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PRIMARY RESEARCH PAPER

A novel, combined approach to assessing species delimitation and biogeography within the well-known desmid species *Micrasterias fimbriata* and *M. rotata* (Desmidiales, Steptophyta)

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Abstract Morphological species of freshwater microalgae often have broad geographic distribution. However, traditional species concepts have been challenged by the results of molecular phylogenetic analyses that mostly indicate higher diversity than was previously recognized by purely morphological approaches. A degree of phenotypic differentiation or different geographic distribution of species defined by molecular data remains largely unknown. In this study, we analyzed a pair of well-known and widely distributed desmid species (*Micrasterias fimbriata* and *M. rotata*) and tested for their phylogenetic and morphological homogeneity as well as their geographic distribution. Geometric morphometric and morphological attributes of cells were used in combination with genetic analysis of the *trnG*^{ucc} sequences of 30 strains isolated from a variety of European locations and obtained from culture collections. *Micrasterias rotata* proved to be phylogenetically homogenous across Europe while *M. fimbriata* turned out to be composed of two firmly delimited lineages, differing by molecular as well as by morphometric and morphological data. Published records of traditional *M. fimbriata* were also included in the classification discrimination analysis and were placed into the newly identified lineages upon comparison to the morphometric data collected from living material. Largely disparate geographic patterns were revealed within traditional *M. fimbriata*. One phylogenetic lineage is frequent in central and eastern Europe, but occurs also in the British Isles. A second lineage has been recorded in North America and in Western Europe, where its distribution is possibly limited to the west of the Rhine River. Interestingly, the morphometric analyses of the published records illustrated that the geographic differences have remained largely unchanged since the 1850s indicating a previously unknown distributional stability among microalgal species groups such as the desmids.

Keywords Microalgae distribution · Desmidiales · Geometric morphometrics · Green algae · Molecular phylogenetics · *Micrasterias* · *trnG*^{ucc}

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Introduction

Although desmids have been recognized as important indicators of water quality, particularly eutrophication and acidification (Coesel, 1982), knowledge of their ecological and geographic distribution relies on purely morphological delimitations of individual taxa (Coesel, 1996). Species concepts of green microalgae have recently been undergoing major conceptual changes in the light of increasing evidence stemming from molecular phylogenetic studies. The artificial nature of traditional species and genera has been demonstrated in various taxonomic groups (Huss et al., 1999; Mikhailyuk et al., 2008; Gontcharov & Melkonian, 2010a, b). Repeated evolution of cryptic, morphologically unrecognizable species has been suggested in green microalgae that frequently occur in freshwater phytoplankton and phytobenthos (Müller et al., 2005; Luo et al., 2006). In the Desmidiales, about 2,500 traditional, morphologically defined species were described from freshwater habitats worldwide (Gontcharov & Melkonian, 2010b). However, the large number of traditionally defined, and—in many cases—doubtful infraspecific taxa considerably obscure the taxonomy of the group (Kouwets, 2008). The genus *Micrasterias* C.Agardh ex Ralfs represents some of the most conspicuous and well-known species of the group. It is also one of the "flagship" freshwater algae constantly attracting attention of amateur scientists and nature-lovers (e.g., Le Naturaliste, 2007; Brochard, 2008). Apparent morphological variability of large and richly ornamented *Micrasterias* cells led the early (e.g., Ralfs, 1848), as well as modern phycologists, to describe about 900 species and infraspecific taxa (Guiry & Guiry, 2010). The genus has been refined by Krieger (1939), and even then about 202 taxa were recognized. Růžička (1981) synonymized many infraspecific taxa, and included 51 species and varieties of *Micrasterias* in his critical revision of the European members of the genus. However, phylogenetic reliability of individual species, and especially of their subspecific taxa, remained questionable.

Members of the genus *Micrasterias* form a single lineage within Desmidiales supported by multigene phylogenetic analyses (Gontcharov & Melkonian, 2008; Hall et al., 2008). However, several morphologically different species, traditionally classified in different genera, such as *Cosmarium ralfsii*, *Staurodesmus dickiei* and *Triploceras gracile*, were found nested within the *Micrasterias*-lineage (Gontcharov & Melkonian, 2008; Hall et al., 2008). Recent species-level studies of *Micrasterias crux-melitensis*/*M. radians* (Neustupa et al., 2010) and *Micrasterias truncata* complexes (Nemjová et al., accepted), employing combined morphological and molecular approaches have revealed that these traditional taxa mostly represent taxonomically meaningful units, but some of the varieties are apparently independent species. Several morphologically defined infraspecific taxa were shown to be artificial, and probably lack taxonomic value (e.g., *M. crux-melitensis* var. *janeira* or *M. truncata* var. *neodamensis*). However, individual species-level phylogenetic lineages were always found to be morphologically identifiable, both by careful microscopic analysis, as well as by quantitative geometric morphometric methods. Consequently, cryptic species have not yet been detected within the genus *Micrasterias* (Neustupa et al., 2010).

In this study, we concentrated on what is probably the most conspicuous *Micrasterias* species—*M. rotata*, together with its close relative *M. fimbriata*. Both of these traditional species occur mostly in the phytobenthos of peatlands. *Micrasterias rotata* has been collected on all continents, excluding Antarctica (Krieger, 1939; Tyler, 1970). On the other hand, collections of traditional *M. fimbriata* are rarer, with specimens being recorded from Europe (Růžička, 1981; Coesel & Meesters, 2007), North America (Prescott et al., 1977), and Northern Asia (Kossinskaja, 1960; Medvedeva, 2001). There is also a single report of *M. fimbriata* var. *brasiliensis* from South America (Borge, 1925; Krieger, 1939). The phylogenetic relation between *M. rotata* and *M. fimbriata* was illustrated by Neustupa & Škaloud (2007) on the basis of 18S rDNA sequence analysis. However, genetic structure and monophyly of these two conspicuous and well-known taxa remained unclear. For this study, we assembled a set of clonal strains, natural samples, and published records (the main focus for which being continental Europe) to test for the monophyly of species and their eventual further phylogenetic and morphological differentiation as well as for geographic distribution of individual taxa. In the past records (e.g., West & West, 1905), *M. fimbriata* has been considered a variety of the broadly defined *M. apiculata* (West & West, 1905). Therefore, we also included sequences of *M. apiculata* var. *apiculata* and the closely similar

M. brachyptera into the study. However, our main attention was paid to the illustration of contrasting species concepts and distribution of traditional *M. rotata* and *M. fimbriata*. Molecular analyses were based upon the group II intron sequences of the plastid gene that encodes transfer RNA-Gly ($trnG^{ucc}$). This plastid-encoded marker was found to be very efficient in species delimitation within the *Micrasterias* lineage of Desmidiales (Neustupa et al., 2010; Nemjová et al., accepted). Qualitative morphological data were obtained by a combination of light microscopy (LM) and scanning electron microscopy (SEM) of samples. Morphological differences in cell shape were quantified using geometric morphometrics to establish a morphospace which spanned the variation between specimens. This was conducted separately for each lineage so that differences between populations and individual taxa could be statistically evaluated (Neustupa et al., 2008, 2010). *Micrasterias* species, being one of the most conspicuous unicellular organisms visible in the light microscope, have been frequently reported and illustrated since the 1850s. In this study, we illustrate that these historical records from the literature may be useful for morphometric reconstruction of the geographic distribution of previously unrecognized taxa.

Materials and methods

Localities and sampling

Sampling locations were chosen to maximize the spread of sites across continental Europe. Three vast regions—Czech Republic, the French departments Landes, and Gironde in Aquitaine, and western regions of Ireland—were chosen for detailed screening. In total, over 1,000 samples from the Czech Republic were searched for *Micrasterias* (Neustupa et al., 2009; Šťastný, 2010). In addition, about 120 samples from Aquitaine and 100 samples from western Ireland were also investigated. Clonal strains were isolated from the natural populations using the single-cell isolating method. Additional strains of the investigated species available in culture collections were also obtained. In total, 30 strains of *M. fimbriata* and *M. rotata* were used in the molecular and morphometric analyses (Table 1). The strains were cultured in MES-buffered DY IV liquid medium at 20C and illuminated at 40 μmol photons m^{-2} s^{-1} from 18 W cool fluorescent tubes (Philips TLD 18W/33, Royal Philips Electronics, Amsterdam, the Netherlands), at a light:dark (L:D) regime of 12:12 h. In addition to the cultured material, natural populations were also used for morphological and morphometric studies. Sampling of natural populations concentrated on reported European records of traditional *M. fimbriata*. These sampling localities were in Denmark, on the Baltic island of Bornholm (Nordstedt, 1888; Burchardt & Kowalski, 2009), the Lake District in northern England (Brodie et al., 2007), several localities in The Netherlands (Coesel & Meesters, 2007), Walchsee bog in Tyrol, Austria (Šťastný & Lenzenweger, 2008), Estonia and Belgium (Supplementary Table 2). The available published figures of *M. fimbriata* were also used for morphological and morphometric comparisons. First, microphotographs available from public domain websites were utilized (Sieralgen in Nederland, 2003; Webber, 2006; Le Naturaliste, 2007, 2010a, b; Brochard, 2008; Photomacrography, 2008; Encyclopedia of Life, 2010; Oyadomari, 2010). Second, specimens published in desmid monographs, and in numerous floristic and taxonomic articles from the U.S.—Alaska (Croasdale, 1956), Louisiana (Förster, 1972), and other different U.S. locations (Wolle, 1892; Prescott et al., 1977); France—Auvergne (Wurtz, 1945; Kouwets, 1987); Vosges Mts. (Comère, 1901); Austria (Lenzenweger, 1981); Belgium (Gysels, 2005); Britain (Ralfs, 1848; Cooke, 1887; West & West, 1905; Brook & Johnson, 2002); Canada, Ontario (Irenee-Marie, 1938); Finland (Kallio, 1953); Germany (Mix, 1970); Poland (Raciborski, 1885); Scotland (Roy & Bisset, 1893); and finally the former Soviet Union (Kossinskaja, 1960). We were also glad to obtain samples collected by Peter F.M. Coesel from De Weerribben, and microphotographs from Koos J. Meesters from Polder Westbroek, The Netherlands. Finally, Alasdair Joyce kindly provided us with the unpublished drawings made by his late father Alan Joyce, originating from different localities in the northwest of Scotland. All the figures used in our analyses are listed in the Supplementary Table 2.

Molecular phylogenetics

For the phylogenetic analyses, the group II intron of the plastid encoded RNA-Gly transfer gene ($trnG^{ucc}$) was chosen. Shaw et al. (2005) illustrated that $trnG^{ucc}$

Table 1 The list of strains used in morphological and molecular analyses

Strain designation	Original identification	Locality	Geographic coordinates	Accession numbers
C1	*Micrasterias fimbriata*	Chvojnov wetland, Czech Republic	49 24′23.39″N 15 25′10.24″E	FR731997
C5	*Micrasterias fimbriata*	Chvojnov wetland, Czech Republic	49 24′23.39″N 15 25′10.24″E	Identical with FR731997
C11	*Micrasterias fimbriata*	Marienteich, Czech Republic	5032′43.53″N 14 40′39.44″E	Identical with FR731997
C14	*Micrasterias fimbriata*	A bog near Rod pond, Czech Republic	49 07′13.99″N 14 45′07.24″E	Identical with FR731997
B1	*Micrasterias fimbriata*	Bastemose, Bornholm, Denmark	55 07′37.63″N 14 56′42.15″E	Identical with FR731997
I5	*Micrasterias fimbriata*	An unnamed pool near Lecknavarna, Ireland	53 34′10.60″N 9 48′29.57″W	Identical with FR731997
I7	*Micrasterias fimbriata*	An unnamed pool near Lecknavarna, Ireland	53 34′10.60″N 9 48′29.57″W	Identical with FR731997
I10	*Micrasterias fimbriata*	Eirk Lough, Ireland	5156′28.21″N 9 37′41.03″W	Identical with FR731997
I11	*Micrasterias fimbriata*	Eirk Lough, Ireland	5156′28.21″N 9 37′41.03″W	Identical with FR731997
W1	*Micrasterias fimbriata*	Schwemm near Walchsee, Tyrol, Austria	47 39′34.52″N 12 17′50.51″E	Identical with FR731997
C9	*Micrasterias apiculata*	Břehyně wetland, Czech Republic	50 34′58.21″N 14 42′11.54″E	FR731998
SVCK 247	*Micrasterias apiculata*	A bog near Zeller See, Austria	4718′15″N 12 48′33″E	Identical with FR731998
SVCK 65	*Micrasterias brachyptera*	Bogs close to Korvanen, Finland	67 56′13″N 27 50′25″E	FR731996
CAUP K608	*Micrasterias fimbriata*	Pools near Hostens, Aquitaine, France	44 29′54.83″N 00 38′19.06″W	FR691070
Q2	*Micrasterias fimbriata*	A bog near Étang Hardy, Aquitaine, France	43 43′08.60″N 01 22′09.42″W	Identical with FR691070
Q10	*Micrasterias fimbriata*	A bog near Étang Hardy, Aquitaine, France	43 43′08.60″N 01 22′09.42″W	Identical with FR691070
Q14	*Micrasterias fimbriata*	A bog near Étang Hardy, Aquitaine, France	43 43′08.60″N 01 22′09.42″W	Identical with FR691070
L1	*Micrasterias fimbriata*	Torver Tarn, Lake District, United Kingdom	54 19′29.57″N 03 06′23.70″W	Identical with FR691070
SAG 162.80	*Micrasterias fimbriata*	Texas, USA	–	Identical with FR691070
CAUP K604	*Micrasterias rotata*	Pools by Cep, Czech Republic	4855′23.65″N 14 50′23.96″E	FR691071
SVCK 1	*Micrasterias rotata*	An unknown locality near Potsdam, Germany	–	Identical with FR691071
SVCK 26	*Micrasterias rotata*	Wildes Moor bei Husum, Germany	54 24′56.11″N 09 14′56.22″E	Identical with FR691071

Table 1 continued

Strain designation	Original identification	Locality	Geographic coordinates	Accession numbers
SVCK 78	*Micrasterias rotata*	Bogs close to Korvanen, Finland	67 56′13″N 27 50′25″E	Identical with FR691071
SVCK 93	*Micrasterias rotata*	Hammerfest, Norway	70 39′33″N 23 41′07″E	Identical with FR691071
SVCK 212	*Micrasterias rotata*	Timmer Moor near Hamburg, Germany	53 39′47.62″N 10 08′25.26″E	Identical with FR691071
SVCK 243	*Micrasterias rotata*	A bog near Sappel close to Millstatt, Kärnten, Austria	46 47′52.60″N 13 37′47.46″E	Identical with FR691071
SVCK 287	*Micrasterias rotata*	Burnham's Swamp near Falmouth, Massachusetts, USA	–	Identical with FR691071
Q1	*Micrasterias rotata*	Pools near Hostens, Aquitaine, France	44 29′54.83″N 00 38′19.06″W	Identical with FR691071
Q6	*Micrasterias rotata*	A bog near Étang Hardy, Aquitaine, France	43 43′08.60″N 01 22′09.42″W	Identical with FR691071
C8	*Micrasterias rotata*	A mountain fen near Nové Hamry, Czech Republic	50 21′50.46″N 12 39′21.90″E	Identical with FR691071
C12	*Micrasterias rotata*	Marienteich, Czech Republic	50 32′43.53″N 14 40′39.44″E	Identical with FR691071
C13	*Micrasterias rotata*	A bog near Rod pond, Czech Republic	49 07′13.99″N 14 45′07.24″E	Identical with FR691071
I6	*Micrasterias rotata*	Muckross Lake, Ireland	52 00′41.33″N 09 31′45.64″W	Identical with FR691071

intron is one of the most variable plastid-encoded molecular phylogenetic markers suitable for species delimitation. Being a low-copy marker, *trnG*^{ucc} overcomes drawbacks of utilizing multiple-copy genes and introns (Álvarez & Wendel, 2003). Recently, *trnG*^{ucc} intron sequences were used in phylogenetic studies of different groups of Streptophytes (Pedersen & Hedenås, 2003; Turmel et al., 2005; Bayer et al., 2009; Neustupa et al., 2010).

Genomic DNA was extracted from the strains (Table 1) according to the following method: After centrifugation, cells were disrupted by shaking for 10 min with glass beads at 1,800 rpm in Retch-MM200. Consequently, genomic DNA was extracted using Invisorb Spin Plant Mini Kit (Invitek) according to the manufacturer's protocol. The polymerase chain reaction was carried out in 20-μl volumes of 13.9 μl of sterile Mili-Q water, 2 μl of $MgCl_2$ (25 μM), 2 μl of PCR Buffer 10× (Applied Biosystems), 0.4 dNTP (10 μM), 0.25 μl of each trnG-ucc primers (Neustupa et al., 2010), 0.2 μl of AmpliTaq GOLD polymerase (5 U/μl), and 1 μl of DNA (not quantified). PCR amplification was set to an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturing at 94°C for 1 min; annealing at 62°C for 1 min; elongation at 72°C for 1.5 min; and final extension at 72°C for 10 min. The PCR products were purified with JetQuick PCR Purification Kit (Genomed) according to manufacturer's protocol. Consequently, they were sequenced using the same primers by Macrogen Inc. on an automatic 3730XL DNA sequencer. Sequencing readings (encompassing 724–772 base pairs) were assembled and edited using the Seqassem software (Hepperle, 2004). The ClustalW algorithm, set to default parameters, was used for aligning sequences in Mega 4.0 (Tamura et al., 2007). Only unique sequences were left in the alignment, and the alignment stability was assessed in SOAP v1.2 alpha 4 (Löytynoja & Milinkovitch, 2001) comparing alignments produced under different gap-

opening and gap-extension penalties (7Ð20/2.5; 2Ð10/1.5). Only stable blocks of alignment were left in the Þnal alignment (see in Supplementary Table 1). The substitution model was selected using the Akaike Information Criterion (AIC) estimated with PAUP/MrMtGui v1.0b (Nylander, 2004). The general reversible model with allowance for invariable sites (GTR+I) was selected as being the most suitable for the data set. The phylogenetic tree was inferred with Bayesian inference (BI) using MrBayes version 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Two parallel runs were carried out for 10,000,000 generations, each with three heated and one cold chain. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was checked, and burn-in was determined using the programÕs ÔÔsumpÕÕ command.

Bootstrap analyses were performed with maximum likelihood (ML) and maximum parsimony (MP) analyses. The ML analyses were performed in GARLI v. 0.951 (Zwickl, 2009) and consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (gentreshfortopoterm = 100,000). The MP bootstrap values were inferred from 10 000 replicates using the CloseÐNeighborÐInterchange algorithm with search level 3 in Mega 4.0 (Tamura et al., 2007). The obtained phylogenetic tree was displayed in FigTree (Rambaut, 2009) and Mega 4.0 (Tamura et al., 2007). Finally, the displayed phylogenetic tree was graphically adjusted in Adobe Illustrator CS3 v13.0.1.

Light and electron microscopy

Microphotographs were taken on an Olympus BX51 light microscope with Olympus Z5060 digital photographic equipment (Olympus Corporation, Tokyo, Japan). The formaldehyde-Þxed samples for SEM were pipetted on acetone-washed glass coverslips that had, subsequently, been coated three times with a poly-L-lysine solution (1:10 in deionized water) to ensure adhesion of cells, and dried on a heating block. Then, samples were transferred in 30% acetone, dehydrated by an acetone series (10 min successively in 30, 50, 70, 90, 95, 99%, and ×2 in 100%), and critical point dried with liquid CO_2. Finally, they were sputter coated with gold and examined using the JEOL 6380 LV scanning electron microscope.

Morphometric methods

For each strain, 20Ð25 adult semicells were randomly chosen for geometric morphometric analysis. The analysis was based on the position of 49 structurally deÞning cell perimeter landmarks (Supplementary Fig. 1). In addition, parallel morphometric analysis of terminal lobules closest to the lateral semicell incision was also conducted in *M. fimbriata* specimens. The lowest terminal lobule of the upper lateral lobule (i.e., the terminal lobule adjacent to the lateral incision) was chosen (Supplementary Fig. 2). In total, there were 11 landmarks depicted on these terminal lobules, including four sliding landmarks, which were used for capturing the lobule outline variation. The TPS-series software (publicly available at http://life.bio.sunysb.edu/morph/) was used (Rohlf, 2008). Positions of landmarks were digitized in TpsDig, ver. 2.12. The landmark conÞgurations were superimposed by generalized Procrustes analysis (GPA) in TpsRelw, ver. 1.42. Correlation between Procrustes and the Kendall tangent space distances was assessed using TpsSmall, ver. 1.20, to ensure that the variation in shape was small enough to allow subsequent analyses (Zelditch et al., 2004). Indeed, this correlation was very high ($r = 0.999$), and so we proceeded with further statistical analyses. The landmark conÞgurations of *Micrasterias* semicells were symmetrized using a standard method of Klingenberg et al. (2002). A principal component analysis (PCA) of geometric morphometric data was conducted on the entire set of 294 semicells acquired from strains subjected to molecular characterization. Scores of the objects on the non-zero principal component (PC) axes were used for two-group linear discrimination analysis (LDA), whose signiÞcance was assessed by the HotellingÕs T^2 test in PAST, ver. 2.01 (Hammer et al., 2001). This analysis was designed for statistical evaluation of differences in shape of individual species. The additional semicells from natural samples, and from the published Þgures, were also landmark-registered for the geometric morphometric analysis. Then, the GPA-aligned conÞgurations of these semicells were subjected to the classiÞcation discrimination analysis using the above-deÞned set based on an independent grouping criterion, i.e., molecular data. This analysis served as a parallel procedure to conÞrm morphological identiÞcation of the newly identiÞed species based

on a qualitative, expert-based, and taxonomic assessment.

Results

Molecular phylogeny

The analyzed *trnG*^{ucc} intron sequences data set consisted of 709 characters, of which 112 were parsimony informative. According to the unrooted Bayesian analysis (Fig. 1), all of the strains were clearly separated from all of the other *Micrasterias*-lineage members, whose *trnG*^{ucc} intron sequences were available in the GenBank database. The *M. fimbriata* strains formed two independent lineages, constituting a moderately supported clade together with *M. brachyptera* (1.00/81/94, Bayesian posterior probability/ML/MP). These lineages of traditional *M. fimbriata* have been tentatively assigned as A-, and B-lineages (ÔÔAÕÕ for Aquitaine, and ÔÔBÕÕ for Bohemia as regions of Þrst isolation). The A-lineage comprised all the *M. fimbriata* strains isolated from Aquitaine (France), Lake District (UK) and a single strain from Texas (USA). Together with *M. brachyptera*, the A-lineage formed a clade with moderate statistical support (1.00/72/86, BI/ML/MP). The B-lineage, composed of strains isolated from Bohemia, Western Ireland, Bornholm and Tyrol, was inferred in a sister position to this clade. The strains

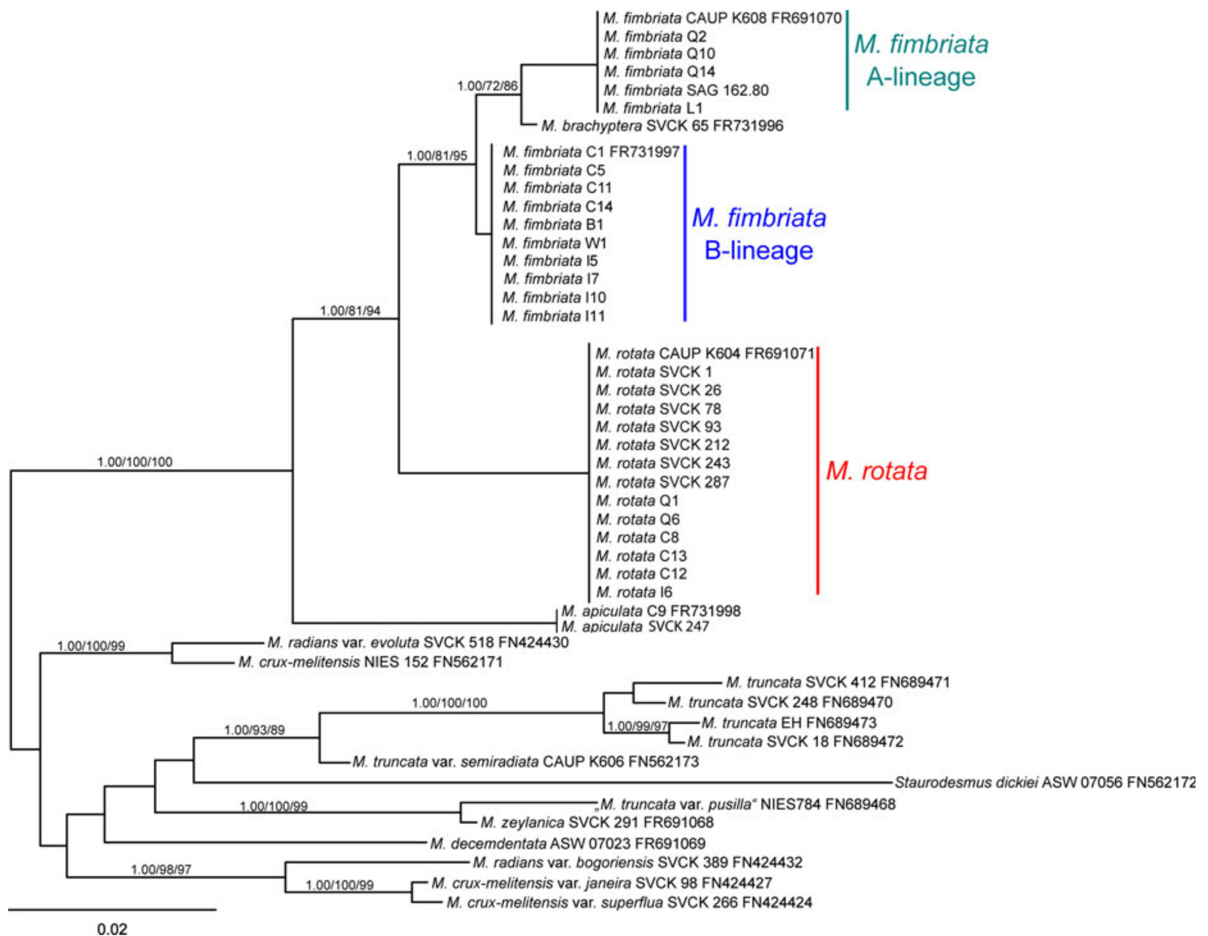

Fig. 1 Unrooted Bayesian phylogenetic tree of *trnG*^{ucc} sequences. The *scale bar* shows the estimated number of substitutions per nucleotide. The posterior probabilities lower then 0.70 and bootstrap support levels below 50% are omitted. The indicators of statistical signiÞcance are provided as follows: Bayesian posterior probability/ML bootstrap support/MP bootstrap support

of *M. rotata* and *M. apiculata* formed independent lineages. Among themselves, all the *M. rotata* strains, as well as all the strains belonging to either the A- or B-lineage of *M. fimbriata*, had identical *trnG^{ucc}* sequences. The strains of *M. apiculata* had the most divergent sequence, differing from the other strains by a unique insertion of 43 nucleotides in its *trnG^{ucc}* intron sequence.

Fig. 2 Light microscopy and SEM pictures of *Micrasterias* ▶ strains. *M. fimbriata*, B-lineage (strain C11), overall morphology (a), apical part of the cell (b), note two subapical spines (*asterisks*) and surface spines (*arrowheads*) on the polar lobe, details of the lateral lobe showing rounded terminal lobules ending with abruptly protruding spines (c, d). *M. fimbriata*, A-lineage (strain CAUP K608), overall morphology (e), apical part of the cell (f), note two subapical spines on the polar lobe (*asterisks*) and numerous surface spines along the major cell incisions (*arrowheads*), details of the lateral lobe showing terminal lobules gradually tapered toward the apex (g, h) *M. rotata* (strain C12) (i), *M. apiculata* (strain SVCK 247) (j), *M. brachyptera* (strain SVCK 65) (k). Scale bars: 20 µm (a, e, i–k), 50 µm (b–d, f–h)

Morphology and geometric morphometrics

The cells of *Micrasterias rotata* (Fig. 2i) had typical unequally divided lateral lobules, and the terminal lobules were usually shortly bidentate. The polar lobe was gradually broadening toward the apex, which always had two bidentate marginal outgrowths. Importantly subapical as well as surface spines were completely lacking. The cell size varied from 205 to 312 µm long (apex–apex) and from 200 to 252 µm broad. In contrast, the investigated strains identified as *M. fimbriata* according to traditional criteria were not homogenous, and formed two morphological groups corresponding to phylogenetic lineages illustrated by our molecular analysis. The members of the B-lineage had slightly unequal lateral lobes and rounded terminal lobules ending with abruptly protruding spines, i.e., so called *fimbriae* (Fig. 2a–d). Apart from two bidentate marginal apices, the polar lobe typically had two subapical spines (Fig. 2b). In some cells, several surface spines were also observed, especially along the major cell incisions (Fig. 2b). The A-lineage cells had unequally divided lateral lobes, but their terminal lobules were not rounded, but instead they gradually tapered toward the apex and did not possess any spines (Fig. 2g–h). However, similarly to B-lineage cells, and contrary to *M. rotata*, they always had two emergent subapical spines on the polar lobes (Fig. 2f). The surface spines along the major cell incisions were present on most cells of the A-lineage (Fig. 2f). The dimensions of cells from the A-lineage varied from 192 to 263 µm in length and from 181 to 228 µm in width. On the other hand, the cells of the B-lineage were slightly larger and varied from 201 to 276 µm in length and from 197 to 248 µm in width. Both the A- and B-lineages clearly differed from *M. apiculata* and *M. brachyptera*, both in cell size as well as in cell shape and lobulation pattern (Fig. 2j, k).

The PCA of geometric morphometric data illustrated that cells belonging to three lineages established on the basis of *trnG^{ucc}* sequence data differed in their overall shape characteristics (Fig. 3a, b). The first PC axis explained 42.9% of the morphometric variation and reflected differences between *Micrasterias rotata* (negative PC1 values) and two lineages of traditional *M. fimbriata*. The second and third PC axes accounted for 12.1 and 10.9% of the variation, respectively. They described shape variation within the phylogenetic groups and, especially in case of the third PC axis the difference between the A-lineage of *M. fimbriata* (positive PC3 values), and other two lineages. The canonical variate analysis (CVA) of scores on the non-zero PC axes illustrated highly significant shape discrimination among groups (Wilk's $\lambda = 0.022$, $F = 133.2$, $P < 0.00001$). The first CV axis (72.1% of the variance) spanned mostly the difference between *M. rotata* and both lineages traditionally assigned to *M. fimbriata*, whereas the second CV axis (27.9%) emphasized differences between both *M. fimbriata* lineages (Fig. 3c). The two-group discrimination analyses confirmed their highly significant shape differences (Hotelling's pairwise comparisons, Bonferroni corrected *P*-values <0.00001 in all the group pairs). The underlying Mahalanobis distances between individual group means indicated that the *M. rotata* cells were more similar to cells of the B-lineage ($D_M = 0.35$), than to cells of the A-lineage ($D_M = 0.51$). The pair of two traditional *M. fimbriata*-assigned lineages had $D_M = 0.38$.

The LDA of geometric morphometric data from *M. fimbriata* strains illustrated 100% correct classification of semicells into their a priori groups based on molecular data (Fig. 4a). Likewise, there was also

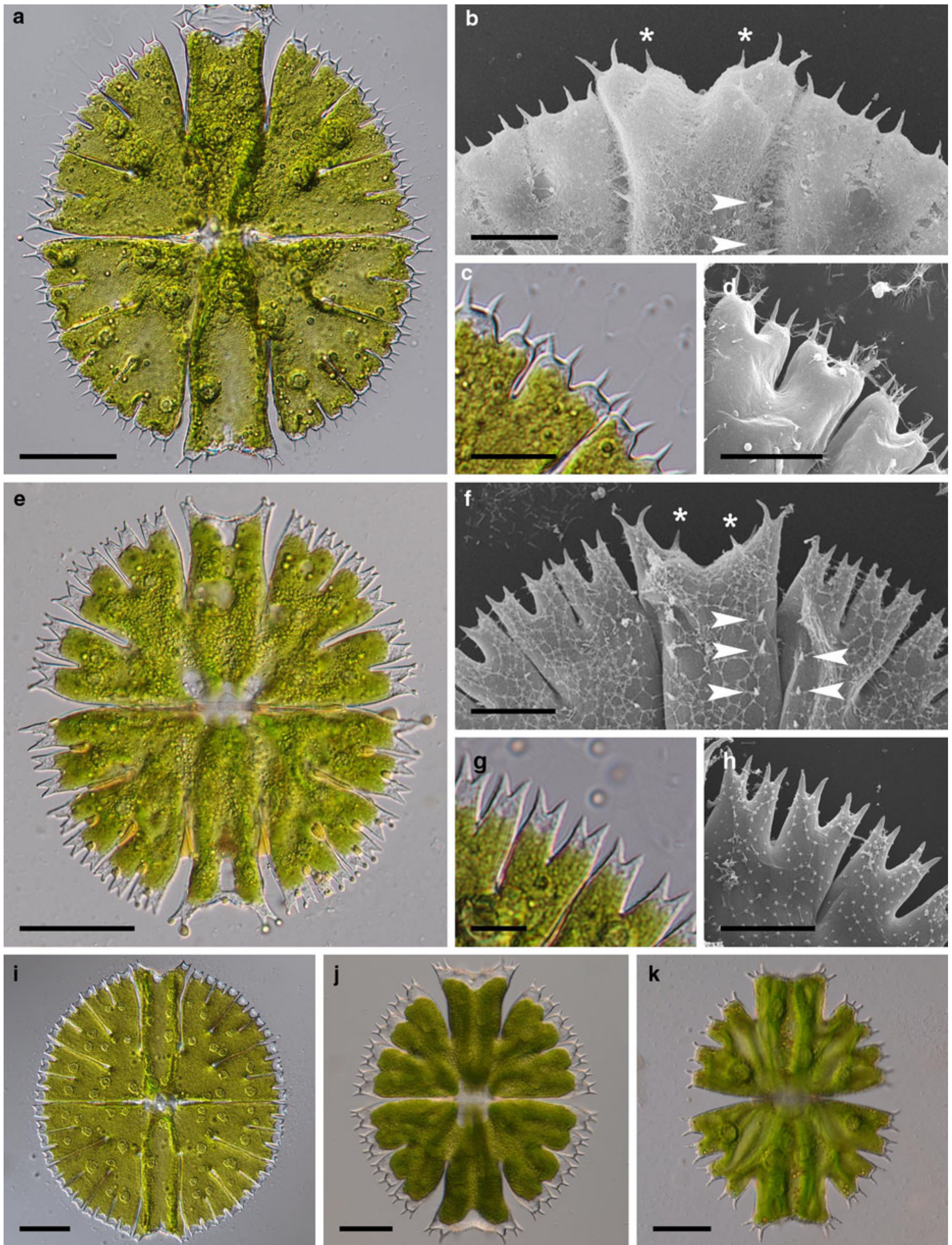
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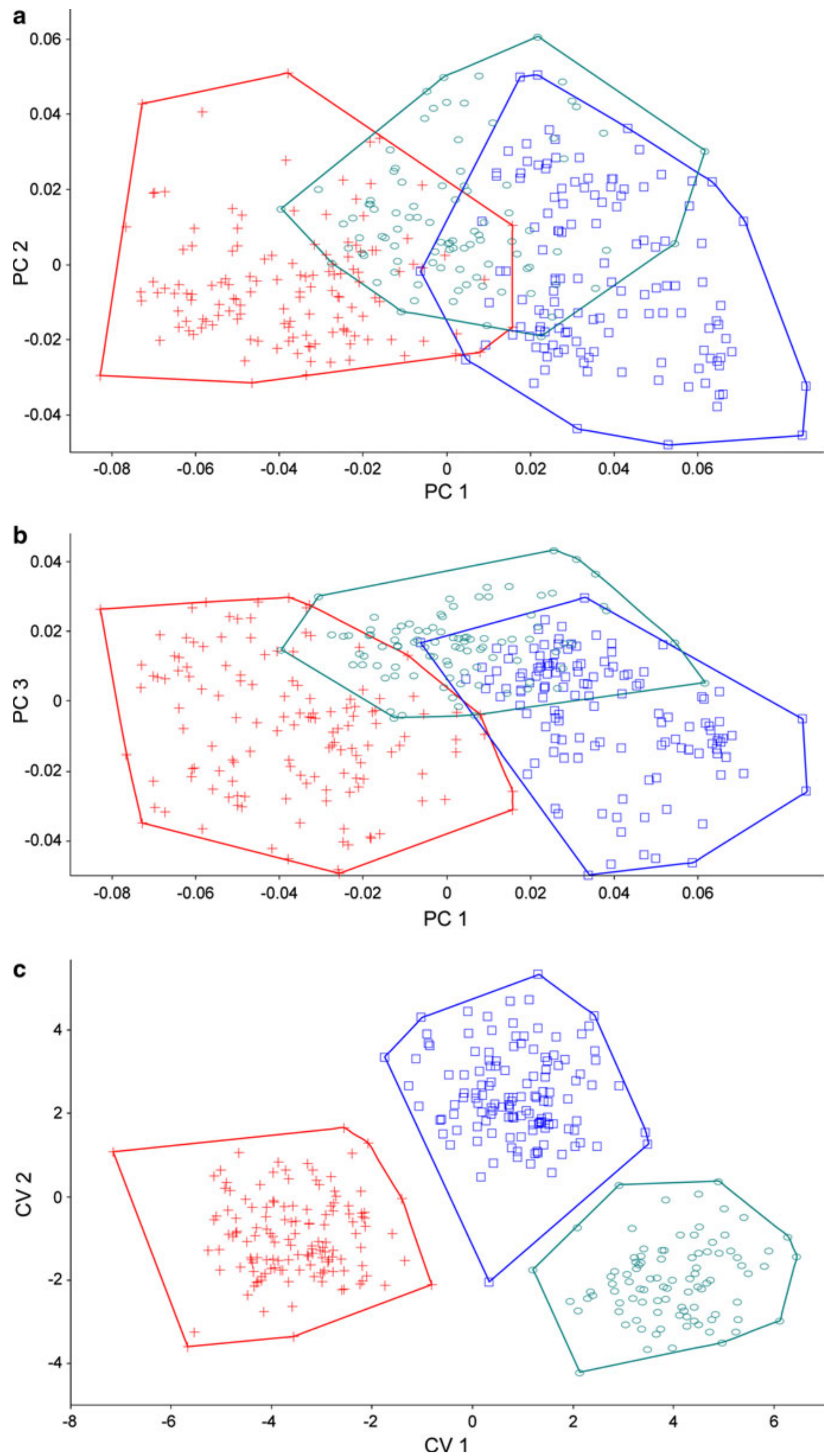
Fig. 3 The PCA and CVA ordination plots of geometric morphometric data of *Micrasterias rotata*, and *M. fimbriata* (A- and B-lineages) semicells. The PC1 versus PC2 (a), PC1 versus PC3 (b), and CV1 versus CV2 plots (c) are depicted. *Crosses*: *M. rotata*, *ellipses*: A-lineage of *M. fimbriata*, *squares*: B-lineage of *M. fimbriata*



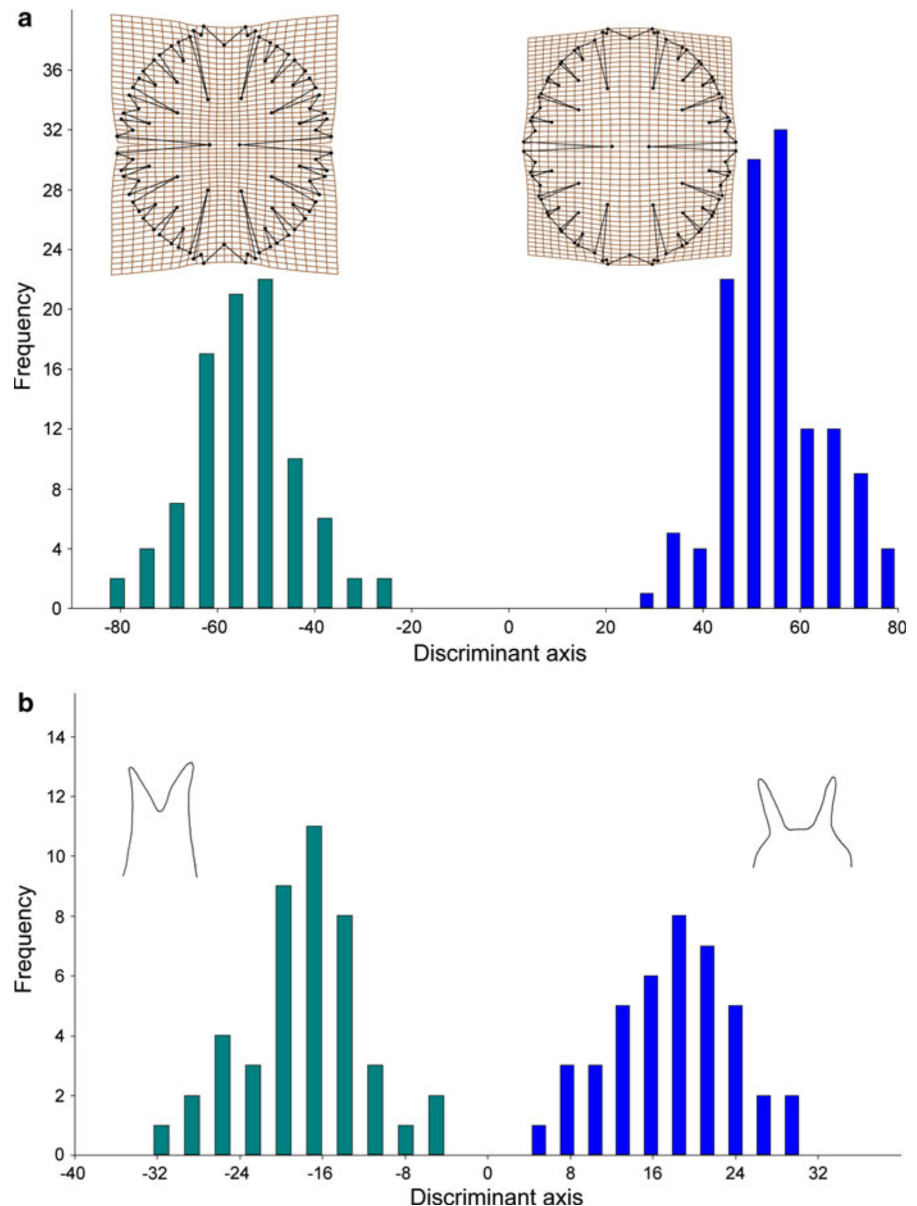


Fig. 4 The linear discrimination analyses of geometric morphometric data of *M. fimbriata* strains. The landmarks analyses of semicells (a), and terminal lobules (b) are depicted. The A-lineage of *M. fimbriata* is depicted in *left bars*, the B-lineage in *right bars*

an unambiguous discrimination of *M. fimbriata* semicells on the basis of their terminal lobule shapes (Fig. 4b). At this stage, we included data from Þgures of natural *M. fimbriata* populations (Supplementary Figs. 3, 4) and from the literature records to the discrimination analysis of terminal lobule shapes. The classiÞcation discrimination analysis was conducted for each cell, and their values on the discriminant axis and subsequently their group assignment were ascertained (Supplementary Table 2). In fact, this analysis was largely conÞrmatory, as the morphological differences in the shape of terminal lobules were readily recognizable by qualitative judgment (see Supplementary Figs. 3, 4). The combined molecular, morphological. and morphometric analyses were used for reconstruction of geographic distribution of two *M. fimbriata* phylogenetic lineages. They illustrated their rather surprising and largely disparate distributional patterns (Fig. 5). In North America, we have not been able to conÞrm any report of the *M. fimbriata* B-lineage morphotype. On the contrary, all the North American literature comprising a span of more than

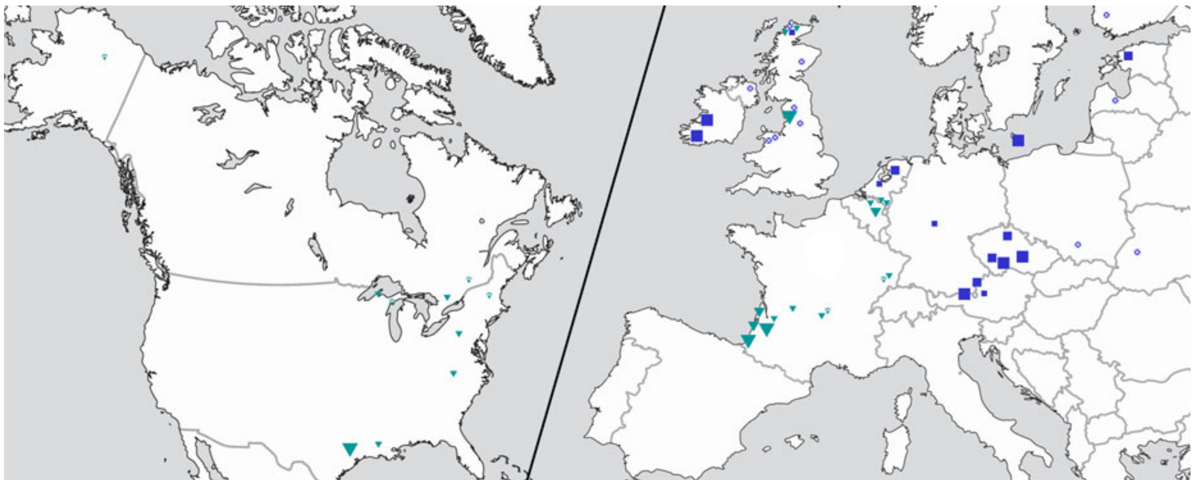


Fig. 5 The *map* illustrating presumptive distribution of A- and B-lineages of *M. fimbriata* in Europe and North America. *Triangles* represent A-lineage and *squares* represent B-lineage findings. *Large symbols* indicate localities of clonal strains, *middle-sized symbols* indicate localities of investigated natural populations, and *small symbols* indicate other published records. The *crossed symbols* indicate records older than 50 years

100 years, and records from Alaska to New England, recently published microphotographs and available strains fitted well into the morphological characteristics of the A-lineage. On the other hand, in Europe, both lineages were encountered. The members of the A-lineage, or the records morphologically corresponding to this lineage, were found in the western parts of the continent, from England to Aquitaine, where they sometimes occurred in abundance. In fact, our broad sampling of desmid assemblages from wetlands and étangs of south-western France resulted in frequent findings of the A-lineage populations, whereas the B-lineage of *M. fimbriata* was not encountered in this region. Similarly, the A-lineage morphotypes were the only ones that were found in our samples from Belgium, and from the Lake District. Surprisingly, there have not been any reported findings of this lineage east of the Rhine River. The two *M. fimbriata* culture strains (SVCK 178 and UTEX LB 766) with an unknown origin also belonged to the A-lineage on the basis of morphological data.

Recent reports of the B-lineage morphotype originate mostly from the Central and Eastern Europe. The B-lineage populations are very probably the only ones of the traditional *M. fimbriata* that occur in the Czech Republic (after examining in excess of 1,000 samples collected from this country). Similarly, the B-lineage is probably the exclusive inhabitant of traditional *M. fimbriata* on Bornholm, where most of the suitable *Micrasterias* habitats were sampled, and the encountered populations analyzed. In addition, all of our findings and the available literature records of *M. fimbriata* from Austria, Germany, and eastern European sites referred to the B-lineage morphotype. However, this lineage has also been encountered in the western parts of Europe. In Western Ireland, where about 100 samples had been collected, only specimens conforming to the B-lineage were found. The natural populations of the B-lineage were also ascertained after material from The Netherlands provided by the courtesy of Peter Coesel and Koos Meesters, and there are also several published reports of this morphotype from England. In addition, Alan Joyce's unpublished records from north-west Scotland sampled between 1985 and 2006 illustrate both A-, and B-lineages. Therefore, to our knowledge, The Netherlands and Scotland are the only parts of Europe where the populations of both lineages co-occur.

Discussion

Remarkable phylogenetic homogeneity of morphologically defined *Micrasterias rotata* across different European region has illustrated its well-defined and robust species concept. The single North American strain of *M. rotata* included in our study was also identical to European populations. Based on these

data, we cannot reject—at least for Europe—the hypothesis that all the populations of *M. rotata* truly represent a homogenous phylogenetic species lineage. This pattern is similar to that illustrated in traditional *M. crux-melitensis* (Neustupa et al., 2010). In this morphospecies, all of the European strains were also found to be identical in their *trnG*^{ucc} and ITS2 sequences. However, the single East-Asian strain of *M. crux-melitensis*—NIES 152—clustered independently, and likely represents a different species. Similarly, we cannot preclude that *M. rotata* may also be found phylogenetically heterogeneous on a global scale.

In *M. fimbriata*, the second traditional species investigated in this study, a more complicated taxonomic structure was revealed. In fact, our results illustrated a previously unrecognized, morphological and phylogenetic differentiation of this traditional species. During our morphological investigation of *M. fimbriata* samples from Aquitaine, we were captivated by their seemingly different morphology from the Central European populations. The molecular analyses confirmed that this traditional species is actually composed of two independent lineages that can also be well defined by careful microscopic observations, as well as by geometric morphometric analysis. The morphological differences between two lineages can be summarized as follows:

(a) Shape of terminal lobules (Fig. 2a–h). This unambiguous character was used in geometric morphometric analysis for discrimination of published figures and natural populations.

(b) The incisions between polar lobe and lateral lobes are shallower in the B-lineage cells. The marginal spines of the A-lineage polar lobes are often long and inwardly bow-shaped (Fig. 2f), whereas they are shorter and straight in the B-lineage.

(c) The surface spine layers (that were used for delimitation of *M. fimbriata* var. *spinosa* in the past) are always present on A-lineage cells (Fig. 2f). On the other hand, they are more rarely encountered on the B-lineage cells (Fig. 2b).

Unfortunately, we do not have abiotic data for all the collection sites available. However, next to the above mentioned morphological differences, there also seems to be a striking contrast in the ecological preferences of the representatives of both lineages. Although the B-lineage specimens in samples from The Czech Republic (Šťastný 2010), Austria (Šťastný & Lenzenweger, 2008), Ireland, and Bornholm generally originate from mesotrophic, slightly acidic wetland habitats, the sites of the A-lineage were mostly distinctly oligotrophic bogs at low pH. The same tendency concerning autecology of both lineages was also observed in the Netherlands (Peter Coesel, pers. comm.). The well-supported morphological and morphometric differentiation of both phylogenetic lineages made it possible to analyze the previously published morphological data for *M. fimbriata*, and to correlate these figures with discrimination patterns based on morphological data of investigated strains. Obviously, we will never be able to acquire sequence data out of the past literature records. However, close and straightforward correlation of morphological and phylogenetic data enabled us to establish the morphology-based discriminative framework for the identification of a presumptive phylogenetic affiliation of published illustrations of morphotypes. Although microphotographs objectively reflect the morphology of cells, the accuracy of drawings can never be assured. Nevertheless, the robustness of our presumptive geographic pattern of distribution, constructed from correlations between the older published data, and more recent findings from the same regions generally confirmed very good reliability of traditional desmidiological drawings. Our geometric morphometric analysis of the published records produced a rather interesting pattern of possible geographic structure for both lineages. In North America, not a single analyzed report corresponded to the B-lineage morphotypes. Moreover, Prescott et al. (1977) illustrated solely the A-lineage specimens in their treatise of North American desmids, but they did not indicate their exact locations. Interestingly, the single South American record of *M. fimbriata* from Brazil (Borge, 1925; Krieger, 1939) also fitted into the A-lineage. As this study was primarily designed for investigation of European data, we did not obtain a more significant amount of North American samples. However, the available strains, recently published microphotographs, as well as all the older literature records support the hypothesis that the A-lineage may be the only American form of traditional *M. fimbriata*. In Europe, tentative distribution of the A-lineage seems to be limited to oceanic

parts of the continent, west of the Rhine. However, outright climatic control of the European A-lineage distribution was supported neither by the American data, where the A-lineage morphotypes were reported also from cold temperate and subarctic regions, such as Ontario or Alaska, nor by its occurrence under rather harsh conditions of the Vosges Mts., at an altitude of more than 900 m above sea level (Lac de Lipsach). Our data also did not support the hypothesis of a recent invasion from North America, as the morphologically well-fitting A-lineage specimens were reported from Western Europe at least twice in the last 100 years (Comère, 1901; Wurtz, 1945). Moreover, these records originated from regions where the A-lineage of *M. fimbriata* was also recently recorded: Vosges Mts. (Le Naturaliste, 2007) and Auvergne (Kouwets, 1987).

The B-lineage seems to be more frequent in central and eastern parts of the European continent, where the A-lineage has not been detected. Interestingly, the B-lineage has never been reported from regions south of the Alps, not even from countries with detailed local accounts and detailed recent checklists of desmids (e.g., Spain—Cambra Sánchez et al., 1998, Italy—Abdelahad et al., 2003 or Romania—Căraus, 2002). However, it also occurs in areas of Western Europe, such as Ireland, or the Netherlands. It seems to have also been relatively widely distributed in Britain at the end of the nineteenth century, as Ralfs (1848), Cooke (1887), Roy & Bisset (1893), and West & West (1905) unanimously recorded morphotypes corresponding to this lineage. In addition, the unpublished drawings by Alan Joyce also include two apparent findings of the B-lineage from Scotland. However, our investigation of the Lake District samples yielded only the A-lineage populations. In addition, solely the A-lineage morphotype of *M. fimbriata* was illustrated (with no collecting locality specified) by Brook & Johnson (2002) in their review of British desmids. Kossinskaja (1960), Gontcharov (1998), and Medvedeva (2001) reported *M. fimbriata* populations from the Far East regions of Russia. However, no original published figures specifically tied to Far Eastern localities are available. At the same time, there are many published records of *M. fimbriata* from different European countries with no original figures included. The species has apparently been considered so well known by traditional desmidiologists that they—unfortunately—did not deem it necessary to draw or photograph their findings anymore. That is why our notion on past distributional patterns of these two *Micrasterias* lineages in Europe will necessarily remain fragmentary, despite the fact that the traditional species was recorded on many occasions. For the analysis of recent data, we used, apart from our own findings, a number of microphotographs published on the internet pages of amateur microscopists who are nowadays often equipped with good light microscopes and produce excellent figures e.g., André Advocat (Le Naturaliste, 2007), Christophe Brochard (Brochard (2008), Wim van Egmond (Sieralgen in Nederland, 2003), and others. We believe that in this study we have illustrated that this relatively new phenomenon of increasing interest of amateur nature-lovers in freshwater algae, coupled with fast publication of their findings on the internet, makes a valuable and accessible contribution to scientific investigation. Therefore, we anticipate that publication of our data on two-fold structure of traditional *M. fimbriata* may soon result in many new findings and localities of both lineages, completing their detailed continentwide distribution. Relative importance of environmental (e.g., climatic) versus historical (e.g., spatial isolation) factors in geographic distribution of microalgae has recently been the subject of intense debates (for a review see e.g., Foissner, 2008). Coesel (1996) and Coesel & Krienitz (2008) suggested that some *Micrasterias* species (such as *M. hardyi* in Australia or *M. sudanensis* in tropical Africa) may represent fine examples of historically constrained geographic distribution areas in unicellular algae. Our data generally concur with these findings and the presently known distributional areas of both *M. fimbriata* lineages in Europe are strikingly similar to phytogeographic patterns of vascular plants taxa (see e.g., Cox & Moore, 2005). Therefore, we cannot exclude that some of the large *Micrasterias* species with low dispersal frequencies may also have largely vicariant and stable distributions, similar to different macroscopic groups.

Differentiation of two *M. fimbriata* lineages warrants their description as separate species. They formed clearly delimited lineages on the *trnG*^{ucc} tree, even with the *Micrasterias brachyptera* strain nested within this clade. At the same time, morphological discriminative characters readily distinguish cells belonging to both lineages. At this point, we should note that the original drawing of *M. fimbriata*

(Ralfs, 1848, Table 8, Fig. 2) apparently corresponds to the B-lineage. At the same time, most of the A-lineage findings were referred to as *M. fimbriata* var. *spinosa*, because of more conspicuous surface spines on these cells (e.g., Wurtz, 1945; Croasdale, 1956; Kouwets, 1987; Engels, 2002). However, the type of this variety—originally described from Scotland—clearly belongs to the B-lineage (Roy & Bisset, 1893) and the presence of surface spine layers can by no means be taken as a discriminative character between the A-, and B-lineages. While these are usually more conspicuous on the cells of the A-lineage, they can also be found on B-lineage specimens. Other varieties (such as var. *obtusiloba*, var. *elefanta*, var. *caudata*, or var. *nuda*) were considered synonymous with the type by Růžička (1981). We certainly have no reason to doubt his taxonomic opinion on the basis of our observations. Our data suggest that the twofold phylogenetic division of *M. fimbriata* was not reflected by any of the traditional subspecific taxa. Therefore, description of the A-lineage as a new *Micrasterias* species would probably be necessary. However, the multigenic phylogenetic revision of the genus is ongoing, and may probably result in some quite far-reaching taxonomic conclusions. Therefore, we think that description of the A-lineage as a separate species—supported by multigenic phylogenies—should be undertaken together with other nomenclatoric changes. Even if the ongoing genuswide study will probably yield more complex insight into the interspecific phylogenetic structure, still, we can now conclude that our A-, and B-lineages are separate, paraphyletic species. *Micrasterias brachyptera* was recovered as a sister species to the A-lineage. However, both these closely related species differ by a number of conspicuous morphological features, such as cell size, degree of lobulation, or the overall cell shape (see e.g., Fig. 2). Based on these data, we can conclude that morphological features of individual *Micrasterias* species can evolve relatively rapidly and, therefore, phylogenetic inferences at the among-species level should be based on molecular data. The main focus of this study was the illustration of the concerted use of molecular and geometric morphometric analyses, as well as of detailed morphological observations, and a combination of these techniques may yield more complex results on the species structure of desmids than could possibly be achieved by applying any of these techniques in isolation.

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References

Abdelahad, N., G. Bazzichelli & R. D'Archino, 2003. Catalogo delle Desmidiaceae (Chlorophyta, Zygnematophyceae) segnalate in Italia. Academia Nazionale delle Science, Rome.

Álvarez, I. & J. F. Wendel, 2003. Ribosomal ITS sequences and plant phylogenetic inference. Molecular Pyhlogenetics and Evolution 29: 417–434.

Bayer, R. J., D. J. Mabberley, C. Morton, C. H. Miller, I. K. Sharma, B. E. Pfeil, S. Rich, R. Hitchcock & S. Sykes, 2009. A molecular phylogeny of the orange subfamily (Rutaceae: Aurantioideae) using nine cpDNA sequences. American Journal of Botany 96: 668–685.

Borge, O., 1925. Die von Dr. F. C. Hoehne während der Expedition Roosevelt-Rondon gesammelten Süsswasseralgen. Arkiv för Botanik 19: 1–56.

Brochard, C., 2008. Cristophe Brochard Nature Photography [available on Internet at http://www.cbrochard.com].

Brodie, J., D. M. John, I. Tittley, M. J. Holmes & D. B. Williamson, 2007. Important Plant Areas for Algae: A Provisional Review of Sites and Areas of Importance for Algae in the United Kingdom. Plantlife International, Salisbury.

Brook, A. J. & L. R. Johnson, 2002. Order Zygnematales. In John, D. M., B. A. Whitton & A. J. Brook (eds), The Freshwater Algal Flora of the British Isles. An Identification Guide to Freshwater and Terrestrial Algae. Cambridge University Press, Cambridge: 479–593.

Burchardt, L. & W. W. A. Kowalski, 2009. Diversity of the algae, habitats, and aquatic ecosystems of Bornholm Island. Oceanological and Hydrobiological Studies 38(Supplement 2): 13–20.

Cambra Sánchez, J., M. Álvarez Cobelas & M. Aboal Sanjurjo, 1998. Lista florística y bibliográfica de los clorófitos (Chlorophyta) de la Península Ibérica, Islas Baleares e Islas Canarias. Asociación Española de Limnología, Burgos.

Cărăuş, I., 2002. The algae of Romania. Studii şi Cercetări, Biologie 7: 1–694.

Coesel, P. F. M., 1982. Structural characteristics and adaptations of desmid communities. Journal of Ecology 70: 163–177.

Coesel, P. F. M., 1996. Biogeography of desmids. Hydrobiologia 336: 41–53.

Coesel, P. F. M. & L. Krienitz, 2008. Diversity and geographic distribution of desmids and other coccoid green algae. Biodiversity and Conservation 17: 381–392.

Coesel, P. F. M. & J. Meesters, 2007. Desmids of the Lowlands. KNNV Publishing, Zeist.

Comère, J., 1901. Les Desmidiées de France. Paul Klincksieck, Paris.

Cooke, M. C., 1887. British Desmids. A Supplement to British Fresh-Water Algae. Williams & Norgate, London.

Cox, C. B. & P. D. Moore, 2005. Biogeography. An Ecological and Evolutionary Approach. John Willey & Sons, New York.

Croasdale, H., 1956. Freshwater algae of Alaska I. Some desmids from the interior. Part 2: *Actinotaenium*, *Micrasterias* and *Cosmarium*. Transactions of the American Microscopical Society 75: 1–70.

Encyclopedia of Life, 2010. *Micrasterias fimbriata* Ralfs [available on Internet at http://www.eol.org/pages/922464].

Engels, M., 2002. Liste der Sammlung von Conjugaten-Kulturen (SVCK) am Institut für Allgemeine Botanik der Universität Hamburg. Institut für Allgemeine Botanik der Universität Hamburg [available on Internet at http://www.biologie.uni-hamburg.de/b-online/d44_1/44_1.htm].

Foissner, W., 2008. Protist diversity and distribution: some basic considerations. Biodiversity and Conservation 17: 235–242.

Förster, K., 1972. Desmidieen aus dem Südosten der Vereinigten Staaten von America. Nova Hedwigia 23: 515–644.

Gontcharov, A. A., 1998. Contribution to the desmid flora of the Primorsky Territory, Russia. Bulletin of the National Science Museum, Tokyo, Series B 23: 59–80.

Gontcharov, A. A. & M. Melkonian, 2008. In search of monophyletic taxa in the family Desmidiaceae (Zygnematophyceae, Viridiplantae): the genus *Cosmarium*. American Journal of Botany 95: 1079–1095.

Gontcharov, A. A. & M. Melkonian, 2010a. Molecular phylogeny and revision of the genus *Netrium* (Zygnematophyceae, Streptophyta): *Nucleotaenium* gen. nov. Journal of Phycology 46: 346–362.

Gontcharov, A. A. & M. Melkonian, 2010b. A study of conflict between molecular phylogeny and taxonomy in the Desmidiaceae (Streptophyta, Viridiplantae): analyses of 291 *rbc*L sequences. Protist. doi:10.1016/j.protis.2010.08.003

Guiry, M. D. & G. M. Guiry, 2010. AlgaeBase. National University of Ireland, Galway [available on Internet at http://www.algaebase.org].

Gysels, J., 2005. Ridderkruisen (*Micrasterias* Ag. ex Ralfs): oude en nieuwe vindplaatsen in de provincie Antwerpen. Antwerpse Koepel voor Natuurstudie, Antwerp.

Hall, J. D., K. G. Karol, R. M. McCourt & C. F. Delwiche, 2008. Phylogeny of the conjugating green algae based on chloroplast and mitochondrial nucleotide sequence data. Journal of Phycology 44: 467–477.

Hammer, Ø., D. A. T. Harper & P. D. Ryan, 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4: 1–9.

Hepperle, D., 2004. SeqAssem©. A Sequence Analysis Tool, Contig Assembler and Trace Data Visualisation Tool for Molecular Sequences [available on Internet at http://www.sequentix.de/software_seqassem.php].

Huelsenbeck, J. P. & F. Ronquist, 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 754–755.

Huss, V. A. R., C. Frank, R. C. Hartmann, M. Hirmer, A. Klouboucek, B. M. Seidel, P. Wenzeler & E. Kessler, 1999. Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* (Chlorophyta). Journal of Phycology 35: 587–598.

Irenee-Marie, F. I. C., 1938. Flore Desmidiale de la Region de Montreal. Laprairie, Montreal.

Kallio, P., 1953. The effect of continued illumination on the desmids. Annales Botanici Societatis Zoologicae Botanicae Fennicae Vanamo 8: 58–74.

Klingenberg, C. P., M. Barluenga & A. Meyer, 2002. Shape analysis of symmetric structures: quantifying variation among individuals and asymmetry. Evolution 56: 1909–1920.

Kossinskaja, E. K., 1960. Flora Sporovych Rastenii SSSR. Tom V. Desmidievye vodorosli. Izdatelstvo Akademii Nauk SSSR, Moscow.

Kouwets, F., 1987. Desmids from the Auvergne (France). Hydrobiologia 146: 193–263.

Kouwets, F., 2008. The species concept in desmids: the problem of variability, infraspecific taxa and the monothetic species definition. Biologia 63: 877–883.

Krieger, W., 1939. Die Desmidiaceen. Kryptogamen Flora Bd. XIII, Teil 2. Akademisches Verlag, Leipzig.

Le Naturaliste, 2007. *Micrasterias fimbriata* Ralfs. Posted by André [available on Internet at http://www.lenaturaliste.net/forum/viewtopic.php?f=91&t=68].

Le Naturaliste, 2010a. *Micrasterias* (identification). Posted by Bruno Dosso [available on Internet at http://www.lenaturaliste.net/forum/viewtopic.php?f=39&t=5125].

Le Naturaliste, 2010b. *Micrasterias*, j'en ai trouvé une! Posted by Kévin Pounot [available on Internet at http://www.lenaturaliste.net/forum/viewtopic.php?f=39&t=6446].

Lenzenweger, R., 1981. Heimische Zieralgen (*Desmidiaceen*)—gefährdete Mikroflora. Öko-L 3: 15–19.

Löytynoja, A. & M. C. Milinkovitch, 2001. SOAP, cleaning multiple alignments from unstable blocks. Bioinformatics 17: 573–574.

Luo, W., S. Pflugmacher, T. Pröschold, N. Walz & L. Krienitz, 2006. Genotype versus phenotype variability in *Chlorella* and *Micractinium* (Chlorophyta, Trebouxiophyceae). Protist 157: 315–333.

Medvedeva, L. A., 2001. Biodiversity of aquatic algal communities in the Sikhote-Alin biosphere reserve (Russia). Cryptogamie Algologie 22: 65–100.

Mikhailyuk, T. I., H. J. Sluiman, A. Massalski, O. Mudimu, E. M. Demchenko, S. Y. Kondratyuk & T. Friedl, 2008. New streptophyte green algae from terrestrial habitats and an assessment of the genus *Interfilum* (Klebsormidiophyceae, Streptophyta). Journal of Phycology 44: 1586–1603.

Mix, M., 1970. Die Desmidiaceen des Zeller Lochs bei Fulda. Mitteilungen aus dem Staatsinstitut für Allgemeine Botanik in Hamburg 13: 71–92.

Müller, J., T. Friedl, D. Hepperle & M. Lorenz, 2005. Distinction between multiple isolates of *Chlorella vulgaris* (Chlorophyta, Trebouxiophycee) and testing for conspecifity using amplified fragment length polymorphism and

ITS rDNA sequences. Journal of Phycology 41: 1236Ð1247.

Nemjová, K., J. Neustupa, J. ŠtÕastný, P. Škaloud & J. Veselá, accepted. Species concept and morphological differentiation of strains traditionally assigned to *Micrasterias truncata*. Phycological Research.

Neustupa, J. & P. Škaloud, 2007. Geometric morphometrics and qualitative patterns in the morphological variation of Þve species of *Micrasterias* (Zygnemophyceae, Viridiplantae). Preslia 79: 401Ð417.

Neustupa, J., J. ŠtÕastný & L. Hodač, 2008. Temperature-related phenotypic plasticity in the green microalga *Micrasterias rotata*. Aquatic Microbial Ecology 51: 77Ð86.

Neustupa, J., K. Černá & J. ŠtÕastný, 2009. Diversity and morphological disparity of desmid assemblages in Central European peatlands. Hydrobiologia 630: 243Ð256.

Neustupa, J., P. Škaloud & J. ŠtÕastný, 2010. The molecular phylogenetic and geometric morphometric evaluation of *Micrasterias crux-melitensis*/*M. radians* species complex. Journal of Phycology 46: 703Ð714.

Nordstedt, C. F. O., 1888. Desmidieer från Bornholm, samlade och delvis deskrifvada af R. T. Hoff. Videnskabelige meddelelser fra den Naturhistoriske forening i Kjøbenhavn 1888: 182Ð213.

Nylander, J. A. A., 2004. MrModeltest v2. Evolutionary Biology Centre, Uppsala University [available on Internet at http://www.abc.se/~nylander/].

Oyadomari, J. K., 2010. Freshwater Algae (and Protozoans) of the Keweenaw Peninsula, MI [available on Internet at http://www.keweenawalgae.mtu.edu/].

Pedersen, N. & L. Hedenäs, 2003. Phylogenetic investigations of a well supported clade within the acrocarpous moss family Bryaceae: evidence from seven chloroplast DNA sequences and morphology. Plant Systematics and Evolution 240: 115Ð132.

Photomacrography, 2008. Photography Through the Microscope [available on Internet at http://www.photomacrography.net/forum/viewtopic.php?t=5266&highlight=micrasterias].

Prescott, G. W., H. T. Croasdale & W. C. Vinyard, 1977. A Synopsis of North American Desmids, Part II. Desmidiaceae: Placodermae, Sect. 2. University of Nebraska Press, Lincoln.

Raciborski, M., 1885. De nonnulis Desmidiaceis novis vel minus cognitis, quae in Polonia inventae sunt. Pamiętnik wydziału matematyczno-przyrodniczego Akademii Umiejętności 10: 57Ð100.

Ralfs, J., 1848. The British Desmidieae. Reeve, Benham & Reeve, London.

Rambaut, A., 2009. FigTree. Tree Figure Drawing Tool. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh.

Rohlf, F. J., 2008. Tps Series. Department of Ecology and Evolution, State University of NewYork at Stony Brook, New York.

Ronquist, F. & J. P. Huelsenbeck, 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572Ð1574.

Roy, J. & J. P. Bisset, 1893. On Scottish Desmidieae I. The Annals of Scottish Natural History 5: 106Ð111.

Růžička, J., 1981. Die Desmidiaceen Mitteleuropas, Band 1, 2. Lieferung. E. SchweizerbartÕsche Verlagsbuchhandlung, Stuttgart.

Shaw, J., E. B. Lickey, J. T. Beck, S. B. Farmer, W. Liu, J. Miller, K. C. Siripun, C. T. Winder, E. E. Schilling & R. L. Small, 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. American Journal of Botany 92: 142Ð166.

Sieralgen in Nederland, 2003. Desmid of the Month, July 2003. *Micrasterias fimbriata* [available on Internet at http://www.desmids.nl/maand/english/juleng03.html].

ŠtÕastný, J., 2010. Desmids (Conjugatophyceae, Viridiplantae) from the Czech Republic; new and rare taxa, distribution, ecology. Fottea 10: 1Ð74.

ŠtÕastný, J. & R. Lenzenweger, 2008. A supplement to the desmid ßora (Desmidiales, Zygnematophyceae) of the ÔÔSchwemmÕÕ near Walchsee in Northern Tyrol (Austria). PhytonÑAnnales Rei Botanicae 48: 37Ð49.

Tamura, K., J. Dudley, M. Nei & S. Kumar, 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596Ð1599.

Turmel, M., C. Otis & C. Lemieux, 2005. The complete chloroplast DNA sequences of the charophycean green algae *Staurastrum* and *Zygnema* reveal that the chloroplast genome underwent extensive changes during the evolution of the Zygnematales. BMC Biology 3: 22Ð34.

Tyler, P. A., 1970. Taxonomy of Australian freshwater algae I. The genus *Micrasterias* in south-eastern Australia. British Phycological Journal 5: 211Ð234.

Webber, M., 2006. Personal www pages of Mark Webber [available on Internet at http://www.markwebber.org/images/dcc0607/index.html].

West, W. & G. S. West, 1905. A Monograph of the British Desmidiaceae, Vol. 2. Ray Society, London.

Wolle, F., 1892. Desmids of the United States and List of American Pediastrums. Moravian Publication OfÞce, Bethlehem.

Wurtz, A., 1945. Recherches ecologiques sur les lacs et tourbières et étangs dÕAuvergne. Annales de la Station Centrale dÕHydrobiologie Appliquée 1: 89Ð199.

Zelditch, M. L., D. L. Swiderski, D. H. Sheets & W. L. Fink, 2004. Geometric Morphometrics for Biologists: A Primer. Elsevier Academic Press, London.

Zwickl, D. J., 2009. Garli. Phylogenetic Analysis of Molecular Sequence Data Using the Maximum-Likelihood Criterion [available on Internet at http://code.google.com/p/garli/].