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1 A POLYPHASIC APPROACH TO THE STUDY OF THE GENUS *NITZSCHIA*  
2 (BACILLARIOPHYTA): THREE NEW PLANKTONIC SPECIES FROM THE ADRIATIC  
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28 Running title: Adriatic *Nitzschia* species

29 **Abstract**

30 The paraphyletic diatom genus *Nitzschia* comprises over 1000 morphologically distinct  
31 pennate taxa, known from the benthos and plankton of freshwater, brackish and marine  
32 environments. The principal diagnostic characters for delimitation of *Nitzschia* species  
33 include valve shape, the position and structure of the raphe, presence/absence and shape of the  
34 proximal raphe endings and terminal raphe fissures, areola structure, and specific  
35 morphometric features such as cell size, and stria and fibula density. In this study, we isolated  
36 12 diatom strains into culture from samples collected at the surface or greater depths of the  
37 southeastern Adriatic Sea. Morphological analyses included LM, SEM and TEM  
38 observations, which, along with specific morphometric features, allowed us to distinguish  
39 three new *Nitzschia* species. These findings were congruent with the results of phylogenetic  
40 analyses performed on nuclear-encoded SSU (18S) rDNA and chloroplast-encoded *rbcL* and  
41 *psbC* genes. One of the new species (*Nitzschia dalmatica* sp. nov.) formed a lineage within a  
42 clade of Bacillariaceae containing members of the *Nitzschia* sect. *Dubiae*, which was sister to  
43 *Psammodictyon*. A second lineage was part of a novel clade that is significantly distinct from  
44 other *Nitzschia* species sequenced so far and includes *Nitzschia adhaerens* sp. nov. and *N.* cf.  
45 *adhaerens*. A further new species was found, *Nitzschia inordinata* sp. nov., which appeared as  
46 the sister group to the *N. adhaerens* clade and the conopeoid *Nitzschia* species in our  
47 phylogenetic trees. Our findings contribute to the overall diversity of genus *Nitzschia*,  
48 especially in identifying some deep branches within the Bacillariaceae, and highlight under-  
49 scoring of this genus in marine plankton.

50

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

52 **Abbreviations:** *rbcL*, ribulose-1,5-bisphosphate

53 carboxylase/oxygenase large subunit; *psbC*, photosystem II CP43 protein; ML, maximum

54 likelihood; BI, Bayesian inference; BPP, Bayesian posterior probability; BS, Bootstrap

## 55 **Introduction**

56 Diatoms (Bacillariophyta) are mostly photoautotrophic, unicellular, eukaryotic,  
57 heterokont, algae with a uniquely ornamented siliceous cell wall. They inhabit both the  
58 plankton and benthos of marine, brackish, and freshwater habitats worldwide. Diatom  
59 biodiversity is huge, though there have been different estimates of species numbers (e.g.  
60 ~100,000 species according to Mann and Vanormelingen 2013; 12,000 described and 8,000  
61 yet to be described species estimated by Guiry 2012), and numerous new taxa are described  
62 every year. Introduction of molecular methods into diatom research, such as in specific gene  
63 phylogenies or metabarcoding studies using 18S rDNA or chloroplast-encoded *rbcL*, has  
64 definitely helped to enlarge our knowledge of diatom diversity (Nealson and Venter 2007,  
65 Agusti et al. 2015, de Vargas et al. 2016, Ruck et al. 2016, Dąbek et al. 2017, Mejdandžić et  
66 al. 2018, Lobban et al. 2019, Rimet et al. 2019, etc.).

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

75 Phylogenetic analyses have shown *Nitzschia* to be paraphyletic, with species of other genera –  
76 *Bacillaria*, *Cylindrotheca*, *Cymbellonitzschia*, *Denticula*, *Fragilariopsis*, *Hantzschia*,  
77 *Psammodictyon*, *Pseudo-nitzschia*, *Simonsenia* and *Tryblionella* – nested within it (e.g.  
78 Lundholm et al. 2002, Rimet et al. 2011 [in their maximum likelihood analysis of SSU rDNA

79 aligned by Clustal], Stepanek et al. 2016, Witkowski et al. 2016, Carballeira et al. 2017). At  
80 first, the family or order (Bacillariaceae or Bacillariales) comprised by these genera appeared  
81 to be monophyletic (references as above and Ruck and Theriot 2011) but more recent studies  
82 (e.g. Ashworth et al. 2017, Lobban et al. 2019) have suggested that the Bacillariales might be  
83 paraphyletic, also containing the genera *Craspedostauros*, *Staurotropis* and *Achnanthes*; these  
84 genera lack fibulae and differ from traditional Bacillariales in several other aspects of  
85 morphology, including raphe position and structure, and areola structure. Introducing more  
86 sequences into phylogenies can often change the placements of certain genera and/or species,  
87 and it is therefore important to expand our molecular sampling effort to bridge our knowledge  
88 gaps regarding taxonomy. Moreover, polyphasic approaches combining morphology and  
89 phylogeny have been fruitful in descriptions of several novel Bacillariales species, mostly of  
90 *Pseudo-nitzschia* and *Nitzschia* (Lundholm et al. 2002, Quijano-Scheggia et al. 2009, Smida  
91 et al. 2014, Witkowski et al. 2016, Carballeira et al. 2017, Barkia et al. 2019, Lobban et al.  
92 2019).

93 *Nitzschia* is ubiquitous, occupying freshwater, brackish and marine habitats (e.g. Cleve  
94 and Grunow 1880, Lange-Bertalot et al. 2017, Lobban et al. 2019). In freshwater lakes,  
95 *Nitzschia* can be common in the phytoplankton, especially in East African lakes (Sitoki et al.  
96 2013, Grady et al. 2020). Most of these *Nitzschia* species are needle-shaped (*N. lacustris*, *N.*  
97 *bacata*, *N. nyassensis*, *N. kavirondoensis*, *N. rusingae*, *N. fenestralis*, *N. aequalis*, *N.*  
98 *mediocris*, etc.), and some of them can comprise >30% or even 100% of the total diatom  
99 community (Sitoki et al. 2013, Grady et al. 2020). In marine phytoplankton, *Nitzschia* has  
100 historically been camouflaged within a category of ‘small pennate diatoms’. Among these are  
101 a number of small, single-celled, more or less bicapitate *Nitzschia* species (including *N.*  
102 *bicapitata*, *N. curvilineata*, *N. bifurcata*, *N. braarudii*, *N. capitata*, *N. ikeanae*, *N. reimersenii*,  
103 *N. schauinslandii* and *N. subinflata* (Kaczmarek et al. 1986, Lee and Fryxell 1996)), which

104 can reach very high relative abundances sometimes accounting for 60%, 70% or even 90% of  
105 the total number of diatom cells (Semina and Mokeeva 1994). These bicapitate *Nitzschia*  
106 species vary in their size and shape and have been recorded in equatorial and subantarctic  
107 regions, including the coasts of West Africa, the Gulf of California, the Indian Ocean and the  
108 North Atlantic (Hustedt 1958, Hasle 1960, 1964, Simonsen 1974, Kaczmarska and Fryxell  
109 1986, Kaczmarska et al. 1986).

110 The Adriatic Sea is an enclosed basin in the northernmost Mediterranean Sea,  
111 characterized by extreme oligotrophy. It is divided bathymetrically into three areas: the  
112 shallow North, shallow to deep Middle and deep South Adriatic Sea (Gačić et al. 2001,  
113 Poulain 2001). The South Adriatic represents a physically dynamic habitat, in which  
114 phytoplankton thrives in seasonal blooms and diatom cells sink and enrich deep water column  
115 layers with carbon (Batistić et al. 2012, Bosak et al. 2016). Research on *Nitzschia* in the  
116 Adriatic Sea has been scarce, especially in marine plankton, where most of the studies have  
117 focused on regularly blooming, potentially harmful, toxin-producing species of the genus  
118 *Pseudo-nitzschia* (Burić et al. 2008, Ljubešić et al. 2011, Marić et al. 2011, Penna et al. 2012).  
119 One *Nitzschia* species that has been reported as ‘blooming’ in the oligotrophic waters of  
120 South Adriatic Sea is *N. sicula*, aggregating on (mini) faecal pellets of microzooplankton  
121 Nauplii; it was recorded in high abundances of 14,000–19,000 cells L<sup>-1</sup> (Viličić et al. 1994).

122 The three new species reported in this paper – *N. adhaerens*, *N. dalmatica* and *N.*  
123 *inordinata* – were found in plankton of the southeastern Adriatic Sea (Croatian coastal and  
124 open waters), with frequencies of occurrence of 20%, 5% and 22%, respectively, among the  
125 65 samples counted (unpublished data from BIOTA [Bio-tracing Adriatic Water Masses]  
126 2016 cruise). The aim of this study is to classify and describe the three new *Nitzschia* species,  
127 using a polyphasic approach combining extensive morphological and phylogenetical analyses.  
128 By using both light and electron (scanning and transmission) microscopy and constructing a

129 phylogeny based on three genes – nuclear SSU rDNA (further on SSU) and plastid-encoded  
130 *rbcL* and *psbC* – this study aligns with recent research on raphid diatoms, and contributes to  
131 the phylogeny of the genus *Nitzschia* and other genera positioned within the Bacillariales.

132

## 133 **Materials and methods**

### 134 *Culture establishment*

135 Samples containing *Nitzschia* cells were collected during the BIOTA (Bio-tracing  
136 Adriatic Water Masses) project in March 2016 at four stations in the southeast Adriatic Sea:  
137 P150 (42° 32' E 17° 59'); P300 (N 42°27' E 17°55'); P600 (N 42°24' E 17°55') and P1000 (N  
138 42°20' E 17°49'). Samples were taken with phytoplankton nets (20 µm pore-size mesh) or 5-L  
139 Niskin bottles. Those collected with Niskin bottles were taken at various depths (30, 100, 250  
140 and 400 m) filtered through 20 µm nitrocellulose and 3 µm polycarbonate filters.

141 Phytoplankton net samples were taken by dragging the net vertically from 20 m depth to  
142 surface. Both phytoplankton net and seawater samples were immediately inoculated into 0.22-  
143 µm filtered seawater taken from the collection site and enriched with f/2 nutrients (Guillard's  
144 f/2 Marine Water Enrichment Solution, Sigma–Aldrich, United Kingdom). Upon returning to  
145 the laboratory, xenic monoclonal cultures of 12 different strains (PMFBION1, PMFBION2,  
146 PMFBION3, PMFBIONA1, BIOTAI-3, BIOTAI-18, BIOTAI-23, BIOTAI-44, BIOTAI-  
147 59, BIOTAI-60, BIOTAI-74 and BIOTAI-84) were isolated by micropipette under the light  
148 microscope (Olympus CKX41, Olympus, Tokyo, Japan). Strains were maintained in plastic  
149 culture flasks (Jet Biofil®, China) in 30 mL of f/2 liquid medium and transferred weekly  
150 through a period of 4 months. Culture conditions were: temperature 18–19°C, a light intensity  
151 of 30 µmol photons m<sup>-2</sup> s<sup>-1</sup> and a photoperiod of 16h:8h of light and dark.

### 152 *Type designation*



153 Holotype slides of representative strains for each described species are deposited in the  
154 Croatian National Diatom Collection, University of Zagreb, Faculty of Science, Zagreb,  
155 Croatia under accession numbers with herbarium acronym 'HRNDC' (Thiers 2020). Isotype  
156 slides have been deposited at the Royal Botanic Garden Edinburgh, Edinburgh, UK, as  
157 Diatom Collection slides under accession numbers with herbarium acronym 'E' (Thiers  
158 2020). Designated strains and full accession numbers are given after species description.

### 159 *Microscopy*

160 Cultures were treated to remove the organic matter from diatom frustules using  
161 Simonsen's cleaning method (Simonsen 1974, Hasle 1978). In this, formaldehyde-fixed (final  
162 conc. 4%) and sedimented samples of cultures collected during a 4-month period of growth  
163 (approx. 5 mL) were first rinsed with distilled water, followed by addition of an equal amount  
164 of saturated KMnO<sub>4</sub> (or diluted 50%) for oxidation of organic matter and allowed to react for  
165 24 h. The next day an equal amount of concentrated HCl was added, gently heated over an  
166 alcohol burner flame, and then rinsed with distilled water five times until the solution reached  
167 neutral pH. Permanent slides were prepared by drying cleaned material on coverslips and  
168 mounting in Naphrax (Brunel microscopes, Chippenham) following Hasle (1978). Light  
169 microscopy was performed with a Zeiss Axio Imager A2 light microscope (Carl Zeiss,  
170 Oberkochen, Germany) equipped with DIC and phase contrast, combined with an AxioCam  
171 305 camera, or with an Olympus BX51 microscope (Olympus, Tokyo, Japan). Permanent  
172 slides chosen for holotype materials of new species are deposited in the Croatian National  
173 Diatom Collection, University of Zagreb, Faculty of Science, Croatia, while isotypes are  
174 deposited at Diatom Collection, Royal Botanic Garden Edinburgh, Edinburgh, United  
175 Kingdom (herbarium abbreviation E).

176 For SEM, parts of the oxidized suspensions were filtered and rinsed with deionized  
177 water through a 3- $\mu$ m Isopore™ polycarbonate membrane filter (Merck Millipore); the filters

178 were mounted on aluminium stubs and coated with platinum using a BAL–TEC MED 020  
179 Modular High Vacuum Coating System for 30 s at 100 mA. An ultra-high-resolution  
180 analytical field emission Hitachi SU-70 scanning electron microscope (Hitachi High-  
181 Technologies Corporation, Tokyo, Japan) was used for the analysis, operated at 5 kV and with  
182 10 mm working distance. When needed, specimens were tilted to 35° inclination. SEM  
183 images were taken using the lower (SE-L) secondary electron detector signal. For TEM,  
184 cleaned material was directly deposited onto Formvar–carbon-coated copper grids, air-dried,  
185 and examined with a FEI Morgagni 268D microscope (Eindhoven, The Netherlands). The  
186 general diatom terminology used for the morphological descriptions follows Ross et al.  
187 (1979), Hustedt (1930) and Round et al. (1990).

#### 188 *DNA isolation, PCR amplification and sequencing*

189 Genomic DNA was isolated from 50 mL of cell cultures obtained in the exponential  
190 phase of growth using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's  
191 instructions. The purity of the extracted DNA was assessed with the NanoDrop™  
192 spectrophotometer (BioSpec–nano [Shimadzu]). The nuclear gene (18S rDNA) and two  
193 chloroplast-encoded genes (*rbcL*, *psbC*) were amplified using the EmeraldAmpMax PCR  
194 Master Mix® (Takara Bio, USA) following the PCR protocol described in Ruck and Theriot  
195 (2011). When necessary, a nested PCR reaction was done with PCR product from the first  
196 reaction as the template for the second reaction. The primers used for amplification are listed  
197 in Table S1 in the Supporting information. PCR products were visualized in a 1% agarose gel  
198 and then purified with Macherey–Nagel NucleoSpin® Gel and PCR Clean-up kit (Macherey–  
199 Nagel, Düren, Germany). The purified products were sent for Sanger sequencing (Macrogen®,  
200 Amsterdam, the Netherlands). All sequences were checked and paired (5'–3' and 3'–5' ends)  
201 using Sequencher 4.1.4 (Gene Code Corporation, Ann Arbor, Michigan, USA). Blast analysis  
202 was done for all sequences with the blastn tool available at

203 <http://blast.ncbi.nlm.nih.gov/Blast.cgi> and 30 sequences belonging to 11 Adriatic *Nitzschia*  
204 strains were deposited in GenBank (accession numbers available in Appendix S1 in the  
205 Supporting information).

#### 206 *Multiple sequence alignment and phylogeny inference*

207 A total of 340 taxa were included in the phylogenetic analyses, of which 162 belonged  
208 to the genus *Nitzschia*. Four separate datasets were defined and analysed: (1) a concatenated  
209 alignment of nuclear-encoded SSU and chloroplast-encoded *rbcL* and *psbC* for 67 taxa; (2) a  
210 concatenated SSU and *rbcL* alignment (169 taxa); (3) a single-gene *rbcL* alignment (340  
211 taxa); and (4) a single-gene *psbC* alignment (70 taxa). The sequences used, with voucher  
212 strain information and GenBank, Thonon Culture Collection, and BOLD accession numbers,  
213 are listed in datasheet Appendix S1 in the Supporting information. *Eunotia* was selected for  
214 the outgroup, since it represents the group of diatoms sister to all other raphids, together with  
215 selected species of *Diploneis*, *Amphora*, *Pleurosigma*, *Trachyneis*, which are members of the  
216 likely sister group of the Bacillariales-plus clade (defined as all Bacillariales taxa plus  
217 *Craspedostauros*, *Staurotropis*, *Achnanthes* and *Undatella*) (sources of phylogenetic data on  
218 raphid diatoms included Stepanek and Kociolek 2014, Witkowski et al. 2016, Ashworth et al.  
219 2017, Lobban et al. 2019). Alignment of 18S rDNA was done with ssu-align software  
220 (Version 0.1.1; eddylib.org/software/ssu-align, © 2016 Howard Hughes Medical Institute;  
221 Nawrocki 2009) following the default settings for aligning sequences according to eukaryotic  
222 SSU secondary structure and masking poorly aligned and unsupported parts of the alignment.  
223 Chloroplast-encoded *rbcL* and *psbC* genes were aligned based on their conceptual translations  
224 into amino acid sequences in Mesquite (Version 3.04; Maddison and Maddison 2015). The  
225 alignments are available at <https://zenodo.org/record/1322635>.

226 Phylogenetic analyses of each dataset first included identification of an appropriate  
227 model of nucleotide substitution and rate variation across sites using a model selection routine

228 available in the IQ-TREE v. 1.5.5. (Nguyen et al. 2015). In addition, we performed a  
229 partition-merging procedure that joined two or more alignment partitions when the merge did  
230 not incur a substantial cost to the model likelihood. Model and partition selection were done  
231 using the Bayesian information criterion (BIC), which penalizes for the number of parameters  
232 in a model. The initial partition models split the single-gene alignments into codons, and the  
233 concatenated alignments were split first into genes and then into codons. Phylogenies were  
234 reconstructed using maximum likelihood (ML) and Bayesian inference (BI) in IQ-TREE  
235 (Nguyen et al. 2015) and MrBayes v. 3.2.6. (Ronquist et al. 2012), respectively. We  
236 performed a total of 200 ML optimizations, 50 for each single-gene alignment and 50 for  
237 concatenated matrices, and finally chose the one with smallest BIC score as the ‘best’ tree  
238 (treefiles available at <https://zenodo.org/deposit/1322635>). ML optimizations were performed  
239 under default settings in IQ-TREE, each starting from a different random seed number, i.e.  
240 different point in parameter space, for a more exhaustive search of the likelihood surface. We  
241 varied the strength of perturbation of the nearest neighbour interchange during tree  
242 rearrangement, repeating the optimization many times, which is helpful for avoiding local  
243 optima during the likelihood optimization (Nguyen et al. 2015). Clade support was assessed  
244 using IQ-TREE’s UltraFast bootstrap routine (Minh et al. 2013) with 1000 pseudoreplicates.

245 Bayesian analyses were carried out in the same fashion for each dataset, with the best  
246 set of partitions as identified by IQ-TREE, but with different parametrization for the  
247 substitution rate matrix. Instead of the models identified as optimal by IQ-TREE, we used the  
248 Generalized Time-Reversible model (GTR). Among-site rate variation in MrBayes was  
249 accommodated via a  $\Gamma$  distribution with four rate categories and by estimating the proportion  
250 of invariant sites. We ran four simultaneous Markov chain Monte Carlo (MCMC)  
251 simulations, each composed of one cold and three heated chains, for a total of 10 million  
252 generations with a sampling frequency of one thousand generations. Stationarity and

253 convergence among the MCMC runs were assessed from the MrBayes output (standard  
254 deviation of split frequencies and potential scale reduction factor) and by inspecting the  
255 posterior distributions in the program Tracer v. 1.6. (Rambaut and Drummond 2007). The  
256 burn-in fraction was 25% of the sampled posterior distributions. Majority rule phylograms of  
257 the post-burn-in distributions of four MrBayes runs available as .tre files are at  
258 <https://zenodo.org/record/1322635>.

259

## 260 **Results**

### 261 *Phylogeny of three new Nitzschia species*

262 All of the Bayesian inference and Maximum Likelihood (BI/ML) trees generated from  
263 all four datasets (concatenated SSU+*rbcL*+*psbC*, concatenated SSU+*rbcL*, and single-gene  
264 *rbcL* and *psbC* datasets) recovered *Nitzschia* as paraphyletic, spread out among other  
265 Bacillariales (*Bacillaria*, *Cylindrotheca*, *Denticula*, *Hantzschia*, *Psammodictyon* and  
266 *Tryblionella*) (Fig. 1, A and B; Figs. S1–S3, A and B). The Bacillariales-plus clade (B-plus),  
267 consisting of Bacillariales together with *Achnanthes*, *Craspedostauros*, *Staurotropis* and  
268 *Undatella* was recovered as monophyletic, supported with high Bayesian posterior probability  
269 value/Bootstrap values (BPP/BS) = 1/95 in the three-gene (SSU+*rbcL*+*psbC*) phylogeny (Fig.  
270 1 A and B); node support in phylogenies constructed with two or one genes, therefore based  
271 on fewer nucleotide positions, decreased from SSU+*rbcL* to *rbcL*, and further to the *psbC*  
272 phylogeny (BPP/BS = 0.85/91, 0.84/63 and 0.81/62 , respectively: Figs. S1-S3 A and B).  
273 Genera outside the Bacillariales-plus clades (*Amphora*, *Diploneis*, *Pleurosigma* and  
274 *Trachyneis*) showed different positioning in respect to the Bacillariales-plus clade. In the  
275 SSU+*rbcL* and *rbcL* phylogenies all of these genera were outside the Bacillariales-plus clade,  
276 while in the *psbC* phylogeny some *Amphora* taxa were nested within the Bacillariales-plus  
277 clade (Fig. S3 A and B). *Eunotia* (Eunotiales) was the monophyletic sister to other raphids, as

278 expected from previous analyses (see Introduction) and served as an outgroup with  
279 BPP/BS=1/100 (Fig. 1 A and B; Figs. S1–S3 A and B).

280 Within the paraphyletic genus *Nitzschia*, the 10 new isolates from the Adriatic Sea  
281 were clearly separated from all previously sequenced taxa and formed three clades that were  
282 widely separated in the phylogeny: the three new species are *Nitzschia dalmatica* sp. nov.  
283 (strains PMFBIONA1, PMFBION3, BIOTAI-74 and BIOTAI-84), *Nitzschia adhaerens* sp.  
284 nov. (strains PMFBION1, PMFBION2, BIOTAI-18, BIOTAI-59 and BIOTAI-60) and  
285 *Nitzschia inordinata* sp. nov. (strain BIOTAI-44) (Fig. 1 A and B; Figs. S1–S3 A and B).

286 The *N. dalmatica* clade (four strains) was monophyletic with BPP/BS = 1/100, and  
287 sister to *Nitzschia* sp. strain UTKSA0111 (strain information given in Appendix S1; BPP/BS  
288 = 1/100) in all datasets (Fig. 1 A and B; Figs. S1–S3 A and B). These two species branched  
289 off within a ‘*dubiiiformis*’ group that (using information from all the trees included here)  
290 contains *N. dubiiiformis*, *N. traheaformis*, *N. pellucida* and *N. dubia*, as well as some  
291 unidentified *Nitzschia* species (Figs. S1, S2). The combined *dalmatica* + ‘*dubiiiformis*’ group  
292 was resolved as monophyletic in the SSU+*rbcL* dataset (BPP/BS = 1/97, Fig. S1 A and B) and  
293 sister to *Psammodictyon* (BPP/BS = 1/100, Fig. S1 A and B). The same was found in the BI  
294 tree of the *rbcL* dataset, but the ML tree did not resolve the ‘*dubiiiformis*’ group as  
295 monophyletic (Fig. S2B).

296 The *N. adhaerens* clade (five strains) was monophyletic in all datasets with BPP/BS =  
297 1/100 in three-gene phylogeny (Fig. 1 A and B), 0.61/96 in SSU+*rbcL* phylogeny (Fig. S1 A  
298 and B), 0.99/100 in *rbcL* phylogeny (Fig. S2 A and B) and 1/100 in *psbC* phylogeny (Fig. S3,  
299 A and B). The closest relatives to *N. adhaerens* in all datasets were *Nitzschia* sp. UTKSA0106  
300 and *N. cf. adhaerens* BIOTAI-23 strain (Fig. 1 A and B; Figs. S1–S3 A and B). In the three-  
301 gene and *psbC* phylogenies, *N. cf. adhaerens* BIOTAI-23 and *Nitzschia* sp. UTKSA0106  
302 comprised the sister clade to *N. adhaerens* with BPP/BS support = 0.70/67 and 0.96/96,

303 respectively (Fig. 1 A and B; Fig. S3 A and B). BIOTAI-23 showed morphological  
304 similarities with *N. adhaerens* in morphometry (Table 1, Figure S4 in the Supporting  
305 information), the lanceolate valve shape, its continuous raphe positioned on an elevated,  
306 discrete keel, and the tiny round to rectangular areolae occluded by finely perforated hymens;  
307 however, its phylogenetic position prevents inclusion in *N. adhaerens*. Beyond *N. cf.*  
308 *adhaerens* and *Nitzschia* sp. UTKSA0106, the relationships of *N. adhaerens* are rather unclear  
309 and inconsistent; for example, the next closest relatives in the three-gene tree are conopeum-  
310 bearing species ('tholophora' species: Lobban et al. 2019), such as *N. cf. volvendirostrata* and  
311 *N. celaenoi*, but *Nitzschia inordinata* sp. nov. and *Bacillaria* sp. SH349 in the SSU+*rbcL* and  
312 *rbcL* phylogenies (Figs. S1–S2, A and B).

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

### 319 *Morphology and description of new taxa*

320 The three new *Nitzschia* species are presented below, and morphometric data for all  
321 strains [valve length (VL), valve width (VW), fibula density in 10  $\mu\text{m}$  (FD), stria density in 10  
322  $\mu\text{m}$  (SD) and the areola density in 1  $\mu\text{m}$  (AD)] are given in Table 1. For *N. adhaerens* and *N.*  
323 *dalmatica*, one strain each was chosen to provide holotype material according to the criteria of  
324 having at least a *rbcL* sequence, well-preserved cleaned material, and measurements made in  
325 both LM and EM. The ranges of the measured parameters given in the species descriptions  
326 are drawn from all the strains and also from natural material (original net and phytoplankton  
327 samples from which the strains were derived and measured).

328

329 *Nitzschia dalmatica* Mucko & Bosak, sp. nov. (Figures 2A–K, 3A–H)

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

350 *Representative DNA sequences:* SSU: MH734172; *rbcL*: MH687908; *psbC*:  
351 MH687897



352 *Holotype*: HRNDC 000010 permanent slide of strain BIOTAI-84 (illustrated in Fig. 2  
353 B–D).

354 *Isotype*: E 5897 permanent slide of strain BIOTAI-84.

355 *Type locality*: Croatia: southeast Adriatic Sea (P150 station, 30 m of depth; 42°32' N;  
356 17°59' E). Cells isolated from the Niskin bottle sample collected on 8<sup>th</sup> March 2016 onboard  
357 RV *Naše More* by M. Mucko.

358 *Etymology*: This species has been named after the historical region of the southeastern  
359 Adriatic Sea coast, 'Dalmatia', where the species was discovered.

360 *Comparisons with similar species*: In the sectional classification of *Nitzschia*  
361 formulated by Grunow (in Cleve and Grunow 1880), *N. dalmatica* would have been classified  
362 in the sect. *Dubiae*, because of its only moderately eccentrically placed raphe system and  
363 somewhat constricted centre. Among the species assigned to this group by Grunow (ibid.),  
364 Hustedt (1939, 1955, 1957) and Krammer and Lange-Bertalot (1988), there are several that  
365 bear some resemblance to *N. dalmatica* in having finely striated valves (>30 in 10 µm). These  
366 include (in date order of publication): *N. pellucida* Grunow, *N. normanii* Grunow, *N.*  
367 *subhybrida* Hustedt, *N. dubiiformis* Hustedt, *N. thermaloides* Hustedt, *N. pseudohybrida*  
368 Hustedt, *N. hybridaeformis* Hustedt, *N. aestuarii* Hustedt, *N. translucida* Hustedt and *N.*  
369 *traheiformis* Chunlian Li, Witkowski & Shu-xian Yu. Most of these have coarser striation  
370 than *N. dalmatica* (39–45 striae in 10 µm) and can be separated from it rather easily,  
371 providing care is taken to ensure that the light microscope is properly set up to resolve striae  
372 with densities between 30 and 40 in 10 µm. This applies to *N. pellucida* and *N. normanii* [c.  
373 32 and 30–32 striae in 10 µm in the original description of Cleve and Grunow (1880) and  
374 Krammer and Lange-Bertalot (1988), respectively]; *N. subhybrida* [c. 32 striae in 10 µm in  
375 the photographs by Simonsen (1987)]; *N. hybridaeformis* Hustedt [c. 35 striae in 10 µm  
376 according to Hustedt (1955) and measured by us as 34–36 in 10 µm from the illustrations of

377 the holotype provided by Simonsen (1987) and online at <http://hustedt.awi.de>]; *N.*  
378 *pseudohybrida* Hustedt and *N. thermaloides* [in both we measured c. 34 striae in 10 µm in  
379 illustrations by Simonsen (1987) or online, in contrast to c. 40 in 10 µm in the original  
380 descriptions given by Hustedt (1955)]; *N. aestuarii* and *N. translucida* [in both we measured  
381 34–35 striae in 10 µm in the photographs by Simonsen (1987), which is slightly higher than  
382 was reported by Hustedt (1959) for *aestuarii* and slightly lower than for *translucida*]; and *N.*  
383 *traheiformis* [with 32–34 striae in 10 µm but otherwise very similar to *N. dalmatica*  
384 (Witkowski et al. 2016)]. In addition, *N. thermaloides* has a noticeably smaller central  
385 interspace than *N. dalmatica* (it is about twice the width of other interspaces in *N.*  
386 *thermaloides* but three times the width in *N. dalmatica*) and a less constricted centre. *N.*  
387 *normanii*, *N. aestuarii* and *N. translucida* are also less constricted than *N. dalmatica* and  
388 hence appear more linear; in contrast, *N. subhybrida* and *N. pellucida* have a much *more*  
389 constricted centre than *N. dalmatica* [compare the illustrations of Simonsen (1987, pl. 99, figs  
390 8–12) and Cleve and Grunow (1880, pl. 5, fig. 96), respectively, with our Fig. 2D), partly  
391 reflecting the more central keel in these species. *N. hybridaeformis* is a larger diatom than *N.*  
392 *dalmatica* (60–93 × 6–8 µm) and has more widely spaced fibulae (5–10 in 10 µm).

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

400       *Species diagnosis:* *Nitzschia dalmatica* is identified and distinguished from similar  
401 taxa by the following character states: eccentric and elevated keel indented in the middle;

402 dense (39–45 striae in 10  $\mu\text{m}$ ) transapical striae uniseriate, separated with elevated and  
403 thickened virgae; a row of round keel areolae with elevated silicified rings present on both  
404 sides of the raphe; external proximal raphe endings slightly curved and droplet-like.

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

406 *Description:* Frustules linear-lanceolate in girdle view; live cells with two plate-like  
407 yellow-brown plastids, one in each half of the cell (Fig. 4A). Cells have several porose girdle  
408 bands per theca, but the details are unclear (Fig. S5 in the Supporting information).  
409 Valvocopula open, with two or three rows of round pores enclosed by finely perforated  
410 hymens (Fig. 5 H and I). Valves lanceolate, 10–34  $\mu\text{m}$  long and 2–5  $\mu\text{m}$  wide, with apices  
411 that are cuneate in valve view and apparently very slightly spatulate in girdle view (Fig. 4 C,  
412 D and G); there are 16–25 fibulae and 48–56 striae in 10  $\mu\text{m}$  (hence the striae cannot be  
413 resolved in LM). Keel narrow, discrete and elevated about valve face (i.e. there is an abrupt  
414 transition from valve face to keel: Figs. 4 B–E and H, 5B), almost central (Fig. 4 E, F and H).  
415 Terminal raphe fissures curved (Fig. 5 A–C). External proximal raphe endings absent (Figs.  
416 4H and 5D). Striation of the valve very fine and delicate, not resolvable in LM (Fig. 4 B–D),  
417 comprising uniseriate striae of tiny round to rectangular areolae (Fig. 4 F–I, 5B); the areolae  
418 (5–6 in 1  $\mu\text{m}$ ) occluded by finely perforated hymens (Fig. 5G); these lie at the outer apertures  
419 of the areolae, so that the external valve face appears smooth (Figs. 4H and 5C). Each  
420 uniseriate transapical stria ends up by two areolae within the keel (Fig. 5 A, C–E and G).  
421 Virgae flat, never bifurcating (Fig. 4 F–I). Fibulae relatively dense (16–25 in 10  $\mu\text{m}$ ),  
422 regularly spaced throughout keel (Fig. 4 F and H); sometimes two fibulae are fused together  
423 (Fig. 4I, arrowhead).

424 *Representative DNA sequences:* SSU: MH734165; *rbcL*: MH687900; *psbC*:  
425 MH687889

426 *Holotype*: HRNDC 000011 permanent slide of strain BIOTAI-18 (illustrated in Fig. 4  
427 B–D).

428 *Isotype*: E 5898 permanent slide of strain BIOTAI-18.

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

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

433 *Comparisons with similar species*: In Grunow's *Nitzschia* classification (in Cleve and  
434 Grunow 1880), *Nitzschia adhaerens* would almost certainly have been placed in section  
435 *Bacillaria*. Subsequently it would have been put in the section *Dissipatae* when the original  
436 concept of *Bacillaria* (as an independent genus characterized by its unique motile colonies)  
437 was restored by Hustedt (e.g. see 1939, p. 661). The characteristic that would have led to  
438 these hypothetical assignments is the almost central position of raphe system and the absence  
439 of longitudinal lines on either side of the keel in LM (though in fact the type of the  
440 *Dissipatae*, *N. dissipata*, does have these, which reflect the presence of external conopea in  
441 this species and its relatives, e.g. *N. sigmoidea*, *N. recta*: Mann 1978, 1986, Lobban et al.  
442 2019). Rather few *Nitzschia* species have been described with near-central raphes and those  
443 that do exist [e.g. *N. longa* Grunow, *N. praelonga* Cleve and *N. cursoria* (Donkin) Grunow]  
444 are mostly coarsely structured, with striation densities < 20 in 10 µm. The only one known to  
445 us with finer striation is *N. linkei* Hustedt, with c. 33 striae in 10 µm according to the original  
446 description (Hustedt 1939). However, this striation density is much lower than in *N.*  
447 *adhaerens* (>48 in 10 µm) and *N. linkei* is a larger diatom (40–55 × 7–9 µm rather than 17–34  
448 × 2.5–5 µm); therefore confusion is very unlikely.

449 *Species diagnosis*: *Nitzschia adhaerens* is identified and distinguished from similar  
450 taxa by the following character states: keel narrow, discrete and elevated about valve face,

451 almost central; transapical striae uniseriate and not resolvable in LM composed of tiny round  
452 to rectangular areolae enclosed by finely perforated hymens; each stria ends up by two areolae  
453 within the keel; proximal raphe endings absent.

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

455 *Description:* Live cells containing two plate-like yellow-brown plastids, one in each  
456 half of the cell (Fig. 6A). Valves sigmoid, 91–152 µm long and 4–8 µm wide, with a  
457 moderately eccentric keel and strongly drawn-out subcapitate apices (Fig. 6 B, D and E); with  
458 7–10 fibulae and 20–24 striae in 10 µm. Girdle bands open and sigmoid (Fig. 6C), perforated  
459 by one row of round pores (Fig. 7G). Keel elevated above the valve face (Fig. 6 D and E) and  
460 enclosed internally by thick fibulae (Figs 6F and 7B). External proximal raphe endings absent  
461 (Fig. 7A). Terminal raphe fissures sharply bent (about 30°) (Fig. 6D). Valve striation  
462 interrupted, starting on the elevated keel, absent in a depressed area of the valve face parallel  
463 to the raphe, and then resuming and continuing to the valve margin (Figs. 6F, 7 A–D). Each  
464 stria uniseriate, containing round areolae (Fig. 7 A–E) occluded by finely perforated hymens  
465 with pores in a hexagonal array (Fig. 7F). Virgae thickened and slightly elevated externally,  
466 especially in a depressed area without striation adjacent to the bases of the fibulae (Fig. 7C).  
467 Fibulae regularly spaced along the keel, slender and riblike at the centre (Fig. 6F) but  
468 becoming proportionately more massive towards the apices (Fig. 6E).

469 *Representative DNA sequences:* SSU: MH734171; *rbcL*: MH687906; *psbC*:  
470 MH687895

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

473 *Isotype:* E 5899 permanent slide of strain BIOTAI-44.

474           *Type locality:* Croatia: Southeast Adriatic Sea (P150 station, 30 m of depth; 42°32' N;  
475 17°59' E). Cells isolated from Niskin bottle sample collected on 8<sup>th</sup> March 2016 onboard RV  
476 *Naše More* by M. Mucko.

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

479           *Comparisons with similar species:* A variety of marine *Nitzschia* species have been  
480 described with sigmoid frustules. Among them are some classified in the section *Obtusae*  
481 (Cleve and Grunow 1880, Krammer and Lange-Bertalot 1988) because of their highly  
482 distinctive proximal raphe endings, which are deflected inwards and end in convergent or  
483 almost parallel transapical grooves (e.g. Mann 1978, figs 865, 875, 881). *N. inordinata* cannot  
484 be confused with these, nor with other sigmoid species that possess proximal raphe endings.  
485 In LM, *N. inordinata* resembles *N. lorenziana*: the two have similar dimensions (*N.*  
486 *lorenziana* valves have lengths of 37–190 µm and widths of 3–7 µm according to Krammer  
487 and Lange-Bertalot 1988) and the ranges of fibula densities overlap (6–10 in 10 µm in  
488 *lorenziana*, 7–10 in *inordinata*). However, the stria densities differ, those of *lorenziana* being  
489 coarser (13–19 in 10 µm rather than 20–24) and SEM (Poulin et al. 1990) reveals that, in  
490 *lorenziana*, the striae are biseriate, central raphe endings are present, the terminal fissures are  
491 forked rather than being bent to one side), and the fibulae are elongated apically (each one  
492 subtending two of the biseriate transapical striae) rather than being narrow ribs as in *N.*  
493 *inordinata*. Among sigmoid species without proximal raphe endings, few have striation as  
494 coarse as in *N. inordinata* ( $\leq 25$  in 10 µm). The first one is *N. perlonga* Pantocsek (1902),  
495 which has extremely long (>480 µm) non-attenuate linear valves and occurs in freshwater.  
496 The most similar brackish or marine species seem to be those within the *N. sigma* complex, as  
497 their valves also taper from centre to poles. However, the fibula structure differs: *N.*  
498 *inordinata* has relatively simple riblike fibulae over most of the valve (though becoming

499 relatively more massive towards the poles) that flare slightly at the entrance to the keel (Fig.  
500 7B), whereas in the *N. sigma* complex the fibulae expand (and delimit portulae) at two levels  
501 (at the opening into the cell lumen and at the entrance to the keel), so that the space between  
502 them is partially enclosed to form a chamber (Mann 1978, figs 845, 846, 849, 850). In  
503 addition, in *N. sigma* the terminal raphe fissures continue almost straight to the valve margin  
504 (ibid., figs 841, 851), contrasting with the sharply bent fissures in *N. inordinata*; furthermore,  
505 the striae are not interrupted near the bases of the fibulae, unlike in *N. inordinata*.

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

## 512 **Discussion**

513         Species delimitation within *Nitzschia* is problematic due to the lack of unique  
514 morphological characters to group them and because DNA sequence data are only available  
515 for a small minority of species (Witkowski et al. 2004, Trobajo et al. 2013, Rimet et al. 2014).  
516 Furthermore, some characters, such as fibula and stria densities or cell width, may change due  
517 to daily or seasonal variation of environmental parameters (Trobajo et al. 2004, 2011), or  
518 within the life cycle, such as in *N. inconspicua* (Mann et al. 2013). Nowadays, we are  
519 observing an increase in newly described *Nitzschia* species, most likely due to higher  
520 culturing efforts and the combination of morphological and phylogenetic investigations  
521 (Smida et al. 2014, Witkowski et al. 2016, Barkia et al. 2019, Lobban et al. 2019). The same  
522 trend in using both morphology and phylogeny for descriptions of new pennate species and  
523 genera, sometimes also involving sequencing of complete genomes or detailed genus

524 overviews, is recorded for other diatoms, such as *Proschkinia* (Gastineau et al. 2019,  
525 Majewska et al. 2019, Kim et al. 2020), *Entomoneis* (Mejdandžić et al. 2017b, 2018),  
526 *Amphora* and *Halamphora* (Stepanek and Kociolek 2019), *Dorofeyukea* (Kulikovskiy et al.  
527 2019) and *Simonsenia* (Kim et al. 2019). This study follows the methodology in combining  
528 morphology with phylogeny to describe three new *Nitzschia* species.

#### 529 *Phylogenetic relationships and morphology comparisons*

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

533 In the SSU+*rbcL* and *rbcL* trees, *N. dalmatica* was resolved within a monophyletic  
534 group of morphologically similar species with near-central or moderately eccentric raphe  
535 system. This grouping of *Nitzschia* species is evident in some previously published molecular  
536 phylogenies of Bacillariaceae (e.g. Witkowski et al. 2016, An et al. 2017) and corresponds to  
537 *Nitzschia* section *Dubiae* as amended by Hustedt (1955) or the *Dubiae–Bilobatae* of Krammer  
538 and Lange-Bertalot (1988). The species that appears most similar to *N. dalmatica* in LM and  
539 metrics is *N. dubiiformis*, but the two are clearly separated in the gene trees, where *N.*  
540 *dalmatica*'s nearest relative is *Nitzschia* sp. UTKSA0111 (the two are sister lineages  
541 supported with high BPP/BS values: 100/100 in all trees; Figs. 1 A and B, S1–S3 A and B).  
542 The morphology of this strain is not fully known; however an LM image is available online  
543 ([http://www.protistcentral.org/Photo/get/photo\\_id/6413](http://www.protistcentral.org/Photo/get/photo_id/6413)) and shows a single valve that, like *N.*  
544 *dalmatica*, belongs to the '*Dubiae–Bilobatae*' group and measures  $41 \times 4.6 \mu\text{m}$  with  
545 imperceptible striation and 8–9 fibulae in  $10 \mu\text{m}$ . It is therefore much longer than the  
546 specimens of *N. dalmatica* we observed (though this could reflect different stages in a size  
547 reduction cycle) and, perhaps more importantly, it has a much lower fibula density. In  
548 addition, the UTKSA0111 valve is much more strongly constricted centrally than in *N.*



549 *dalmatica*. Striation of UTKSA0111 is resolvable with SEM, counting 39–41 stria in 10  $\mu\text{m}$   
550 and 6–7 areolae in 1  $\mu\text{m}$ . Additionally, both *N. dalmatica* and strain UTKSA0111 have well  
551 silicified central nodule without areolae and keel with two rows of areolae which are  
552 externally surrounded by elevated silicified rings (Matt P. Ashworth, personal  
553 communication).

554         The second novel species, *N. adhaerens*, is part of a separate lineage, also containing  
555 our clone BIOTAI-23 and *Nitzschia* sp. UTKSA0106, that seems (three-gene and SSU+*rbcL*  
556 trees) to be related to a group characterized by the possession of conopea, i.e. external silica  
557 flaps extending out laterally from the keel (Mann 1978, 1986). The latter group includes *N.*  
558 *dissipata*, *N. volvendirostrata*, *N. nanodissipata* and the type species of *Nitzschia*, *N.*  
559 *sigmoidea*, which also share with each other delicate striation (relative to the size of the  
560 valves), a moderately eccentric or nearly central raphe, and an absence of central raphe  
561 endings. This suite of characters has been called the ‘tholophora’ morphology of *Nitzschia* by  
562 Lobban et al. (2019), who described 14 new *Nitzschia* species within this group, all with  
563 conopea and all phylogenetically close to each other; we omitted their new sequences from  
564 our analyses, considering that the 162 *Nitzschia* taxa represented in our trees are enough to  
565 give good sequence sampling depth. *N. adhaerens*, although it is a delicately structured  
566 species like those in the ‘tholophora’ group, does not possess conopea, but it is unclear  
567 whether this is symplesiomorphic or whether conopea have been lost secondarily. Important  
568 for resolving this point is to know whether or not the diatoms closest to *N. adhaerens* also  
569 lack conopea. The most closely related species (strain) to *N. adhaerens* in all trees is yet  
570 undescribed *Nitzschia* sp. UTKSA0106. The morphology of this strain is not fully known and  
571 it may or may not have conopea, but its valves are clearly differentiated from *N. adhaerens* by  
572 their slightly spatulate ends in girdle view (i.e. a polar expansion of the keel) and their  
573 narrow, protracted poles (Matt P. Ashworth, personal communication, and LM images online

574 at [http://www.protistcentral.org/Photo/get/photo\\_id/6410](http://www.protistcentral.org/Photo/get/photo_id/6410)). UTKSA0106 valves also differ  
575 from *N. adhaerens* in being larger ( $54\text{--}57 \times 7.7 \mu\text{m}$  in the online photographs) and having  
576 much lower fibula density (9–11 in  $10 \mu\text{m}$ ). The other diatom related to *N. adhaerens* in our  
577 molecular datasets is our BIOTAI-23, which sometimes appears as the sister to *N. adhaerens*  
578 (SSU+*rbcL*, *rbcL*), and sometimes as sister to UTKSA0106 (three-gene, *psbC*). BIOTAI-23  
579 definitely lacks conopea and is morphologically similar to the strains we include within *N.*  
580 *adhaerens*. The fibula densities in strain BIOTAI-23 are within the range measured in the *N.*  
581 *adhaerens* strains but with different lower and upper limits (17–20 in  $10 \mu\text{m}$  in BIOTAI-23  
582 and 16–25 in  $10 \mu\text{m}$  in *N. adhaerens*), and the valves seem wider (4–6  $\mu\text{m}$  rather than the 3–4  
583 of most *adhaerens*); other morphometrics – length and stria density – match. However,  
584 genetically these two are separated with high support (three-gene phylogeny). For the moment  
585 BIOTAI-23 is referred to as *Nitzschia cf. adhaerens*.

586 In the phylogenetic trees, the third new species, *N. inordinata* (for which we have only  
587 a single isolate), is either sister to the clade containing both the ‘tholophora’ *Nitzschia* and the  
588 *adhaerens* group (three-gene, *psbC*), or on a separate branch within it, together with  
589 ‘*Bacillaria*’ SH349 (SSU+*rbcL*, *rbcL*). Because of its sigmoid shape, lack of proximal raphe  
590 endings, and rather coarse striation, *N. inordinata* would probably have been allocated to sect.  
591 *Sigmata* by Grunow, but our SSU+*rbcL* and *rbcL* trees show *N. inordinata* and *N. sigma* to be  
592 not only distinct (see Results) but also distantly related. The close relative of *N. inordinata*,  
593 ‘*Bacillaria*’ strain SH349, was sequenced and its LM morphology described by An et al.  
594 (2017). The valve length and width given for ‘*Bacillaria*’ SH349 (115.7 and  $10 \mu\text{m}$ ,  
595 respectively) are within the range of *N. inordinata*, as are the stria and fibula densities (20 and  
596 10 fibulae in  $10 \mu\text{m}$ , respectively). We obtained unmounted material of strain SH349 from  
597 Professor J.H. Noh and examined it under SEM. It showed a similar morphology to *N.*  
598 *inordinata*: the fibula and keel structure are the same, both species have irregularly arranged

599 areolae towards the valve face margin, and both have an interruption to the striae at the bases  
600 of the fibulae (unpublished observations: images available from D.G. Mann on request).  
601 However, *N. inordinata* is easily separated from ‘*Bacillaria*’ SH349 because it is clearly  
602 sigmoid, whereas SH349 is not (An et al. 2017, fig. 3g).

603         The valve shape, raphe position and metric characters reported for ‘*Bacillaria*’ SH349  
604 (lanceolate outline, nearly central raphe, measuring  $115.7 \times 10 \mu\text{m}$ , with 10 fibulae and 20  
605 striae in  $10 \mu\text{m}$ ) fall within Grunow’s concept (in Cleve and Grunow 1880) of *Nitzschia*  
606 *socialis*, a species described by Gregory (1857) and named for its tendency to be found, even  
607 after acid cleaning, in groups of cells orientated parallel to each other. Ralfs (in Prichard  
608 1861, p. 784) transferred *N. socialis* to *Bacillaria* and, in doing so, implied that the groups of  
609 cells Gregory observed reflect the existence of motile colonies (as in *Bacillaria paxillifera*),  
610 because Ralfs explicitly prescribed motile colonies as a defining characteristic of *Bacillaria*  
611 (his description of the genus stated “frustules ... united into a short band, moving on each  
612 other by a sliding motion without separation”). However, as far as we know, the existence of  
613 motile colonies in *N. socialis* has never been confirmed and strain SH349 too was not seen to  
614 form motile colonies (Prof. J.H. Noh, personal communication 22 May 2019). Assignment to  
615 *Bacillaria* is consistent with the near-central position of the raphe in SH349 and the close  
616 match of its SSU with “*Bacillaria cf. paxillifer*” strain BA14c (GenBank HM805020).  
617 However, strain BA14c too does not form the special motile colonies that are supposed to  
618 characterize the genus *Bacillaria* (observations of strain BA14c by Dr. F. Pniewski and  
619 ourselves; see also the photographs of BA14 clones available online at  
620 <https://ccba.ug.edu.pl/>), though its SSU sequence matches another “*Bacillaria paxillifer*”  
621 sequence in GenBank (M87325) with 98.48% identity (BLAST). It is therefore unclear  
622 whether the special motile colonies supposed to be characteristic of *Bacillaria* are indeed a  
623 synapomorphy for a monophyletic group.

624 Summarizing: there is insufficient information about several of the ‘*Bacillaria*’ clones  
625 that have been sequenced to be able to judge how they differ from *N. inordinata*  
626 morphologically and whether they should be assigned to *Bacillaria*, given that they do not  
627 form motile colonies. The closest known relative to *N. inordinata* is strain SH349, which  
628 seems to be identifiable as *Nitzschia* (*Bacillaria*?) *socialis* sensu Grunow (in Cleve and  
629 Grunow 1880). These two diatoms occupy an interesting position in phylogenetic trees, on a  
630 deep branch that may be basal to the ‘tholophora’ and *adhaerens* groups. Neither are close to  
631 ‘true’ *Bacillaria* (i.e. the species with motile colonies: cf. Jahn and Schmid 2007).

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

#### 643 *Small pennate Nitzschia diversity in marine plankton*

644 Relative to the number of benthic species, not many *Nitzschia* species are planktonic,  
645 especially in marine waters. Most of the few that are – such as *N. longissima* (Hasle and  
646 Syvertsen 1996), *N. bicapitata* (Fryxell, 2000) and two of the species described here (*N.*  
647 *dalmatica* and *N. inordinata*) – occur as single cells, even though theoretical considerations  
648 and experimental data (e.g. Reynolds 2006) confirm that the formation of stellate or chainlike

649 colonies (like those of *N. asterionelloides*, *Fragilariopsis* and *Pseudo-nitzschia*: Hustedt  
650 1942, Hasle and Syvertsen 1996) can often be considered adaptive in relation to  
651 sedimentation. Although discovered in the plankton, a different habitat (i.e. benthos) for *N.*  
652 *dalmatica* cannot be ruled out due to drifting of diatom flora from coastal to open waters  
653 systems by currents and waves. According to Fryxell (2000), cells of small bicapitate  
654 *Nitzschia* species occurring in the plankton frequently aggregate on substrates, thus  
655 representing a major food source for some grazers, as was observed with our new species *N.*  
656 *adhaerens*, which aggregated on large chains of *Chaetoceros*. *Nitzschia adhaerens* like *N.*  
657 *dalmatica* therefore can probably have two habitats – one existing as an epiphyte on large  
658 *Chaetoceros* chains, and one existing solely in plankton. Spicular cell shape can also be  
659 considered to be an adaptation to planktonic existence, because of the high surface area to  
660 volume ratio (Reynolds 2006), and it is noticeable that most of the planktonic *Nitzschia*  
661 species occurring in tropical freshwaters (e.g. Kilham et al. 1986, Grady et al. 2020) are  
662 spicular, as is *N. inordinata* among the species described here. Less elongate and squat  
663 *Nitzschia* species are generally benthic and either adhere to surfaces, or live free, moving  
664 through sediments.

665         Single-celled pennate diatoms in the Adriatic Sea were previously reported by Batistić  
666 et al. 2012 and Bosak et al. 2016 as ‘shade-flora’, part of a larger, deep-dwelling  
667 phytoplankton community found at the bottom of the photic zone or below it (~150 up to 500  
668 m of depth). Most of those single-celled pennates were taxonomically assigned to *Navicula* cf.  
669 *distans*, *N. cf. directa* and species belonging to *Nitzschia* cf. *bicapitata* complex (Bosak et al.  
670 2016). Successful culturing from samples collected at those depths has not yet been  
671 attempted, as far as we know, although survival of diatoms in low light conditions has already  
672 been confirmed (Smayda and Mitchell-Innes 1974, Waite and Harrison 1992, Jochem 1999,  
673 etc.). This is important to emphasize as most of the *N. adhaerens* and *N. cf. adhaerens* strains

674 were isolated from 100, 250 or even 400 m of depth (BIOTAI-59, BIOTAI-60, BIOTAI-18  
675 and BIOTAI-23, respectively). General phytoplankton investigations conducted both in the  
676 Adriatic Sea and elsewhere (Baltic Sea, Atlantic Ocean, Indian Ocean and Pacific Ocean)  
677 have usually classified ‘unidentified pennate diatoms’ into size classes [nano (>2 and <20µm)  
678 and micro (>20 and <200µm)], which are abundant and frequently occurring in coastal and  
679 open waters (Piiparinen et al. 2010, Cerino et al. 2012, Brandini et al., 2014, Estrada et al.  
680 2016). Investigating these solitary pennate diatoms in the marine plankton has become of  
681 great importance, because of their high contribution to the overall phytoplankton community,  
682 microbial loop, and carbon fluxes from surface to bottom layers in the oceans. In this study,  
683 we have isolated strains of small *Nitzschia* species from net phytoplankton samples, but also  
684 from 30, 100, 250 and 400 m of depth. Mejdandžić et al. (2018) successfully cultivated  
685 diatoms from *Entomoneis* from the same samples, as well as undescribed *Haslea* species  
686 (Mejdandžić et al. 2017a) and some other unpublished strains from other pennate diatom  
687 genera (*Navicula*, *Psammodictyon*, *Diploneis*); thus showing that marine pennate planktonic  
688 diatoms are subjected to sinking and can survive harsh environmental conditions. These cells  
689 can be returned to the surface through intense vertical convection in the South Adriatic Sea  
690 during the winter period (Batistić et al. 2012; Korlević et al. 2015), which gives cells a new  
691 opportunity to increase their numbers in the photic zone.

692         It is not surprising to discover new species of marine planktonic pennate diatoms,  
693 especially those belonging to the paraphyletic genus *Nitzschia*, since most investigations of  
694 marine phytoplankton do not make a detailed examination of diatom cells in samples, and  
695 *Nitzschia* species need especially careful LM and SEM observations to determine their  
696 morphology and identity. Additionally, there are a lot of different criteria to take into  
697 consideration when delimiting a species within marine planktonic pennate diatoms, such as  
698 morphological variations, sexual reproduction (if observed), variation in genetic material

699 within different populations of same species (which is difficult to obtain), and phenotypic  
700 plasticity and changes in culturing conditions. With that in mind, this study shows that an  
701 investigation combining most of the wanted criteria, including fine-grained morphological  
702 observations and multigene phylogenies of related species and genera, is needed to resolve  
703 planktonic pennate diatoms.

704

## 705 **Acknowledgments**

706 This material is based in part upon the work supported by Croatian Science  
707 Foundation under the project BIOTA [Bio-tracing Adriatic Water Masses], UIP-2013-11-  
708 6433 and in part under the project TurtleBIOME [Loggerhead sea turtle (*Caretta caretta*)  
709 microbiome: insight into endozoic and epizoic communities], UIP-2017-05-5635. The authors  
710 are grateful to the crew of RV "Naše more" for their help during the fieldwork. The authors  
711 declare there is no conflict of interests. MM is grateful to Dr I. Sviličić Petrić and A. Kolda  
712 for providing facilities for molecular laboratory work and M. Vugrin for culturing efforts. We  
713 are very grateful to Professor J.H. Noh for very kindly supplying us with material of clone  
714 SH349 ('Unidentified *Bacillaria* sp. 1' in An et al. 2017) and Dr F. Pniewski for a culture of  
715 clone BA14c ('*Bacillaria* cf. *paxillifera*'); detailed observations of these will be published  
716 elsewhere. RT acknowledges support from the CERCA Programme/Generalitat de Catalunya.  
717 The Royal Botanic Garden Edinburgh is supported by the Scottish Government's Rural and  
718 Environment Science and Analytical Services Division. We are kindly grateful to Dr M.P.  
719 Ashworth for supplying us with LM and SEM images of *Nitzschia* sp. strains UTKSA0106  
720 and UTKSA0111.

721

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998 **Tables and Figure captions**

999 **Table Captions**

1000 **Table 1.** Morphometric parameters measured under light (LM) and electron microscopy (EM)  
 1001 in 11 strains belonging to three new Adriatic *Nitzschia* species, strain BIOTAI-23  
 1002 representing *N. cf. adhaerens* and natural material where new species cells were observed. VL  
 1003 – valve length; VW – valve width; SN – stria density in 10 µm; FN – fibula density in 10 µm;  
 1004 AN – areola density in 1 µm.

Species	Strain name	Measured cells (no.)	VL (µm)	VW (µm)	FN	SN	AN
<i>N. dalmatica</i>	BIOTAI-74	LM n=26	23-29	4-6	14-15		
	BIOTAI-74	EM n=18	23-26	4-5	16-19	39-44	5-7
	BIOTAI-84	LM n=31	18-23	3-6	14-15		
	BIOTAI-84	EM n=16	21-22	4-5	14	39-44	5-6
	PMFBION3	LM n=31	21-27	3-5	14-16		
	PMFBION3	EM n=14	20-26	3-5	14-16	39-44	5-6
	PMFBIONA1	LM n=31	21-27	3-5	14-16		
	PMFBIONA1	EM n=13	22-26	3-5	14-16	39-45	
	<i>Natural material</i>	LM n=22	12-41	3-5	10-18		
<i>N. adhaerens</i>	BIOTAI-3	LM n=49	21-31	3-4	16-19		
	BIOTAI-3	EM n=10	23-27	3-4	23-24	48-55	5-6
	BIOTAI-18	LM n=28	21-24	3-4	21-23		
	BIOTAI-18	EM n=9	20-27	3	23-24	48-54	5-6
	BIOTAI-59	LM n=24	24-26	3-4	21-24		
	BIOTAI-60	LM n=31	24-26	3-5	20-22		
	BIOTAI-60	EM n=15	22-34	3-4	20-25	50-56	6
	PMFBION1	LM n=6	26-27	3-4	18-19		
	PMFBION2	LM n=21	17-25	3-4	19-24		
	PMFBION2	EM n=5	21-24	2-3	22	49-50	
	<i>Natural material</i>	LM n=20	10-25	2-3	16-18		
	<i>N. cf. adhaerens</i> (BIOTAI-23)	LM n=33	21-29	4-6	16-19		
	EM n=20	17-27	4-6	17-20	49-51	5-6	
<i>N. inordinata</i>	BIOTAI-44	LM n=24	140-152	6-8	7-10	21-24	3
	<i>Natural material</i>	LM n=9	91-141	4-6	7	20-22	

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1018 **Figure Captions**

1019 **Figure 1. A:** Majority rule phylogram of the post-burn-in distributions of the four MrBayes  
1020 runs inferred from a concatenated dataset of three markers: SSU, *rbcL* and *psbC*. Branch  
1021 support is summarized above branches as Bayesian posterior probability. **B:** "Best" Maximum  
1022 Likelihood tree inferred from a concatenated dataset of three markers: SSU, *rbcL* and *psbC*.  
1023 Branch support is summarized above branches as Maximum Likelihood bootstrap values.  
1024 Three new species of the Adriatic strains are in bold and highlighted.

1025

1026 **Figure 2.** *Nitzschia dalmatica* sp. nov. Mucko & Bosak (A–D) LM; (E–I) SEM; (J–K) TEM;  
1027 (A–D, F, I) strain BIOTAI-84; (E, G, H) strain BIOTAI-73; (J–K) strain PMFBIONA1. (A)  
1028 Live broadly linear cell containing two yellow-brown plate-like plastids. (B–K) Cleaned  
1029 material. (B, C) Linear-lanceolate valves with constricted raphe-bearing margin in the central  
1030 area and cuneate to slightly capitate apices. (D) A cell in girdle view with numerous girdle  
1031 bands. (E, F) External and internal valve views with eccentric and elevated keel. (G, I)  
1032 External view of the valve with cuneate to capitate apex with slightly curved terminal raphe  
1033 fissure; note the rimmed areolae in the keel. (H) Internal valve view with cuneate to capitate  
1034 apex, dense striation and coarse irregularly spaced fibulae. (J, K) Straight to slightly curved

1035 terminal raphe fissures. Scale bars (A–F) 10  $\mu\text{m}$ ; (G, H) 2  $\mu\text{m}$ ; (I) 1  $\mu\text{m}$ ; (J) 0.2  $\mu\text{m}$ ; (K) 0.5  
1036  $\mu\text{m}$ .

1037

1038 **Figure 3.** *Nitzschia dalmatica* sp. nov. Mucko & Bosak (A, B, D, E) SEM; (C, F, G, H) TEM;  
1039 (A, B, D) strain BIOTAI-74; (E) strain BIOTAI-84; (C, F, G, H) strain PMFBIONA1. (A–  
1040 C) External and internal valve view with proximal raphe endings curved and droplet-like.  
1041 Note the bifurcated virga (C, arrowhead). (D) Thickened and elevated virgae and keel areolae  
1042 with raised rims; note the occasional presence of two areolae opposite a single valve stria  
1043 (arrowhead). (E) Internal valve view with round areolae within striae and robust, irregularly  
1044 spaced, riblike fibulae. (F) Finely perforate hymenate areolae. (G, H) Open numerous girdle  
1045 bands, each perforated by two or three rows of round pores. Scale bars (A–E) 2  $\mu\text{m}$ ; (F) 0.2  
1046  $\mu\text{m}$ ; (G, H) 2  $\mu\text{m}$ .

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1048 **Figure 4.** *Nitzschia adhaerens* sp. nov. Mucko & Bosak (A–D) LM; (E, G–I) SEM; (F) TEM;  
1049 (A–E, H, I) strain BIOTAI-18; (F) strain BIOTAI-3; (G) strain BIOTAI-60. (A) Linear-  
1050 lanceolate living cells with two yellow-brown plate-like plastids. (B–I) Cleaned material. (B)  
1051 Spindle-shaped valve with cuneate apices in valve view. (C) A valve in girdle view, showing  
1052 the very slightly spatulate apices. (D) Frustule in girdle view. (E) External valve view with  
1053 slightly eccentric, narrow keel. (F) Slightly eccentric keel with regularly spaced fibulae. (G)  
1054 Internal valve view revealing fine, uniseriate striae and riblike fibulae. (H) External valve  
1055 view showing the fine hymenate, round to rectangular areolae; note (by comparison with I)  
1056 that the hymens lie near the external apertures of the areolae. (I) Internal valve view with  
1057 regularly spaced riblike fibulae, which are sometimes fused together (arrowhead). Scale bars  
1058 (A–G) 10  $\mu\text{m}$ ; (H, I) 2  $\mu\text{m}$ .

1059

1060 **Figure 5.** *Nitzschia adhaerens* sp. nov. Mucko & Bosak (A, D, G, H) TEM; (B, C, E, F, I)  
1061 SEM; (A, D, G, H) strain BIOTAI-3; (B, C) strain BIOTAI-60; (E, D, I) strain BIOTAI-18.  
1062 (A) Curved terminal raphe fissure and finely hymenate areolae. (B, C) External valve view of  
1063 apex with curved terminal raphe fissure ending in an elliptical terminal pore. (D) Central area  
1064 of the valve showing continuous raphe and regularly spaced fibulae. (E, F) Valve apex curved  
1065 to one side of cell. (G) Details of round to rectangular hymenate areolae with fine  
1066 perforations. (H, I) Details of valvocopulae with two or three rows of areolae like those of the  
1067 valve. Scale bars (A–F) 1  $\mu\text{m}$ ; (G, H) 0.5  $\mu\text{m}$ ; (I) 5  $\mu\text{m}$ .

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1069 **Figure 6.** *Nitzschia inordinata* sp. nov. Mucko & Bosak (A–C) LM; (D–F) SEM; (A–F)  
1070 strain BIOTAI-44. (A) Two live cells after cell division with two yellow-brown plate-like  
1071 plastids and pronounced lipid globules. (B–F) Cleaned material. (B) Sigmoid valve with  
1072 moderately eccentric keel and strongly drawn-out apices. (C) Open sigmoid girdle band. (D)  
1073 Exterior of the protracted and subcapitate apex with elevated keel and abruptly bent terminal  
1074 raphe fissure. (E) Internal view of valve apex with thick fibulae enclosing the keel. (F)  
1075 External valve view of central area showing continuous raphe and irregularly spaced areolae  
1076 within the striae. Scale bars (A–C) 20  $\mu\text{m}$ ; (D–F) 5  $\mu\text{m}$ .

1077

1078 **Figure 7.** *Nitzschia inordinata* sp. nov. Mucko & Bosak (A–C) SEM; (D–G) TEM; (A–G)  
1079 strain BIOTAI-44. (A) Internal view of central valve area showing riblike fibulae, a  
1080 longitudinal area devoid of pores adjacent to the fibula bases, and striae containing irregularly  
1081 spaced round areolae. (B) Internal valve view with  $\pm$ regularly spaced riblike fibulae. (C)  
1082 Details of external valve view showing elevated keel, valve depression without areolae, only  
1083 thickened virgae, and irregularly spaced areolae towards the valve margin. (D) Round,  
1084 irregularly spaced areolae within uniseriate striae. (E, F) Detail of the round areolae and the



1085 hymen (F) with tiny pores in a hexagonal array. (G) One row of pores on a girdle band. Scale  
1086 bars (A–D) 5  $\mu\text{m}$ ; (D, E, G) 1  $\mu\text{m}$ ; (F) 100 nm.

1087

1088 **Supplementary Material:**

1089 **Table S1.** Primers used to amplify SSU, *rbcL* and *psbC* fragments in this study. Primers in  
1090 bold were used for nested PCR reaction.

1091 **Appendix S1.** Datasheet containing information about taxa (strains) used for phylogeny. Taxa  
1092 and strain name, other strain names (if mentioned in other databases), location of isolation and  
1093 associated SSU, *rbcL* and *psbC* sequences deposited either in GenBank, r-Syst or BOLD  
1094 database are provided for all taxa (if data were available in the literature). Taxa described in  
1095 this study are in bold.

1096 **Figure S1.** Majority rule phylogram of post-burn-in distributions of the four MrBayes runs  
1097 (A) and ‘best’ Maximum Likelihood phylogram (B) constructed from concatenated  
1098 SSU+*rbcL* alignment containing 169 taxa. Bayesian posterior probability and bootstrap values  
1099 are indicated above branches or with arrows. New *Nitzschia* species are highlighted.

1100 **Figure S2.** Majority rule phylogram of post-burn-in distributions of the four MrBayes runs  
1101 (A) and ‘best’ Maximum Likelihood phylogram (B) constructed from *rbcL* alignment  
1102 containing 340 taxa. Bayesian posterior probability and bootstrap values are indicated above  
1103 branches or with arrows. New *Nitzschia* species are highlighted.

1104 **Figure S3.** Majority rule phylogram of post-burn-in distributions of the four MrBayes runs  
1105 (A) and ‘best’ Maximum Likelihood phylogram (B) constructed from *psbC* alignment  
1106 containing 70 taxa. Bayesian posterior probability and bootstrap values are indicated above  
1107 branches or with arrows. New *Nitzschia* species are highlighted.

1108 **Figure S4.** *Nitzschia cf. adhaerens* strain BIOTAI-23 SEM images showing valve features.

1109 **Figure S5.** *Nitzschia adhaerens* strain BIOTAI-3 SEM showing two joined valves and girdle  
1110 structure.