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Observations of two marine members of the genus *Cymbellonitzschia* (Bacillariophyta) from Tokyo Bay, Japan, with the description of the new species *Cymbellonitzschia banzuensis*

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SUMMARY

Cymbellonitzschia is a small genus of canal raphid diatoms within the Bacillariales reported from both freshwater and coastal marine environments. The genus is characterized by its distinct dorsiventral or 'cymbelloid' valve outline with the canal raphe either on the dorsal or, more commonly, along the ventral margin. Of the six currently described species only two freshwater representatives, the type of the name of the genus *C. minima* and the widely reported *C. diluviana*, have received detailed morphological treatments. This lack of understanding of the morphological diversity within the group hampers any efforts at understanding the relationships between species in this small but ecologically diverse genus. Presented here are observations and descriptions of two marine representatives from the genus *Cymbellonitzschia*, *C. szulczewskii* and the new species *C. banzuensis* sp. nov., based on light and scanning electron microscopy and a preliminary investigation of the phylogenetic position of marine *Cymbellonitzschia* within the Bacillariales. These species were collected co-occurring in low tide sand samples from the Banzu Flats, Tokyo Bay, Japan. The morphology of these marine species will be compared to the documented freshwater species to better understand the morphological diversity and relationships within the genus.

Key words: *Cymbellonitzschia*, diatom, new species, Tokyo Bay, Japan.

INTRODUCTION

Cymbellonitzschia Hustedt is a small genus of canal raphid diatoms, belonging to the Bacillariales, first described from Lake Tanganyika as *Cymbellonitzschia minima* Hustedt (Hustedt 1924). Since the original description, only five additional species have been encountered: the freshwater species *C. cataractorum* Kufferath, *C. diluviana* Hustedt, and *C. manguini* Maillard and two marine species *C. hossamedinii* Salah and *C. szulczewskii* Witkowski *et al*. Aside from the type of the name of the genus, *C. minima*, and the widely reported *C. diluviana*, the morphology of species within this genus have not been well investigated, with most known only from a small number of specimens (Salah 1955; Kufferath 1957; Maillard 1967). This situation is further complicated by the poor illustration of several of these species (Kufferath 1957; Maillard 1967), making their inclusion in the genus questionable at best.

Although not specieous, taxa within the genus inhabit a broad range of habitat types from tropical African rift lakes (*C. minima* and *C. cataractorum*; Hustedt 1924; Kufferath 1957; Vyverman & Cocquyt 1993), oligotrophic (*C. diluviana*; Sovereign 1958; Stoermer & Kreis 1978) and eutrophic (*C. diluviana*; Stoermer 1964; Jewson & Lowry 1993) freshwaters and estuarine and coastal marine habitats (*C. hossamedinii* and *C. szulczewskii*; Salah 1955; Witkowski *et al*. 2000). Although ecologically diverse, when habitat type is reported, species of *Cymbellonitzschia* are reported from sandy substrates or with an epipsammic growth form (Cocquyt & Jewson 1994; Kawashima & Mayama 2003; Jewson *et al*. 2006).

As the name would suggest, the genus shares a close affinity with the genus *Nitzschia* Hassall having both a bacillarioid canal raphe system and chloroplast arrangement, as well as similar striae and uniseriate areolae (Round *et al*. 1990; Jewson & Lowry 1993; Cocquyt & Jewson 1994). What distinguishes *Cymbellonitzschia* from other bacillarioid diatoms is its distinct 'cymbelloid' asymmetry across the apical plane (arched dorsal margin and straight ventral margin, referring to the dorsiventral valve outline of the genus *Cymbella* Agardh). Aside from symmetry, few investigated features unify the genus as currently prescribed. In fact, it has been speculated that differences in raphe position between *C. minima* and *C. diluviana*, with the raphe predominantly along the dorsal margin in *C. minima* and along the ventral margin in *C. diluviana*, may be an indication of their non-monophyly (Ross 1983). This distinction has been weakened somewhat by the observation that in some *C. diluviana* valves the raphe can be positioned along the dorsal margin (Jewson & Lowry 1993). However, the difference in the expression of this orientation, with approximately 58% of *C. minima* valves exhibiting a dorsal raphe orientation (Cocquyt & Jewson

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1994) compared with only 3% of *C. diluviana* cells in this orientation (Jewson & Lowry 1993) may indicate that this feature has phylogenetic significance. Certainly, recent systematic investigations have demonstrated the pitfalls of diatom classification systems based on symmetry (e.g. Kociolek & Stoermer 1988; Cox & Williams 2006; Stepanek & Kociolek 2014) and may be confounded by similar growth forms between unrelated taxa.

Hampering our understanding of these relationships is the few reported species that have been investigated in detail. Whereas the freshwater *C. minima* and *C. diluviana* have been well examined by both light microscope (LM) and scanning electron microscope (SEM) (Round *et al*. 1990; Jewson & Lowry 1993; Cocquyt & Jewson 1994; Kawashima & Mayama 2003), little is known about the fine morphological features of the taxa described from marine habitats. Of the two marine species, *C. hossamedinii* is known only from a single line drawing from very few specimens (Salah 1955) and *C. szulczewskii*, while documented with several LM images, has been illustrated with SEM by only a single image of the interior of an incomplete valve (Witkowski *et al*. 2000).

We present here the detailed morphological observations of two marine *Cymbellonitzschia* species collected from the Banzu Flats, Tokyo Bay, Japan, including the description of one species, *C. banzuensis* as new to science and a preliminary examination of the phylogenetic position of marine *Cymbellonitzschia* within the Bacillariales. The morphology of these two species will be compared to that of previously examined freshwater representatives and a discussion of shared morphological features follows.

MATERIALS AND METHODS

Taxon collection

Sand samples containing *C. banzuensis* and *C. szulczewskii* were collected from the Banzu Flats in Tokyo Bay, Tokyo Prefecture, Japan (35.44081°N, 139.91078°E). At the time of collection the sampling site had a salinity of approximately 33 ppt, determined using an optical refractometer. Collections were made at low tide from submerged sand by Stepanek and Mayama in June 2013.

For LM and SEM observations, organic material was removed by boiling samples in concentrated sulphuric acid with the addition of potassium permanganate, followed by repeated rinses with distilled water until a neutral pH was reached. For all LM observations, cleaned material was air dried onto glass coverslips and permanent slides were prepared using Naphrax (Brunel Microscopes Ltd., Chipenham, UK) mounting medium. All LM observations were performed using an Olympus BX-51 light microscope (Olympus America Inc., Center Valley, PA, USA) with ×100 oil immersion objective (numerical aperture 1.40). Light micrographs were taken using an Olympus DP71 digital camera. For SEM observations, cleaned material was air dried onto glass coverslips, mounted onto aluminum stubs and sputter coated with ca. 1.5 nm gold using a Cressington 108 sputter coater (Cressington Scientific Instruments Ltd., Watford, UK). Scanning electron microscope observations were made using a JEOL JSM 7401 field emission SEM (JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 3 kV. All frustule measurements and light and scanning electron micrographs were obtained from collected wild material unless otherwise stated.

Cymbellonitzschia banzuensis was isolated into monoculture through micropipette serial dilution into Daigo's Artificial Seawater medium (Nihon Pharmaceutical Company, Tokyo, Japan). Due to the epipsammic growth form of *C. banzuensis*, samples containing sand from the flats were shaken vigorously to dislodge cells from sand grains before isolation. Cultures of *C. banzuensis* were maintained at approximately 25°C under a 12:12 light regime with and irradiance of approximately 50 μmol m^{-2} s^{-1}.

DNA extraction, amplification and sequencing

DNA was extracted from pelleted culture material using a Chelex 100 extraction method (Richlen & Barber 2005). An approximately 1400 bp region of the chloroplast encoded large subunit of RUBISCO (*rbc*L) was chosen as the barcode marker for *C. banzuensis*. The 'full' as well as the shorter *rbc*L-3P (748 bp) region have both been shown to be universal and powerful in discriminating between diatom species (Hamsher *et al*. 2011). As the *rbc*L marker is also often used in diatom phylogenetic studies (Bruder & Medlin 2008; Ruck & Theriot 2011; Stepanek & Kociolek 2014; Stepanek *et al*. 2015), the longer *rbc*L region is included here to increase its phylogenetic utility in future studies. In an effort to provide multiple lines of evidence for the phylogenetic position of this taxon, as well as to enhance its utility in future research, the often utilized 18S small subunit rDNA (SSU) region was sequenced as well. The primers used for the amplification and sequencing of the *rbc*L and SSU markers are listed in Table 1.

The *rbc*L and SSU markers were polymerase chain reaction (PCR) amplified using GE healthcare Illustra Ready-To-Go™ PCR beads (GE Healthcare Biosciences, Pittsburgh, PA, USA) following the manufacturer's protocol. The PCR profile used was 94°C initial denaturation for 3 min 30 s, followed by 36 cycles of 94°C for 50 s, 52°C for 50 s, 72°C for 80 s, with a final extension at 72°C for 15 min. Amplified PCR products were purified using ExoSap-it (Affymetrix, Santa Clara, CA, USA) following the manufacturer's protocol. Sequencing was performed by Functional Biosciences (Madison, WI, USA) and raw sequence files were assembled and edited using Geneious ver. 5.6 (Drummond *et al*. 2012).

Sequence alignment and phylogenetic analysis

To confirm the phylogenetic position of *C. banzuensis* within the Bacillariales and test for appropriate outgroup taxa, sequence data for *C. banzuensis* was first added to the *rbc*L alignment used by Stepanek and Kociolek (2014). This alignment includes a broad sampling of raphid diatom lineages including canal raphid diatoms from the Bacillariales, Surirellales and Rhopalodiales. Maximum likelihood estimation performed on this alignment returned a tree with *C. banzuensis* nested within the Bacillariales (data not shown). For simplicity, all further analyses were performed only with

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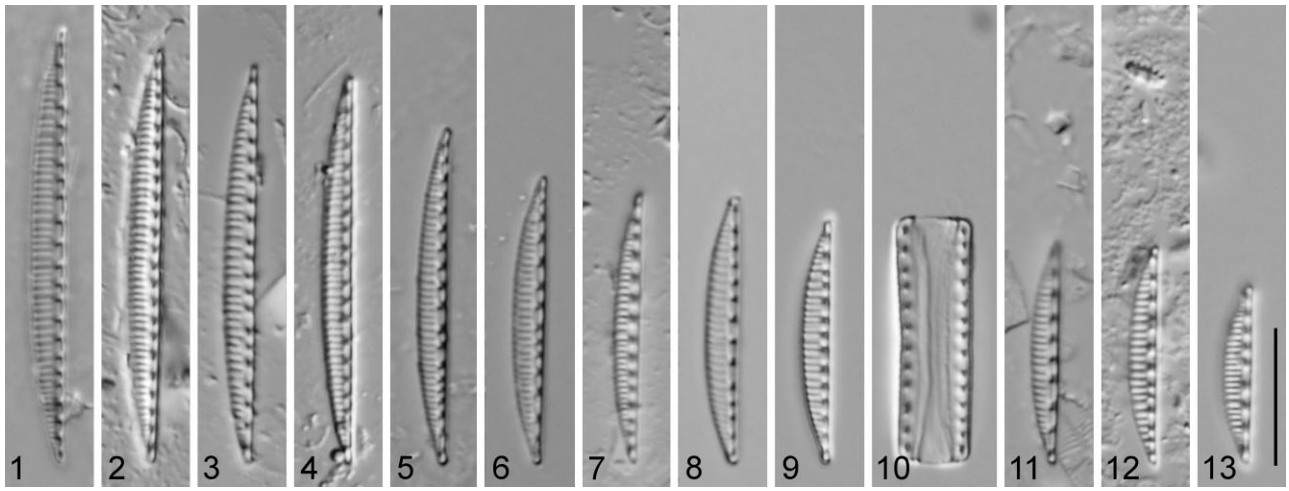


Figs 1–13. Light microscopy (LM) of *Cymbellonitzschia banzuensis* sp. nov. from collection material. Single valves (1–9, 11–13) and girdle view of the whole frustule (10) showing observed size range. 5. Holotype specimen. Scale bar = 10 μm.

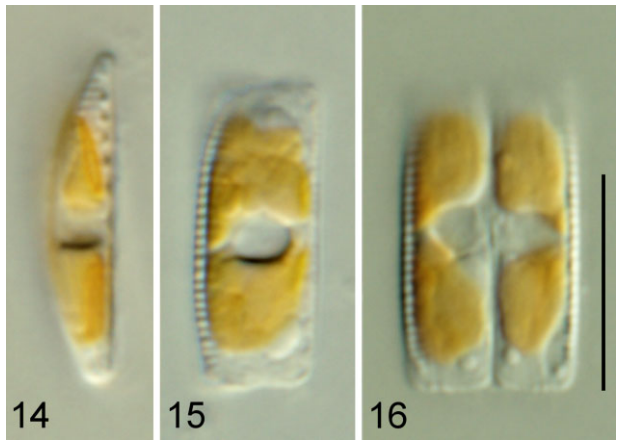
taxa from within the Bacillariales clade with two *Amphora* Ehrenberg ex Kützing taxa (sister to the Bacillariales; Stepanek & Kociolek 2014) as outgroup taxa. For the phylogenetic analysis the markers *rbc*L and SSU were analyzed independently. For *rbc*L, 29 sequences were obtained from GenBank, including taxa from the Bacillariales genera *Bacillaria* Gmelin, *Cylindrotheca* Rabenhorst, *Denticula* Kützing, *Fragilariopsis* Hustedt in Schmidt, *Hantzschia* Grunow, *Nitzschia*, *Pseudonitzschia* Peragallo in Peragallo & Peragallo, *Tryblionella* Smith, and the outgroup taxa. For SSU, 43 sequences were obtained from GenBank with the inclusion of two taxa from the genus *Psammodictyon* Mann. See Table S1 for a complete list of taxa and GenBank accession numbers.

Sequences were aligned in Geneious ver. 5.6 using a muscle alignment algorithm (Edgar 2004). The ends of the alignment were trimmed to minimize missing data, resulting in a final alignments of 30 taxa and 1405 bp for *rbc*L (Alignment S1) and of 44 taxa and 1673 bp for SSU (Alignment S2). Maximum likelihood estimation (ML) was performed using a general time reversible (GTR) model of evolution with a gamma distribution (Γ) and a proportion of invariable sites (I) with PhyML version 3.0 (Guindon *et al.* 2010) implemented in SeaView version 4.3.4 (Gouy *et al.* 2010). Node support was estimated using 500 bootstrap replicates. Bayesian estimation was conducted with MrBayes version 3.2.1 (Ronquist *et al.* 2012) using the GTR+Γ + I model of evolution. The Bayesian estimation was run for 10 million generations, with a 2 million generation burn-in, with chains sampled every 1000 generations.

RESULTS

Cymbellonitzschia banzuensis sp. nov. (Figs 1–22)

Holotype: TNS-AL-58911, slide prepared from collected material deposited at the National Museum of Nature and Science, Tsukuba, Japan (TNS). Holotype specimen is represented by Figure 5.



Figs 14–16. LM of *C. banzuensis sp. nov.* living cells from culture material containing two ventrally appressed plate-like plastids. 14. Cell in valve view. 15, 16. Cells in girdle view. Scale bar = 10 μm.

Isotypes: TNS-AL-58911m, cleaned collection material deposited at TNS. JPK10928 slide and cleaned material deposited at the University of Colorado Boulder (COLO), Kociolek collection.

Type locality: 35.44081°N, 139.91078°E, epipsammic, Banzu Flats, Tokyo Bay, Japan. The deposited material, as well as the material from which cultures were grown, was collected 23 June 2013.

Etymology: The species epithet refers to the type location, the Banzu Flats in Tokyo Bay.

GenBank accession numbers: KT693309 (*rbc*L barcode), KT693310 (SSU).

Description

Valves with hantzschioid symmetry. In valve view, valves narrowly semi-lanceolate with the dorsal margin smoothly arched. Ventral margin straight. Valves 13.0–33.0 μm long and 2.0–

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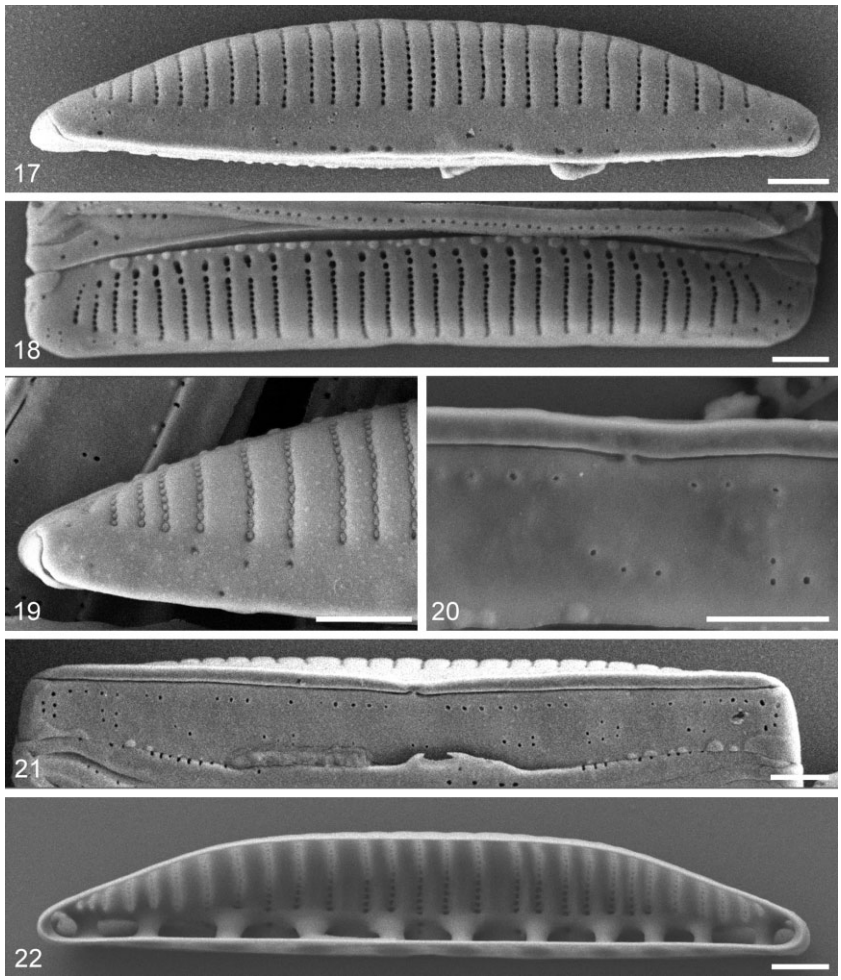

Figs 17–22. Scanning electron microscopy (SEM) of *C. banzuensis sp. nov.* from culture material. 17. External whole valve view. 18. Dorsal girdle view of valve. 19. Detail of valve end, showing areolae occlusions and dorsally curved distal raphe ends. 20. Detail of the ventral margin showing closely spaced proximal raphe ends. 21. Ventral girdle view of valve. 22. Internal whole valve view. Scale bars = 1 μm.

3.0 μm wide with slightly protracted acutely rounded to weakly rostrate apices. Canal raphe on the ventral mantle appears continuous and is not raised on a keel, fibulae 7–9 in 10 μm. Striae parallel throughout, somewhat irregularly spaced in some specimens, 16–22 in 10 μm. In girdle view, frustules are rectangular. Live cells with two, transapically positioned, plate-like chloroplasts (Figs 14–16). Both chloroplasts ventrally appressed and extending more or less up the valve face (Fig. 14).

SEM observations

In SEM of cultured material, a broad hyaline area, infrequently ornamented with small irregularly placed pores, runs longitudinally near the ventral margin (Figs 17, 19). This hyaline area corresponds, internally, to the canal raphe and covers approximately one third of the valve face (Fig. 17). The raphe lies almost entirely along the ventral mantle with only the dorsally hooked distal raphe ends visible on the valve face (Figs 17, 19–21). The raphe is discontinuous with proximal raphe ends very closely spaced and only slightly inflated (Figs 20, 21). The striae composed of rows of small closely spaced areolae separated by thick virgae and extend uninterrupted onto the dorsal mantle (Figs 17–19). The areolae appear externally occluded by a simple velum in some specimens (Fig. 19). No striae present on the ventral mantle but, similar to the hyaline area on the valve face, it is dotted with small irregularly placed puncta (Figs 20, 21). Internally, the canal raphe system is subtended by fibulae that widen only slightly to the valve face to form a mostly open canal without portulae (Fig. 22). Fibulae are relatively evenly spaced throughout the valve with 1–3 striae between fibulae. Internally, the proximal and distal raphe ends were not easily observable due to their position and depth of the mantle.

Comparison with congeneric species

Cymbellonitzschia banzuensis can be distinguished from the common freshwater species *C. diluviana* by its relatively narrower valve outline and protracted acutely rounded apices, as well as by its coarser striae (Hustedt 1954 gives length 10–20 μm, breadth 2.5–4.0 μm, 22–26 striae in 10 μm for *C. diluviana*). Of the known marine or estuarine species, *C. banzuensis* can be distinguished from *C. hossamedinii* by its narrow valves (Salah 1955 gives a breadth of 4–5 μm for *C. hossamedinii*), and from *C. szulczewskii* by its more coarse striae.

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Cymbellonitzschia szulczewskii Witkowski, Lange-Bertalot & Metzeltin (2000; p.868, pl. 213, figs 1–7) (our Figs 23–39)

JPK10928 slide and material deposited at the University of Colorado Boulder (COLO), Kociolek collection.

Description

Valves with hantzschioid symmetry. In valve view, valves with a smoothly arched dorsal margin and a straight ventral margin. Valve length 17.0–22.0 μm, valve breadth 2.0–2.5 μm (Witkowski *et al.* 2000 gives length 19–25 μm, breadth 2.0 μm for their populations). The valve apices are protracted and acutely rounded. The striae are very fine, numbering 31–32 in 10 μm (Witkowski *et al.* 2000 gives 28–31 striae in 10 μm for their populations). The canal raphe runs along the ventral margin. Fibulae are somewhat irregularly spaced and number 8–11 in 10 μm.

SEM observations

In SEM, a prominent hyaline area runs along the ventral margin the length of the valve, corresponding internally to the canal (Fig. 36). The valve ends are protracted and appear nearly subcapitate in some specimens. Externally, the raphe is discontinuous and is nearly completely on the ventral mantle (Figs 36–38). The distal raphe ends weakly deflect dorsally onto the valve face near the apices (Figs 36, 37). The proximal raphe ends terminate very closely on the ventral mantle (Fig. 38). Striae are composed of rows of small, closely-spaced areolae and extend uninterrupted onto the dorsal mantle and in some specimens may end ventrally in a single larger and more widely-spaced areola positioned within the ventral hyaline area (Fig. 37). The areolae appear to be occluded externally (Fig. 37), although this thin occlusion is absent in some specimens. The ventral mantle is unornamented (Figs 37, 38). Internally, the canal raphe system is subtended by fibulae forming an open canal without portulae (Fig. 39).

Phylogenetic analysis

The result of the ML estimation, inferred from the chloroplast marker *rbc*L, is shown in Figure 40. Both the ML and Bayesian estimation returned identical topologies with log likelihood values of –9632.53 and –9059.98, respectively. Both analyses returned a tree with *C. banzuensis* in a weakly supported (23/72, ML bootstrap (BS)/Bayesian posterior probability (BPP), as a percentage) position sister to *Nitzschia longissima* (Brébisson in Kützing) Grunow. These two taxa are part of a weakly supported (14/72) larger clade containing a monophyletic *Cylindrotheca* clade.

The results of the ML and Bayesian estimation inferred from the nuclear marker SSU provided no further resolution as to the position of *C. banzuensis* within the Bacillariales. Both estimations returned trees with low support throughout, with the ML tree placing *C. banzuensis* sister to a clade containing taxa from the genera *Cylindrotheca, Nitzschia, Denticula, Fragilariopsis*, and *Pseudo-nitzschia* (BS 23) (Supplemental Fig. S1), and a largely unresolved Bayesian tree placing *C. banzuensis* sister to the genus *Cylindrotheca* (BPP 51) (Supplemental Fig. S2). The better supported *rbc*L tree is presented here (Fig. 40).

DISCUSSION

Morphologically, the co-occurring marine species *C. banzuensis* and *C. szulczewskii*, although differing in striae and fibulae counts and the position of the distal raphe ends, show more similarity to each other than to the remaining described *Cymbellonitzschia* species. Both *C. banzuensis* and *C. szulczewskii* have a similar narrow valve shape with protracted acute ends and exhibit the canal raphe along the ventral mantle in all valves observed. In SEM, both have striae composed of small externally-occluded areolae, a distinct external hyaline area running the length of the ventral margin, and a raphe fully on the ventral mantle with dorsally curved distal ends. It is difficult to make detailed comparisons between these species and the only other described marine taxon, *C. hossamedinii*, as only a single line drawing of this species exists (pl. 2, fig. 9 in Salah 1955). The specimen described by Salah (1955) is relatively broad compared to *C. banzuensis* and *C. szulczewskii* (Salah 1955 gives length 20–25 μm and breadth of 4–5 μm for the few specimens observed). However, *C. hossamedinii* does exhibit protracted, acutely-rounded valve ends similar to the currently investigated species.

When the two marine species are compared to the type of the name of the genus, *C. minima*, which has been reported

Table 1. Amplification and sequencing primers for the chloroplast marker *rbc*L and the nuclear marker small subunit rDNA (SSU) used in this study

Primer name	Primer sequence†	Reference
rbcL66+‡	TTA AGG AGA AAT AAA TGT CTC AAT CTG	Alverson *et al.* 2007
rbcL404+	GCT TTA CGT TTA GAA GAT ATG	Ruck & Theriot 2011
rbcL1255–	TTG GTG CAT TTG ACC ACA GT	Alverson *et al.* 2007
dp7–§	AAA SHD CCT TGT GTW AGT YTC	Daugbjerg & Andersen 1997
SSU1‡	AAC CTG GTT GAT CCT GCC AGT	Medlin *et al.* 1988
SSU11+	TGA TCC TGC CAG TAG TCA TAC GCT	Alverson *et al.* 2007
SSU850+	GGG ACA GTT GGG GGT ATT CGT A	Ruck & Theriot 2011
SSU870–	TAC GAA TAC CCC CAA CTG TCC C	Ruck & Theriot 2011
ITS1DR§	CCT TGT TAC GAC TTC ACC TTC C	Edgar & Theriot 2004

†All primer sequence is presented in the 5′ to 3′ direction.
‡Forward amplification primer used in PCR reaction.
§Reverse amplification primer used in PCR reaction.

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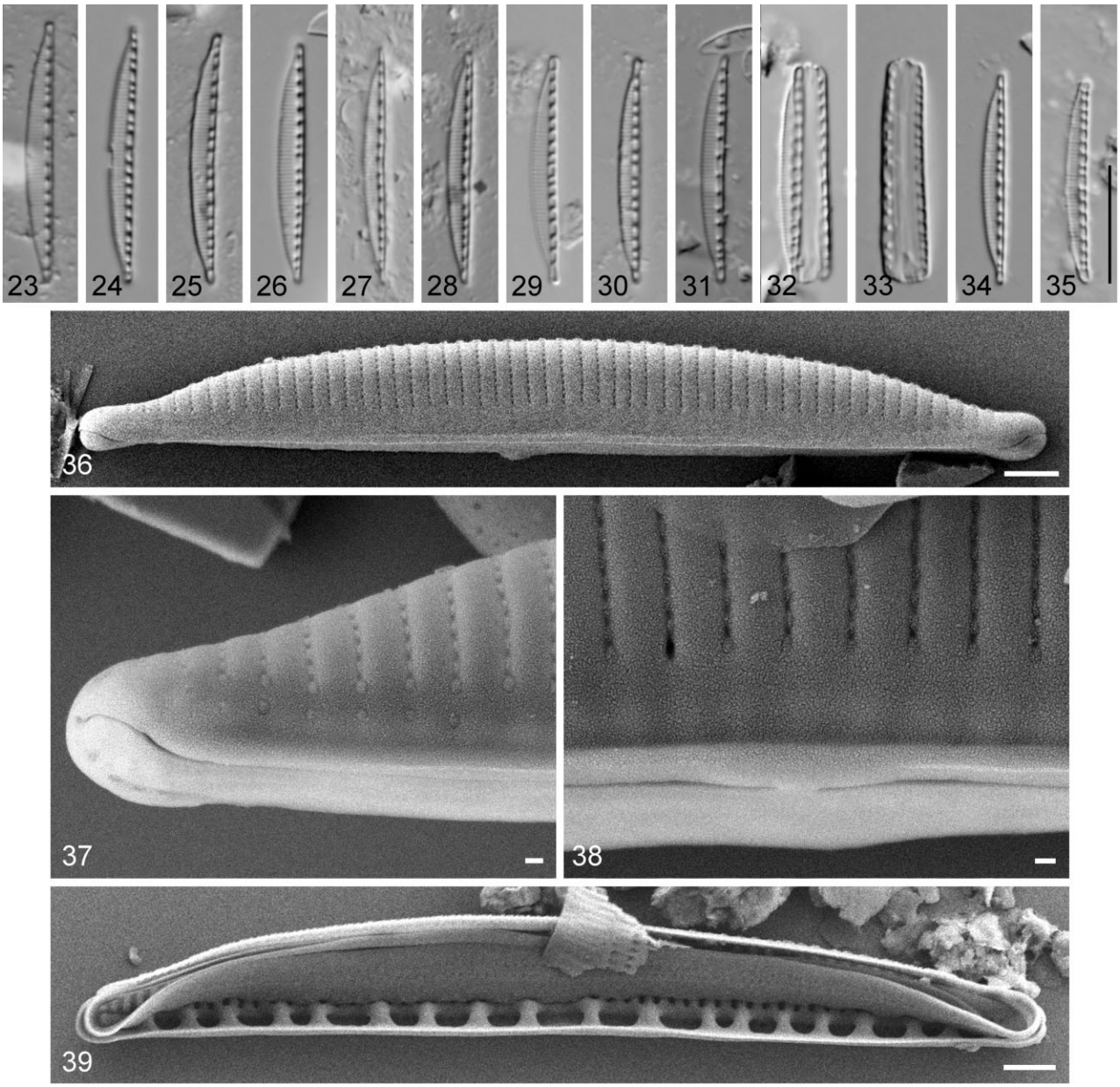

Figs 23–39. *C. szulczewskii*. 23–35. LM of collection material. Single valves (23–31, 34, 35) and girdle view whole frustules (32, 33) showing observed size range. 36–39. SEM of collection material. 36. External whole valve view. 37. Detail of external valve end showing dorsally curved distal raphe end and occluded striae. 38. Detail of ventral valve center showing closely spaced proximal raphe ends. 39. Interior whole valve view. Scale bars: 10 μm in Figures 23–35, 1 μm in Figures 36, 39, 100 nm in Figures 37, 38.

only from east African rift lakes (Hustedt 1924; Vyverman & Cocquyt 1993; Cocquyt & Jewson 1994), there are several apparent differences. The most obvious, even in the LM, is the position of the canal raphe with respect to overall valve symmetry. A dorsal position of the raphe, often observed in *C. minima*, has not been observed in collected materials of *C. banzuensis* ($n = 35$) and *C. szulczewskii* ($n = 30$), or in cleaned culture material of *C. banzuensis* ($n > 500$). This observation indicates consistent protoplast movement during the cell cycle producing only *cis* type valve orientations (Mann & Stickle 1988). This is compared to *C. minima* that must exhibit *trans* frustule symmetry to produce the nearly equal number of observed raphe orientations (Cocquyt & Jewson 1994). Aside from differences in valve morphology, *cis* or *trans* orientations are indicators of very different ontologies with regard to nuclear movement during mitosis (Mann & Stickle 1988). In addition to the position of the raphe in relation to valve symmetry, in *C. minima* the raphe is either on the valve face (when dorsally positioned) or at the valve margin (when ventrally positioned) and in either position the distal ends hook ventrally. In contrast, *C. banzuensis* and *C. szulczewskii* have the raphe positioned on the ventral mantle and the distal ends hook dorsally onto the valve face.

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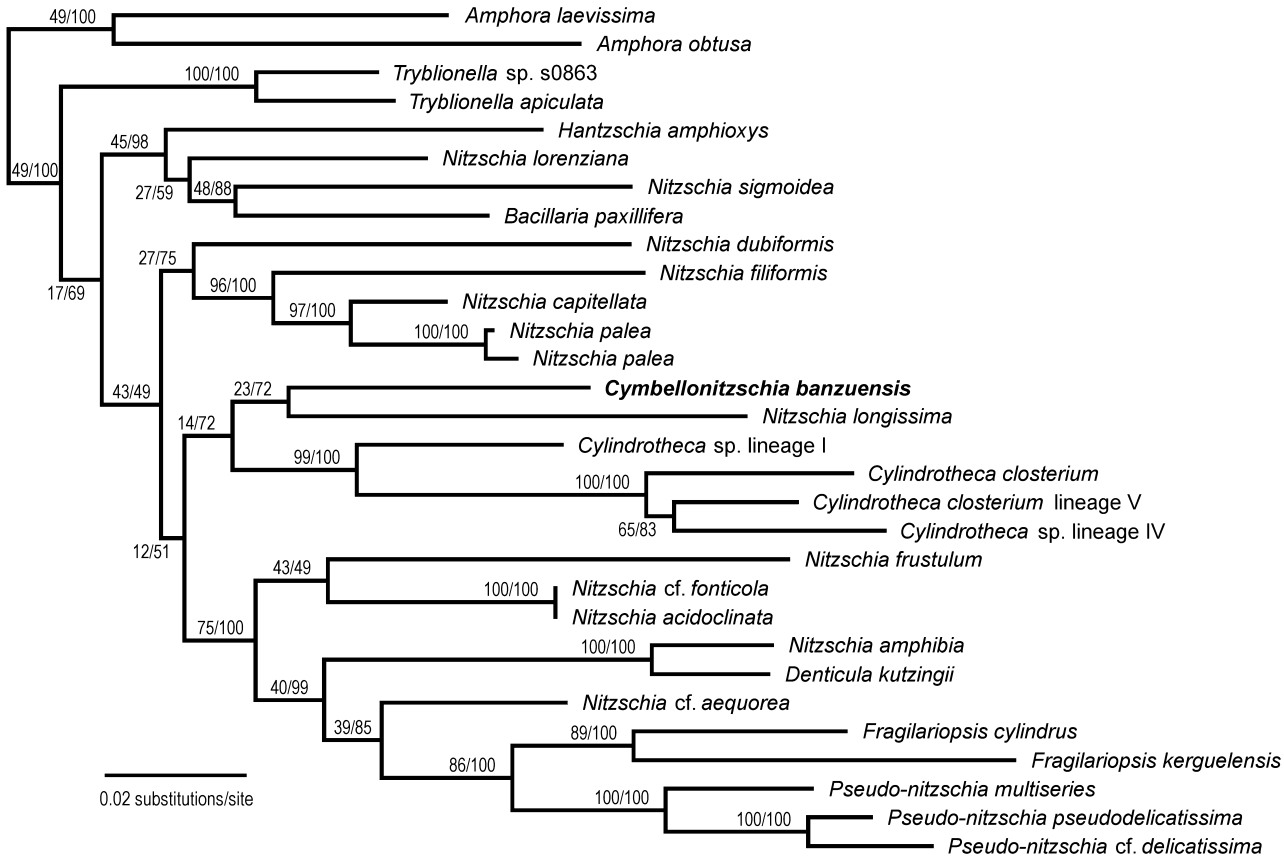

Fig. 40. Maximum likelihood phylogram inferred from the chloroplast encoded marker *rbc*L. Node support is given as maximum likelihood bootstrap values (500 bootstrap replicates)/ Bayesian posterior probability (as a percent).

Several areolae features are also quite different between *C. minima* and the marine species investigated here. In *C. minima* the areolae are medially occluded and although interrupted by a marginal ridge, are present on both the dorsal and ventral margins (figs 3–13 in Cocquyt & Jewson 1994). In the marine taxa, the areolae are externally occluded and the striae are uninterrupted onto the dorsal margin and absent on the ventral margin (Figs 17–21, 36–38).

For most of these features *C. banzuensis* and *C. szulczewskii* appear more similar to *C. diluviana*. All three taxa have the raphe predominantly on the ventral side, striae composed of small externally occluded areolae and lack striae on the ventral margin. Although it has been noted that *C. diluviana* exhibits the raphe along the dorsal valve margin in approximately 3% of observed valves (Jewson & Lowry 1993), this orientation is expressed in the majority of the time in *C. minima*, with 58% of observed valves with the raphe along the dorsal margin (Cocquyt & Jewson 1994). It is also worth noting that the dorsal raphe orientation observed in *C. diluviana* was only found in cells at the smallest end of their size diminution series, and may correspond to curvature of the ventral margin, not the movement of the raphe to the dorsal side during cell development (Jewson & Lowry 1993). This is contrasted to *C. minima*, which exhibits a dorsal raphe at a range of cell sizes, and this movement of the raphe is accompanied by different internal fibulae structures not seen in *C. diluviana* (longitudinal ridges connecting fibulae when positioned on the dorsal side) (Cocquyt & Jewson 1994).

As with *C. minima*, the raphe of *C. diluviana* runs along the valve margin and the distal ends always hook ventrally onto the ventral mantle (Round *et al.* 1990), features that are in contrast with the taxa examined here. Additionally, it appears that the striae in *C. diluviana* are restricted to the valve face and do not continue onto the dorsal margin (Round *et al.* 1990; Jewson *et al.* 2006), although, as with the ventral margin, small irregular pores may be present.

Of the four species from the genus *Cymbellonitzschia* that have now been investigated with the SEM, clear morphological differences exist between them. It would appear that *C. banzuensis* and *C. szulczewskii* are morphologically most similar to *C. diluviana*. With that said, the differences between these taxa are not trivial, with *C. diluviana* having a raphe with distal ends hooking ventrally onto the margin and the marine species having raphe ends hooking dorsally onto the valve face. As with the *cis* or *trans* frustule arrangements, the direction of the distal raphe ends have a deeper ontological basis with ends hooked towards the secondary side of the valve in most raphid diatoms (Round *et al.* 1990). Along with these morphological differences is a clear divide in ecology, with *C. diluviana* widely reported from oligotrophic and eutrophic freshwaters (Sovereign 1958; Stoermer 1964; Stoermer & Kreis 1978; Jewson & Lowry 1993) and *C. banzuensis* and *C. szulczewskii* reported only from coastal

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marine habitats (salinity 33 ppt in this study; Witkowski *et al*. 2000). This difference in early valve formation along with the extreme difference in ecology raises the possibility that, although sharing some morphological features, they are more distantly related.

The larger issue seems to be whether any of these taxa are closely related to the type of the name of the genus, *C. minima*. Aside from the predominant dorsal position of the raphe, which has previously been cited as evidence for the non-monophyly of the genus (Ross 1983), the striae and areolae features mentioned above clearly separate *C. minima* from all of the other species investigated. The problems with using symmetry as the primary character for a genus has been repeatedly demonstrated (Kociolek & Stoermer 1988; Cox & Williams 2006; Stepanek & Kociolek 2014), and the distinct symmetry of *Cymbellonitzschia* may end up having more to do with a shared epipsammic growth form than with a shared evolutionary history. The 'cymbelloid' symmetry allows both raphe systems to be in contact with the substrate allowing for much greater adhesion in the often abrasive epipsammic environment (Jewson *et al*. 2006). A further example of this potential convergence would be the genus *Catenula* Mereschkowsky, which also grows in short chains attached to sand grains and has a very similar valve outline and raphe position (Round *et al*. 1990). As with *C. diluviana*, the ecology of *C. minima*, known only from African rift lakes, differs greatly from the coastal marine species presented here. Given the limited known taxonomic diversity of this group (seven described species), the ecological diversity across these species is astounding. With the morphological differences discussed above, this difference in ecology raises additional uncertainty concerning the monophyly of the genus. If the genus is a natural group, the great ecological breadth between species would add to growing evidence (Alverson *et al*. 2007, 2011; Potapova 2011) that diatom lineages move between freshwater and marine habitats at a much greater frequency than previous assumed (Round & Sims 1980; Mann 1999).

Aside from confirming its position within the Bacillariales, the preliminary phylogeny, as it currently stands, does little to resolve the relationships between the genera within the Bacillariales. The position of *C. banzuensis* as sister to *N. longissima* (Nitzschiella Section of *Nitzschia sensu* Grunow 1862) within a larger clade containing *Cylindrotheca* are weakly supported in the *rbc*L tree (23/72 and 14/72, respectively) and only supported in the Bayesian tree inferred from SSU (BPP 51), and aside from a shared marine ecology there is little similarity between *C. banzuensis* and the rest of the clade. In addition, *C. banzuensis* has more shared morphological features with the Lanceolatae Section of *Nitzschia* than the Nitzschiella Section to which it is sister, with both groups having simple externally occluded areolae but subsection Nitzschiella defined by long produced apices (e.g. *N. reversa* Smith) (Grunow 1862). The generally poor backbone support within this phylogeny and previous investigations of the Bacillariales (Rimet *et al*. 2011) may, in large part, be a reflection of the small taxon sampling within the group. Although 27 taxa from nine genera were included here, when compared to the over 3500 currently described species (Fourtanier & Kociolek 2011) from 15 genera within the Bacillariales (Round *et al*. 1990), the limitations of the available dataset are apparent. Furthermore, given the morphological differences between *Cymbellonitzschia* species, any investigation into the systematic position and monophyly of the genus must include *C. minima* and *C. diluviana*.

What is apparent, if the tree presented here and by Rimet *et al*. (2011) are taken at face value, is that the Bacillariales is in need of systematic revision. Furthermore, this revision is likely to bring significant changes to the classification system of this large and diverse group. Although the morphological data presented here continue to call into question the monophyly of *Cymbellonitzschia*, it seems unwise to split members of this genus into two or more genera without further evidence. Doing so without a clearer understanding of the relationships within the genus and, more importantly, the larger relationships within the rest of the Bacillariales, will likely necessitate further taxonomic changes when a systematic revision of the Bacillariales is conducted.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Maximum likelihood phylogram inferred from the nuclear encoded marker SSU.
Fig. S2. Bayesian estimation phylogram inferred from the nuclear encoded marker SSU.
Table S1. List of taxa included in the phylogenetic analysis, including culture ID and GenBank accession numbers.

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