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A POLYPHASIC APPROACH TO THE STUDY OF THE GENUS *NITZSCHIA*

(BACILLARIOPHYTA): THREE NEW PLANKTONIC SPECIES FROM THE ADRIATIC SEA

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Running title: Adriatic Nitzschia species
Abstract

The paraphyletic diatom genus *Nitzschia* comprises over 1000 morphologically distinct pennate taxa, known from the benthos and plankton of freshwater, brackish and marine environments. The principal diagnostic characters for delimitation of *Nitzschia* species include valve shape, the position and structure of the raphe, presence/absence and shape of the proximal raphe endings and terminal raphe fissures, areola structure, and specific morphometric features such as cell size, and stria and fibula density. In this study, we isolated 12 diatom strains into culture from samples collected at the surface or greater depths of the southeastern Adriatic Sea. Morphological analyses included LM, SEM and TEM observations, which, along with specific morphometric features, allowed us to distinguish three new *Nitzschia* species. These findings were congruent with the results of phylogenetic analyses performed on nuclear-encoded SSU (18S) rDNA and chloroplast-encoded *rbcL* and *psbC* genes. One of the new species (*Nitzschia dalmatica* sp. nov.) formed a lineage within a clade of Bacillariaceae containing members of the *Nitzschia* sect. *Dubiae*, which was sister to *Psammodictyon*. A second lineage was part of a novel clade that is significantly distinct from other *Nitzschia* species sequenced so far and includes *Nitzschia adhaerens* sp. nov. and *N*. cf. *adhaerens*. A further new species was found, *Nitzschia inordinata* sp. nov., which appeared as the sister group to the *N. adhaerens* clade and the conopeoid *Nitzschia* species in our phylogenetic trees. Our findings contribute to the overall diversity of genus *Nitzschia*, especially in identifying some deep branches within the Bacillariaceae, and highlight underscoring of this genus in marine plankton.

Key index words: Adriatic Sea, diatoms, morphology, *Nitzschia*, phylogeny, phytoplankton

Abbreviations: *rbcL*, ribulose–1,5–bisphosphate carboxylase/oxygenase large subunit; *psbC*, photosystem II CP43 protein; ML, maximum
Introduction

Diatoms (Bacillariophyta) are mostly photoautotrophic, unicellular, eukaryotic, heterokont algae with a uniquely ornamented siliceous cell wall. They inhabit both the plankton and benthos of marine, brackish, and freshwater habitats worldwide. Diatom biodiversity is huge, though there have been different estimates of species numbers (e.g. ~100,000 species according to Mann and Vanormelingen 2013; 12,000 described and 8,000 yet to be described species estimated by Guiry 2012), and numerous new taxa are described every year. Introduction of molecular methods into diatom research, such as in specific gene phylogenies or metabarcoding studies using 18S rDNA or chloroplast-encoded rbcL, has definitely helped to enlarge our knowledge of diatom diversity (Nealson and Venter 2007, Agusti et al. 2015, de Vargas et al. 2016, Ruck et al. 2016, Dąbek et al. 2017, Mejdandžić et al. 2018, Lobban et al. 2019, Rimet et al. 2019, etc.).

The taxonomically intriguing and diverse genus *Nitzschia* is the second largest diatom genus, with approx. 1500 species described so far, the largest genus being *Navicula* (although the total for this genus is artificially high since many of its species are already known to need transfer elsewhere). Morphologically, *Nitzschia* is recognized by cells living individually or in colonies, with linear or lanceolate (more rarely broadly elliptical), not infrequently sigmoid cells, and a more-or-less transapically displaced (rarely almost central) keel (raphe canal) supported by siliceous bridges (fibulae) (Hustedt 1930). Most *Nitzschia* cells have two plastids, one in each half of the cell (in a ‘fore and aft’ arrangement).

Phylogenetic analyses have shown *Nitzschia* to be paraphyletic, with species of other genera – *Bacillaria, Cylindrotheca, Cymbellonitzschia, Denticula, Fragilariopsis, Hantzschia, Psammodictyon, Pseudo-nitzschia, Simonsenia and Tryblionella* – nested within it (e.g. Lundholm et al. 2002, Rimet et al. 2011 [in their maximum likelihood analysis of SSU rDNA...
aligned by Clustal], Stepanek et al. 2016, Witkowski et al. 2016, Carballeira et al. 2017). At first, the family or order (Bacillariaceae or Bacillariales) comprised by these genera appeared to be monophyletic (references as above and Ruck and Theriot 2011) but more recent studies (e.g. Ashworth et al. 2017, Lobban et al. 2019) have suggested that the Bacillariales might be paraphyletic, also containing the genera Craspedostauros, Staurotropis and Achnanthes; these genera lack fibulae and differ from traditional Bacillariales in several other aspects of morphology, including raphe position and structure, and areola structure. Introducing more sequences into phylogenies can often change the placements of certain genera and/or species, and it is therefore important to expand our molecular sampling effort to bridge our knowledge gaps regarding taxonomy. Moreover, polyphasic approaches combining morphology and phylogeny have been fruitful in descriptions of several novel Bacillariales species, mostly of Pseudo-nitzschia and Nitzschia (Lundholm et al. 2002, Quijano-Scheggia et al. 2009, Smida et al. 2014, Witkowski et al. 2016, Carballeira et al. 2017, Barkia et al. 2019, Lobban et al. 2019).

Nitzschia is ubiquitous, occupying freshwater, brackish and marine habitats (e.g. Cleve and Grunow 1880, Lange-Bertalot et al. 2017, Lobban et al. 2019). In freshwater lakes, Nitzschia can be common in the phytoplankton, especially in East African lakes (Sitoki et al. 2013, Grady et al. 2020). Most of these Nitzschia species are needle-shaped (N. lacustris, N. bacata, N. nyassensis, N. kavirondoensis, N. rusingae, N. fenestralis, N. aequalis, N. mediocris, etc.), and some of them can comprise >30% or even 100% of the total diatom community (Sitoki et al. 2013, Grady et al. 2020). In marine phytoplankton, Nitzschia has historically been camouflaged within a category of ‘small pennate diatoms’. Among these are a number of small, single-celled, more or less bicapitate Nitzschia species (including N. bicapitata, N. curvilineata, N. bifurcata, N. braarudii, N. capitata, N. ikeanae, N. reimersenii, N. schaunslandii and N. subinflata (Kaczmarska et al. 1986, Lee and Fryxell 1996)), which
can reach very high relative abundances sometimes accounting for 60%, 70% or even 90% of the total number of diatom cells (Semina and Mokeeva 1994). These bicapitate *Nitzschia* species vary in their size and shape and have been recorded in equatorial and subantarctic regions, including the coasts of West Africa, the Gulf of California, the Indian Ocean and the North Atlantic (Hustedt 1958, Hasle 1960, 1964, Simonsen 1974, Kaczmarska and Fryxell 1986, Kaczmarska et al. 1986).

The Adriatic Sea is an enclosed basin in the northernmost Mediterranean Sea, characterized by extreme oligotrophy. It is divided bathymetrically into three areas: the shallow North, shallow to deep Middle and deep South Adriatic Sea (Gačić et al. 2001, Poulain 2001). The South Adriatic represents a physically dynamic habitat, in which phytoplankton thrives in seasonal blooms and diatom cells sink and enrich deep water column layers with carbon (Batistić et al. 2012, Bosak et al. 2016). Research on *Nitzschia* in the Adriatic Sea has been scarce, especially in marine plankton, where most of the studies have focused on regularly blooming, potentially harmful, toxin-producing species of the genus *Pseudo-nitzschia* (Burić et al. 2008, Ljubešić et al. 2011, Marić et al. 2011, Penna et al. 2012).

One *Nitzschia* species that has been reported as ‘blooming’ in the oligotrophic waters of South Adriatic Sea is *N. sicula*, aggregating on (mini) faecal pellets of microzooplankton Nauplii; it was recorded in high abundances of 14,000–19,000 cells L⁻¹ (Viličić et al. 1994).

The three new species reported in this paper – *N. adhaerens*, *N. dalmatica* and *N. inordinata* – were found in plankton of the southeastern Adriatic Sea (Croatian coastal and open waters), with frequencies of occurrence of 20%, 5% and 22%, respectively, among the 65 samples counted (unpublished data from BIOTA [Bio-tracing Adriatic Water Masses] 2016 cruise). The aim of this study is to classify and describe the three new *Nitzschia* species, using a polyphasic approach combining extensive morphological and phylogenetical analyses. By using both light and electron (scanning and transmission) microscopy and constructing a
phylogeny based on three genes – nuclear SSU rDNA (further on SSU) and plastid-encoded
*rbcL* and *psbC* – this study aligns with recent research on raphid diatoms, and contributes to
the phylogeny of the genus *Nitzschia* and other genera positioned within the Bacillariaceae.

**Materials and methods**

**Culture establishment**

Samples containing *Nitzschia* cells were collected during the BIOTA (Bio-tracing
Adriatic Water Masses) project in March 2016 at four stations in the southeast Adriatic Sea:

P150 (42° 32′ E 17° 59′); P300 (N 42°27′ E 17°55′); P600 (N 42°24′ E 17°55′) and P1000 (N
42°20′ E 17°49′). Samples were taken with phytoplankton nets (20 μm pore-size mesh) or 5-L
Niskin bottles. Those collected with Niskin bottles were taken at various depths (30, 100, 250
and 400 m) filtered through 20 μm nitrocellulose and 3 μm polycarbonate filters.

Phytoplankton net samples were taken by dragging the net vertically from 20 m depth to
surface. Both phytoplankton net and seawater samples were immediately inoculated into 0.22-
μm filtered seawater taken from the collection site and enriched with f/2 nutrients (Guillard’s
f/2 Marine Water Enrichment Solution, Sigma–Aldrich, United Kingdom). Upon returning to
the laboratory, xenic monoclonal cultures of 12 different strains (PMFBION1, PMFBION2,
PMFBION3, PMFBIONA1, BIOTAII-3, BIOTAII-18, BIOTAII-23, BIOTAII-44, BIOTAII-
59, BIOTAII-60, BIOTAII-74 and BIOTAII-84) were isolated by micropipette under the light
microscope (Olympus CKX41, Olympus, Tokyo, Japan). Strains were maintained in plastic
culture flasks (Jet Biofil®, China) in 30 mL of f/2 liquid medium and transferred weekly
through a period of 4 months. Culture conditions were: temperature 18–19°C, a light intensity
of 30 μmol photons m⁻² s⁻¹ and a photoperiod of 16h:8h of light and dark.

**Type designation**
Holotype slides of representative strains for each described species are deposited in the Croatian National Diatom Collection, University of Zagreb, Faculty of Science, Zagreb, Croatia under accession numbers with herbarium acronym ‘HRNDC’ (Thiers 2020). Isotype slides have been deposited at the Royal Botanic Garden Edinburgh, Edinburgh, UK, as Diatom Collection slides under accession numbers with herbarium acronym ‘E’ (Thiers 2020). Designated strains and full accession numbers are given after species description.

**Microscopy**

Cultures were treated to remove the organic matter from diatom frustules using Simonsen’s cleaning method (Simonsen 1974, Hasle 1978). In this, formaldehyde-fixed (final conc. 4%) and sedimented samples of cultures collected during a 4-month period of growth (approx. 5 mL) were first rinsed with distilled water, followed by addition of an equal amount of saturated KMnO₄ (or diluted 50%) for oxidation of organic matter and allowed to react for 24 h. The next day an equal amount of concentrated HCl was added, gently heated over an alcohol burner flame, and then rinsed with distilled water five times until the solution reached neutral pH. Permanent slides were prepared by drying cleaned material on coverslips and mounting in Naphrax (Brunel microscopes, Chippenham) following Hasle (1978). Light microscopy was performed with a Zeiss Axio Imager A2 light microscope (Carl Zeiss, Oberkochen, Germany) equipped with DIC and phase contrast, combined with an Axiocam 305 camera, or with an Olympus BX51 microscope (Olympus, Tokyo, Japan). Permanent slides chosen for holotype materials of new species are deposited in the Croatian National Diatom Collection, University of Zagreb, Faculty of Science, Croatia, while isotypes are deposited at Diatom Collection, Royal Botanic Garden Edinburgh, Edinburgh, United Kingdom (herbarium abbreviation E).

For SEM, parts of the oxidized suspensions were filtered and rinsed with deionized water through a 3-μm Isopore™ polycarbonate membrane filter (Merck Millipore); the filters
were mounted on aluminium stubs and coated with platinum using a BAL–TEC MED 020 Modular High Vacuum Coating System for 30 s at 100 mA. An ultra-high-resolution analytical field emission Hitachi SU-70 scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) was used for the analysis, operated at 5 kV and with 10 mm working distance. When needed, specimens were tilted to 35° inclination. SEM images were taken using the lower (SE-L) secondary electron detector signal. For TEM, cleaned material was directly deposited onto Formvar–carbon-coated copper grids, air-dried, and examined with a FEI Morgagni 268D microscope (Eindhoven, The Netherlands). The general diatom terminology used for the morphological descriptions follows Ross et al. (1979), Hustedt (1930) and Round et al. (1990).

**DNA isolation, PCR amplification and sequencing**

Genomic DNA was isolated from 50 mL of cell cultures obtained in the exponential phase of growth using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. The purity of the extracted DNA was assessed with the NanoDrop™ spectrophotometer (BioSpec–nano [Shimadzu]). The nuclear gene (18S rDNA) and two chloroplast-encoded genes (rbcL, psbC) were amplified using the EmeraldAmpMax PCR Master Mix® (Takara Bio, USA) following the PCR protocol described in Ruck and Theriot (2011). When necessary, a nested PCR reaction was done with PCR product from the first reaction as the template for the second reaction. The primers used for amplification are listed in Table S1 in the Supporting information. PCR products were visualized in a 1% agarose gel and then purified with Macherey–Nagel NucleoSpin® Gel and PCR Clean-up kit (Macherey–Nagel, Düren, Germany). The purified products were sent for Sanger sequencing (Macrogen®, Amsterdam, the Netherlands). All sequences were checked and paired (5′–3′ and 3′–5′ ends) using Sequencher 4.1.4 (Gene Code Corporation, Ann Arbor, Michigan, USA). Blast analysis was done for all sequences with the blastn tool available at
and 30 sequences belonging to 11 Adriatic *Nitzschia* strains were deposited in GenBank (accession numbers available in Appendix S1 in the Supporting information).

*Multiple sequence alignment and phylogeny inference*

A total of 340 taxa were included in the phylogenetic analyses, of which 162 belonged to the genus *Nitzschia*. Four separate datasets were defined and analysed: (1) a concatenated alignment of nuclear-encoded SSU and chloroplast-encoded *rbcL* and *psbC* for 67 taxa; (2) a concatenated SSU and *rbcL* alignment (169 taxa); (3) a single-gene *rbcL* alignment (340 taxa); and (4) a single-gene *psbC* alignment (70 taxa). The sequences used, with voucher strain information and GenBank, Thonon Culture Collection, and BOLD accession numbers, are listed in datasheet Appendix S1 in the Supporting information. *Eunotia* was selected for the outgroup, since it represents the group of diatoms sister to all other raphids, together with selected species of *Diploneis*, *Amphora*, *Pleurosigma*, *Trachyneis*, which are members of the likely sister group of the Bacillariales-plus clade (defined as all Bacillariales taxa plus *Craspedostauros*, *Staurotropis*, *Achnanthes* and *Undatella*) (sources of phylogenetic data on raphid diatoms included Stepanek and Kociolek 2014, Witkowski et al. 2016, Ashworth et al. 2017, Lobban et al. 2019). Alignment of 18S rDNA was done with ssu-align software (Version 0.1.1; eddylab.org/software/ssu-align, © 2016 Howard Hughes Medical Institute; Nawrocki 2009) following the default settings for aligning sequences according to eukaryotic SSU secondary structure and masking poorly aligned and unsupported parts of the alignment. Chloroplast-encoded *rbcL* and *psbC* genes were aligned based on their conceptual translations into amino acid sequences in Mesquite (Version 3.04; Maddison and Maddison 2015). The alignments are available at https://zenodo.org/record/1322635.

Phylogenetic analyses of each dataset first included identification of an appropriate model of nucleotide substitution and rate variation across sites using a model selection routine.
available in the IQ-TREE v. 1.5.5. (Nguyen et al. 2015). In addition, we performed a partition-merging procedure that joined two or more alignment partitions when the merge did not incur a substantial cost to the model likelihood. Model and partition selection were done using the Bayesian information criterion (BIC), which penalizes for the number of parameters in a model. The initial partition models split the single-gene alignments into codons, and the concatenated alignments were split first into genes and then into codons. Phylogenies were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) in IQ-TREE (Nguyen et al. 2015) and MrBayes v. 3.2.6. (Ronquist et al. 2012), respectively. We performed a total of 200 ML optimizations, 50 for each single-gene alignment and 50 for concatenated matrices, and finally chose the one with smallest BIC score as the ‘best’ tree (treefiles available at https://zenodo.org/deposit/1322635). ML optimizations were performed under default settings in IQ-TREE, each starting from a different random seed number, i.e. different point in parameter space, for a more exhaustive search of the likelihood surface. We varied the strength of perturbation of the nearest neighbour interchange during tree rearrangement, repeating the optimization many times, which is helpful for avoiding local optima during the likelihood optimization (Nguyen et al. 2015). Clade support was assessed using IQ-TREE’s UltraFast bootstrap routine (Minh et al. 2013) with 1000 pseudoreplicates. Bayesian analyses were carried out in the same fashion for each dataset, with the best set of partitions as identified by IQ-TREE, but with different parametrization for the substitution rate matrix. Instead of the models identified as optimal by IQ-TREE, we used the Generalized Time-Reversible model (GTR). Among-site rate variation in MrBayes was accommodated via a $\Gamma$ distribution with four rate categories and by estimating the proportion of invariant sites. We ran four simultaneous Markov chain Monte Carlo (MCMC) simulations, each composed of one cold and three heated chains, for a total of 10 million generations with a sampling frequency of one thousand generations. Stationarity and
convergence among the MCMC runs were assessed from the MrBayes output (standard deviation of split frequencies and potential scale reduction factor) and by inspecting the posterior distributions in the program Tracer v. 1.6. (Rambaut and Drummond 2007). The burn-in fraction was 25% of the sampled posterior distributions. Majority rule phylograms of the post-burn-in distributions of four MrBayes runs available as .tre files are at https://zenodo.org/record/1322635.

**Results**

*Phylogeny of three new Nitzschia species*

All of the Bayesian inference and Maximum Likelihood (BI/ML) trees generated from all four datasets (concatenated SSU+rbcL+psbC, concatenated SSU+rbcL, and single-gene rbcL and psbC datasets) recovered *Nitzschia* as paraphyletic, spread out among other Bacillariales (*Bacillaria, Cylindrotheca, Denticula, Hantzschia, Psammo dictyon and Tryblionella*) (Fig. 1 A and B; Figs. S1–S3, A and B). The Bacillariales-plus clade (B-plus), consisting of Bacillariales together with *Achnanthes, Craspedostauros, Staurotropis and Undatella* was recovered as monophyletic, supported with high Bayesian posterior probability value/Bootstrap values (BPP/BS) = 1/95 in the three-gene (SSU+rbcL+psbC) phylogeny (Fig. 1 A and B); node support in phylogenies constructed with two or one genes, therefore based on fewer nucleotide positions, decreased from SSU+rbcL to rbcL, and further to the psbC phylogeny (BPP/BS = 0.85/91, 0.84/63 and 0.81/62, respectively: Figs. S1-S3 A and B).

Genera outside the Bacillariales-plus clades (*Amphora, Diploneis, Pleurosigma and Trachyneis*) showed different positioning in respect to the Bacillariales-plus clade. In the SSU+rbcL and rbcL phylogenies all of these genera were outside the Bacillariales-plus clade, while in the psbC phylogeny some *Amphora* taxa were nested within the Bacillariales-plus clade (Fig. S3 A and B). *Eunotia* (Eunotiales) was the monophyletic sister to other raphids, as
expected from previous analyses (see Introduction) and served as an outgroup with BPP/BS=1/100 (Fig. 1 A and B; Figs. S1–S3 A and B).

Within the paraphyletic genus *Nitzschia*, the 10 new isolates from the Adriatic Sea were clearly separated from all previously sequenced taxa and formed three clades that were widely separated in the phylogeny: the three new species are *Nitzschia dalmatica* sp. nov. (strains PMFBION1, PMFBION3, BIOTAII-74 and BIOTAII-84), *Nitzschia adhaerens* sp. nov. (strains PMFBION1, PMFBION2, BIOTAII-18, BIOTAII-59 and BIOTAII-60) and *Nitzschia inordinata* sp. nov. (strain BIOTAII-44) (Fig. 1 A and B; Figs. S1–S3 A and B).

The *N. dalmatica* clade (four strains) was monophyletic with BPP/BS = 1/100, and sister to *Nitzschia* sp. strain UTKSA0111 (strain information given in Appendix S1; BPP/BS = 1/100) in all datasets (Fig. 1 A and B; Figs. S1–S3 A and B). These two species branched off within a ‘*dubiiformis*’ group that (using information from all the trees included here) contains *N. dubiiformis*, *N. traheaformis*, *N. pellucida* and *N. dubia*, as well as some unidentified *Nitzschia* species (Figs. S1, S2). The combined *dalmatica* + ‘*dubiiformis*’ group was resolved as monophyletic in the SSU+*rbcL* dataset (BPP/BS = 1/97, Fig. S1 A and B) and sister to *Psammodictyon* (BPP/BS = 1/100, Fig. S1 A and B). The same was found in the BI tree of the *rbcL* dataset, but the ML tree did not resolve the ‘*dubiiformis*’ group as monophyletic (Fig. S2B).

The *N. adhaerens* clade (five strains) was monophyletic in all datasets with BPP/BS = 1/100 in three-gene phylogeny (Fig. 1 A and B), 0.61/96 in SSU+*rbcL* phylogeny (Fig. S1 A and B), 0.99/100 in *rbcL* phylogeny (Fig. S2 A and B) and 1/100 in *psbC* phylogeny (Fig. S3, A and B). The closest relatives to *N. adhaerens* in all datasets were *Nitzschia* sp. UTKSA0106 and *N. cf. adhaerens* BIOTAII-23 strain (Fig. 1 A and B; Figs. S1–S3 A and B). In the three-gene and *psbC* phylogenies, *N. cf. adhaerens* BIOTAII-23 and *Nitzschia* sp. UTKSA0106 comprised the sister clade to *N. adhaerens* with BPP/BS support = 0.70/67 and 0.96/96,
respectively (Fig. 1 A and B; Fig. S3 A and B). BIOTAII-23 showed morphological
similarities with *N. adhaerens* in morphometry (Table 1, Figure S4 in the Supporting
information), the lanceolate valve shape, its continuous raphe positioned on an elevated,
discrete keel, and the tiny round to rectangular areolae occluded by finely perforated hymens;
however, its phylogenetic position prevents inclusion in *N. adhaerens*. Beyond *N. cf. adhaerens* and *Nitzschia* sp. UTKSA0106, the relationships of *N. adhaerens* are rather unclear
and inconsistent; for example, the next closest relatives in the three-gene tree are conopeum-bearing species (‘tholophora’ species: Lobban et al. 2019), such as *N. cf. volvendirostrata* and
*N. celaenoi*, but *Nitzschia inordinata* sp. nov. and *Bacillaria* sp. SH349 in the SSU*+rbcL* and
*rbcL* phylogenies (Figs. S1–S2, A and B).

*N. inordinata* was represented by one strain (BIOTAII-44), which was recovered as
sister to a clade containing *N. adhaerens*, *Nitzschia* sp. UTKSA0106 and *Nitzschia* cf.
*adhaerens*. In some analyses (three-gene, *psbC*) this clade also included conopeum-bearing
*Nitzschia* species (e.g. *N. cf. volvendirostrata*, *N. dissipata*: Fig. 1 A and B; Fig. S3 A and B).
In the SSU*+rbcL* and *rbcL* phylogenies, *N. inordinata* grouped with *Bacillaria* sp. strain
SH349 (BI/BS = 1/100; Figs. S1–S2 A and B).

**Morphology and description of new taxa**

The three new *Nitzschia* species are presented below, and morphometric data for all
strains [valve length (VL), valve width (VW), fibula density in 10 μm (FD), stria density in 10
μm (SD) and the areola density in 1 μm (AD)] are given in Table 1. For *N. adhaerens* and *N. dalmatica*, one strain each was chosen to provide holotype material according to the criteria of
having at least a *rbcL* sequence, well-preserved cleaned material, and measurements made in
both LM and EM. The ranges of the measured parameters given in the species descriptions
are drawn from all the strains and also from natural material (original net and phytoplankton
samples from which the strains were derived and measured).
*Nitzschia dalmatica* Mucko & Bosak, sp. nov. (Figures 2A–K, 3A–H)

**Description:** Living cells with two plate-like plastids, one in each apical half of the cell (Fig. 2A). Frustules broadly linear in girdle view, tapering towards rounded poles and somewhat constricted in the middle (Fig. 2B), with numerous girdle bands (Fig. 2D). Girdle bands open and perforated by two or three rows of round pores (Fig. 3 G and H). Valves linear-lanceolate, 12–41 µm long and 3–6 µm wide, with 10–19 fibulae in 10 µm, 39–45 striae in 10 µm and 5–7 areolae in 1 µm (Table 1); apices cuneate to slightly capitate (Fig. 2 B–C). External and internal valve views reveal an eccentric and elevated keel indented in the middle (Fig. 2 E and F). Terminal raphe fissures straight or slightly curved (Fig. 2 G–K). Internal terminal raphe fissure simple, finishing in a helictoglossa (Fig. 2H, arrowhead). Central nodule well silicified, external proximal raphe endings slightly curved and droplet-like (Fig. 3 A–C). Transapical striae uniseriate, parallel and relatively dense, extending uninterrupted from the bottom of the keel to the valve margin (Fig. 3 A–E). Virgae elevated and thickened (Fig. 3D), sometimes bifurcating towards the valve margin (Fig. 3C, arrowhead). Keel containing two rows of areolae, one on each side of the raphe (very occasionally there are two areolae instead of one: e.g. at arrowhead in Fig. 3D). Each keel areola surrounded externally by an elevated silicified ring (Fig. 2 G and I, Fig. 3 A and D). Areolae within the striae round, very small, occluded with finely perforated hymens (Fig. 3F). Fibulae relatively coarse, rib-like, present along the whole length of the keel except for a wide central interspace opposite the central nodule, irregularly spaced (Fig. 2F and H, Fig. 3 B and E).

**Representative DNA sequences:** SSU: MH734172; *rbcL*: MH687908; *psbC*: MH687897
Holotype: HRNDC 000010 permanent slide of strain BIOTAII-84 (illustrated in Fig. 2 B–D).

Isotype: E 5897 permanent slide of strain BIOTAII-84.

Type locality: Croatia: southeast Adriatic Sea (P150 station, 30 m of depth; 42°32’ N; 17°59’ E). Cells isolated from the Niskin bottle sample collected on 8th March 2016 onboard RV Naše More by M. Mucko.

Etymology: This species has been named after the historical region of the southeastern Adriatic Sea coast, ‘Dalmatia’, where the species was discovered.

Comparisons with similar species: In the sectional classification of Nitzschia formulated by Grunow (in Cleve and Grunow 1880), N. dalmatica would have been classified in the sect. Dubiae, because of its only moderately eccentrically placed raphe system and somewhat constricted centre. Among the species assigned to this group by Grunow (ibid.), Hustedt (1939, 1955, 1957) and Krammer and Lange-Bertalot (1988), there are several that bear some resemblance to N. dalmatica in having finely striated valves (>30 in 10 µm). These include (in date order of publication): N. pellucida Grunow, N. normanii Grunow, N. subhybrida Hustedt, N. dubiiformis Hustedt, N. thermaloides Hustedt, N. pseudohybrida Hustedt, N. hybridaeformis Hustedt, N. aestuarii Hustedt, N. translucida Hustedt and N. traheaformis Chunlian Li, Witkowski & Shu-xian Yu. Most of these have coarser striaion than N. dalmatica (39–45 striae in 10 µm) and can be separated from it rather easily, providing care is taken to ensure that the light microscope is properly set up to resolve striae with densities between 30 and 40 in 10 µm. This applies to N. pellucida and N. normanii [c. 32 and 30–32 striae in 10 µm in the original description of Cleve and Grunow (1880) and Krammer and Lange-Bertalot (1988), respectively]; N. subhybrida [c. 32 striae in 10 µm in the photographs by Simonsen (1987)]; N. hybridaeformis Hustedt [c. 35 striae in 10 µm according to Hustedt (1955) and measured by us as 34–36 in 10 µm from the illustrations of
the holotype provided by Simonsen (1987) and online at http://hustedt.awi.de; *N. pseudohybrida* Hustedt and *N. thermaloides* [in both we measured c. 34 striae in 10 µm in illustrations by Simonsen (1987) or online, in contrast to c. 40 in 10 µm in the original descriptions given by Hustedt (1955)]; *N. aestivalii* and *N. translucida* [in both we measured 34–35 striae in 10 µm in the photographs by Simonsen (1987), which is slightly higher than was reported by Hustedt (1959) for *aestivalii* and slightly lower than for *translucida*]; and *N. traeaiformis* [with 32–34 striae in 10 µm but otherwise very similar to *N. dalmatica* (Witkowski et al. 2016)]. In addition, *N. thermaloides* has a noticeably smaller central interspace than *N. dalmatica* (it is about twice the width of other interspaces in *N. thermaloides* but three times the width in *N. dalmatica*) and a less constricted centre. *N. normanii*, *N. aestivalii* and *N. translucida* are also less constricted than *N. dalmatica* and hence appear more linear; in contrast, *N. subhybrida* and *N. pellucida* have a much more constricted centre than *N. dalmatica* [compare the illustrations of Simonsen (1987, pl. 99, figs 8–12) and Cleve and Grunow (1880, pl. 5, fig. 96), respectively, with our Fig. 2D), partly reflecting the more central keel in these species. *N. hybridaeformis* is a larger diatom than *N. dalmatica* (60–93 × 6–8 µm) and has more widely spaced fibulae (5–10 in 10 µm).

Possibly the most difficult species to separate from *N. dalmatica* (39–45 striae in 10 µ) is *N. dubiiformis* since, unlike the species discussed in the previous paragraph, *N. dubiiformis* has very finely striated valves, with c. 43–44 striae in 10 µm according to Simonsen (1987, p. 260). Furthermore, the valves and frustules have a similar shape to *N. dalmatica*. However, *N. dubiiformis* is a larger diatom (the original description gives 40–50 × 5–7 µm) and the fibulae appear smaller and more evenly spaced than in *N. dalmatica*, forming a rather neat marginal row in girdle view (Hustedt 1939, figs 111, 112; Simonsen 1987, pl. 383, figs 1–7).

*Species diagnosis:* *Nitzschia dalmatica* is identified and distinguished from similar taxa by the following character states: eccentric and elevated keel indented in the middle;
dense (39–45 striae in 10 µm) transapical striae uniseriate, separated with elevated and
thickened virgae; a row of round keel areolae with elevated silicified rings present on both
sides of the raphe; external proximal raphe endings slightly curved and droplet-like.

*Nitzschia adhaerens* Mucko & Bosak, sp. nov. (Figures 4A–I, 5A–I)

**Description:** Frustules linear-lanceolate in girdle view; live cells with two plate-like
yellow-brown plastids, one in each half of the cell (Fig. 4A). Cells have several porose girdle
bands per theca, but the details are unclear (Fig. S5 in the Supporting information).
Valvocopula open, with two or three rows of round pores enclosed by finely perforated
hymens (Fig. 5 H and I). Valves lanceolate, 10–34 µm long and 2–5 µm wide, with apices
that are cuneate in valve view and apparently very slightly spathulate in girdle view (Fig. 4 C,
D and G); there are 16–25 fibulae and 48–56 striae in 10 µm (hence the striae cannot be
resolved in LM). Keel narrow, discrete and elevated about valve face (i.e. there is an abrupt
transition from valve face to keel: Figs. 4 B–E and H, 5B), almost central (Fig. 4 E, F and H).
Terminal raphe fissures curved (Fig. 5 A–C). External proximal raphe endings absent (Figs.
4H and 5D). Striation of the valve very fine and delicate, not resolvable in LM (Fig. 4 B–D),
comprising uniseriate striae of tiny round to rectangular areolae (Fig. 4 F–I, 5B); the areolae
(5–6 in 1 µm) occluded by finely perforated hymens (Fig. 5G); these lie at the outer apertures
of the areolae, so that the external valve face appears smooth (Figs. 4H and 5C). Each
uniseriate transapical stria ends up by two areolae within the keel (Fig. 5 A, C–E and G).
Virgae flat, never bifurcating (Fig. 4 F–I). Fibulae relatively dense (16-25 in 10 µm),
regularly spaced throughout keel (Fig. 4 F and H); sometimes two fibulae are fused together
(Fig. 4I, arrowhead).

*Representative DNA sequences:* SSU: MH734165; *rbcL*: MH687900; *psbC*:
MH687889
Holotype: HRNDC 000011 permanent slide of strain BIOTAII-18 (illustrated in Fig. 4 B–D).

Isotype: E 5898 permanent slide of strain BIOTAII-18.

Type locality: Croatia: Southeast Adriatic Sea (P600, 250 m of depth; 42°24′ N; 17°55′ E). Niskin bottle sample collected on 8th March 2016 onboard RV Naše More by M. Mucko.

Etymology: The specific epithet refers to observations of the cells in net samples sticking to (adhering to) the setae of the colonial planktonic diatom Chaetoceros.

Comparisons with similar species: In Grunow’s Nitzschia classification (in Cleve and Grunow 1880), Nitzschia adhaerens would almost certainly have been placed in section Bacillaria. Subsequently it would have been put in the section Dissipatae when the original concept of Bacillaria (as an independent genus characterized by its unique motile colonies) was restored by Hustedt (e.g. see 1939, p. 661). The characteristic that would have led to these hypothetical assignments is the almost central position of raphe system and the absence of longitudinal lines on either side of the keel in LM (though in fact the type of the Dissipatae, N. dissipata, does have these, which reflect the presence of external conopea in this species and its relatives, e.g. N. sigmoidea, N. recta: Mann 1978, 1986, Lobban et al. 2019). Rather few Nitzschia species have been described with near-central raphes and those that do exist [e.g. N. longa Grunow, N. praelonga Cleve and N. cursoria (Donkin) Grunow] are mostly coarsely structured, with striation densities < 20 in 10 μm. The only one known to us with finer striation is N. linkei Hustedt, with c. 33 striae in 10 μm according to the original description (Hustedt 1939). However, this striation density is much lower than in N. adhaerens (>48 in 10 μm) and N. linkei is a larger diatom (40–55 × 7–9 μm rather than 17–34 × 2.5–5 μm); therefore confusion is very unlikely.

Species diagnosis: Nitzschia adhaerens is identified and distinguished from similar taxa by the following character states: keel narrow, discrete and elevated about valve face,
almost central; transapical striae uniseriate and not resolvable in LM composed of tiny round
to rectangular areolae enclosed by finely perforated hymens; each stria ends up by two areolae
within the keel; proximal raphe endings absent.

*Nitzschia inordinata* Mucko & Bosak, sp. nov. (Figures 6A–G, 7A–F)

*Description:* Live cells containing two plate-like yellow-brown plastids, one in each
half of the cell (Fig. 6A). Valves sigmoid, 91–152 µm long and 4–8 µm wide, with a
moderately eccentric keel and strongly drawn-out subcapitate apices (Fig. 6 B, D and E); with
7–10 fibulae and 20–24 striae in 10 µm. Girdle bands open and sigmoid (Fig. 6C), perforated
by one row of round pores (Fig. 7G). Keel elevated above the valve face (Fig. 6 D and E) and
enclosed internally by thick fibulae (Figs 6F and 7B). External proximal raphe endings absent
(Fig. 7A). Terminal raphe fissures sharply bent (about 30°) (Fig. 6D). Valve striation
interrupted, starting on the elevated keel, absent in a depressed area of the valve face parallel
to the raphe, and then resuming and continuing to the valve margin (Figs. 6F, 7 A–D). Each
stria uniseriate, containing round areolae (Fig. 7 A–E) occluded by finely perforated hymens
with pores in a hexagonal array (Fig. 7F). Virgae thickened and slightly elevated externally,
especially in a depressed area without striation adjacent to the bases of the fibulae (Fig. 7C).
Fibulae regularly spaced along the keel, slender and riblike at the centre (Fig. 6F) but
becoming proportionately more massive towards the apices (Fig. 6E).

*Representative DNA sequences:* SSU: MH734171; *rbcL:* MH687906; *psbC:

MH687895

*Holotype:* HRNDC 000012 permanent slide of strain BIOTAII-44 (illustrated in Fig. 6
B and C).

*Isotype:* E 5899 permanent slide of strain BIOTAII-44.
Type locality: Croatia: Southeast Adriatic Sea (P150 station, 30 m of depth; 42°32’ N; 17°59’ E). Cells isolated from Niskin bottle sample collected on 8th March 2016 onboard RV Naše More by M. Mucko.

Etymology: The specific epithet refers to the irregular spacing of the areolae within the striae.

Comparisons with similar species: A variety of marine Nitzschia species have been described with sigmoid frustules. Among them are some classified in the section Obtusae (Cleve and Grunow 1880, Krammer and Lange-Bertalot 1988) because of their highly distinctive proximal raphe endings, which are deflected inwards and end in convergent or almost parallel transapical grooves (e.g. Mann 1978, figs 865, 875, 881). N. inordinata cannot be confused with these, nor with other sigmoid species that possess proximal raphe endings.

In LM, N. inordinata resembles N. lorenziana: the two have similar dimensions (N. lorenziana valves have lengths of 37–190 µm and widths of 3–7 µm according to Krammer and Lange-Bertalot 1988) and the ranges of fibula densities overlap (6–10 in 10 µm in lorenziana, 7–10 in inordinata). However, the stria densities differ, those of lorenziana being coarser (13–19 in 10 µm rather than 20–24) and SEM (Poulin et al. 1990) reveals that, in lorenziana, the striae are biseriate, central raphe endings are present, the terminal fissures are forked rather than being bent to one side), and the fibulae are elongated apically (each one subtending two of the biseriate transapical striae) rather than being narrow ribs as in N. inordinata. Among sigmoid species without proximal raphe endings, few have striation as coarse as in N. inordinata (≤25 in 10 µm). The first one is N. perlonga Pantocsek (1902), which has extremely long (>480 µm) non-attenuate linear valves and occurs in freshwater.

The most similar brackish or marine species seem to be those within the N. sigma complex, as their valves also taper from centre to poles. However, the fibula structure differs: N. inordinata has relatively simple riblike fibulae over most of the valve (though becoming
relatively more massive towards the poles) that flare slightly at the entrance to the keel (Fig. 7B), whereas in the *N. sigma* complex the fibulae expand (and delimit portulae) at two levels (at the opening into the cell lumen and at the entrance to the keel), so that the space between them is partially enclosed to form a chamber (Mann 1978, figs 845, 846, 849, 850). In addition, in *N. sigma* the terminal raphe fissures continue almost straight to the valve margin (ibid., figs 841, 851), contrasting with the sharply bent fissures in *N. inordinata*; furthermore, the striae are not interrupted near the bases of the fibulae, unlike in *N. inordinata*.

*Species diagnosis:* *Nitzschia inordinata* is identified and distinguished from similar taxa by the following character states: Valves sigmoid; keel moderately eccentric; apices subcapitate and strongly drawn-out; proximal raphe endings absent; transapical striae uniseriate; areolae occluded by finely perforated hymens with pores in a hexagonal array; valve striation interrupted in depressed valve face area; fibulae slender and riblike at the centre but more massive towards the apices.

**Discussion**

Species delimitation within *Nitzschia* is problematic due to the lack of unique morphological characters to group them and because DNA sequence data are only available for a small minority of species (Witkowski et al. 2004, Trobajo et al. 2013, Rimet et al. 2014). Furthermore, some characters, such as fibula and stria densities or cell width, may change due to daily or seasonal variation of environmental parameters (Trobajo et al. 2004, 2011), or within the life cycle, such as in *N. inconspicua* (Mann et al. 2013). Nowadays, we are observing an increase in newly described *Nitzschia* species, most likely due to higher culturing efforts and the combination of morphological and phylogenetic investigations (Smida et al. 2014, Witkowski et al. 2016, Barkia et al. 2019, Lobban et al. 2019). The same trend in using both morphology and phylogeny for descriptions of new pennate species and genera, sometimes also involving sequencing of complete genomes or detailed genus
overviews, is recorded for other diatoms, such as *Proschkinia* (Gastineau et al. 2019,
*Amphora* and *Halamarphyora* (Stepanek and Kociolek 2019), *Dorofeyukea* (Kulikovskiy et al.
2019) and *Simonsenia* (Kim et al. 2019). This study follows the methodology in combining
morphology with phylogeny to describe three new *Nitzschia* species.

**Phylogenetic relationships and morphology comparisons**

Our results show that *Nitzschia* is paraphyletic, which is congruent with similar
previous studies (e.g. Rimet et al. 2011, Witkowski et al. 2015, 2016, Barkia et al. 2019,
Lobban et al. 2019).

In the SSU+rbcL and rbcL trees, *N. dalmatica* was resolved within a monophyletic
group of morphologically similar species with near-central or moderately eccentric raphe
system. This grouping of *Nitzschia* species is evident in some previously published molecular
phylogenies of Bacillariaceae (e.g. Witkowski et al. 2016, An et al. 2017) and corresponds to
*Nitzschia* section *Dubiae* as amended by Hustedt (1955) or the *Dubiae–Bilobatae* of Krammer
and Lange-Bertalot (1988). The species that appears most similar to *N. dalmatica* in LM and
metrics is *N. dubiiformis*, but the two are clearly separated in the gene trees, where *N.
dalmatica*’s nearest relative is *Nitzschia* sp. UTKSA0111 (the two are sister lineages
supported with high BPP/BS values: 100/100 in all trees; Figs. 1 A and B, S1–S3 A and B).
The morphology of this strain is not fully known; however an LM image is available online
(http://www.protistcentral.org/Photo/get/photo_id/6413) and shows a single valve that, like *N.
dalmatica*, belongs to the ‘*Dubiae–Bilobatae*’ group and measures 41 × 4.6 µm with
imperceptible striation and 8–9 fibulae in 10 µm. It is therefore much longer than the
specimens of *N. dalmatica* we observed (though this could reflect different stages in a size
reduction cycle) and, perhaps more importantly, it has a much lower fibula density. In
addition, the UTKSA0111 valve is much more strongly constricted centrally than in *N.
*dalmatica*. Striation of UTKSA0111 is resolvable with SEM, counting 39–41 stria in 10 µm and 6–7 areolae in 1 µm. Additionally, both *N. dalmatica* and strain UTKSA0111 have well-silicified central nodule without areolae and keel with two rows of areolae which are externally surrounded by elevated silicified rings (Matt P. Ashworth, personal communication).

The second novel species, *N. adhaerens*, is part of a separate lineage, also containing our clone BIOTAII-23 and *Nitzschia* sp. UTKSA0106, that seems (three-gene and SSU+rbcL trees) to be related to a group characterized by the possession of conopea, i.e. external silica flaps extending out laterally from the keel (Mann 1978, 1986). The latter group includes *N. dissipata*, *N. volvendirostrata*, *N. nanodissipata* and the type species of *Nitzschia*, *N. sigmoidea*, which also share with each other delicate striaion (relative to the size of the valves), a moderately eccentric or nearly central raphe, and an absence of central raphe endings. This suite of characters has been called the ‘tholophora’ morphology of *Nitzschia* by Lobban et al. (2019), who described 14 new *Nitzschia* species within this group, all with conopea and all phylogenetically close to each other; we omitted their new sequences from our analyses, considering that the 162 *Nitzschia* taxa represented in our trees are enough to give good sequence sampling depth. *N. adhaerens*, although it is a delicately structured species like those in the ‘tholophora’ group, does not possess conopea, but it is unclear whether this is symplesiomorphic or whether conopea have been lost secondarily. Important for resolving this point is to know whether or not the diatoms closest to *N. adhaerens* also lack conopea. The most closely related species (strain) to *N. adhaerens* in all trees is yet undescribed *Nitzschia* sp. UTKSA0106. The morphology of this strain is not fully known and it may or may not have conopea, but its valves are clearly differentiated from *N. adhaerens* by their slightly spathulate ends in girdle view (i.e. a polar expansion of the keel) and their narrow, protracted poles (Matt P. Ashworth, personal communication, and LM images online
UTKSA0106 valves also differ from *N. adhaerens* in being larger (54–57 × 7.7 µm in the online photographs) and having much lower fibula density (9–11 in 10 µm). The other diatom related to *N. adhaerens* in our molecular datasets is our BIOTAII-23, which sometimes appears as the sister to *N. adhaerens* (SSU+*rbcL*, *rbcL*), and sometimes as sister to UTKSA0106 (three-gene, *psbC*). BIOTAII-23 definitely lacks conopea and is morphologically similar to the strains we include within *N. adhaerens*. The fibula densities in strain BIOTAII-23 are within the range measured in the *N. adhaerens* strains but with different lower and upper limits (17–20 in 10 µm in BIOTAII-23 and 16–25 in 10 µm in *N. adhaerens*), and the valves seem wider (4–6 µm rather than the 3–4 of most *adhaerens*); other morphometrics – length and stria density – match. However, genetically these two are separated with high support (three-gene phylogeny). For the moment BIOTAII-23 is referred to as *Nitzschia cf. adhaerens*.

In the phylogenetic trees, the third new species, *N. inordinata* (for which we have only a single isolate), is either sister to the clade containing both the ‘tholophora’ *Nitzschia* and the *adhaerens* group (three-gene, *psbC*), or on a separate branch within it, together with ‘*Bacillaria*’ SH349 (SSU+*rbcL*, *rbcL*). Because of its sigmoid shape, lack of proximal raphe endings, and rather coarse striation, *N. inordinata* would probably have been allocated to sect. *Sigmata* by Grunow, but our SSU+*rbcL* and *rbcL* trees show *N. inordinata* and *N. sigma* to be not only distinct (see Results) but also distantly related. The close relative of *N. inordinata*, ‘*Bacillaria*’ strain SH349, was sequenced and its LM morphology described by An et al. (2017). The valve length and width given for ‘*Bacillaria*’ SH349 (115.7 and 10 µm, respectively) are within the range of *N. inordinata*, as are the stria and fibula densities (20 and 10 fibulae in 10 µm, respectively). We obtained unmounted material of strain SH349 from Professor J.H. Noh and examined it under SEM. It showed a similar morphology to *N. inordinata*: the fibula and keel structure are the same, both species have irregularly arranged
areolae towards the valve face margin, and both have an interruption to the striae at the bases of the fibulae (unpublished observations: images available from D.G. Mann on request).

However, *N. inordinata* is easily separated from ‘*Bacillaria*’ SH349 because it is clearly sigmoid, whereas SH349 is not (An et al. 2017, fig. 3g).

The valve shape, raphe position and metric characters reported for ‘*Bacillaria*’ SH349 (lanceolate outline, nearly central raphe, measuring 115.7 × 10 μm, with 10 fibulae and 20 striae in 10 μm) fall within Grunow’s concept (in Cleve and Grunow 1880) of *Nitzschia socialis*, a species described by Gregory (1857) and named for its tendency to be found, even after acid cleaning, in groups of cells orientated parallel to each other. Ralfs (in Prichard 1861, p. 784) transferred *N. socialis* to *Bacillaria* and, in doing so, implied that the groups of cells Gregory observed reflect the existence of motile colonies (as in *Bacillaria paxillifera*), because Ralfs explicitly prescribed motile colonies as a defining characteristic of *Bacillaria* (his description of the genus stated “frustules … united into a short band, moving on each other by a sliding motion without separation”). However, as far as we know, the existence of motile colonies in *N. socialis* has never been confirmed and strain SH349 too was not seen to form motile colonies (Prof. J.H. Noh, personal communication 22 May 2019). Assignment to *Bacillaria* is consistent with the near-central position of the raphe in SH349 and the close match of its SSU with “*Bacillaria cf. paxillifer*” strain BA14c (GenBank HM805020).

However, strain BA14c too does not form the special motile colonies that are supposed to characterize the genus *Bacillaria* (observations of strain BA14c by Dr. F. Pniewski and ourselves; see also the photographs of BA14 clones available online at [https://ccba.ug.edu.pl/](https://ccba.ug.edu.pl/), though its SSU sequence matches another “*Bacillaria paxillifer*” sequence in GenBank (M87325) with 98.48% identity (BLAST). It is therefore unclear whether the special motile colonies supposed to be characteristic of *Bacillaria* are indeed a synapomorphy for a monophyletic group.
Summarizing: there is insufficient information about several of the ‘Bacillaria’ clones that have been sequenced to be able to judge how they differ from *N. inordinata* morphologically and whether they should be assigned to *Bacillaria*, given that they do not form motile colonies. The closest known relative to *N. inordinata* is strain SH349, which seems to be identifiable as *Nitzschia (Bacillaria?) socialis* sensu Grunow (in Cleve and Grunow 1880). These two diatoms occupy an interesting position in phylogenetic trees, on a deep branch that may be basal to the ‘tholophora’ and *adhaerens* groups. Neither are close to ‘true’ *Bacillaria* (i.e. the species with motile colonies: cf. Jahn and Schmid 2007).

The deep branch represented by *N. inordinata* and *N. adhaerens*, together with the ‘tholophora’ *Nitzschia* species, exists alongside some other known deep branches (e.g. Carballeira et al. 2017, Kim et al. 2019, Lobban et al. 2019), viz. *Bacillaria, Hantzschia*, and a few isolated ‘*Nitzschia*’ species (including *N. lorenziana* Grun. and the *Nitzschia* clone TCC886, identified currently as *N. palea*: see Figs S1 and S2) that do not obviously group with any others. The discovery of the *adhaerens–inordinata* clade suggests that, for understanding the early evolution and diversification of Bacillariaceae, it is important to make further studies of the *Bacillaria*-like isolates SH349 and BA14c (see above) and to target other marine *Nitzschia* species with near-central raphe systems, such as two others that Grunow included in his section ‘*Bacillaria*’, namely *N. longa* (which has an unusual keel structure: Hustedt 1955) and *N. praelonga*.

Small pennate *Nitzschia* diversity in marine plankton

Relative to the number of benthic species, not many *Nitzschia* species are planktonic, especially in marine waters. Most of the few that are – such as *N. longissima* (Hasle and Syvertsen 1996), *N. bicapitata* (Fryxell, 2000) and two of the species described here (*N. dalmatica* and *N. inordinata*) – occur as single cells, even though theoretical considerations and experimental data (e.g. Reynolds 2006) confirm that the formation of stellate or chainlike
colonies (like those of *N. asterionelloides*, *Fragilariopsis* and *Pseudo-nitzschia*: Hustedt 1942, Hasle and Syvertsen 1996) can often be considered adaptive in relation to sedimentation. Although discovered in the plankton, a different habitat (i.e. benthos) for *N. dalmatica* cannot be ruled out due to drifting of diatom flora from coastal to open waters systems by currents and waves. According to Fryxell (2000), cells of small bicapitate *Nitzschia* species occurring in the plankton frequently aggregate on substrates, thus representing a major food source for some grazers, as was observed with our new species *N. adhaerens*, which aggregated on large chains of *Chaetoceros*. *Nitzschia adhaerens* like *N. dalmatica* therefore can probably have two habitats – one existing as an epiphyte on large *Chaetoceros* chains, and one existing solely in plankton. Spicular cell shape can also be considered to be an adaptation to planktonic existence, because of the high surface area to volume ratio (Reynolds 2006), and it is noticeable that most of the planktonic *Nitzschia* species occurring in tropical freshwaters (e.g. Kilham et al. 1986, Grady et al. 2020) are spicular, as is *N. inordinata* among the species described here. Less elongate and squat *Nitzschia* species are generally benthic and either adhere to surfaces, or live free, moving through sediments.

Single-celled pennate diatoms in the Adriatic Sea were previously reported by Batistić et al. 2012 and Bosak et al. 2016 as ‘shade-flora’, part of a larger, deep-dwelling phytoplankton community found at the bottom of the photic zone or below it (~150 up to 500 m of depth). Most of those single-celled pennates were taxonomically assigned to *Navicula cf. distans*, *N. cf. directa* and species belonging to *Nitzschia cf. bicapitata* compex (Bosak et al. 2016). Successful culturing from samples collected at those depths has not yet been attempted, as far as we know, although survival of diatoms in low light conditions has already been confirmed (Smayda and Mitchell-Innes 1974, Waite and Harrison 1992, Jochem 1999, etc.). This is important to emphasize as most of the *N. adhaerens* and *N. cf. adhaerens* strains
were isolated from 100, 250 or even 400 m of depth (BIOTAII-59, BIOTAII-60, BIOTAII-18
and BIOTAII-23, respectively). General phytoplankton investigations conducted both in the
Adriatic Sea and elsewhere (Baltic Sea, Atlantic Ocean, Indian Ocean and Pacific Ocean)
have usually classified ‘unidentified pennate diatoms’ into size classes [nano (>2 and <20µm)
and micro (>20 and <200µm)], which are abundant and frequently occurring in coastal and
open waters (Piiparinen et al. 2010, Cerino et al. 2012, Brandini et al., 2014, Estrada et al.
2016). Investigating these solitary pennate diatoms in the marine plankton has become of
great importance, because of their high contribution to the overall phytoplankton community,
microbial loop, and carbon fluxes from surface to bottom layers in the oceans. In this study,
we have isolated strains of small Nitzschia species from net phytoplankton samples, but also
from 30, 100, 250 and 400 m of depth. Mejdandžić et al. (2018) successfully cultivated
diatoms from Entomoneis from the same samples, as well as undescribed Haslea species
(Mejdandžić et al. 2017a) and some other unpublished strains from other pennate diatom
genera (Navicula, Psammodictyon, Diploneis); thus showing that marine pennate planktonic
diatoms are subjected to sinking and can survive harsh environmental conditions. These cells
can be returned to the surface through intense vertical convection in the South Adriatic Sea
during the winter period (Batistić et al. 2012; Korlević et al. 2015), which gives cells a new
opportunity to increase their numbers in the photic zone.

It is not surprising to discover new species of marine planktonic pennate diatoms,
especially those belonging to the paraphyletic genus Nitzschia, since most investigations of
marine phytoplankton do not make a detailed examination of diatom cells in samples, and
Nitzschia species need especially careful LM and SEM observations to determine their
morphology and identity. Additionally, there are a lot of different criteria to take into
consideration when delimiting a species within marine planktonic pennate diatoms, such as
morphological variations, sexual reproduction (if observed), variation in genetic material
within different populations of same species (which is difficult to obtain), and phenotypic plasticity and changes in culturing conditions. With that in mind, this study shows that an investigation combining most of the wanted criteria, including fine-grained morphological observations and multigene phylogenies of related species and genera, is needed to resolve planktonic pennate diatoms.

**Acknowledgments**

This material is based in part upon the work supported by Croatian Science Foundation under the project BIOTA [Bio-tracing Adriatic Water Masses], UIP-2013-11-6433 and in part under the project TurtleBIOME [Loggerhead sea turtle (*Caretta caretta*) microbiome: insight into endozoic and epizoic communities], UIP-2017-05-5635. The authors are grateful to the crew of RV "Naše more" for their help during the fieldwork. The authors declare there is no conflict of interests. MM is grateful to Dr I. Sviličić Petrić and A. Kolda for providing facilities for molecular laboratory work and M. Vugrin for culturing efforts. We are very grateful to Professor J.H. Noh for very kindly supplying us with material of clone SH349 (‘Unidentified *Bacillaria* sp. 1’ in An et al. 2017) and Dr F. Pniewski for a culture of clone BA14c (‘*Bacillaria cf. paxillifera*’); detailed observations of these will be published elsewhere. RT acknowledges support from the CERCA Programme/Generalitat de Catalunya. The Royal Botanic Garden Edinburgh is supported by the Scottish Government’s Rural and Environment Science and Analytical Services Division. We are kindly grateful to Dr M.P. Ashworth for supplying us with LM and SEM images of *Nitzschia* sp. strains UTKSA0106 and UTKSA0111.
References


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### Table 1. Morphometric parameters measured under light (LM) and electron microscopy (EM) in 11 strains belonging to three new Adriatic *Nitzschia* species, strain BIOTAII-23 representing *N. cf. adhaerens* and natural material where new species cells were observed. VL – valve length; VW – valve width; SN – stria density in 10 μm; FN – fibula density in 10 μm; AN – areola density in 1 μm.

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<th>Species</th>
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<th>Measured cells (no.)</th>
<th>VL (μm)</th>
<th>VW (μm)</th>
<th>FN</th>
<th>SN</th>
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Figure Captions

**Figure 1.** A: Majority rule phylogram of the post-burn-in distributions of the four MrBayes runs inferred from a concatenated dataset of three markers: SSU, *rbcL* and *psbC*. Branch support is summarized above branches as Bayesian posterior probability. B: “Best” Maximum Likelihood tree inferred from a concatenated dataset of three markers: SSU, *rbcL* and *psbC*. Branch support is summarized above branches as Maximum Likelihood bootstrap values.

Three new species of the Adriatic strains are in bold and highlighted.

**Figure 2.** *Nitzschia dalmatica* sp. nov. Mucko & Bosak (A–D) LM; (E–I) SEM; (J–K) TEM; (A–D, F, I) strain BIOTAI1-84; (E, G, H) strain BIOTAI1-73; (J–K) strain PMFBIONA1. (A) Live broadly linear cell containing two yellow-brown plate-like plastids. (B–K) Cleaned material. (B, C) Linear-lanceolate valves with constricted raphe-bearing margin in the central area and cuneate to slightly capitate apices. (D) A cell in girdle view with numerous girdle bands. (E, F) External and internal valve views with eccentric and elevated keel. (G, I) External view of the valve with cuneate to capitate apex with slightly curved terminal raphe fissure; note the rimmed areolae in the keel. (H) Internal valve view with cuneate to capitate apex, dense striation and coarse irregularly spaced fibulae. (J, K) Straight to slightly curved
terminal raphe fissures. Scale bars (A–F) 10 µm; (G, H) 2 µm; (I) 1 µm; (J) 0.2 µm; (K) 0.5 µm.

**Figure 3.** *Nitzschia dalmatica* sp. nov. Mucko & Bosak (A, B, D, E) SEM; (C, F, G, H) TEM; (A, B, D) strain BIOTAII-74; (E) strain BIOTAII-84; (C, F, G, H) strain PMFBIONA1. (A–C) External and internal valve view with proximal raphe endings curved and droplet-like. Note the bifurcated virga (C, arrowhead). (D) Thickened and elevated virgae and keel areolae with raised rims; note the occasional presence of two areolae opposite a single valve stria (arrowhead). (E) Internal valve view with round areolae within striae and robust, irregularly spaced, riblike fibulae. (F) Finely perforate hymenate areolae. (G, H) Open numerous girdle bands, each perforated by two or three rows of round pores. Scale bars (A–E) 2 µm; (F) 0.2 µm; (G, H) 2 µm.

**Figure 4.** *Nitzschia adhaerens* sp. nov. Mucko & Bosak (A–D) LM; (E, G–I) SEM; (F) TEM; (A–E, H, I) strain BIOTAII-18; (F) strain BIOTAII-3; (G) strain BIOTAII-60. (A) Linear-lanceolate living cells with two yellow-brown plate-like plastids. (B–I) Cleaned material. (B) Spindle-shaped valve with cuneate apices in valve view. (C) A valve in girdle view, showing the very slightly spathulate apices. (D) Frustule in girdle view. (E) External valve view with slightly eccentric, narrow keel. (F) Slightly eccentric keel with regularly spaced fibulae. (G) Internal valve view revealing fine, uniseriate striae and riblike fibulae. (H) External valve view showing the fine hymenate, round to rectangular areolae; note (by comparison with I) that the hymens lie near the external apertures of the areolae. (I) Internal valve view with regularly spaced riblike fibulae, which are sometimes fused together (arrowhead). Scale bars (A–G) 10 µm; (H, I) 2 µm.
**Figure 5.** *Nitzschia adhaerens* sp. nov. Mucko & Bosak (A, D, G, H) TEM; (B, C, E, F, I) SEM; (A, D, G, H) strain BIOTAII-3; (B, C) strain BIOTAII-60; (E, D, I) strain BIOTAII-18. (A) Curved terminal raphe fissure and finely hymenate areolae. (B, C) External valve view of apex with curved terminal raphe fissure ending in an elliptical terminal pore. (D) Central area of the valve showing continuous raphe and regularly spaced fibulae. (E, F) Valve apex curved to one side of cell. (G) Details of round to rectangular hymenate areolae with fine perforations. (H, I) Details of valvocopulae with two or three rows of areolae like those of the valve. Scale bars (A–F) 1 µm; (G, H) 0.5 µm; (I) 5 µm.

**Figure 6.** *Nitzschia inordinata* sp. nov. Mucko & Bosak (A–C) LM; (D–F) SEM; (A–F) strain BIOTAII-44. (A) Two live cells after cell division with two yellow-brown plate-like plastids and pronounced lipid globules. (B–F) Cleaned material. (B) Sigmoid valve with moderately eccentric keel and strongly drawn-out apices. (C) Open sigmoid girdle band. (D) Exterior of the protracted and subcapitate apex with elevated keel and abruptly bent terminal raphe fissure. (E) Internal view of valve apex with thick fibulae enclosing the keel. (F) External valve view of central area showing continuous raphe and irregularly spaced areolae within the striae. Scale bars (A–C) 20 µm; (D–F) 5 µm.

**Figure 7.** *Nitzschia inordinata* sp. nov. Mucko & Bosak (A–C) SEM; (D–G) TEM; (A–G) strain BIOTAII-44. (A) Internal view of central valve area showing riblike fibulae, a longitudinal area devoid of pores adjacent to the fibula bases, and striae containing irregularly spaced round areolae. (B) Internal valve view with ± regularly spaced riblike fibulae. (C) Details of external valve view showing elevated keel, valve depression without areolae, only thickened virgae, and irregularly spaced areolae towards the valve margin. (D) Round, irregularly spaced areolae within uniseriate striae. (E, F) Detail of the round areolae and the
hymen (F) with tiny pores in a hexagonal array. (G) One row of pores on a girdle band. Scale bars (A–D) 5 μm; (D, E, G) 1 μm; (F) 100 nm.

**Supplementary Material:**

**Table S1.** Primers used to amplify SSU, *rbcL* and *psbC* fragments in this study. Primers in bold were used for nested PCR reaction.

**Appendix S1.** Datasheet containing information about taxa (strains) used for phylogeny. Taxa and strain name, other strain names (if mentioned in other databases), location of isolation and associated SSU, *rbcL* and *psbC* sequences deposited either in GenBank, r-Syst or BOLD database are provided for all taxa (if data were available in the literature). Taxa described in this study are in bold.

**Figure S1.** Majority rule phylogram of post-burn-in distributions of the four MrBayes runs (A) and ‘best’ Maximum Likelihood phylogram (B) constructed from concatenated SSU+*rbcL* alignment containing 169 taxa. Bayesian posterior probability and bootstrap values are indicated above branches or with arrows. New *Nitzschia* species are highlighted.

**Figure S2.** Majority rule phylogram of post-burn-in distributions of the four MrBayes runs (A) and ‘best’ Maximum Likelihood phylogram (B) constructed from *rbcL* alignment containing 340 taxa. Bayesian posterior probability and bootstrap values are indicated above branches or with arrows. New *Nitzschia* species are highlighted.

**Figure S3.** Majority rule phylogram of post-burn-in distributions of the four MrBayes runs (A) and ‘best’ Maximum Likelihood phylogram (B) constructed from *psbC* alignment containing 70 taxa. Bayesian posterior probability and bootstrap values are indicated above branches or with arrows. New *Nitzschia* species are highlighted.

**Figure S4.** *Nitzschia cf. adhaerens* strain BIOTAII-23 SEM images showing valve features.
Figure S5. *Nitzschia adhaerens* strain BIOTAI-3 SEM showing two joined valves and girdle structure.