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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* (Kaczmarska 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⁻¹ (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⁻² s⁻¹ 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 KMnO₄ (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- μ 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; eddylab.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 Γ 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 μm (FD), stria density in 10
322 μm (SD) and the areola density in 1 μm (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 μm long and 3–6 μm wide, with 10–19 fibulae in 10 μm , 39–45
335 striae in 10 μm and 5–7 areolae in 1 μm (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 8th 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 μm) 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 μm long and 2–5 μm 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 μm (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 μm) 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 μm),
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 8th 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 8th 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* (≤ 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) 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 μm
550 and 6–7 areolae in 1 μm . 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). 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 μm 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

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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. inordinata</i>	<i>N. cf. adhaerens</i>	LM n=33	21-29	4-6	16-19	
(BIOTAI-23)		EM n=20	17-27	4-6	17-20	49-51	5-6
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 μm ; (G, H) 2 μm ; (I) 1 μm ; (J) 0.2 μm ; (K) 0.5
1036 μm .

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 μm ; (F) 0.2
1046 μm ; (G, H) 2 μm .

1047

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 μm ; (H, I) 2 μm .

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 μm ; (G, H) 0.5 μm ; (I) 5 μm .

1068

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 μm ; (D–F) 5 μm .

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 μm ; (D, E, G) 1 μm ; (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 BIOTAIL-3 SEM showing two joined valves and girdle
1110 structure.