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Morphological and molecular phylogenetic studies on *Fistulifera saprophila*

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Accurate taxonomic identification provides the foundation for a number of diatom applications, such as the ecological monitoring of waters and the reconstruction of past environments. Despite significant recent developments in diatom taxonomy and phylogenetics, to date, only a few taxa have been studied extensively using a wide range of techniques. In this paper, data gained from intensive research on the morphology of live and cleaned diatom cells of two *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot strains isolated from the Gulf of Gdańsk are discussed. This study suggests that because specimens of *F. saprophila* have very delicate frustules prone to dissolution, the species has not been fully investigated and, therefore, a revised description is presented. Data on live cells and colony morphology, as well as interesting findings on the discrepancies between measurements of wet-mounted, dried and Naphrax-mounted diatom cells are also shown. The geographical distribution and ecological tolerance of *F. saprophila* is probably much wider than previously reported, but needs further investigation. The results of molecular phylogenetic analyses confirm the clear separation of *Fistulifera* Lange-Bertalot from *Navicula* Bory, also showing that its sister genera are *Craticula* Grunow, *Eolimna* Lange-Bertalot and *Stauroneis* Ehrenberg. Surprisingly, genera considered to have similar morphological characteristics, such as *Mayamaea* Lange-Bertalot and *Sellaphora* Merechowsky fall on a separate phylogenetic branch.

**Keywords:** Fistulifera, diatoms, morphology, phylogenetics, Baltic Sea, taxon identification

**Introduction**

*Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot was first described as *Navicula saprophila* Lange-Bertalot & Bonik, and was frequently included in standard floras by Krammer & Lange-Bertalot (1985, 1986) and Lange-Bertalot (2001). The species has been recorded from various localities in Europe, Japan and the USA (Gevrey et al. 2004, Rimet et al. 2005, Szabó et al. 2005, Vouilloud et al. 2005, Della Bella et al. 2007, Michelutti et al. 2007, Stancheva et al. 2007, Ishihara 2009, Pilny 2009, Torrisi et al. 2010). According to Lange-Bertalot (2001), *Fistulifera* species commonly occur in highly eutrophic, meso- to polysaprobic waters, as reported by Gevrey et al. (2004), Szabó et al. (2005), Michelutti et al. (2007) and Stancheva et al. (2007), although brackish waters have also been suggested as potential habitats (Della Bella et al. 2007).

The genus *Fistulifera* was erected by Lange-Bertalot (1997) for three small naviculoid species: *Navicula saprophila* as the generitype, *N. pelliculosa* (Brébisson ex Kützing) Hilse and *N. iranensis* Hustedt. *Fistulifera saprophila* is not a particularly problematic species, although its description has been modified several times (Lange-Bertalot & Bonik 1976, Krammer & Lange-Bertalot 1985, Lange-Bertalot 1997, 2001). The main difficulties with the genus are that its species are very small and delicate, require careful preparation, and must be examined using high-powered microscopy. The frustules are weakly silici- fied, thus the valve outlines are rather indistinct and almost no ornamentation is visible in light microscopy. In permanent slides only the raphe can be clearly distinguished (Lange-Bertalot 1997). The study of cultured strains of *F. saprophila* using confocal laser scanning microscopy (CLSM) and high-resolution scanning electron microscopy (SEM) has allowed us to document, for the first time, their live structures and to obtain improved and better SEM images of the frustule components.

Before molecular phylogenetic investigations started at the end of the 1980s, studies into the phylogeny and taxonomy of diatoms were based almost exclusively on the morphological features of the silica frustules (Beszteri et al. 2001). Currently, molecular analyses have become a standard approach in diatom systematics, with the nuclear-encoded small subunit (18S) rRNA gene sequence being one of the most frequently used genetic markers; this has proved useful in reconstructing diatom evolution at higher taxonomical levels (Alverson 2008). To explore more closely related taxa, the use of faster evolving genes, e.g., nuclear-encoded large subunit (LSU or 28S; D1–D3 regions) and internal transcribed spacer (ITS) rDNA or rcbL (gene encoding the large subunit of
ribose-1,5-bisphosphate carboxylase oxygenase), is more appropriate (Bruder & Medlin 2007, Alverson 2008, Medlin & Kooistra 2010). In this paper, in addition to the results of morphological studies, a comparative phylogenetic survey of *F. saprophila* is presented, and some of the uncertainties in the species delineation and distribution raised by Lange-Bertalot (2001) are discussed.

**Materials and methods**

Two strains of *F. saprophila*, BA-55 and BA-56, were isolated from inshore brackish water (salinity: 7–8 psu) in the Sopot area of the Gulf of Gdański, Baltic Sea, in April 2003 (Pniewski et al. 2010), and maintained in the Culture Collection of Baltic Algae (CCBA) at the Institute of Oceanography, University of Gdańsk, Poland (http://ccba.ug.edu.pl/pages/en/home.php; Latala et al. 2006). In order to study valve morphology in detail, both strains of *F. saprophila* were cultured at 8 psu and 17.5°C in f/2 medium (Guillard 1975) under continuous light at an irradiance of 40 μmol photons m⁻² s⁻¹ for four weeks.

**Microscopy examination**

Live specimens were examined and documented in water mounts using four different light microscopes: a Zeiss Axio Scope, a Leica DFC400, a Nikon 80i and a CLSM Leica TCS/SP1. Samples for light (LM), scanning (SEM) and transmission electron microscope (TEM) analyses were cleaned with nitric acid (Round et al. 1990) to remove the organic matter and rinsed several times with distilled water. The best results were achieved when the material was cleaned with 50% nitric acid, gently warmed on a hot plate at ca. 90°C for 30 min and washed at least four times with distilled water using a vortex mixer at 2000 rpm before and after oxidation. Permanent slides were mounted in Naphrax® and examined under LM using a Nikon 80i with Nomarski contrast optics. The detailed morphology of the strains was examined with SEM (Philips XL 30; Natural History Museum (NHM), London, UK), VP SEM (Carl Zeiss Ultra Confocal Laser Scanning Microscopy Plus; Natural History Museum) and TEM (Hitachi H-7100; Natural History Museum). Part of the cleaned suspension was pipetted onto formvar-coated grids and air-dried. Treated samples and permanent slides are stored in the CCBA, Poland. SEM stubs and TEM grids were deposited in the Natural History Museum, London, UK.

In this paper, in addition to the results of morphological studies, a comparative phylogenetic survey of *F. saprophila* is presented, and some of the uncertainties in the species delineation and distribution raised by Lange-Bertalot (2001) are discussed.

Morphological features and biometric data were gathered from 238 and 298 frustules from *F. saprophila* strains BA-55 and BA-56, respectively. In addition, further measurements were made in LM using a Nikon 80i to test for the influence of the mounts, i.e., water and Naphrax, on the frustule morphometrics. In this case, the length and width of 31 cells from each treatment: (1) live cells in water, (2) the same cells dried at room temperature, and (3) the same dried cells mounted in Naphrax, were measured at ×400 and ×1000. The differences in morphometric data were examined by a Kruskal–Wallis test using Statistica v.10 (StatSoft, Inc. 2011).

In addition, to compare the fistula in the genus *Proschkinia* N.I. Karajeva, some selected species were examined using VP SEM: *P. complanatoides* (Hustedt ex Simonsen) D.G. Mann (type material from Hustedt collection ASI465, and Newhaven, Kent from the NHM collection, London, UK) and *P. hyalosirella* (Hustedt ex Simonsen) D.G. Mann (material from Porlock Weir, Devon from the NHM collection, London, UK).

**Phylogenetic analyses**

Phylogenetic analyses were carried out using previously published diatom SSU rDNA sequences (e.g., Medlin et al. 1996, Beszteri et al. 2001, Sinninghe-Damsté et al. 2004, Rimet et al. 2011) and the sequences generated by this study for the *F. saprophila* strains BA-55 (1749 base pairs (bp), GenBank accession number HM805032) and BA-56 (1761 bp, GenBank accession number HM805033) (Pniewski et al. 2010). Forty-two SSU rDNA naviculoid diatom sequences were retrieved from GenBank and aligned using BioEdit v. 7.0.5.2 (Hall 1999). The alignment was then corrected manually using the Align Manual Sequence Alignment Editor (Hepperle 2004). The final data set was 1854 positions long, of which 395 were parsimony informative. *Ellerbeckia sol* (Ehrenberg) Crawford & Sims was selected to root the data set.

Two independent types of analysis, Bayesian inference (BI) and maximum parsimony (MP), were used to construct the 18S rDNA phylogenies. Bayesian analysis was carried out using Mr Bayes 3.1 software (Ronquist & Huelsenbeck 2003) and was run with the GTR + G + I
model (nuc model = 4by4, nst = 6, rates = invgamma) for 4,500,000 Markov chain Monte Carlo (MCMC) generations, saving every 1000th tree. The Bayesian search consisted of four parallel runs with four chains each (three heated, one cold), and the convergence of the runs was checked using the Tracer MCMC Trace Analysis Tool v. 1.5.0 (Rambaut & Drummond 2007). All chains mixed well, traversing their posterior space and quickly reached their stationary distribution. For all four runs combined, the mean likelihood was $-\ln L = 13430.91$ (SE = 0.27) and the effective sample size (ESS) reached 1765.12. The 50% majority rule consensus tree was constructed using a burn-in of 10%. To measure tree strength, posterior probabilities (PPs) were calculated. MP analysis was implemented with PAUP* V4.0b10 (Swofford 2001). Gaps were treated as missing data and characters were treated as multistate and unordered. MP trees were obtained using the tree bisection–reconnection (TBR) branch-swapping algorithm and an heuristic search with 10 random taxon additions. The stability of the recovered clades was evaluated by bootstrapping (BS) using 1000 replicates. The trees were displayed using FigTree v. 1.3.1 (Rambaut 2009).

To analyse the sequences belonging to *F. saprophila* AJ867025, HM805032, HM805033, and *F. pelliculosa* AJ544657, AY485454, EU260468, they were aligned with BioEdit v. 7.0.5.2 (Hall 1999) and manually corrected in the Align Manual Sequence Alignment Editor (Hepperle 2004) to construct a separate data set. The resulting matrix was used to calculate the number of base pair differences between sequences and their genetic variability using uncorrected pair-wise ($p$) distances with pair-wise deletion of gaps option in MEGA 4 (Tamura et al. 2007).

**Results**

**Morphological studies**

The growth of *F. saprophila* in Petri dishes was clearly visible to the naked eye after 14 days (Fig. 1). In LM, the cells were grouped into irregular colonies or long chains (Figs 2–3) and were surrounded by thick mucilage (Fig. 4, arrow). Through LM and CLSM, each cell showed two parietal chloroplasts lying along each side of the girdle or curved around the apices (Figs 4–6); no pyrenoid was visible in the examined specimens of *F. saprophila*.

Figs 1–4. Different colonies of live *Fistulifera saprophila*. **Fig. 1.** Macroscopic brownish colonies in a Petri dish. **Figs 2–3.** Some colonies in water mount, LM. **Fig. 4.** Thick mucilage (arrow) surrounding a four-celled colony, LM. Scale bars = 1 cm (Fig. 1); 10 μm (Figs 2–4).
Our initial examination of the material using SEM showed that many valves were strongly dissolved (e.g., only the raphe sternum area was present) and/or covered with a thin film or unidentified particles (Figs 7–9). The poor image quality encouraged us to develop a technique that would produce satisfactory images of $Fistulifera$ valves. Even careful boiling with hydrogen peroxide following the method of Battarbee (1986) resulted in damaged frustules, with the fragile valve margins frequently being lost (Fig. 7). The best results of undissolved and clean valves (Figs 10–11) were obtained when the material was cleaned following the protocol described above (see Materials and methods).

Use of the VP SEM, which does not require any oxidation of the diatom material, allowed the examination of live cells and showed that the cells of $F. saprophila$ are covered by a thick organic coating (Figs 12–15). In addition, the fimbriate valve margins, usually described as being part of the...
valve face, actually constitute the valve mantle. Individual copulae were only visible when the material was oxidized and examined in SEM and TEM (Figs 10–11). Girdle bands are usually narrow and lack pores (Figs 14–15). The isolated central pore or fistula has a linear or slightly curved, slit-like external opening and a raised, covered internal
Figs 17–25. Details of the fistula in *Fistulifera* and *Proschkinia*, SEM external (Figs 17, 22, 24) and internal (Figs 18–19, 23, 25) views, and TEM (Figs 20–21). Fig. 17. Very short and straight fistula between the central raphe endings of *F. saprophila*. Fig. 18. Small and rounded internal opening of the fistula in *F. saprophila*. Fig. 19. Internal opening of fistula covered with a raised wart-like structure in *F. saprophila*. Figs 20–21. Structure of the fistula in *F. saprophila*. Fig. 22. Position of the fistula on one side of the valve just besides the central raphe endings in *P. hyalosirella*. Fig. 23. Internal opening covered with a raised wart-like structure in *P. hyalosirella*. Fig. 24. Long and slightly wavy fistula on one side of the central valve region in *P. complanatoides*. Fig. 25. Transapically elongated covered opening of the fistula in line with the short central stria in *P. complanatoides*. Scale bars = 1 μm (Figs 17–19, 22–25); 200 nm (Figs 20–21).

wart-like structure (Figs 16–21). It resembles the internally occluded, isolated pore in *Proschkinia* Karayeva, although there is currently no other morphological or genetic evidence that these genera are closely related. However, no comparative molecular phylogenetic analyses including both genera have been conducted. For comparison,
Table 1. Comparison of the main valve features and biometric data (size range and mean) of *Fistulifera saprophila* from the literature and this study. Strains BA-55 and BA-56 maintained in culture at a salinity of 8 psu.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>Live material</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valve length (μm)</td>
<td>Live material: n.d.</td>
<td>8.2–9.3 (8.8)</td>
<td>5.7–7.2 (6.3)</td>
</tr>
<tr>
<td></td>
<td>Oxidized material: 4.5–7.6 (3.8)</td>
<td>4.9–7.6 (6.1)</td>
<td>4.7–6.8 (5.8)</td>
</tr>
<tr>
<td></td>
<td>Valve width (μm)</td>
<td>2.1–4.1 (3.1)</td>
<td>2.6–5.0 (3.7)</td>
</tr>
<tr>
<td></td>
<td>Striae in 10 μm</td>
<td>48–81</td>
<td>55–95</td>
</tr>
<tr>
<td>Striae</td>
<td>Radiate, usually straight not sinuous, at an angle of 75–80°</td>
<td>Radiate, straight to slightly curved, at an angle of 43–90°</td>
<td></td>
</tr>
<tr>
<td>Areolae in 10 μm</td>
<td>80–110</td>
<td>80–120</td>
<td></td>
</tr>
<tr>
<td>Valve shape</td>
<td>Elliptical with bluntly rounded apices</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Appearance in LM</td>
<td>Live valve hyaline and structureless, except for the median raphe sternum</td>
<td>Valve hyaline and structureless, except for the median raphe sternum</td>
<td></td>
</tr>
<tr>
<td>Ecology</td>
<td>Highly eutrophic waters, of higher saprobic level up to polysaprobic, resistant towards organic pollution, disappearing with strong industrial pollution</td>
<td>Eutrophic waters of mesosaprobic level, salinity 6–8 psu but in cultures was grown successfully between 0 and 40 psu</td>
<td></td>
</tr>
<tr>
<td>Distribution</td>
<td>Cosmopolitan, very abundant in Europe, can be locally dominant</td>
<td>Gulf of Gdańsk, southern Baltic Sea</td>
<td></td>
</tr>
</tbody>
</table>

Note: n.d.: no data.

the position and structure of the fistula are presented for *P. hyalosirella* (Figs 22–23) and *P. complanatoides* (Figs 24–25).

Some variation in the valve dimensions, depending on the pretreatment of the material, was noted (Table 1). The observed difference in the length of live cells between studied strains was statistically significant (Kruskal–Wallis test, *p* < 0.05). Cells from strain BA-55 were longer, and therefore appeared narrower than cells of strain BA-56, i.e., linear-elliptical rather than elliptical to almost circular. When the cells are cleaned to remove the organic content, the valves flatten on the surface (Figs 10–11) and appear wider than the live cells (Figs 12–13). When the material was treated following the protocol for the VP SEM, the cells did not break open and the valve widths were about half that of measurements made for cleaned valves following standard treatment.

On average, morphometric data for live cells in a water mount were larger than those of cleaned cells, suggesting a pretreatment or mountant effect (e.g., water or Naphrax) on frustule size. To investigate this phenomenon, length and width were measured at ×400 and ×1000 on live cells in the water mount, the same cells after drying and those dried cells mounted in Naphrax. For measurements at the same magnification there was a statistical difference between live and dried cells (except for width under ×400, *p* = 0.065), and live and Naphrax-mounted cells (Kruskal–Wallis test, *p* < 0.05) (Fig. 26), suggesting that the differences in the refractive index of the mountant affect the valve metrics.

**Phylogenetic analyses**

The topologies of the Bayesian and parsimony trees were similar, revealing the same general branching order, but with some variation within particular clades. The comparison of support values, i.e., BS and PPs, showed that the latter was consistently higher.

Neither a monophyletic origin of the order Naviculales Bessey nor monophyly of the Naviculaceae Kützing were resolved in the phylogenetic analyses. However, they showed *Fistulifera* as distinct from *Navicula* Bory (Fig. 27), supporting its morphologically based separation.

The first major clade (I) contained a group of *Navicula* species, with taxa from the genus *Haslea* Simon-sen and the family Pleurosigmataceae D.G. Mann (68% BS, 100% PPs). The first branch (subclade 2a) of the second major clade (II) comprised species from the orders Achnanthales Silva, Cymbellales D.G. Mann and Naviculales, after which there was a divergence into two subclades: 2b and 2c (89% BS, 100% PPs). Sub-clade 2b (90% BS, 100% PPs) contained species of *Sellaphora, Pinnularia* Ehrenberg and *Mayamaea* Lange-Bertalot, along with *Eolimna minima* (Grunow) Lange-Bertalot and *Fistulifera/Navicula pelliculosa* (Brébisson) Hilse sequences (AJ544657 and EU260468). Subclade
Fig. 26. Variation in the valve length (a) and width (b) of *Fistulifera saprophila* strain BA-56 in relation to water, dry and Naphrax mount observed at \( \times 400 \) and \( \times 1000 \). The boxes represent the 25–75% quartiles, the median is shown with a horizontal line inside the box, and minimum and maximum values are shown with short horizontal lines (whiskers).

2c (52% BS, 93% PPs) diverged into two with the first branch containing the well-supported *Fistulifera* lineage (100% BS, 100% PPs; including *F. saprophila* AJ867025, HM805032, HM805033 and *F. pelliculosa* AY485454) in an unresolved polytomy with *Stauroneis* Ehrenberg and *Craticula* Grunow//E. subminuscula* (Manguin) Moser,
Fig. 27. Fifty percent majority rule Bayesian tree showing the phylogenetic relationships of the sequenced taxa. Posterior probabilities (PPs) and bootstrap values (BS) >50% recovered in both BI and MP analyses are shown at the nodes (PPs/BS). -: support values <50%.

Pair-wise comparison of *F. saprophila* sequences showed differences of up to 8 bp (for genetic distance, \( p \) varied between 0.001 and 0.005 difference per site; Tables 2–3). Two sequences, HM805033 (BA-56) and AJ867025 differed by only 1 bp (1/1542 sites = 0.1%; \( p = 0.001 \)). Strain BA-55 (HM805032) differed from BA-56 (HM805033) by 8 bp (8/1745 sites = 0.5%; \( p = 0.005 \)) and from AJ867025 by 6 bp (6/1542 sites = 0.4%; \( p = 0.004 \)). There was a 5-bp difference between AY485454 and
Table 2. Estimates of uncorrected $p$-distances between sequences of tested Fistulifera taxa.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>$p$-distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F. saprophila$ HM805032</td>
<td>0.005</td>
</tr>
<tr>
<td>$F. saprophila$ HM805033</td>
<td>0.0004</td>
</tr>
<tr>
<td>$F. saprophila$ AJ867025</td>
<td>0.003</td>
</tr>
<tr>
<td>$F. pelliculosa$ AY485454</td>
<td>0.0060</td>
</tr>
<tr>
<td>$N. pelliculosa$ EU260468</td>
<td>0.0058</td>
</tr>
<tr>
<td>$F. pelliculosa$ AJ544657</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 3. Estimates of base pair differences between sequences of tested Fistulifera taxa.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Base pair differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F. saprophila$ HM805032</td>
<td>8</td>
</tr>
<tr>
<td>$F. saprophila$ HM805033</td>
<td>6</td>
</tr>
<tr>
<td>$F. saprophila$ AJ867025</td>
<td>5</td>
</tr>
<tr>
<td>$F. pelliculosa$ AY485454</td>
<td>5</td>
</tr>
<tr>
<td>$N. pelliculosa$ EU260468</td>
<td>92</td>
</tr>
<tr>
<td>$N. pelliculosa$ EU260468</td>
<td>101</td>
</tr>
</tbody>
</table>

HM805032, and HM805033 and AJ867025 (Table 3). The number of differences between $F. pelliculosa$ AJ544657 and $N. pelliculosa$ EU260468 was 2 bp (2/1750 sites = 0.1%; $p = 0.001$; Tables 2–3) confirming their close relationship. The comparison with $F. saprophila$ sequences revealed that they differed in up to 106 bp ($F. saprophila$ HM805033 and $F. pelliculosa$ AJ544657) (106/1750 sites = 6.1%; $p = 0.061$; Tables 2–3).

Discussion
Our initial SEM examination of hydrogen-peroxide-cleaned material confirmed previously published observations that $F. saprophila$ valves are prone to dissolution during laboratory preparation using standard oxidation techniques (e.g., Round et al. 1990) and our images were comparable with similar ones from Lange-Bertalot (1997, fig. 33; 2001, pl. 111, fig. 6). Consequently, a technique leading to more satisfactory images of Fistulifera valves was developed. The best results were achieved when the material was gently warmed with ca. 50% nitric acid on a hot plate at ca. 90°C for 30 min and washed at least four times with distilled water using vortex mixer at 2000 rpm before and after the oxidation process (as described in Material and methods).

Measurements of cleaned valves corroborated the data given by Krammer & Lange-Bertalot (1986) and Lange-Bertalot (1997) only in the case of length. The differences between valve width metrics for cleaned valves from this study and those reported previously (Krammer & Lange-Bertalot 1986, Lange-Bertalot 2001) were probably caused by the assumption that the fringe belongs to the valve face, not to the fimbriate mantle. Such an approach could not be supported unless microscopic techniques enabling the observation of unbroken cells were used, allowing investigation of frustule details. Based on these observations, it is suggested that measurement of the valve width should be the distance between opposite fringes.

On average, morphometric data of live cells mounted in water were larger than those of cleaned valves, suggesting a pretreatment or mountant effect (e.g., water or Naphrax) on frustule size. Both media have a different refractive index, 1.333 for water (Hecht 2001) and 1.73 for Naphrax, resulting in different refraction angles influencing the size of the optical image. Thus for morphological studies, it is suggested that the medium used when the measurements were taken is clearly stated. To our knowledge, this is the first time that test results comparing diatom cell measurements taken in different mediums have been presented. The size difference between the strains was smaller for cleaned material than for live specimens, but because the measurements were made at different times, they may have been at different stages in the life cycle and further studies are needed to confirm whether there is a consistent size difference between the strains. Both strains had the same stria and areola densities, as well as stria organization.

Previous phylogenetic studies included only a single sequence of Fistulifera (e.g., Behnke et al. 2004, Sinninghe-Damsté et al. 2004), but Sorhannus (2007) and Pniewski et al. (2010) used more sequences, allowing them to test for and demonstrate the monophyly of the genus. The addition of further sequences of purported Fistulifera taxa in Rimet et al. (2011) suggested that the genus was not monophyletic. As shown in this last study, $F. saprophila$
Comparison of Cox (2009), it is suspected that physiological description and micrographs reported by Ki et al. were misidentified. For example, based on the short morphological description and micrographs reported by Ki et al. (2009), it is suspected that N. pelliculosa EU260468 is probably the nominate variety of Mayamaea atomus (Kützing) Lange-Bertalot. Moreover, to our knowledge, there are no available LM or SEM illustrations of N. pelliculosa A544657 from which its identity can be confirmed. For example, based on the short morphological description and micrographs reported by Ki et al. (2009), it is suspected that N. pelliculosa EU260468 is probably the nominate variety of Mayamaea atomus (Kützing) Lange-Bertalot. Moreover, to our knowledge, there are no available LM or SEM illustrations of N. pelliculosa A544657 from which its identity can be confirmed. The strain was isolated in 1950 (Lewin 1955), 50 years before it was sequenced (Behnke et al. 2004, Thamatrakoln et al. 2006, Epp et al. 2011). Considering that the long-term maintenance of diatoms can lead to not only size reduction, but also morphological variation, unambiguous identification of this taxon was not possible.

Despite the fact that four Fistulifera sequences always grouped together in subclade 2c, no clear phylogenetic relationships were recovered. Because SSU rDNA is less variable than the other genetic markers, such as rbcL or cox1, it is less suited to revealing inter- and intraspecific variability (Evans et al. 2007). However, pairwise comparison of F. saprophila sequences showed that the genetic distance (p) varied between 0.001 and 0.005 differences per site (Tables 2–3). Furthermore, it would be interesting to verify the identity of the only F. pelliculosa (AY485454) that grouped with the F. saprophila sequences. Calculations of the genetic distance showed a close relationship between this isolate and the other F. saprophila strains (p = 0.003; Table 2), suggesting that they might be conspecific.

When the small size and the lack of distinctive LM features of these genera, Fistulifera, Eolimna or Mayamaea, are considered, it is perhaps less surprising that the molecular analyses appear to give contradictory results. Without SEM comparison of voucher specimens for GenBank sequences, it is often difficult to be certain of the identities of such diatoms, particularly when LM identifications are inevitably made by different individuals based on limited published data. Although the species identifications of submitted sequences in GenBank are usually taken on trust, they may be wrong and the taxonomic inferences based on them should therefore be regarded with caution, unless the identity of the sequenced strains can be confirmed. The phylogenetic analyses suggest also that two strains described as N. pelliculosa, i.e., AJ544657 and EU260468, were misidentified.

The ultrastructural similarity between the fistula of F. saprophila and the internally occluded isolated pore of Proschkinia species is intriguing (Cox 2012). Proschkinia currently occupies a rather isolated position in the Naviculales, with some other distinctive morphological features (Cox 2011) and although it would seem unlikely that it is closely related to Fistulifera, it would be interesting to generate 18S rDNA sequences for Proschkinia and include them in a broad phylogenetic analysis of naviculoid taxa, including Fistulifera.

Previous reports suggest that F. saprophila is a widely distributed taxon with broad ecological tolerances, but more research is needed to show both species ranges and preferences. It is recorded from the periphyton, epiphyton and epipelon, and from the high Arctic to subtropical regions in the southern hemisphere (Di Dato et al. 2005, Szabó et al. 2005, Vouilloud et al. 2005, Michelutti et al. 2007, Stancheva et al. 2007, Ishihara 2009). However, its range is possibly much wider, because its delicate frustules might be overlooked due to damage during standard laboratory treatment. For example, it has been relatively easily isolated from a number of stations in the Gulf of Gdańsk, but was not found on permanent slides from those sites.

Fistulifera saprophila is most frequently found in eutrophic, α-mesosaprobic to polysaprobic waters (Gevrey et al. 2004, Rimet et al. 2005, Michelutti et al. 2007, Stancheva et al. 2007), but some studies also suggest that it has a high salinity tolerance (Lemke et al. 2011). Adaptation to different habitats exposed to anthropogenic pollution might explain its colonization success, especially given the range of contemporary introduction vectors. It should be also stressed that the strains are easy to culture and can be maintained for a long time without apparent morphological change. This also makes them ideal for further studies on physiological and genetic diversity.

Since it is clear that our strains belong to F. saprophila but differ from published accounts, a revised and expanded description is given based on our observations.

Fistulifera saprophila (Lange-Bertalot & Bonik) Lange-Bertalot emend

Description. Cells small, elliptical to broadly elliptical, 5.7–9.3 μm long, 2.1–5.0 μm wide. Forming long chains or irregular colonies surrounded by thick mucilage. Each cell has two parietal chloroplasts, located asymmetrically along each side of the girdle, curving around the apices. Pyrenoids not visible.

Under LM, the frustules appear hyaline, without any structure other than the simple straight raphe sternum. When the cells are cleaned of organic matter, the valves flatten on the surface and appear wider than live cells; they are elliptical in shape with bluntly rounded apices or almost circular. In material treated so that cells did not break open, the valves are 4.7–7.6 μm long and 1.4–2.5 μm wide compared with (3.8) 4.5–7.6 μm long and 2–4 μm wide reported by Lange-Bertalot (2001).

Under electron microscopy, the raphe is filiform and almost straight, with simple central endings and hooked polar endings (Fig. 10). The striae are radiate, straight to
slightly curved, with an angle of 43–90° to the raphe and vary from 55 to 95 in 10 μm (48–81 in 10 μm according to Lange-Bertalot 2001). Each stria is composed of a single row of almost circular areolae (80–120 in 10 μm) closed by hymenes (Figs 20–21). At the valve margin, each stria transforms into a slit, the virgae split into 1–3 fimbriae (Fig. 11, f arrow). The isolated central pore or fistula is linear or slightly curved, with a slit-like external opening and a raised, covered internal wart-like structure. Individual numerous copulae visible only in oxidized material are narrow and lack pores. The fimbriate margins of the mantle are easily damaged and corroded during laboratory treatment, and may therefore be missing in permanent preparations, which can cause some identification problems.

**Distribution.** The species is cosmopolitan, abundant and can be dominant locally.

**Ecology.** The species is probably eurybiontic. It has been recorded from oligo- to highly eutrophic waters, with meso- to polysaprobic levels of organic pollution, but disappearing under strong industrial waste water influence (Lange-Bertalot 2001). The species is not common in good quality waters but it occurs across lower quality classes (Kelly et al. 2008). Similarly, Lecointe et al. (1999) classified it as a pollution-tolerant species, with a large distribution. The species is known from fresh and brackish waters but it grew successfully in culture over a wide salinity range (0–32 psu) for at least 6 weeks and at 40 psu for 2 weeks in our experiments.

On the basis of the phylogenetic studies, *Cricricula, Eolimna* and *Stauroneis* are sister genera to *Fistulifera*. Surprisingly, the genera that are considered morphologically similar, such as *Mayamaea* and *Sellaphora*, form a separate phylogenetic branch.

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