudo-nitzschia galaxiae*.

Strain isolation

The isolation of single '*Nitzschiella*' cells was performed using a capillary pipette on an inverted microscope (Hoshaw & Rosowski 1973). Cultures were maintained at $21 \pm 0.1^\circ\text{C}$ with a 12:12 h (light:dark) photoperiod and an irradiance of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, in sterile-filtered seawater enriched with f/2 nutrients (Guillard & Ryther 1962). Every month, the algal cultures were reinoculated in fresh culture medium. A total of six strains of the taxon studied were established in July 2019.

DNA extraction, PCR amplification and sequencing

Algal cultures were harvested during late exponential growth phase and centrifuged at $4000 \times g$ for 15 min. Pellets were extracted using CTAB (N-cetyl-N,N,N-trimethylammoniumbromide) buffer (2% CTAB, 1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, 5M NaCl, 1%) modified from Doyle & Doyle (1987).

Extracted DNA was amplified by Polymerase Chain Reaction (PCR) with a SimpliAmpTM Thermal Cycler (Thermo Fisher Scientific). PCR amplification of ITS (ITS1–5.8S–ITS2) and LSU (region D1–D3) ribosomal genes has been conducted as described in Accoroni *et al.* (2020).

Sanger sequencing was carried out by MacroGen Europe using the same couple of primer used for PCR amplification (D1R–D3Ca and ITS1–ITS4; Accoroni *et al.* 2020).

Molecular analyses

Forward and reverse sequences were aligned and manually adjusted for the presence of double peaks and missing or incorrect insertions of bases by eye with BioEdit (Hall 1999).

The ambiguities in forward and reverse sequences were resolved avoiding the use of degenerate nucleotides (e.g. R, Y and K).

The alignment of LSU sequences included the four sequences from this study and 48 sequences from GenBank, with the purpose of adding closely related sequences retrieved from a BLAST tool search ($\geq 98\%$ query coverage, $>90\%$ identity; Table S1) in which some species of *Nitzschia* sect. *Nitzschiella* (*Nitzschia draveillensis* M. Coste & Ricard, *N. varelae* Carballeira, D.G. Mann & Trobajo) and *Cylindrotheca closterium*, and some DA-producing diatoms, viz. *Nitzschia navis-varingica* Lundholm & Moestrup, *Pseudo-nitzschia cuspidata* (Hasle) Hasle and *P. pseudodelicatissima* (Hasle) Hasle, were included. A maximum of two representatives of each group of identical sequences with identical names were chosen. The last closely related sequences retrieved from the BLAST search were chosen as root, viz. *Pseudo-nitzschia americana* (Hasle) G.A. Fryxell, *P. cuspidata* and *P. pseudodelicatissima*. Alignments were made with ClustalW (Thompson *et al.* 1994) (<https://www.genome.jp/tools-bin/clustalw>) with default settings. In particular, the strain *Cylindrotheca* sp. (MT489381) contained complete sequences of SSU-ITS1-5.8s-ITS2-LSU and the alignment resulted in $>3,000$ nucleotides; the alignment was manually adjusted with Bioedit in order to exclude gaps resulting from non-aligned sequences.

The final alignment yielded 684 characters, of which 477 conserved and 203 variable sites, and 160 were parsimony informative.

Two independent analyses were used to infer LSU phylogenies: Maximum Likelihood (ML) and Bayesian Inference (BI). The best nucleotide substitution model was tested with Partitionfinder 2 (Lanfear *et al.* 2017). The construction of both ML and BI was conducted

with generalized time-reversible evolution model (GTR) with the addition of gamma distribution and invariant sites for BI (GTR+G+I).

ML analyses were carried out with RAxML (Stamatakis *et al.* 2008) with 1,000 pseudo replicates. Bayesian analyses were carried out using MrBayes 3.2 (Ronquist *et al.* 2012) with 3,000,000 Markov chain Monte Carlo generations, and sample frequency of 1,500 and diagnosing frequency of 1,000. The statistical validity of the Bayesian analysis was checked with Tracer v.1.7 (Rambaut *et al.* 2018). The 50% majority rule consensus tree was constructed discarding the first 25% of samples. Posterior probabilities were calculated to measure tree strength. Both ML and BI analyses were carried out through Cipress portal (Miller *et al.* 2011).

The outputs from the analyses were visualized by FigTree v1.4.4 (Rambaut 2018). The genetic *p*-distance was calculated using MEGA7 (Kumar *et al.* 2016). Moreover, in order to detect individual differences, LSU and ITS sequences of *N. gobbii* were aligned in two separate datasets and then *p*-distance was tested. The resulting datasets from LSU and ITS sequence alignments yielded 676 and 639 characters, respectively.

Morphological characterization

Field and cultured '*Nitzschia*' cells were measured for their length (apical axis) in LM at 1000× magnification using a micrometric ocular on an inverted microscope (ZEISS Axiovert 135) equipped with phase contrast.

A total of four strains were used for ultrastructural analysis by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Subsamples were collected from cultures during exponential phase. The collected strains were acid-cleaned following the von Stosch's protocol (Hasle & Syvertsen 1997). Briefly, samples were centrifuged, the supernatant was removed, then the pellet was resuspended with 1 ml of distilled water to

remove marine salt. After that, cells were gently cleaned (i.e. in bain-marie instead of directly on the Bunsen as indicated in the protocol) with HNO₃ and H₂SO₄ (1/4 v/v, respectively) to remove organic material and preserve the delicate frustules of this species, and washed with distilled water (four times, at least). A drop of the cleaned material was placed on a grid and another on a stub for observation with a Philips TEM 400 microscope and a Zeiss Supra 40 FE-SEM (Carl Zeiss AG, Oberkochen, Germany), respectively. Several cells were measured (see Table 1) for width (transapical axis), fibulae, striae and poroids density in both valves and cingular bands.

Valve shape and morphometric data of morphologically similar species were recovered from the literature (references in Table 1) in order compare *N. gobbii sp. nov.* with similar species.

Toxin content

CHEMICALS AND STANDARDS

The acetonitrile (MeACN) and formic acid (FA) were of LC-MS grade, and the methanol (MeOH) was of HPLC grade. Water was distilled and passed through a MilliQ water purification system (DIW) (Millipore Ltd., Bedford, MA, USA).

Certified reference material for DA, CRM-DA-g (103.3 µg ml⁻¹), was purchased from the Institute of Biotoxin Metrology at the National Research Council of Canada (NRCC, Halifax, Nova Scotia, Canada). Calibration solutions of DA were prepared from serial dilutions of the reference material in DIW.

DOMOIC ACID EXTRACTION

Chemical analysis to assess the toxin content requires a large quantity of cells, so each strain was grown in an increasing volume, up to a maximum of 2 litres to achieve abundances ranging from 2.0×10^8 to 3.8×10^8 cells.

The strains were grown in the same culture conditions reported above. Cells were harvested from the early stationary growth phase. Algal pellets of three *Nitzschia gobbii* strains were extracted using a mixture of MeOH/H₂O (50:50 v/v), following the official EU-RL RP-LC-UV method (EURLMB 2008) for the determination of DA in shellfish and finfish.

The entire culture volume (2 litres) was centrifuged for 20 min at 2500 × g (4°C) in 40 centrifuge tubes (50 ml volume). Pellets were combined and extracted with 5 ml of MeOH/H₂O (50:50 v/v), vortex-mixed for 1 min, and bath-sonicated for 10 min. After sonication, the aliquot was centrifuged for 10 min at 2500 × g (4°C), and the supernatant was transferred to a 100 ml evaporation flask. Pellet extraction was repeated three times, and the supernatants were combined and evaporated to dryness. The residue was reconstituted in 1 ml of MeOH/H₂O (50:50 v/v) and filtered through a 0.2-µm syringe filter (Minisart, Sartorius, Germany) for LC-MS/MS analysis.

LC-MS/MS ANALYSIS

LC-MS/MS analyses were performed using a hybrid triple-quadrupole/linear ion trap 3200 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbo V source and an electrospray ionization (ESI) probe. The mass spectrometer was coupled to an Agilent model 1200 LC instrument (Palo Alto, CA, USA), which included a solvent reservoir, inline degasser, quaternary pump, refrigerated autosampler and column oven.

The method was implemented following the conditions described by Mafra *et al.* (2009), which were properly modified. LC separation was performed using a Gemini[®] NX-C18 column (2 mm × 100 mm, 3 µm particle size; Phenomenex, Torrance, CA, USA), set at 40°C, with a flowrate of 0.4 ml min⁻¹. Mobile phase A was DIW and B MeACN, both containing 0.2% of FA. Gradient elution was adopted, as described below: from 10% to 20%

B in 5 min, from 20% to 35% B in 1 min, then hold for 6 min, return to the original conditions at 13 min, and hold for 7 min before the next injection.

Infusion experiments were performed using CRM-DA-g to set the turbo IonSpray source parameters as follows: Nebulizer Gas (GS1) 50 psi, Auxiliary Gas (GS2) 60 psi, Temperature (TEM) 600°C, Ion Spray Voltage (IS) 5000 V, Curtain Gas (CUR) 20 psi.

DA was detected using Multiple Reaction Monitoring (MRM) in positive ion mode by selecting the following transitions: m/z 312.2→266.1, m/z 312.2→220.1 and m/z 312.2→161.1. In addition, the pseudotransition m/z 334.2→334.2 of sodium adduct [DA + Na]⁺ was monitored to investigate ion suppression due to salts. A declustering potential (DP) of 60 V and a collision energy (CE) of 30 V were used for all transitions.

The limit of quantitation (LOQ), calculated assuming a signal to noise (S/N) ratio of 10, was 10 ng ml⁻¹, while the limit of detection (LOD) (S/N ratio of 3) was 3 ng ml⁻¹.

Statistical analyses

The Shapiro-Wilk test was used to check data for normal distribution, and the Bartlett's test was used to assess homogeneity of variances. Data were not normally distributed, nor homogeneity of variances were respected, therefore Kruskal-Wallis test was performed to test seasonality. When significant differences were detected ($p < 0.05$), a Pairwise *t*-test comparison was performed.

RESULTS

Nitzschia gobbii Accoroni, Romagnoli, Giulietti & Totti *sp. nov.*

Figs 1–26

DESCRIPTION: 1) Light microscopy observations: Cells solitary, not forming colonies, 27.0–97.5 µm in length (apical axis) and 0.7–2.5 µm in width (transapical axis) (Table 1). Frustules lanceolate-fusiform, weakly silicified and expanded at the centre for ½–⅓ of the total length, with two thin rostra. Rostrate parts either straight or slightly curved (Figs 1–8). In the latter case, rostra could be

curved either in the same or in different directions. Live cells with two yellow chloroplasts (Figs 1–8). Cells in field samples appear thinner in central part and with longer rostra (Figs 1–4) than in culture (Figs 5–8). In cultured cells, the expanded central part is longer with respect to the overall cell length than in field material. In cultured cells, rostra are often straight. 2) Electron microscopy observations: Valves lanceolate-fusiform. Raphe more or less eccentric, sometimes sinuous (Figs 13, 22) not elevated into a prominent keel (Figs 9–13). Central nodule present (Figs 14–16). At the poles, raphe ends are straight or slightly curved (Figs 17–19). Raphe observed either on the same side on both valves (hantzschoid symmetry) or on opposite sides (nitzschoid symmetry) (Figs 20, 21). Conopeum not present. Wall of outer raphe canal perforated by one row of poroids on both margins (Fig. 23). Fibulae unequally distributed, largely spaced at the central nodule and with a different distribution pattern between epi- and hypovalve (Figs 20–22). Fibulae crossing both valve margins and becoming more regularly spaced toward the apices (Fig. 23). Density of fibulae higher on rostra (10–43 in 10 µm) than on the central part (2–20 in 10 µm) (Figs 20–23). Transapical striae (36–42 in 5 µm) uniseriate and incomplete (Figs 20–22), with areolae characterized by irregular distribution pattern and hymenation. Poroids with variable arrangements, including striae appearing as strips of perforations where poroids are not distinguishable (Figs 24–25). In girdle view, two (three) girdle bands, with two rows of poroids and 56–64 band striae in 5 µm. Valvocopula and second cingular band have identical pattern with poroids perforated in dots, sometimes circularly arranged (Fig. 26). The presence of a third band was detected only once. Domoic acid was not detected. D1–D3 of LSU rDNA: GenBank accession number MW891545; ITS rDNA: GenBank accession number MW891543.

HOLOTYPE: Acid-cleaned material of strain NT07191 deposited as stub (Stub_007_ANC) at the Botanical Museum of the Università Politecnica delle Marche, Ancona Italy (ANC), and bearing many specimens.

ISOTYPE: Acid-cleaned material of strain NT07191 on permanent slide (BM 81885) has been deposited at the Natural History Museum in London, with many specimens.

TYPE LOCALITY: station SG01 (LTER Senigallia-Susak transect) northern Adriatic Sea, Italy (43°45.86'N, 13°13.00'E), 18th July 2019.

ETYMOLOGY: The specific epithet honours the technician Luigi Gobbi from Università Politecnica delle Marche, recently retired, who provided valuable help with electron microscopy.

COMPARISON WITH SIMILAR SPECIES (DIAGNOSIS): Several species are morphologically similar to *Nitzschia gobbii* sp. nov. (see comparison in Table 1). *Nitzschia gobbii* can be distinguished from *N. acicularis* (Kützinger) W. Smith, *N. decipiens* Hustedt, *N. draveillensis*, *N. droebakensis* Hasle, *N. gaoi*

Bing Liu, S. Blanco & B.Q. Huang, *N. inordinata* Mucko & Bosak, *Pseudo-nitzschia galaxiae*, *N. kavirondoensis* Sitoki & Rott, *N. rusingae* Rott & Sitoki, *N. varelae*, *N. ventricosa* J.L. Palmer and *N. lecointei* Van Heurck, by the higher number of striae in 10 μm (and also by the higher number of band striae in 10 μm for *N. kavirondoensis* and *N. rusingae*). *Pseudo-nitzschia galaxiae* and *N. varelae* differ from *N. gobbii* in having poroids in the outer canal wall, while *N. acicularis*, *N. decipiens*, *N. gaoi* and *N. rusingae* differ from *N. gobbii* in lacking the central nodule. The thin fusiform valve shape of *N. gobbii* can also be useful for identification: *N. varelae* is arcuate, *N. droebakensis* has longer rostra with respect to the enlarged central part, *N. lecointei* is more lanceolate and often lacks rostra. Compared to *Cylindrotheca closterium*, *N. gobbii* has a smoother expansion in the central part of the valve and the raphe is not twisted. *Nitzschia inordinata* has circular poroids and differs from *N. gobbii* in having a longer and wider frustule with a higher central-valve/rostra ratio. *Nitzschia rusingae* and *N. ventricosa* are longer than *N. gobbii*.

Molecular analyses

Blast results of the 4 LSU and ITS sequences matched with 98.4% (cover 98–99%) and 91.6–91.9% (cover 100%) of identity, respectively, with *Nitzschia* cf. *pusilla* (KT390088) (Table S1). The 4 LSU sequences were all identical (*p*-distance 0.000), while the 4 ITS sequences had 1 variable site (nucleotide position 603/639, C replaced by T) in strain NT07198 (*p*-distance 0.002).

Phylogenetic trees inferred from ML and BI analyses of LSU were mostly congruent. An exception was the node comprising *Nitzschia gobbii* and *Nitzschia* cf. *agnita* (AF417664): in the ML topology *N. gobbii* is resolved alone (98), while in BI one node included both taxa with a long branch separating *Nitzschia* cf. *agnita* from *Nitzschia gobbii* (Figs 27, S1). The ML consensus tree showed that all the four strains were resolved in a clade (0.97/75) with *Nitzschia* cf. *agnita* (AF417664, *p*-distance 0.037) and *Nitzschia* cf. *pusilla* (KT390088, *p*-distance 0.017). The basal node comprising *Nitzschia* spp, *Cylindrotheca* spp and *Bacillaria* spp was strongly supported (1.00/100). Many of the deepest nodes were without significant support but clades comprising *Cylindrotheca* spp (i.e. *C. closterium*, *Cylindrotheca* sp and *C. fusiformis* Reimann & J.C. Lewin, although the latter is now regarded as an uncertain

taxonomical entity) and *Bacillaria* spp were strongly supported (1.00/98 and 1.00/100, respectively). *Cylindrotheca closterium* showed an important divergence from *N. gobbii* (*p*-distance 0.071).

Among *Nitzschia* spp, we could recognize three strongly (clade *a* 1.00/96, *b* 1.00/91 and *d* 1.00/98) and two moderately (clade *c* 1.00/83 and *e* 0.97/75) supported clades. The DA-producer *Nitzschia navis-varingica* formed a well-supported branch (0.99/100) inside clade *a*, showing a consistent divergence from *N. gobbii* (*p*-distance 0.068). *Nitzschia draveillensis* formed a branch supported only in ML analysis (90) inside clade *d*, while *Nitzschia varelae* was not supported in either analysis. These two species clearly diverge from *N. gobbii* (*p*-distance 0.045 and 0.048, respectively).

Seasonal trend

Nitzschia gobbii has been commonly recorded in the study area throughout the study period (1988–2019). Significant differences among seasons were observed (Kruskal-Wallis $p < 0.01$) but the pairwise comparison among seasons did not detect significant differences (Bonferroni correction, $p > 0.05$). The mean annual cycle, calculated on a monthly basis, showed that it has been recorded mainly in spring-summer with the highest mean abundance of $1.0 \times 10^5 \pm 7.1 \times 10^4$ cells l^{-1} recorded in July. During the rest of the year, *Nitzschia gobbii* never exceeded the mean abundance of 2.6×10^4 cells l^{-1} (Fig. 28). The highest abundance of 3.2×10^6 cells l^{-1} was recorded in July 2019 (89% of the total diatoms community) during the bloom from which the species was isolated.

Toxin content

None of the tested strains by LC-MS/MS produced DA in detectable amounts; the LOD varied between 0.0079 and 0.015 fg cell $^{-1}$.

DISCUSSION

In this study we described and named a planktonic pennate diatom belonging to *Nitzschia* sect. *Nitzschiella*, i.e. species with an expanded central part and long protruding rostra, as *Nitzschia gobbii* sp. nov. Although never described until now, this is a common component of the phytoplankton community in the coastal station of Senigallia-Susak transect, where its presence has been noticed for 30 years.

Nitzschia gobbii described in this study belongs to the genus *Nitzschia* given its morphological features, i.e. solitary cells, lanceolate valve shape, nitzschioid symmetry, poroids in the outer canal raphe. This species has a more or less eccentric raphe, showing both nitzschioid and hantzschoid symmetry. For a long time *Nitzschia* species were thought to be distinguishable from species of *Hantzschia* Grunow on the basis of the position of the eccentric raphe, representing either a nitzschioid or a hantzschoid symmetry (Grunow 1877). However, this classification was later revised by Mann (1980) and Pickett-Heaps (1983), and *Nitzschia* species were recognized to have both symmetries, whereas *Hantzschia* species produce sibling valves with raphe systems with only hantzschoid symmetry. Later studies revealed that two unrelated lineages (i.e. *Cymbellonitzschia banzuensis* Stephanek, Hamscher, S.Mayama, Jewson & Kociolek and *Nitzschia varelae*) showed hantzschoid symmetry (Mann & Trobajo 2014; Stepanek *et al.* 2016; Carballeira *et al.* 2017).

Morphometric data of *Nitzschia gobbii* compared to those of species with *Nitzschiella* morphology are summarized in Table 1. Although, *N. gobbii* has notable features, such as the unequal distribution of poroids within striae and the irregular hymenation pattern, this species shares some morphometrical data with some others of the section. For example, its length is close to that of *N. acicularis*, *N. decipiens*, *N. droebakensis* and *Pseudo-nitzschia galaxiae*, and the number of striae in 10 μ m is comparable with that of *Cylindrotheca closterium*. Although *N. gobbii* has been observed with rostra curved in opposite directions as in *N.*

reversa W. Smith (1853) and in *N. inordinata*, the new species can be also found with rostra curved in the same directions, with only one curved rostrum or almost straight rostra, as in *N. acicularis*, in which, however, the central nodule is absent.

In the Mediterranean Sea, species showing ‘*Nitzschiella*’ morphology are *Cylindrotheca closterium*, *Pseudo-nitzschia galaxiae* and *Nitzschia inordinata*. The valve shape of *C. closterium* resembles to that of *N. gobbii* (Fig. 29), but, in the latter, frustule is more delicate and the central valve expansion is smoother (Fig. 30). Furthermore, *C. closterium* has a clearly twisted raphe (although visible only with EM) unlike *N. gobbii*, which shows sometimes a sinuous raphe. Due to the widespread distribution of *C. closterium*, we suspect that a large proportion of misidentifications of *N. gobbii* may have been as *C. closterium*.

The valve shape of *Nitzschia gobbii* is very similar to that of *Pseudo-nitzschia galaxiae*, a well-known DA producer (Lundholm & Moestrup 2002), and maybe these two species have also been misidentified in LM (Figs 29, 31), especially because *P. galaxiae* is frequently observed as solitary cells rather than as colonies, unlike most other *Pseudo-nitzschia* species (Lundholm & Moestrup 2002; Cerino *et al.* 2005). It is noteworthy that *N. gobbii* field and cultured cells look like the long and medium morphotypes of *P. galaxiae* (*sensu* Cerino *et al.* 2005), respectively. However, the ultrastructure of *P. galaxiae* has clearly distinguishable characteristics in comparison to *N. gobbii*: in valve view, transapical striae are less dense and are formed of hymenate perforations (3–4 nm in diameter), and cells have no poroids in the outer canal wall (as in all *Pseudo-nitzschia* species) (Lundholm & Moestrup 2002).

Molecular analysis confirmed the presence of *P. galaxiae* in the NE Adriatic Sea (Turk Dermastia *et al.* 2020). In the western Adriatic coastal waters, studies focused on *Pseudo-nitzschia* populations and based only on molecular analyses never detected its presence (Penna *et al.* 2013; Pugliese *et al.* 2017; Giulietti *et al.* 2021), although *P. galaxiae* has been

sometimes reported by monitoring based on morphological analysis (Caroppo *et al.* 2005; Regione Veneto 2007; Regione Emilia-Romagna 2015, 2017).

Nitzschia gobbii is similar to the recently described species *N. inordinata* in possessing incomplete uniseriate striae with unequal distribution of poroids (Mucko *et al.* 2020). However, *N. inordinata* is very silicified and has well-defined, circular poroids, unlike *N. gobbii*, which has irregular poroids. Moreover, *N. inordinata* is longer, wider and has a lower density of transapical striae in 10 µm than *N. gobbii* (Table 1).

The four LSU sequences of *Nitzschia gobbii* are resolved in a well-supported clade, and in the ML tree they clustered alone. According to ML and BI analysis, the closest relatives of *N. gobbii* are *Nitzschia* cf. *pusilla* (KT390088) and *Nitzschia* cf. *agnita* (AF417664), collected by Fan *et al.* (2015) in South China Sea and Lundholm *et al.* (2002) in Denmark, respectively.

Unfortunately, Fan *et al.* (2015) did not supply morphological information for *Nitzschia* cf. *pusilla* (KT390088), and Lundholm *et al.* (2002) supplied little information about *Nitzschia* cf. *agnita* (AF417664), which differs from *N. gobbii* by the absence of the central nodule and by the circular poroids in valve striae.

Among the closest relatives of *Nitzschia gobbii* highlighted by the molecular analyses in this study, only *C. closterium*, *N. draveillensis* and *N. varelae* show ‘*Nitzschiella*’ morphology, confirming that this section is not a molecularly supported group (Lundholm & Moestrup 2002; Mann *et al.* 2021). A significant genetic distance was detected between *N. gobbii* and those genera considered monophyletic such as *Pseudo-nitzschia* and *Cylindrotheca* (Lundholm *et al.* 2002; Carballeira *et al.* 2017; Mann *et al.* 2021).

Nitzschia is a paraphyletic assemblage and molecularly defined subgroups were highlighted in several studies (Rovira *et al.* 2015; Carballeira *et al.* 2017; Mann *et al.* 2021) and confirmed by clades *a-e* from this study. In particular, the DA-producer *Nitzschia navis-*

varingica formed a strongly supported branch (as in Lundholm *et al.* 2002; Mann *et al.* 2021) well separated from *N. gobbii*, with a high *p*-distance supporting the divergence.

In the Adriatic Sea, planktonic bacillariacean diatoms, such as *Cylindrotheca closterium* and *Pseudo-nitzschia galaxiae* have been commonly identified in both western (Caroppo *et al.* 1999; Sabetta *et al.* 2005; Bandelj *et al.* 2008; Bernardi Aubry *et al.* 2018; Totti *et al.* 2019) and eastern (Mozetič *et al.* 1998; Najdek *et al.* 2005; Bosak *et al.* 2009; Cabrini *et al.* 2012; Marić *et al.* 2012; Turk Dermastia *et al.* 2019) coastal areas. *Nitzschia gobbii* may have been confused with delicate and finely striated species of *C. closterium* or with *P. galaxiae* (particularly the long morphotype, Cerino *et al.* 2005) when not in colony. During the 30 years of the study period, *Nitzschia gobbii* appeared throughout the year, mainly in summer, sometimes with high abundance, and was a regular inhabitant of mucilage aggregates where, although it was named '*Nitzschiella*' during counting, it was later reported as *C. closterium* (Pettine *et al.* 1993; Totti *et al.* 2005). The species is probably widespread in the Adriatic Sea: it has been recorded also in the Venice Lagoon (Bernardi Aubry, personal communication) and in the Gulf of Trieste (Cerino, personal communication).

Several species of Bacillariaceae belonging to both *Nitzschia* (i.e. *N. bizertensis* B.Smida, N.Lundholm, A.S.Hlaili & H.H.Mabrouk and *N. navis-varingica*) and *Pseudo-nitzschia* are known to produce DA (Bates *et al.* 2018). In the Adriatic Sea, DA was recorded for the first time by Ciminiello *et al.* (2005) in *Mytilus galloprovincialis*, with a toxin content that ranged from 63 to 190 ng g⁻¹, i.e. well below the regulatory limit of DA in tissue (20 mg kg⁻¹; European Council 2004) . Since then, DA rarely reached 2 mg kg⁻¹ in tissue (Ljubešić *et al.* 2011; Marić *et al.* 2011; Arapov *et al.* 2017), so the result from this study showing that *N. gobbii* did not produce DA in detectable amounts was not surprising.

In conclusion, results from this study highlighted the presence of a pennate diatom never described before but commonly occurring in the phytoplankton community of the

Adriatic Sea. This new species looks like other common species when observed through LM, some of which are toxic. Hence, the characterization of this new species is relevant in order to avoid erroneous reports of potential DA producers. However, the absence of toxin detection in *N. gobbii* does not definitively exclude that this species may be potentially toxic, as several potentially toxic species have been reported to be sometimes toxic and non-toxic in other occasions, or in other areas (e.g. *Pseudo-nitzschia*, Trainer *et al.* 2012; Zingone *et al.* 2021), and further studies are encouraged on other strains from different areas.

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TABLES AND FIGURES

Table 1. Morphological characters of species showing the '*Nitzschiella*' (*sensu* Cleve & Grunow 1880; Hasle 1964) morphotype. Cell length (apical axis) was measured in LM at 1000x. Cell width (transapical axis), fibulae, striae and poroids density were measured in EM.

Species	Apical axis (μm)	Transapical axis (μm)	Central nodule	Fibulae in 10 μm (body)	Fibulae in 10 μm (rostra)	Striae in 10 μm	Poroids in 1 μm	Band striae in 10 μm	Poroids in outer canal	References
<i>Nitzschia gobbii</i> sp. nov.	27.0–53.1 ^a (44.0 \pm 4.8) n=59	0.7–2.5 (1.1 \pm 0.4) n=46	+	2–20 (8.4 \pm 2.9) n=80	10–43 (27.7 \pm 9.2) n=74	72–84 ^b (77.8 \pm 2.6) ^b n=68	Irregular pattern	112–128 ^b (121.6 \pm 4.0) ^b n=52	+	This study (culture samples)
<i>Nitzschia acicularis</i>	ca. 64	4	-	16–20	n.r.	60	5	n.r.	+	Hasle (1964)
<i>N. decipiens</i>	32–36	2.7	-	7–9	n.r.	44	6?	n.r.	+	Hasle (1964)
<i>N. draveillensis</i>	55–110	3.5–4.5	+	19–21	n.r.	50–60	n.r.	n.r.	n.r.	Coste & Ricard (1980)
<i>N. droebakensis</i>	35–42	3	+	16–22	n.r.	60	7–8	n.r.	+	Hasle (1964)
<i>N. gaoi</i>	83–107	3.8–5.7	-	8–11	8–13	40	5	n.r.	+	Liu <i>et al.</i> (2015)

<i>N. inordinata</i>	91–152	4–8	-	7–10	n.r.	20–24	3	n.r.	+	Mucko <i>et al.</i> (2021)
<i>N. kavirondoensis</i>	32–77	2.8–3.7	+	15–18	n.r.	42–48	n.r.	60–70	n.r.	Sitoki <i>et al.</i> (2013)
<i>N. longissima</i>	ca. 200	6–7	+	10–27	n.r.	52–60	6.5-7	n.r.	+	Hasle & Syvertsen (1997)
<i>N. rusingae</i>	70–165	3.0–4.8	-	11–13	n.r.	46–48	n.r.	50–60	n.r.	Sitoki <i>et al.</i> (2013)
<i>N. varelae</i>	40.4–84.8	2.1–3.2	+	12.7–16	n.r.	54–60	n.r.	n.r.	-	Carballeira <i>et al.</i> (2017)
<i>N. ventricosa</i>	100-650	9-20	+	3-10	n.r.	26-27	n.r.	n.r.	n.r.	Cleve & Grunow (1880)
<i>N. lecointei</i>	21–112	2.5–5	+	5–14	n.r.	51–55	7–8	n.r.	+	Hasle (1964)
<i>Cylindrotheca closterium</i>	30–400	2.5–8	+	10–12	n.r.	70–100	ca. 30	n.r.	+	Hasle (1964)
<i>Pseudo-nitzschia galaxiae</i>	25–41	1.3–1.7	+	16–26	n.r.	56–64	several	n.r.	-	Lundholm & Moestrup (2002)

^a 30–97.5 µm in field samples

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^b measurement taken in 5 μm and reported proportionally

LEGENDS FOR FIGURES

Figs 1–8. *Nitzschia gobbii* sp. nov., LM. Scale bars = 10 μ m.

Figs 1–4. Cells from field material showing slightly curved rostra.

Figs 5–8. Cultured cells showing both slightly curved and straight rostra.

Figs 9–19. *Nitzschia gobbii* sp. nov., SEM. External (Figs 9, 14, 17, 18) and internal (Figs 10–13, 15–16, 19) views.

Figs 9–13. Valve views. Double arrowheads indicate the raphe position. Scale bars = 1 μ m.

Fig. 9. External valve.

Fig. 10. Internal valve showing an eccentric raphe.

Fig. 11. Internal valve showing a less eccentric raphe.

Fig. 12. Internal valve showing an almost centric raphe.

Fig. 13. Internal valve showing a sinuous raphe.

Figs 14–16. Magnification of the central part of the valve with different lengths of the proximal mantle (p). Arrowheads indicate the central nodule. Scale bars = 0.5 μ m.

Figs 17–19. Magnification of the apical part of the valve. Arrowheads indicate the terminal nodule. Scale bars = 1 μ m.

Figs 20–26. *Nitzschia gobbii* sp. nov., TEM.

Figs 20–22. Open cell showing both valves characterized by fibulae largely spaced at the central nodule and with a different distribution pattern between epi- and hypovalve; white arrowheads indicate the raphe position. Scale bars = 1 μ m.

Fig. 20. Nitzschoid symmetry.

Fig. 21. Hantzschoid symmetry.

Fig. 22. A sinuous raphe is evident.

Fig. 23. Cell apex with high density of fibulae; the canal raphe is perforated by one row of poroids in both margins of the raphe slit. Scale bar = 1 μm

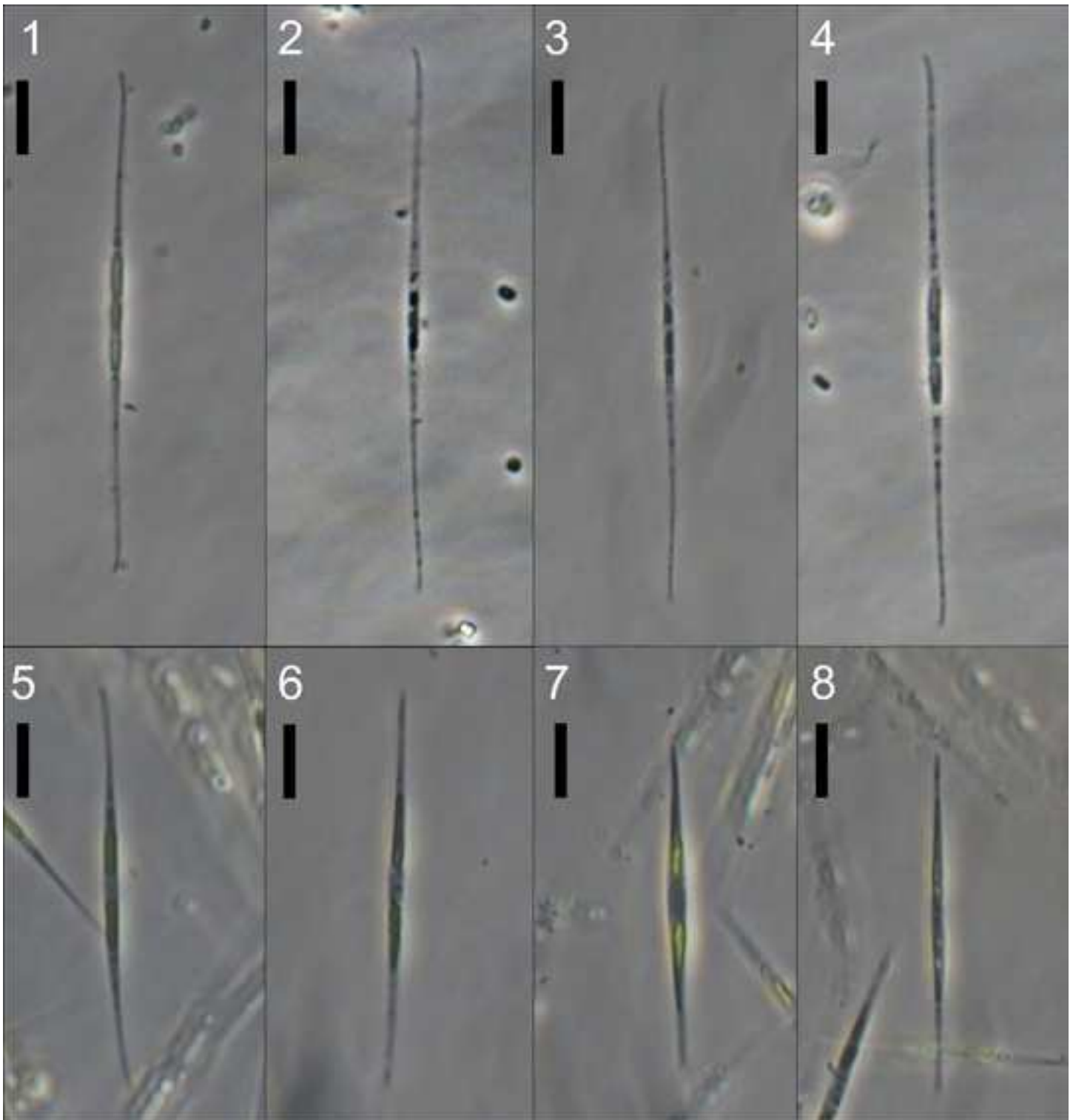
Figs 24, 25. Central part of the cell showing incomplete uniseriate striae in valve view with irregular hymenation of poroids; black arrowhead in Fig. 25 indicates a stria with a strip of perforations where poroids are not distinguishable. Scale bar = 0.5 μm .

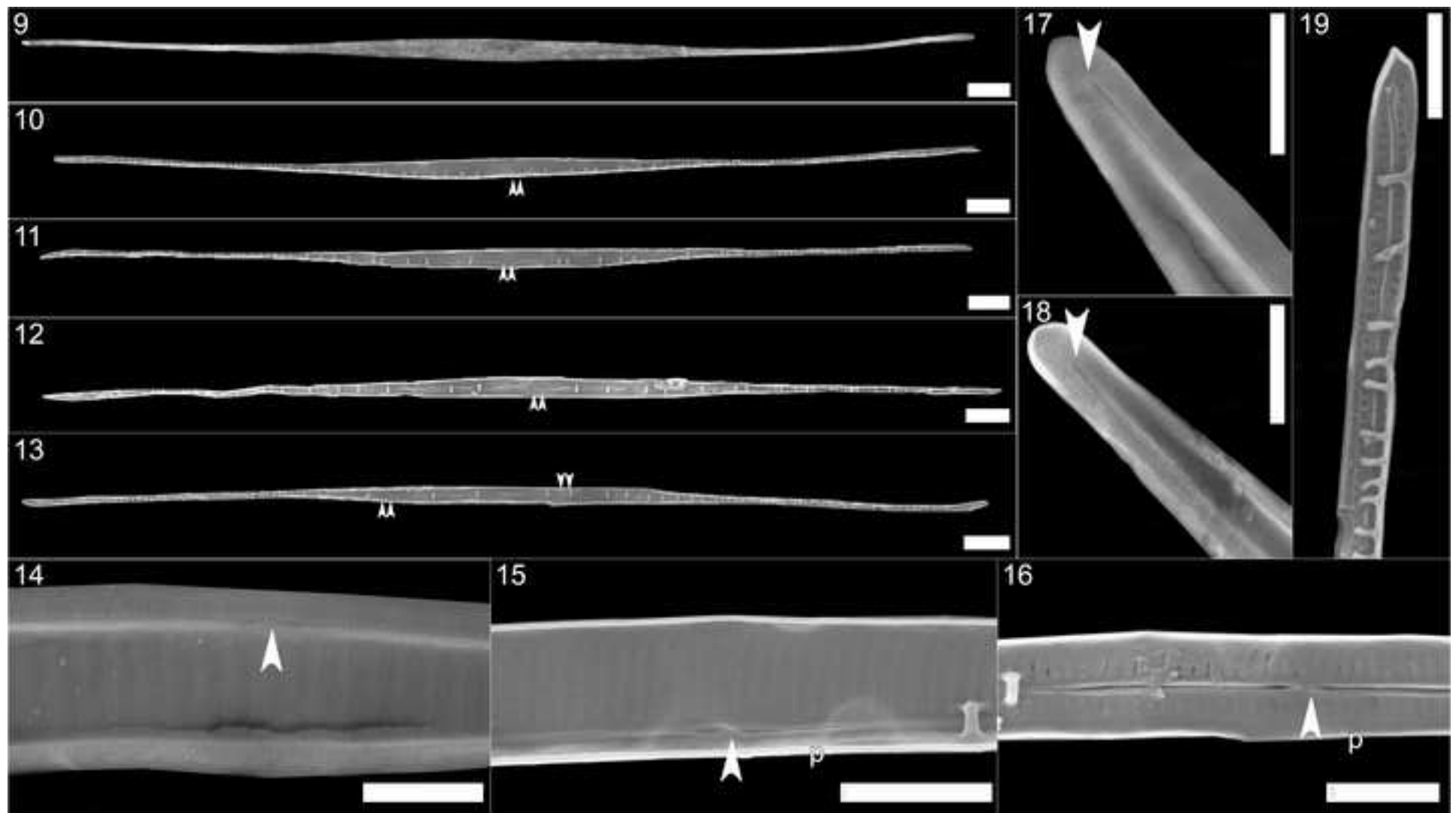
Fig. 26. Cingulum comprising two girdle bands. Each band has two rows of poroids. Scale bar = 0.5 μm .

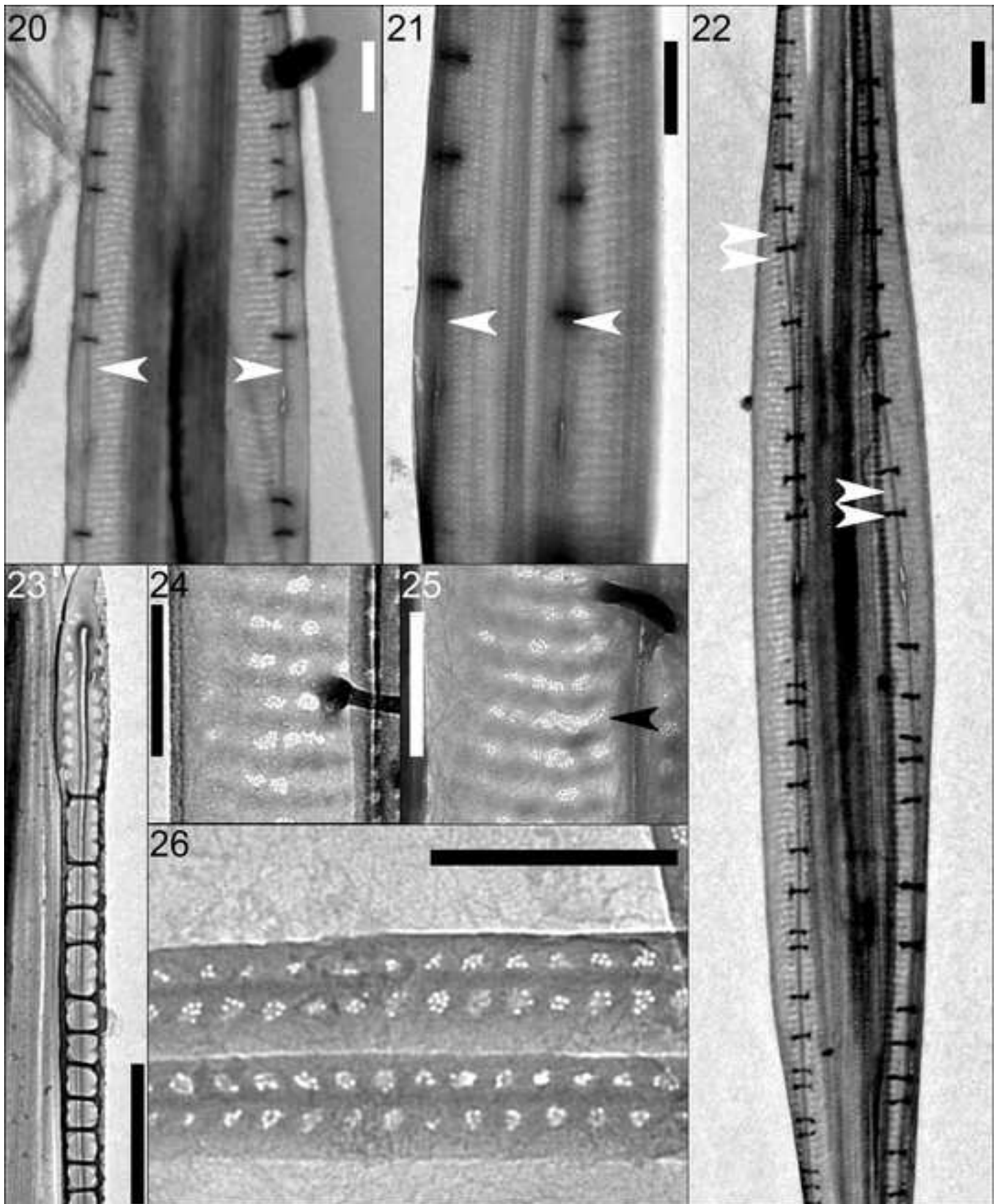
Fig. 27. ML consensus tree of the region D1–D3 of LSU rDNA, with support values of posterior probabilities (PP) and Maximum Likelihood (ML). Values on the nodes are PP/ML (only values higher than 0.95 and 70 for PP and ML, respectively, are shown). Scale bar = substitutions/site.

Fig. 28. Boxplot with the mean annual abundance (cells l^{-1}) of *Nitzschia gobbii* with the mean (circle), median (bold horizontal line), the interquartile range (boxes), and the min–max range (vertical line). The extremes and outliers are not shown.

Figs 29–31. Comparison between *Nitzschia gobbii* sp. nov. (Fig. 29), *Cylindrotheca closterium* (Fig. 30) and *Pseudo-nitzschia galaxiae* (long morphotype) (Fig. 31) as seen in LM. Scale bars = 10 μm . Image of *Pseudo-nitzschia galaxiae* courtesy of Federica Cerino.







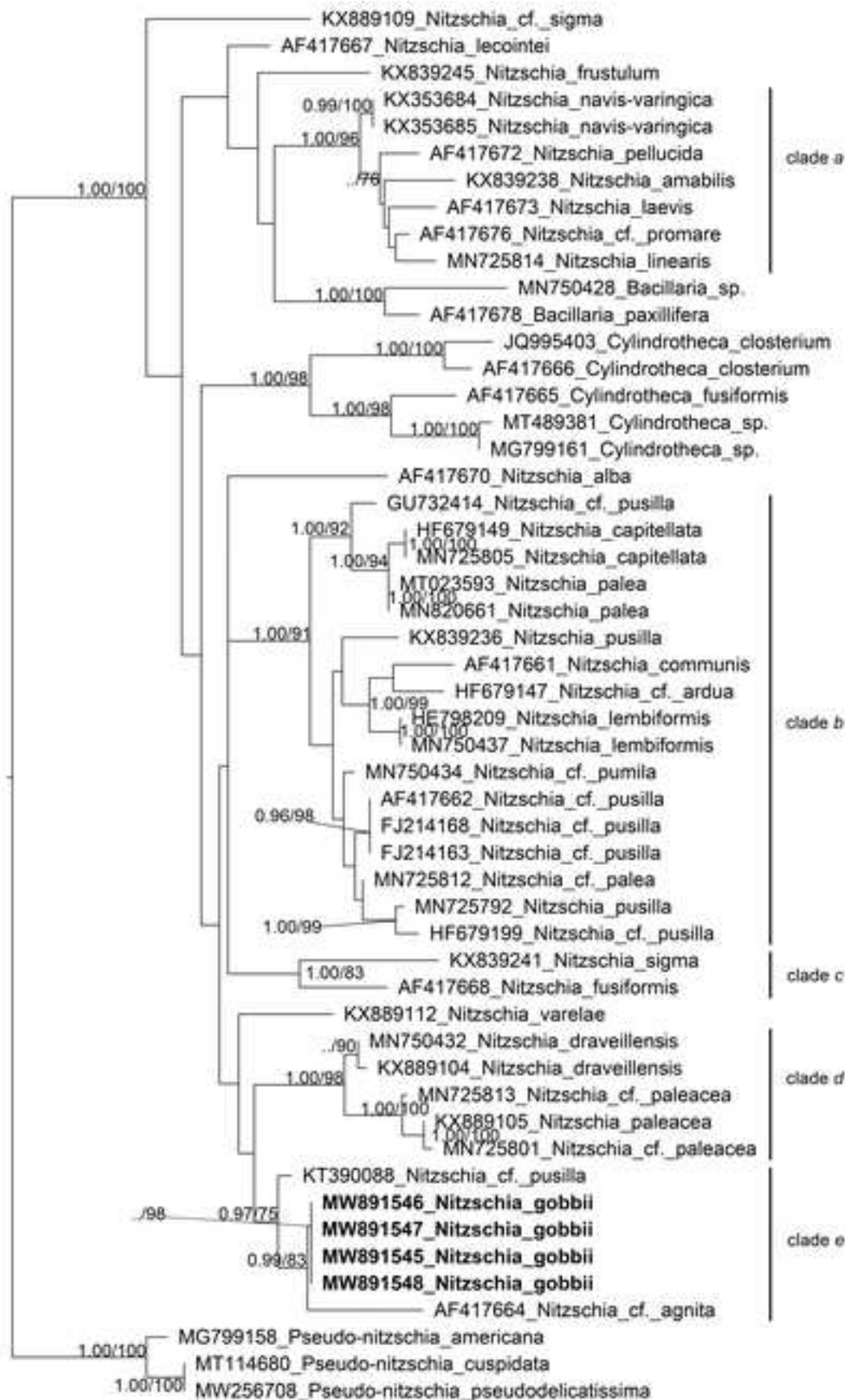
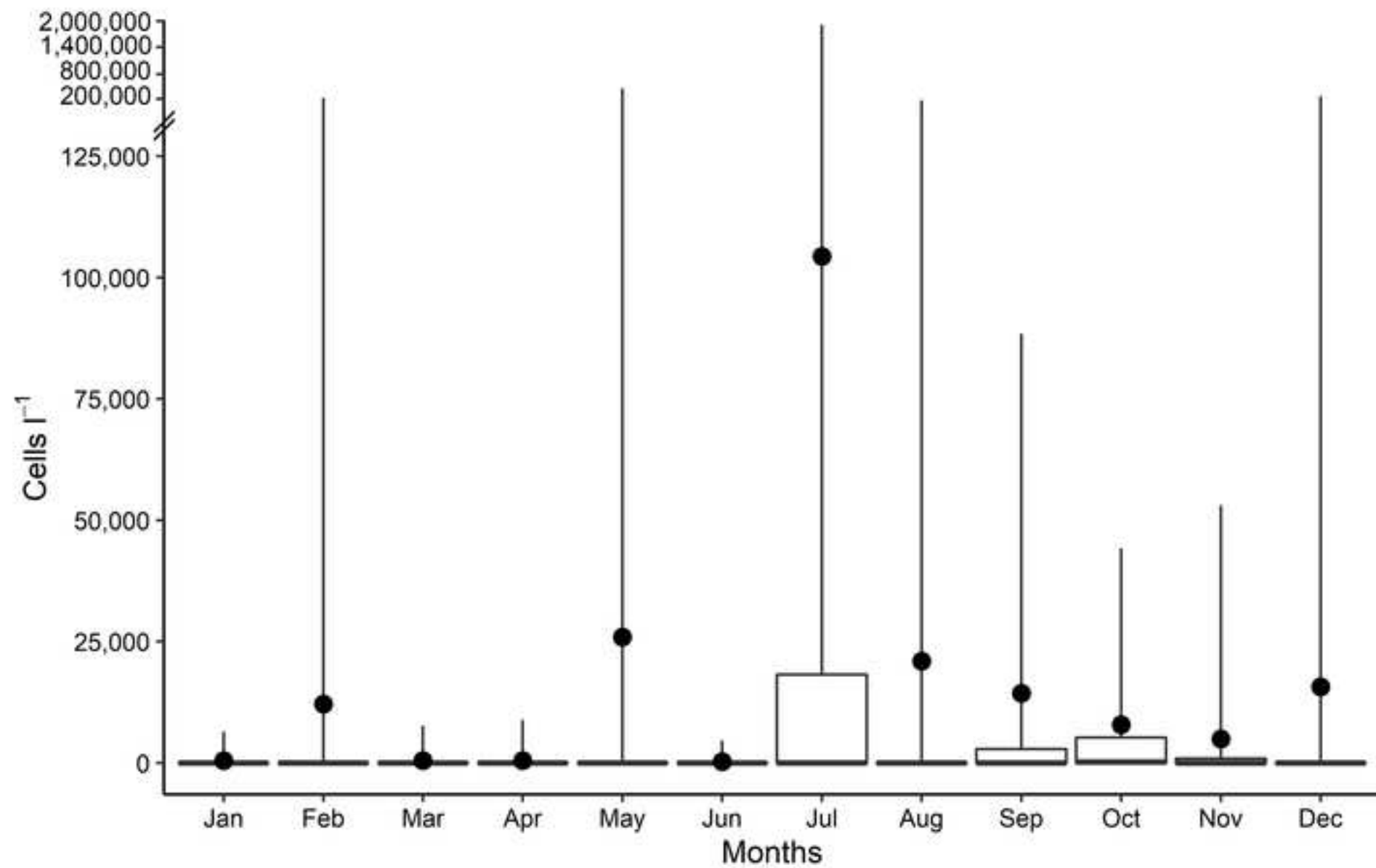
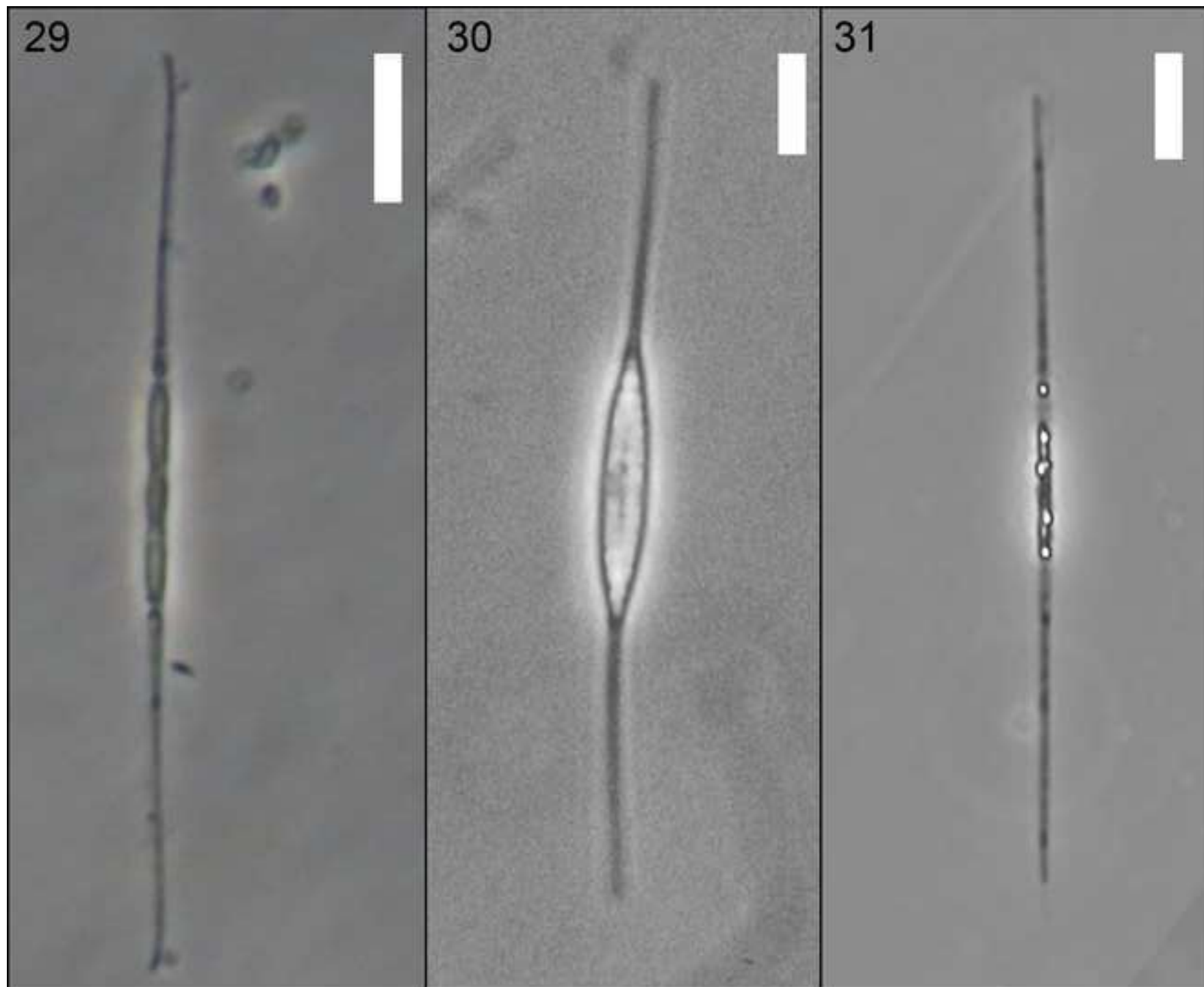
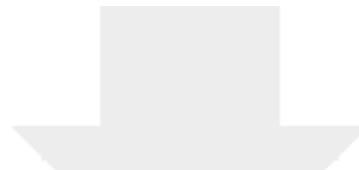


Fig. 28

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Supplemental Material

Suppl materials (Table S1 and Fig. S1).docx

