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ORIGINAL ARTICLE

Molecular phylogeny of baculiform desmid taxa (Zygnematophyceae)

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Abstract The baculiform, rod-like morphotypes belong to several phylogenetic lineages within Desmidiaceae (Zygnematophyceae, Viridiplantae). Some, for example the genus *Pleurotaenium*, form independent lineages, but reductive evolution of complicated desmid cells toward baculiform morphology also occurred in individual lineages, for example *Micrasterias*. In this genus, the rod like *Triploceras* forms evolved from more complex ancestors. In this study, we tested for an independent position of the subtropical and tropical genus *Triplastrum*, previously separated from *Triploceras* on the basis of morphological data. In addition, monophyly of *Pleurotaenium* was also investigated with multiple isolates corresponding to seven species of this genus, including the morphologically dissimilar *P. nodosum* and *P. ovatum*. Finally, two isolates of *Docidium baculum* were also investigated. Molecular phylogenetic analysis of concatenated *rbcL* + *cox*III sequence data implied that the baculiform taxa investigated were in three distantly related positions within Desmidiaceae. The genus *Triplastrum* proved to be unrelated to *Triploceras*, because it clustered in the "omniradiate" lineage of Desmidiaceae among morphologically dissimilar taxa. The genus *Pleurotaenium* was monophyletic, but *P. ovatum* was recovered in a weakly supported sister position to all the other members of the genus. The *trn*G^{ucc} phylogeny of *Pleurotaenium* taxa concurred with the *rbcL* + *cox*III phylogram, and generally revealed the poor morphological concepts of some species in this genus. The most common taxa *P. ehrenbergii* and *P. trabecula* were resolved as polyphyletic because their strains were distributed among several strongly supported clades. However, strains of *P. nodosum* and *P. archeri* formed separate, well supported lineages within the genus.

Keywords Desmids · Molecular phylogeny · Zygnematophyceae · *Docidium* · *Pleurotaenium* · *Triplastrum*

Introduction

Desmidiales are the single most species-rich group of microalgae of the Streptophyta evolutionary lineage of green plants (Viridiplantae). They belong to a group of conjugating green algae (Zygnematophyceae) that have recently been revealed to be the closest evolutionary relatives of embryophytes (Wodniok et al. 2011). More than 6,000 species of desmids have been described in freshwater habitats worldwide (Brook 1981). Desmids are especially abundant in the phytobenthos of acidic wetlands (Coesel and Meesters 2007). Four families of Desmidiales (Desmidiaceae, Gonatozygaceae, Peniaceae, and Closteriaceae) have been recognised on the basis of morphological and cytological data. A single family, Desmidiaceae, contains approximately 90 % of the species described so far. However, the generic taxonomy of this group has recently been virtually deconstructed because molecular phylogenetic studies illustrated non-monophyly of many traditional genera (Gontcharov et al. 2003; Gontcharov and

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Melkonian 2005, 2011; Gontcharov 2008; Hall et al. 2008). Gontcharov and Melkonian (2008, 2011) discovered that Desmidiaceae consists of at least 22 independent phylogenetic lineages that may form the basis of the newly defined monophyletic genera within this important microalgae group. However, molecular data are still not available for several traditional genera with distinct morphology; therefore, their taxonomic status remains uncertain. Moreover, Hall et al. (2008) and Gontcharov and Melkonian (2011) reported that some desmid taxa with distinctly different morphology may form a single phylogenetic group. An interesting example of this phenomenon among desmid genera is represented by the genus *Triploceras* J. W. Bailey. This genus is characterised by elongated baculiform cells dotted with numerous knot-like projections (i.e., verrucae). However, molecular data indicated it is nested within the *Micrasterias* lineage (Škaloud et al. 2011), where most taxa are characterised by flat, richly ornamented cells divided to form polar and lateral lobes (Krieger 1939; Růžička 1981; Coesel and Meesters 2007). Interestingly, the morphology of *Triploceras* is vaguely similar to that of aradiate morphs of *Micrasterias* C. Agardh ex Ralfs, i.e., cells with reduced lateral lobes which may occasionally appear in *Micrasterias* populations exposed to stressful environmental conditions (Kallio and Heikkilä 1969). In addition to *Triploceras*, baculiform desmid morphotypes belong to several other independent lineages within Desmidiaceae. Gontcharov and Melkonian (2011) defined three phylogenetic lineages corresponding to the traditional genera, namely *Pleurotaenium* Nägeli, *Haplotaenium* T. Bando, and *Docidium* Brébisson ex Ralfs, characterised by rod-like morphology of the cells. However, the phylogenetic position of further baculiform desmid genera, for example *Triplastrum* Iyengar and Ramanathan, *Ichthydontum* A. M. Scott and Prescott, or *Ichthyocercus* West and G. S. West remained unknown. These taxa almost exclusively occur in tropical and subtropical habitats and are currently not available in cultures. Molecular data are also lacking for some morphologically very distinct species of the genus *Pleurotaenium*, for example *P. nodosum* (F. M. Bailey) P. Lundell, and *P. ovatum* (Nordstedt) Nordstedt. Monophyly of these species with other *Pleurotaenium* taxa cannot be regarded as straightforward. Moreover, the presence of prominent knot-like projections on the surface of *P. nodosum* cells makes this species somewhat similar to the genus *Triploceras*. Therefore, testing for monophyly of the traditional genus *Pleurotaenium* may definitely be of interest for desmidiacean taxonomy.

The genus *Triplastrum* was introduced by Iyengar and Ramanathan (1942) to accommodate species that were formerly classified into the genus *Triploceras* but lacked typical knot-like projections (verrucae). Populations of this genus so far have only been reported in warmer regions of the Old World (Coesel 1996). Turner (1892) analysed the collections made by G. C. Wallich in India and described *Triploceras abbreviatum* W. B. Turner from samples collected in Raniganj (NW of Kolkata). His specimens had four plastids and 3 or 4 terminal cell lobes, each with a single minute lateral spine. Allorge (1924) described *Triploceras simplex* P. Allorge from Lac de Grand-Lieu in western France. This species was characterised by two plastids and two spines on the tips of terminal lobes. A few years later Kisselev (1930), cited after Kossinskaja (1960), described *Triploceras spinulosum* Kisselev from a rice field in Uzbekistan. This species differed from the two former species by having two plastids but bearing up to four spines on the apical lobes (Kossinskaja 1960). Iyengar and Ramanathan (1942), apparently unaware of Kisselev's study, created a new genus *Triplastrum* and also described a new species *T. indicum* Iyengar and Ramanathan with morphology almost identical to that of *T. spinulosum*. They also transferred *T. abbreviatum* and *T. simplex* into this newly formed genus. Hinode (1952), unaware of the Iyengar and Ramanathan (1942) study, reported *T. simplex* from Shikoku Island in southern Japan. He concluded that morphological differences between *T. abbreviatum* and *T. simplex* were negligible and therefore created a new combination, *T. abbreviatum* var. *simplex*. Hinode (1952) was also the first to illustrate fine punctuation of the cell wall in his specimens, and he also reported irregularly undulated zygospores. Gauthier-Lièvre (1960) reported *Triplastrum spinulosum* from several tropical African localities in Mali, Chad, and Uganda. She concluded that morphological differences between *T. indicum* and *T. spinulosum* are negligible and combined the former name as a variety of the later species *T. spinulosum* var. *indicum*. However, she also created a new variety, *T. spinulosum* var. *africanum*, for her specimens with a somewhat narrower subapical part of semicells. Couté and Rousselin (1975) reported a number of localities of *T. spinulosum* from the Niger river basin. The number of terminal spines in their populations varied, and they concluded that all the varieties of *T. spinulosum* should be regarded as synonymous. However, these varieties were not put into synonymy until Claassen's (1977) study of *T. spinulosum* from the Transvaal, South Africa. She observed cells with three or four terminal lobes, each bearing 2–4 spines. The cells had 2–4 and occasionally even up to six plastids. Islam and Akter (2005) reported *T. abbreviatum* with 2–4 plastids and with two spines on the terminal lobes from tea gardens in Dhaka, Bangladesh. Kouwets (1998) confirmed the occurrence of *Triplastrum* in South West France. He identified his specimens as *T. spinulosum* var. *indicum* and reported that the cells had 3–4 apical lobes, terminating in 3–4 spines. His figure depicted a cell with four plastids.

Coesel and Van Geest (2008) reported *T. abbreviatum* from the Okavango delta in Botswana. Notably, they concluded that there are possibly no essential morphological differences between all the *Triplastrum* species described so far and suggested that the genus may in fact be monospecific.

For this study, we isolated a clonal strain of *Triplastrum* from Lac de Cazaux, Aquitaine, France, in addition to 27 strains of other baculiform desmid species that belong to different traditional genera (*Pleurotaenium* and *Docidium*). Consequently, this study principally addressed two questions:

1. Is the genus *Triplastrum* really different from the genus *Triploceras*? Does it form a separate lineage within Desmidiaceae, warranting its classification as separate genus?
2. Do all the strains classified in the traditional genus *Pleurotaenium* really form a monophyletic lineage? Is there any (pseudo) cryptic species diversity within this frequently occurring genus?

Materials and methods

Isolation and cultivation of strains; light microscope (LM) and scanning electron microscope (SEM) observations

The natural populations of the genus *Triplastrum* were observed in plankton samples taken from the Lac de Cazaux (Aquitaine, France) on 4 October 2009 and 15 September 2010. The origin of all the other baculiform strains isolated in this study is given in Table 1. The strains were isolated by single-cell pipetting into unialgal cultures, which were grown in MES (morpholinoethanesulfonic acid)-buffered DY IV liquid medium (Andersen et al. 1997) at 24 °C and continuously illuminated at 5–15 µmol photons m^{-2} s^{-1} from 18 W cool fluorescent tubes (Philips TLD 18 W/33; Royal Philips Electronics, Amsterdam, The Netherlands). The clonal strain of *Triplastrum simplex* isolated in this study was deposited as strain no. K 1101 in the Culture Collection of Algae of Charles University in Prague (CAUP), see http://botany.natur.cuni.cz/algo/caup.html. Microphotographs of the cells were taken with an Olympus (Tokyo, Japan) BX51 light microscope with an Olympus Z5060 digital camera. For SEM, the acetone-washed glass coverslips were heated and coated three times with a poly(L-lysine) solution (1:10 in deionised water) to ensure appropriate cell adhesion. A drop of the formaldehyde-fixed cell suspension was put on the coverslip, transferred into 30 % acetone, and dehydrated in an acetone series. Subsequently, the cells were dried to a critical point with liquid carbon dioxide (CO_2). Finally, they were sputter coated with gold and examined with a Jeol 6380 LV SEM.

DNA isolation, polymerase chain reaction (PCR), and DNA sequencing

After centrifugation of desmid cells in 2 mL tubes, 100–200 mL of InstaGene matrix (Bio-Rad Laboratories) was added to the pellet. The cells were then mechanically disrupted by shaking for 5 min in the presence of glass beads (3 mm diameter; Sigma–Aldrich) in Mixer Mill MM 400 (Retsch, Haan, Germany). Subsequently, the solution was incubated at 56 °C for 30 min, vortex mixed for 10 s, and heated at 99 °C for 8 min. After vortex mixing a second time, the tubes were centrifuged at 12,000 rpm for 2 min, and the supernatant was directly used as a PCR template. Three molecular markers were amplified by PCR: chloroplast *rbc*L and *trn*G^{ucc} and mitochondrial *cox*III. The PCR reaction in a total volume of 20 µL contained 13.1 µL sterile Milli-Q water, 2 µL AmpliTaq Gold® 360 buffer 10× (Applied Biosystems, Life technologies, Carlsbad, CA, USA), 2.2 µL $MgCl_2$ (25 mM), 0.4 µL dNTP mix (10 mM), 0.25 µL of each primer (25 nM), 0.6 µL 360 GC enhancer, 0.2 µL AmpliTaq Gold® 360 DNA polymerase, and 1 µL DNA (10 ng μL^{-1}). The *rbc*L gene was amplified using newly designed primers rbcL-Pleurot-F (5′-GGT TAA AGA TTA TAG ACT TAC-3′) and *rbcL*-Pleurot-R (5′-CCT TGA CGA GCA AGA TCA CG-3′). The *trn*G^{ucc} marker was amplified using the primers designed by Neustupa et al. (2010): trnG-F (5′-AGC GGG TAT AGT TTA GTG GT-3′) and trnG-R (5′-GGT AGC GGG AAT CGA ACC CGC-3′). Finally, amplification of *cox*III marker was performed using primers designed by Škaloud et al. (2011): COX-ZYG-F3 (5′-TTA CTG GAG GTG GCA CAC TT-3′) and COX-ZYG-R2 (5′-TCC ATG AAA TCC AGT AGC TAA G-3′). The *rbc*L, *trn*G^{ucc}, and *cox*III markers were amplified in either a Touchgene gradient thermal cycler (Krackeler Scientific, Albany, NY, USA) or an XP thermal cycler (Bioer, Tokyo, Japan), starting with initial denaturation at 94 °C for 4/2/2 min, followed by 35/40/37 cycles of denaturing at 94 °C for 1 min, annealing at 54/62/50 °C for 1 min, and elongation at 72 °C for 2.5/1.5/3 min, with a final extension at 72 °C for 10 min, respectively. The PCR products were stained with bromophenol blue loading dye, quantified on 1 % agarose gel, stained with ethidium bromide, and cleaned with the Jetquick PCR purification kit (Genomed, Löhne, Germany) in accordance with the manufacturer's procedure. The purified amplification products were sequenced using an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730xl) at Macrogen in Seoul, Korea. Sequencing reads were assembled and edited by use of SeqAssem software (Hepperle 2004).

Table 1 List of strains used in the study and their origin

Identity	Strain designation	Locality	Geographic coordinates
Docidium baculum	C6	Břehyně wetland, Czech Republic	50°34′58.58″N; 14°42′10.96″E
Docidium baculum	I1	A pool near Derrycunihy, Kerry, Ireland	51°57′57.66″N; 9°35′49.24″W
Pleurotaenium archeri	H50	Schwemm peat bog near Walchsee, Austria	47°39′19.35″N; 12°17′22.71″E
Pleurotaenium archeri	H51	Schwemm peat bog near Walchsee, Austria	47°39′19.35″N; 12°17′22.71″E
Pleurotaenium crenulatum	592	Grand Étang de Biscarrosse, Aquitaine, France	44°23′13.92′'N; 01°11′32.68″W
Pleurotaenium ehrenbergii	579	A pool near Hostens, Aquitaine, France	44°29′54.83″N; 00°38′19.06″W
Pleurotaenium ehrenbergii	737	Lough an Oileáin, Connemara, Ireland	53°27′38.91″N; 9°32′35.90″W
Pleurotaenium ehrenbergii	H1	A pool in Břehyně wetland, Czech Republic	50°35′01.17″N; 14°43′00.68″E
Pleurotaenium ehrenbergii	H13	Étang de Cazaux, Aquitaine, France	44°30′34.49″N; 1°11′39.12″W
Pleurotaenium ehrenbergii	H55	The mesotrophic sandpit pool near Cep, Czech Republic	48°55′04.03″N; 14°52′56.72″E
Pleurotaenium ehrenbergii	Q12	A bog near Étang Hardy, Aquitaine, France	43°43′12.99″N; 1°22′07.93″W
Pleurotaenium ehrenbergii	Q15	A pool close to Lac de Cazaux, Aquitaine, France	44°30′28.70″N; 1°11′57.30″W
Pleurotaenium ehrenbergii	Q19	A pool close to Lac de Cazaux, Aquitaine, France	44°30′28.70″N; 1°11′57.30″W
Pleurotaenium nodosum	I3	Long Range lake, Kerry, Ireland	51°59′51.04″N; 9°33′3.81″W
Pleurotaenium nodosum	I8	Lough na hEasléime, Connemara, Ireland	53°26′15.48″N; 9°32′36.78″W
Pleurotaenium nodulosum	H37	A peaty pool near Loughanillaun lake, Connemara, Ireland	53°27′36.63″N; 9°32′35.27″W
Pleurotaenium sp.	H14	Étang de Cazaux, Aquitaine, France	44°30′34.49″N; 1°11′39.12″W
Pleurotaenium sp.	H38	Bastemose wetland, Bornholm, Denmark	55°07′37.63″N; 14°56′42.15″E
Pleurotaenium sp.	H39	Schwemm peat bog near Walchsee, Austria	47°39′19.35″N; 12°17′22.71″E
Pleurotaenium sp.	H41	Étang Hardy, Aquitaine, France	43°43′01.64″N; 1°21′45.82″W
Pleurotaenium sp.	J-E9	A flower basin in Katano Kamoike, Kaga, Japan	36°19′17.23″N; 136°17′37.26″E
Pleurotaenium sp.	Q18	A littoral zone of Lac de Cazaux, Aquitaine, France	44°31′08.32″N; 1°10′44.69″W
Pleurotaenium ovatum	J2	Midorogaike pond, Kyoto, Japan	35°03′26.66″N; 135°46′06.13′'E
Pleurotaenium trabecula	634	Étang Hardy, Aquitaine, France	43°43′04.19″N; 01°21′57.15″W
Pleurotaenium trabecula	655	A pool near Hostens, Aquitaine, France	44°31′07.08″N; 00°36′51.18″W
Pleurotaenium trabecula	748	A pool near Derrycunihy, Kerry, Ireland	51°57′57.66″N; 9°35′49.24″W
Pleurotaenium trabecula	H40	The mesotrophic sandpit Cep, Czech Republic	48°55′04.03″N; 14°52′56.72″E
Triplastrum simplex	Q13	The littoral zone of Lac de Cazaux, Aquitaine, France	44°31′08.32″N; 1°10′44.69″W

Sequence alignment, model selection, and phylogenetic analysis

Two different alignments were constructed for the phylogenetic analysis:

1. a concatenated *rbc*L + *cox*III alignment of 104 desmid sequences selected to encompass all Desmidiaceae lineages, including *Triplastrum*, *Docidium*, and *Pleurotaenium* sequences, determined in this study; and
2. a *trn*G^{ucc} alignment of 24 *Pleurotaenium* sequences, which were all determined in this study.

The list of all sequences involved in phylogenetic analysis, including the GenBank accession numbers, is given in online resource 1. The *trn*G^{ucc} sequences were manually aligned in MEGA 4 (Kumar et al. 2008). The final *rbc*L + *cox*III alignment was generated as follows. First, we downloaded 82 core *rbc*L + *cox*III sequences from GenBank database to encompass most of the Desmidiales lineages, using the phylogeny published by Hall et al. (2008) as a guide. We then added 35 additional *rbc*L sequences selected according to the desmid phylogeny recently published by Gontcharov and Melkonian (2011) to cover the remaining Desmidiales lineages. Finally, 19 *rbc*L and 20 *cox*III sequences determined in this study together with seven closely related sequences revealed by BLAST searches were added to the alignment and manually aligned in MEGA 4. The final concatenated matrix contained 104 taxa, which was 1,885-bp long, and was 98 % filled for the *rbc*L data and 58 % filled for the *cox*III data. The matrix is available from the corresponding author.

A suitable partitioning strategy and partition-specific substitution models for the *rbc*L + *cox*III dataset were selected in a multi-step process (Verbruggen et al. 2010). Initially, a guide tree was obtained by conducting a second-level maximum-likelihood (ML) search on the unpartitioned dataset with an HKY + Γ_8 model by using

Treefinder (Jobb 2008). The dataset was then divided by four different partitioning strategies, combining different codon position segmentation. For each partition present in these partitioning strategies, 12 different nucleotide substitution models were evaluated (F81, HKY, GTR, and their combinations with Γ, I, and Γ + I). Subsequently, Bayesian information criterion (BIC) calculations were performed for all four potential partitioning strategies, assuming the guide tree and evaluated models for each partition. This BIC-based model selection procedure selected the following partitioning strategy with four partitions (receiving the lowest BIC score):

1. first and second codon position of *rbc*L (GTR + Γ);
2. third codon position of *rbc*L (GTR + Γ);
3. second codon position of *cox*III (HKY + Γ); and
4. third codon position of *cox*III (GTR + Γ).

The most appropriate substitution model for the *trn*G^{ucc} dataset was estimated by use of the Akaike information criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander 2004).

The phylogenetic trees were inferred with Bayesian inference (BI) by using MrBayes version 3.1 (Ronquist and Huelsenbeck 2003). Analysis of the *rbc*L + *cox*III dataset was carried out on a partitioned dataset by using the strategy selected during the multi-step process described above. All parameters were unlinked among partitions. In the BI analyses, two parallel Markov chain Monte Carlo (MCMC) runs were carried out for 3 million generations each with one cold and three heated chains. Trees and parameters were sampled for every 100 generations. Convergence of the two cold chains was checked and "burn-in" was determined by use of the "sump" command. Bootstrap analysis was performed by ML and weighted parsimony (wMP) criteria by using Treefinder and PAUP version 4.0b10 (Swofford 2002), respectively. ML bootstrap values were obtained by running ML bootstrap analysis (100 replicates) under the partitioned *rbc*L + *cox*III and unpartitioned *trn*G^{ucc} datasets. The wMP bootstrapping (1,000 replicates) was performed using heuristic searches with 100 random sequence addition replicates, TBR swapping, and random addition of sequences (the number was limited to 10,000 for each replicate). The weight to the characters was assigned using the rescaled consistency index on a scale of 0–1,000. New weights were based on the mean of the fit values for each character over all of the trees in memory.

Results

The *Triplastrum* cells from Lac de Cazaux had typical baculiform morphology. The cells were 65–90 µm long and 8.5–13.0 µm broad. Cells with subapical narrowing frequently occurred in the natural samples (Fig. 1a). Conversely, the cultured populations had rather straight rod like cells (Fig. 1b), even if some cells with subapical narrowing were also present. There were 2–4 lobed plastids per cell, each with a centrally located pyrenoid (Fig. 1a–c). The cell wall was covered with minute pores (Fig. 1d, e), and a 2–3 µm thick sheath envelope was also observed (Fig. 1c). The apical parts of the cells were typically elongated into three terminal lobes (Fig. 1f–i). However, four apical lobes were observed on many cells (Fig. 1j). Interestingly, some semicells had triradiate outlines down to the isthmus level (Fig. 1f, g). The spines were present on the tips of the terminal lobes. Most often, there were two spines per lobe (Fig. 1g, h, j), but their number varied from one to three in the natural samples and in the cultured clonal populations (Fig. 1g–j).

Morphology of the other investigated strains corresponded to the well-known desmid morphospecies with baculiform cells (for identification, see Table 1). The *Docidium* cells were characterised by elongated cylindrical, more or less straight cells, with a shallow sinus and truncate, or truncately rounded, and smooth apices (Fig. 2a). The whorl of longitudinal, granule-like plications—a characteristic feature of this genus—was also present (Fig. 2b). The cells of the genus *Pleurotaenium* differed by the absence of this whorl of plications, by parietal chloroplasts, and by the whorl of granules or tubercles at the apex (Fig. 2c–k). In total, representatives of seven *Pleurotaenium* species have been found: *P. ovatum* (Fig. 2d), *P. ehrenbergii* (Fig. 2e), *P. crenulatum* (Fig. 2f), *P. trabecula* (Fig. 2g), *P. archeri* (Fig. 2h), *P. nodosum* (Fig. 2i), and *P. nodulosum* (Fig. 2j). *Pleurotaenium ovatum* was clearly distinguished from all the other species of the genus by the thick, oval-shaped cells with 2.5–3.5:1 length-to-width ratio. The cells were 270–320 µm long and 90–130 µm broad. *P. ehrenbergii* had cylinder-shaped semicells and there were 4–6 distinct granules along the semicells apices. The cells of this morphospecies were 250–450 µm long and 15–30 µm broad. *P. crenulatum* had, typically, 350–450 µm long and 35–45 µm broad cells distinctly tapering toward the apex, which bore 6–8 small granules. The strains of *P. trabecula* were typically slightly tapering toward the smooth, rounded apices; their cells were 350–750 µm long and 30–50 µm broad. *P. archeri* was distinguished by a characteristic deep constriction above the basal inflation. The apices of this species had 4–7 marginal granules. The cells of this species were 450–800 µm long and 35–55 µm broad. The two investigated strains of *P. nodosum* were clearly distinguished by 4–5 rings of conspicuous projections in each semicell. Finally, *P. nodulosum* was identified by relatively thick cylinder-shaped cells (450–550 µm long and 45–60 µm

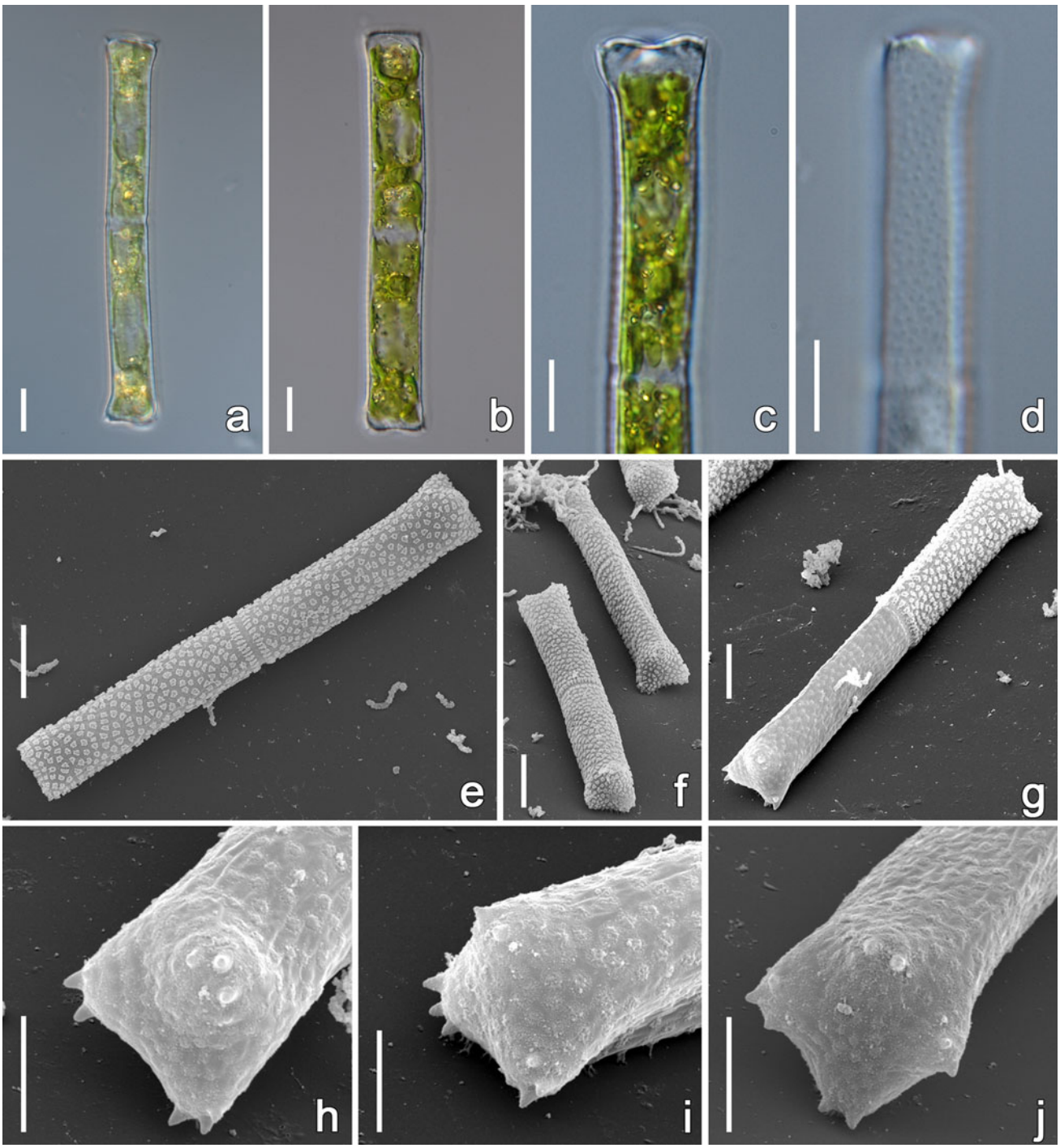


Fig. 1 Morphology of *Triplastrum simplex* (Q13). **a** Morphology of a cell found in the natural sample, **b** morphology of a cultured cell. Note the sheath envelope surrounding the cell, **c** detail of one semicell containing two plastids with distinct pyrenoids, **d** empty semicell showing cell-wall ornamentation, **e** cell-wall ornamentation, **f** apical parts of two *Triplastrum* cells, typically elongated into three terminal lobes (note triradiate outline of the rear cell), **g** triradiate *Triplastrum* cell, **h** three terminal lobes bearing two or three spines, **i** terminal lobes bearing three spines, **j** four terminal lobes. *Scale bar* = 10 μm (Fig. 1a–g) or 5 μm (Fig. 1h–j)

broad) and by a ring of small granules that were typically visible along the semicell apices. Five additional *Pleurotaenium* strains could not be allocated with certainty to any of the previously described species on the basis of the traditional morphological delimitation criteria, for example semicell shape, dimensions, or cellular apex ornamentation (Fig. 2k). Therefore, these strains were tentatively labelled as *Pleurotaenium* sp.

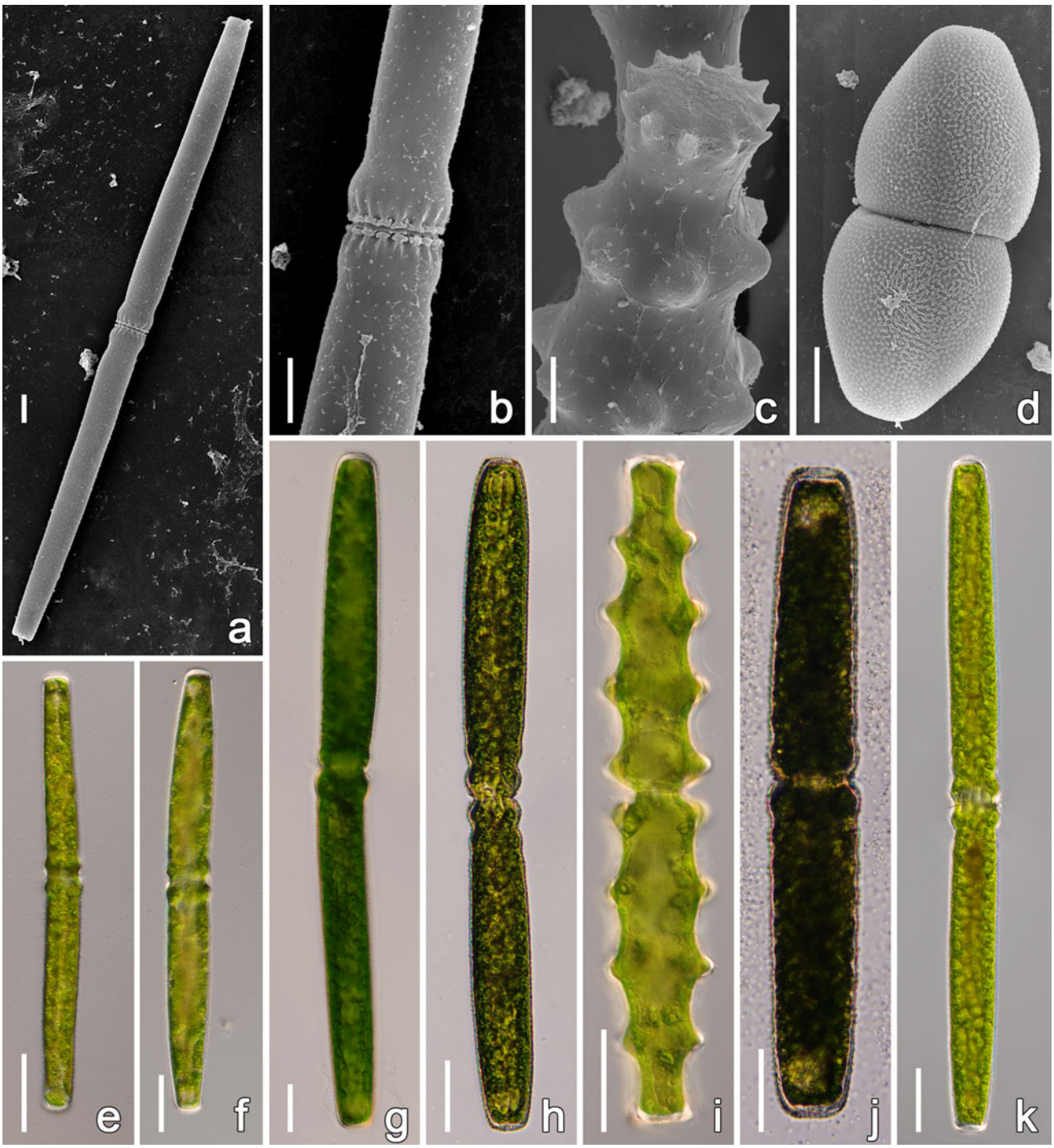


Fig. 2 Morphology of baculiform taxa *Docidium* and *Pleurotaenium*. **a**, **b** *Docidium baculum* (I1), **a** overall morphology, **b** middle of the cell showing a whorl of longitudinal, granule-like plications at the basal semicell inflations. **c**–**k** *Pleurotaenium*, **c** apex of the cell bearing a whorl of tubercles (I3), **d** *P. ovatum* (J2), **e** *P. ehrenbergii* (579), **f** *P. crenulatum* (592), **g** *P. trabecula* (655), **h** *P. archeri* (H50), **i** *P. nodosum* (I8), **j** *P. nodulosum* (H37), **k** *Pleurotaenium* sp. (J–E9). *Scale bar* = 10 μm (Fig. 2**a**–**c**) or 50 μm (Fig. 2**d**–**k**)

The concatenated *rbcL* + *cox*III phylogenetic tree constructed by Bayesian inference on partitioned datasets inferred the investigated baculiform desmid species in three distantly related positions within Desmidiaceae (Fig. 3). *Triplastrum simplex* was nested within the firmly supported "omniradiate" lineage (sensu Gontcharov 2008), in a sister position to a morphologically very different taxon, *Spondylosium luetkemuelleri* Grönblad. The two strains of *Docidium baculum* were highly similar in their nucleotide sequences, forming a distinct lineage inferred

within the *Staurastrum/Micrasterias* clade (Fig. 3). This lineage was distantly related to two other baculiform strains, *Docidium undulatum* and *Haplotaenium minutum*, albeit with relatively low statistical support (BI/ML/MP support 0.86/–/88). Finally, all *Pleurotaenium* strains were inferred as closely related to each other, supporting the monophyly of the genus revealed by Hall et al. (2008) and Gontcharov and Melkonian (2011). With the single exception of the morphologically distinct *P. ovatum*, all the other *Pleurotaenium* strains formed a single, strongly supported lineage within Desmidiaceae. *P. ovatum* was placed in a sister position to the *Pleurotaenium* lineage but with very low statistical support (0.76/–/–).

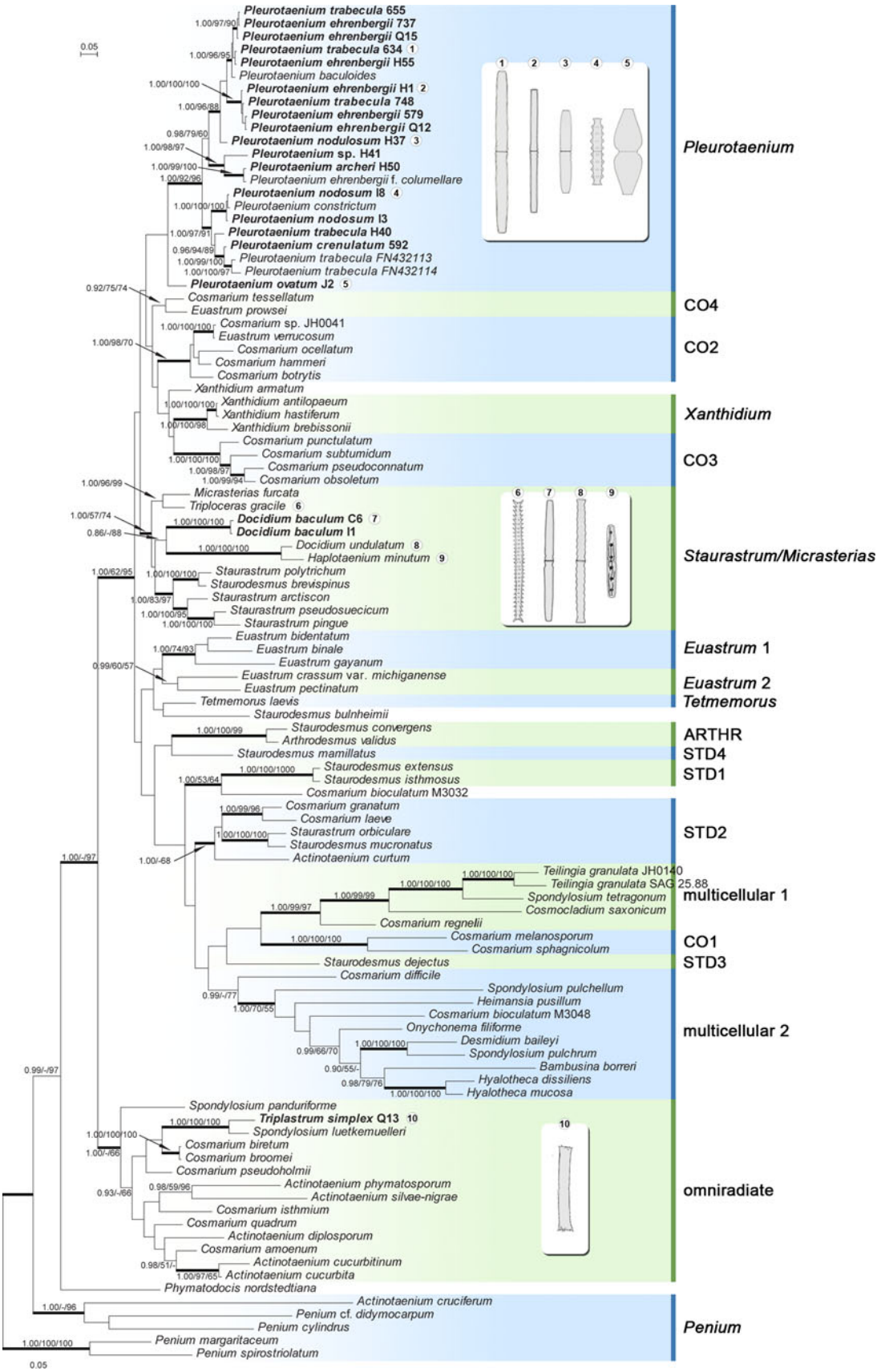
To resolve better phylogenetic relationships among *Pleurotaenium* strains, we also analysed the *trn*G^{ucc} sequences obtained for 24 *Pleurotaenium* strains (Fig. 4). The *trn*G^{ucc} phylogeny was in agreement with that of the *rbcL* + *cox*III phylogram, generally revealing poor species concept in this genus. The most common species of the genus *P. ehrenbergii* and *P. trabecula* were resolved as polyphyletic because their strains were distributed among several strongly supported clades in the tree. However, two strains of *Pleurotaenium nodosum*, typically with the prominent knot-like projections (Fig. 2i), formed a separate firmly supported lineage within the genus. Similarly, the two strains of *Pleurotaenium archeri* also clustered together and formed a distinct clade of the *Pleurotaenium* lineage.

Discussion

In total, four species (i.e., *T. abbreviatum*, *T. indicum*, *T. simplex*, and *T. spinulosum*) have been described within the genus *Triplastrum*. However, their separate status remained unclear because reliable characters for their discrimination are lacking. Růžička (1977) suggested that the number of plastids may possibly be used for species delimitation. However, there were 2–4 plastids in our samples including the clonal strains. Similar variation in plastid number was also observed by Claassen (1977) so this character can hardly be regarded as sufficiently stable for species discrimination. At the same time, overall cell shape, especially subapical narrowing of semicells, was used for distinguishing *T. spinulosum* var. *africanum* by Gauthier-Lièvre (1960). However, Couté and Rousselin (1975) reported high variability of this feature and suggested that *T. spinulosum* var. *africanum* may be conspecific with other varieties of this species. We certainly cannot disprove their view. In our material, the straight rod like cells and the cells with narrowed subapical parts were both present. Most taxonomic value was attributed to the varying number of apical spines on terminal cell lobes among different morphologically defined species (Krieger 1937; Hinode 1952; Gauthier-Lièvre 1960). However, we ascertained that this character was highly variable both in natural populations and cultures. The number of spines varied from one to three, with most lobes bearing two apical spines. Therefore, our strains could be identified as *T. simplex*, in agreement with the original description of Allorge (1924) from Lac de Grand-Lieu close to Nantes (Pays de la Loire, France). However, Kouwets (1998) identified his specimens from nearby Aquitaine as *T. spinulosum* var. *indicum* because the cells frequently had 3–4 terminal spines on the apical lobes. Interestingly, in our samples the cells bearing more than two spines were sometimes also present. Therefore, our observations certainly do not contradict the hypothesis of Coesel and Van Geest (2008) that all findings of *Triplastrum* may be of a single species. However, bearing in mind that cryptic and pseudocryptic species differentiation may apparently not be rare among desmids (Gontcharov and Melkonian 2008; Neustupa et al. 2010; Nemjová et al. 2011; Neustupa et al. 2011), we would not like to formally synonymise these traditional species before molecular data are available for populations from different parts of the world. We should note that this matter may further be complicated by the fact that Iyengar and Ramanathan (1942) did not formally establish a type species for the genus *Triplastrum*.

Our concatenated *rbcL* + *cox*III phylogenetic tree of Desmidiaceae (Fig. 3) generally concurred with previously published desmidiacean phylogenies (Gontcharov 2008; Hall et al. 2008; Gontcharov and Melkonian 2011). The family Desmidiaceae consisted of approximately 20 independent lineages, partly corresponding to individual traditional genera. The traditional genus *Triplastrum* forms a part of the "omniradiate" lineage of Desmidiaceae as defined by Gontcharov and Melkonian (2008, 2011). Because this lineage consists of species with profoundly different morphology, traditional taxonomy placed them into different genera. The only character shared by many of the strains included in this lineage was the omniradiate symmetry of cells in their apical view. The morphology of

Fig. 3 Bayesian analysis based on the combined and partitioned *rbc*L + *cox*III dataset of Desmidiaceae. Four partitions were analysed separately by using a GTR + Γ model for the first and second codon positions of the *rbc*L gene, GTR + Γ model for third codon position of the *rbc*L gene, HKY + Γ model for first and second codon positions of the *cox*III gene, and GTR + Γ model for third codon position of the *cox*III gene. *Values* at the nodes indicate statistical support estimated by three methods—MrBayes posterior-node probability (*left*), maximum-likelihood bootstrap (*middle*), and maximum-parsimony bootstrap (*right*). Thick branches represent nodes receiving the highest PP support (1.00). Species affiliation to 19 desmid clades sensu Gontcharov and Melkonian (2011) is indicated. Morphology of the selected investigated strains is given in the boxes and linked to corresponding sequences by numbers. *Scale bar* shows the estimated number of substitutions per site



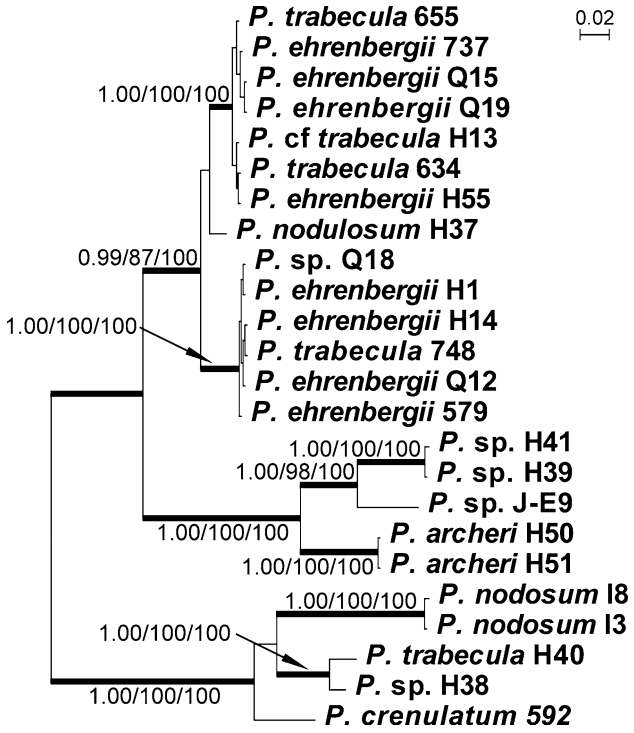


Fig. 4 Bayesian analysis of *Pleurotaenium trn*G^{ucc} sequences, using a GTR + Γ nucleotide substitution model. *Values* at the nodes indicate statistical support estimated by three methods—MrBayes posterior-node probability (*left*), maximum-likelihood bootstrap (*middle*), and maximum-parsimony bootstrap (*right*). *Thick branches* represent nodes receiving the highest PP support (1.00). *Scale bar* shows the estimated number of substitutions per site

the genus *Triplastrum* seems to corroborate this basic morphological delimitation, because the cells usually have cylindrical outlines, differentiating into 3 or 4 terminal lobes (Claassen 1977; Kouwets 1998; this study). However, our SEM investigations occasionally revealed that more or less distinctly triradiate *Triplastrum* semicells may also be formed (Fig. 1f, g). Gontcharov and Melkonian (2008, 2011) suggested that the "omniradiate" lineage may be defined as a separate genus on the basis of their morphology (cylindrical cell outline) and the presence of non-homoplasious synapomorphy consisting of two substitutions in the spacer of helix 25 in the SSU rRNA molecule. However, our morphological investigations did not detect any simple morphological criterion for delimitation of this lineage. Therefore, should the "omniradiate" lineage of Desmidiaceae eventually be described as a separate genus, it may be defined solely on the basis of molecular data. Interestingly, *Triplastrum* was recovered in a sister position to a sequence depicted as *Spondylosium luetkemuelleri* by Gontcharov and Melkonian (2011). It was originally described by Grönblad (1938) from Finland to accommodate natural populations of distinctly *Cosmarium*-like desmids that formed loose filaments resembling other members of the filamentous genus *Spondylosium* Brébisson ex Kützing. However, this species is morphologically entirely different from *Triplastrum* and from the other members of the "omniradiate" clade. Unfortunately, neither clonal strain nor a microphotograph drawing or natural sample of desmid

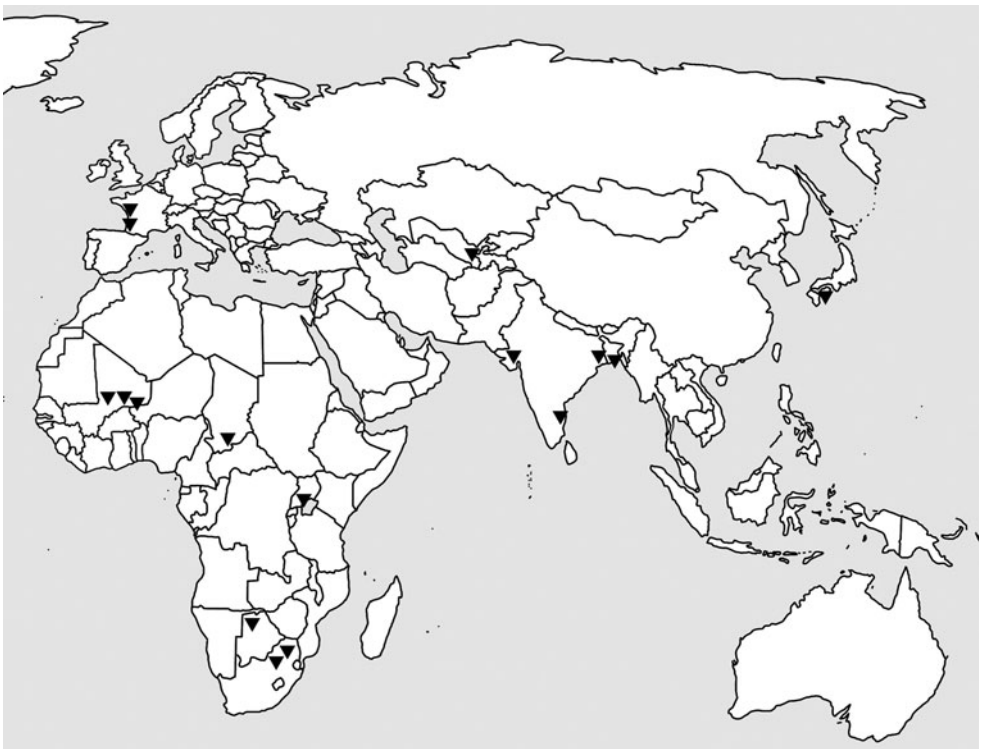


Fig. 5 Geographic distribution of the genus *Triplastrum* showing its presumed occurrence in warmer regions of the old world

related to the *rbcL* sequence (FN432115) deposited by Gontcharov and Melkonian (2011) is available. The original locality of their sampling is also unknown. Therefore, taxonomic affiliation of the closest known relative of *Triplastrum simplex* remains speculative. Molecular characterization of additional *S. luetkemuelleri* strains or natural populations is sorely needed for elucidation of this interesting and rather improbable relationship.

Geographic distribution of many desmids, which is based on numerous floristic accounts, has been well documented (Coesel 1996; Coesel and Krienitz 2008). The genus *Triplastrum*, being distinctly different from other desmids, could possibly not be overlooked for individual investigations. Conversely, it has repeatedly been reported from regions where it is probably widely distributed, for example the Niger basin (Gauthier-Lièvre 1960; Couté and Rousselin 1975), eastern parts of India and Bangladesh (Iyengar and Ramanathan 1942; Islam 1980; Islam and Akter 2005), or western and southwestern France (Allorge 1924; Kouwets 1998; this study). Published records restrict the currently known distribution of *Triplastrum* to Africa, Asia, and Europe (Fig. 5). However, in Europe, a continent with most detailed desmidiacean distribution data available, any future reports of *Triplastrum* outside Aquitaine or other adjacent regions of Western France would be rather surprising. The genus *Triplastrum* is, apparently, a warm-water lineage, and it is probably missing from regions with pronounced annual freezing periods. Even the northern-most findings from France (Allorge 1924; Kouwets 1998; this study), southern Japan (Hinode 1952), or Uzbekistan (Kisselev 1930) originated from localities with warm temperate or subtropical climate. In Lac de Cazaux, it has always been encountered in late summer phytoplankton, i.e., in a period with the highest water temperatures. In cultures, it virtually did not grow at temperatures below 20 °C, and growth was apparently more rapid in temperatures above 25 °C. Undoubtedly, the most natural locations of *Triplastrum* are in tropical habitats. However, in many tropical locations the genus still remains rare and infrequent (Turner 1892; Couté and Rousselin 1975).

The individual lineages of baculiform desmids did not form a clade within Desmidiaceae. Therefore, we can conclude that the rod like morphology evolved repeatedly several times within desmids. Our study confirmed the moderately supported lineage including species traditionally allocated to *Docidium* and *Haplotaenium*. Gontcharov and Melkonian (2011) also illustrated monophyly of these taxa on the basis of *rbcL* sequence data but with no bootstrap support. Interestingly, our strains of *Docidium baculum* from Ireland and the Czech Republic were recovered in a sister position to the firmly supported clades of *Docidium undulatum* and *Haplotaenium minutum*. Therefore, we suggest that the traditional genus *Haplotaenium* may, in fact, be nested within *Docidium*.

In accordance with previous results of Hall et al. (2008) and Gontcharov and Melkonian (2011), the broadly sampled traditional genus *Pleurotaenium* was revealed as a monophyletic lineage within Desmidiaceae. Even the morphologically distinct species *P. nodosum* and *P. ovale* were recovered within this *Pleurotaenium* generic lineage. Interestingly, clearly higher species diversity within *Pleurotaenium* was indicated by the *trn*G^{ucc}-based phylogenetic tree than may be apparent on the basis of morphological data. Two traditional species with relatively few discriminating morphological features, *P. ehrenbergii and P. trabecula*, were found to be polyphyletic, and they will have to be split into several phylogenetic species in the future. There were at least two well-defined phylogenetic species-level lineages including strains with both *P. ehrenbergii* and *P. trabecula* morphology. Likewise, an additional independent clade that included a single strain of *P. trabecula* and an unidentified *Pleurotaenium* sp. strain was also recovered. However, these individual species lineages may also be morphologically discerned after a more detailed study of a large number of strains. Meanwhile, we can conclude that traditional morphological characters distinguishing *P. trabecula* and *P. ehrenbergii*, for example apex morphology and presence or absence of apex granules, are probably not valid. Similar pseudocryptic species diversity was recently identified in the genus *Micrasterias* (Neustupa et al. 2010, 2011). However, individual species-level lineages were defined on the basis of their biogeographic and micro-morphological differences. These characters might also be useful for distinguishing phylogenetic species of the genus *Pleurotaenium*.

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References

Allorge P (1924) Desmidiées du Lac de Grand-Lieu. Rev Algol 1:462–470

Andersen RA, Morton SL, Sexton JP (1997) Provasoli-Guillard national center for culture of marine phytoplankton 1997. List of strains. J Phycol 33(suppl):1–75

Brook AJ (1981) The biology of desmids. Blackwell, Oxford

Claassen MI (1977) Freshwater algae of southern Africa, 2. *Triplastrum spinulosum* from the Transvaal. Bothalia 12:231–237

Coesel PFM (1996) Biogeography of desmids. Hydrobiologia 336:41–53

Coesel PFM, Krienitz L (2008) Diversity and geographic distribution of desmids and other coccoid green algae. Biodivers Conserv 17:381–392

Coesel PFM, Meesters J (2007) Desmids of the lowlands. KNNV, Zeist

Coesel PFM, Van Geest A (2008) Taxonomic and biogeographical notes on Okavango desmids (Zygnematophyceae, Streptophyta). Syst Geogr Pl 78:27–46

Couté A, Rousselin G (1975) Contribution à l'étude des algues d'eau deuce du moyen Niger (Mali). Bull Mus Hist Nat 277:73–175

Gauthier-Lièvre L (1960) Les genres *Ichthyocercus*, *Triploceras* et *Triplastrum* en Afrique. Rev Algol NS 5:55–65

Gontcharov AA (2008) Phylogeny and classification of Zygnematophyceae (Streptophyta): current state of affairs. Fottea 8:87–104

Gontcharov AA, Melkonian M (2005) Molecular phylogeny of *Staurastrum* Meyen ex Ralfs and related genera (Zygnematophyceae, Streptophyta) based on coding and noncoding rDNA sequence comparisons. J Phycol 41:887–899

Gontcharov AA, Melkonian M (2008) In search of monophyletic taxa in the family Desmidiaceae (Zygnematophyceae, Viridiplantae): the genus *Cosmarium*. Am J Bot 95:1079–1095

Gontcharov AA, Melkonian M (2011) A study of conflict between molecular phylogeny and taxonomy in the Desmidiaceae (Streptophyta, Viridiplantae): Analyses of 291 *rbc*L sequences. Protist 162:253–267

Gontcharov AA, Marin B, Melkonian M (2003) Molecular phylogeny of conjugating green algae (Zygnemophyceae, Streptophyta) inferred from SSU rDNA sequence comparisons. J Mol Evol 56:89–104

Grönblad R (1938) Neue und seltene Desmidiaceen. Bot Not 1938:49–66

Hall JD, Karol KG, McCourt RM, Delwiche CF (2008) Phylogeny of the conjugating green algae based on chloroplast and mitochondrial nucleotide sequence data. J Phycol 44:467–477

Hepperle D (2004) SeqAssem©. A sequence analysis tool, contig assembler and trace data visualization tool for molecular sequences. (http://www.sequentix.de)

Hinode T (1952) On some Japanese desmids I. Hikobia 1:145–149

Islam AKMN (1980) Study on *Triplastrum* found in Bangladesh with a note on its species. Bangladesh J Bot 9:1–12

Islam AKMN, Akter N (2005) Desmids of some selected areas of Bangladesh. 3. *Docidium*, *Pleurotaenium*, *Triplastrum* and *Triploceras*. Bangladesh J Plant Taxon 12:11–23

Iyengar MOP, Ramanathan KR (1942) *Triplastrum*, a new member of the Desmidiaceae from south India. J Indian Bot Soc 21:225–229

Jobb G (2008) TREEFINDER version of October 2008. Munich, Germany (http://www.treefinder.de)

Kallio P, Heikkilä H (1969) UV-induced fades changes in *Micrasterias torreyi*. Öster Bot Zeit 116:226–243

Kisselev IA (1930) Periodicity of the "Nau" pond plankton (Old Buchara Usbeckistan). Trans Uzbekistan Inst Trop Med 1

Kossinskaja CC (1960) Desmidiales 1. Flora plantarum cryptogamarum URSS 5

Kouwets FAC (1998) Southern elements in the desmid flora of France. Biologia 53:445–455

Krieger W (1937) Die Desmidiaceen Europas mit Berücksichtigung der aussereuropäischen Arten. K ryptogamenflora, Bd. XIII, Teil 1. Akademisches Verlag, Leipzig

Krieger W (1939) Die Desmidiaceen. Kryptogamenflora, Bd. XIII, Teil 2. Akademisches Verlag, Leipzig

Kumar S, Dudley J, Nei M, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299–306

Nemjová K, Neustupa J, Šťastný J, Škaloud P, Veselá J (2011) Species concept and morphological differentiation of strains traditionally assigned to *Micrasterias truncata*. Phycol Res 59:208–220

Neustupa J, Škaloud P, Šťastný J (2010) The molecular phylogenetic and geometric morphometric evaluation of *Micrasterias crux-melitensis*/*M. radians* species complex. J Phycol 46:703–714

Neustupa J, Šťastný J, Nemjová K, Mazalová P, Goodyer E, Poulíčková A, Škaloud P (2011) A novel, combined approach to assessing species delimitation and biogeography within the well-known desmid species *Micrasterias fimbriata* and *M. rotata* (Desmidiales, Steptophyta). Hydrobiologia 667:223–239

Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University. (http://www.abc.se/~nylander/)

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Růžička J (1977) Die desmidiaceen mitteleuropas band 1, 1. Lieferung, Schweizerbart

Růžička J (1981) Die Desmidiaceen Mitteleuropas, Band 1, 2. Lieferung, Schweizerbart

Škaloud O, Nemjová K, Veselá J, Černá K, Neustupa J (2011) A multilocus phylogeny of the desmid genus *Micrasterias* (Streptophyta): Evidence for the accelerated rate of morphological evolution in protists. Mol Phylogen Evol 61:933–943

Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Turner WB (1892) The freshwater algae of East India. K Svenska Vetensk-Akad Handl 25:1–87

Verbruggen H, Maggs CA, Saunders GW, Le Gall L, Yoon HS, De Clerck O (2010) Data mining approach identifies research priorities and data requirements for resolving the red algal tree of life. BMC Evol Biol 10:1–16

Wodniok S, Brinkmann H, Glockner G, Heidel AJ, Philippe H, Melkonian M, Becker B (2011) Origin of land plants: do conjugating green algae hold the key? BMC Evol Biol 11:104