

Evolutionary relationships between the varieties of green algae *Pediastrum boryanum* and *P. duplex* s.l. (Chlorophyceae, Hydrodictyaceae)

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Abstract: The infraspecific relationships of the two most variable species, *Pediastrum boryanum* and *P. duplex* s.l. identified on the basis of morphological criteria, have been poorly studied so far. The present study focused on 12 original strains isolated from Polish water bodies and 29 strains from the GenBank database, which represent *P. boryanum*, *P. duplex* s.l. varieties and other morphologically similar taxa, against the background of 14 other strains of the family Hydrodictyaceae. In order to estimate the level of congruence between the phylogeny and the classical taxonomic system based on morphological criteria, the strains of *P. boryanum* and *P. duplex* s.l., and other morphologically similar taxa were subjected to parallel phylogenetic analyses applying Bayesian and Maximum Likelihood methods to combined molecular data (26S rDNA and *rbcL* cpDNA) and morphological analyses based on light and scanning electron microscopy, and iconographic documentation of almost all strains published elsewhere. The gene topologies revealed many discrepancies in the morphological features used to delimit the analysed taxa. The polyphyly of both *P. boryanum* and *P. duplex* s.l. was confirmed. *Pseudopediastrum boryanum* var. *cornutum* (formerly *P. boryanum* var. *cornutum*) formed a well supported monophyletic clade, whereas other varieties, including var. *boryanum*, *brevicorne* and *longicorne*, proved to be complex taxa. Previous description of *Lacunastrum gracillimum* (formerly *P. duplex* var. *gracillimum*) was well supported. A clade composed mostly of European strains identified as *P. duplex* var. *rugulosum* should be regarded as this taxon, whereas clades containing strains exhibiting *P. duplex* var. *rugulosum*-like morphology represent new taxa. Taxonomical changes within both *P. boryanum* and *P. duplex* s.l. are required on the basis of a higher number of strains and detailed morphometric data.

Key words: Bayesian phylogenetic analysis, Hydrodictyaceae, Maximum Likelihood, morphological variability, *Pediastrum boryanum*, *Pediastrum duplex*, *Pseudopediastrum*, 26S rDNA, *rbcL* cpDNA

INTRODUCTION

Pediastrum boryanum and *P. duplex* s.l. are microscopic green algal species of the family Hydrodictyaceae, order Sphaeropleales, class Chlorophyceae, which occur commonly worldwide in the phytoplankton, especially of fresh and coastal waters with higher conductivity (KOMÁREK & JANKOVSKÁ 2001; LENARCZYK 2015). Both were described in the first half of the nineteenth century: *P. boryanum* by MENEGHINI (1840), who emended its original description as *Helierella boryana* (TURPIN 1828), and *P. duplex* by MEYEN (1829). As with other representatives of the genus *Pediastrum* MEYEN, the species form flat, star-shaped colonies of cells, called coenobia. As a result of mitotic divisions of a single maternal cell, the cells in a coenobium are all of the same age. The number of cells, usually 8, 16 or 32, is fixed (2ⁿ) and does not change during coenobium growth (KOMÁREK & FOTT 1983; LENARCZYK 2016).

In the most recent key to identification of *Pediastrum* taxa by KOMÁREK & JANKOVSKÁ (2001), nine varieties of *P. boryanum* and four varieties of *P. duplex* are distinguished. What is typical of *P. boryanum* coenobia is a lack of perforations between the U-shaped marginal cells, having two lobes ending with hyaline processes of various length, and the polygonal inner cells, as well as a reticulate wall ornamentation composed of granules situated regularly in the corners of a triangular mesh. In contrast, *P. duplex* always forms perforated coenobia with slender or robust H-shaped cells, its marginal cells have two lobes which end with very short hyaline processes and their wall surface is smooth (without any wall ornamentation), wrinkled or reticulate with irregularly situated granules (PARRA 1979; KOMÁREK & JANKOVSKÁ 2001; LENARCZYK 2014; LENARCZYK & WOŁOWSKI 2016).

According to KOMÁREK & JANKOVSKÁ (2001), both species belong to the most morphologically variable

Pediastrum taxa. The variability does not only follow a genetic basis, but also results from ontogenetic changes – the position of cells in the coenobium – as well as plasticity under different growth environment conditions, such as salinity, composition and concentration of nutrients, and light intensity (CHANG & CHANG–SCHNEIDER 1980; NEUSTUPA & HODAČ 2005; ROJO et al. 2007; LENARCZYK 2014, 2016; LENARCZYK & McMANUS 2016; SUTKOWY 2017). As a consequence of the great variability, several infraspecific taxonomic classifications of *P. boryanum* and *P. duplex* have been introduced, putting different emphasis on individual morphological features (e.g. RACIBORSKI 1889; SULEK 1969; PARRA 1979). These changes in the systematics have resulted in publication of a great number of taxon names, most of them currently regarded as synonyms (PARRA 1979).

In recent years, the phylogenetic studies by McMANUS & LEWIS (2005, 2011), BUCHHEIM et al. (2005), JENA et al. (2014) and LENARCZYK & McMANUS (2016) have thrown little light on the systematics of *P. boryanum* and *P. duplex* s.l.

McMANUS & LEWIS (2005) analysed 26S, 5.8S and ITS–2 rDNA of single strains identified as *P. boryanum* varieties, including the vars. *brevicorne* A. Braun, *cornutum* Sulek and *forcipatum* Parra, two other strains without varietal status, plus eight strains of *P. duplex*, including only one identified to variety level (*asperum* (A. BRAUN) HANSRIG).

BUCHHEIM et al. (2005) published their results on phylogenetic relationships within the genus *Pediastrum* based on 18S, 26S and ITS–2 rDNA regions, analysing only two strains of *P. boryanum*, including one identified as the var. *cornutum*, and two strains of *P. duplex*. They split the genus *Pediastrum* s.l. into five genera, renaming *P. boryanum* as *Pseudopediastrum boryanum* (Turpin) E. Hegewald.

Later, McMANUS & LEWIS (2011) analysed nuclear (26S rDNA) and chloroplast (*rbcL* cpDNA) sequences of *Ps. boryanum* and *P. duplex*. Among the *Ps. boryanum* strains were two identified as the var. *cornutum* forming a common clade, three strains identified as the var. *brevicorne* belonging to separate clades, single strains identified as the var. *boryanum* and var. *forcipatum* related to strains of *Ps. boryanum* or an unidentified *Pseudopediastrum* sp. *Ps. boryanum* was shown to be paraphyletic, with *Ps. kawraiskyi* (Schmidle) E. Hegewald and *P. integrum* Nägeli being nested within a clade composed of most *Ps. boryanum* strains, except for one strain identified as *Ps. boryanum* var. *brevicorne*. Similarly, analysing 36 strains of *P. duplex*, four were identified as the var. *gracillimum* W. West et G.S. West and single strains as vars. *duplex*, *rugulosum* Raciborski and *asperum*, with *P. duplex* being considered nonmonophyletic. Some strains identified as *P. duplex* were more closely related to *P. angulosum* (Ehrenberg) ex Meneghini or *P. cf. alternans* NYGAARD than to other *P. duplex* strains. McMANUS & LEWIS (2011) pointed out that the presence of intercellular perforations and the type of cell wall surface do not

generally correspond with the phylogeny of *P. duplex*. However, the phylogenetic studies on *P. duplex*, supported by precise morphological analyses based on geometric morphometric methods, gave the opportunity to transfer the variety *gracillimum* to a new genus *Lacunastrum*, as *L. gracillimum* (W. West et G. S. West) H. McManus (McMANUS et al. 2011).

In the next studies on *Pediastrum* phylogeny, JENA et al. (2014) sequenced 18S and ITS rDNA regions of five *Ps. boryanum*, including four which were identified as *Ps. boryanum* and one as *Ps. brevicorne* (A. Braun) Jena et C. Bock (formerly *P. boryanum* var. *brevicorne*), forming separate evolutionary lineages, as well as seven strains of *P. duplex* and two *Lacunastrum gracillimum*. A single strain having H-shaped cells typical of *P. duplex*, but with marginal cells situated somewhat out of the plane of the coenobium, was described as *Sorastrum pediastriforme* Jena et C. Bock on the basis of its close relationship with a few strains of the three-dimensional genus *Sorastrum* Kützing.

In the most recent paper on *Pediastrum* phylogeny, LENARCZYK & McMANUS (2016) used phylogenetic and geometric morphometric methods in order to test taxonomical boundaries of *P. alternans* Nygaard. They determined the phylogenetic position, based on three molecular markers (26S, ITS and *rbcL*), of two strains identified as *Ps. alternans* and one as *Ps. boryanum* var. *longicorne* among several other representatives of the family Hydrodictyaceae published elsewhere (McMANUS & LEWIS 2005, 2011).

In all these phylogenetic studies, a low number of strains identified as *P. boryanum* and *P. duplex* varieties were included. The studies did not allow reliable conclusions on phylogenetic relationships of most *P. boryanum* and *P. duplex* varieties and the evolution of their morphology. Consequently, the present study focused on a rich collection of original and published DNA sequences of monoclonal strains identified as *P. boryanum* and *P. duplex* varieties, as well as those with morphology typical of, or close to, the morphology of these species. Detailed morphological analysis consisted in identification of the size of coenobia (number of cells) and perforations between cells, the marginal cell shape and type of the cell wall surface. Phylogenetic analysis was based on two molecular markers (26S nuclear DNA and *rbcL* chloroplast DNA) used jointly. The aim of the present study was to estimate the level of congruence between the phylogeny of *P. boryanum*, *P. duplex* s.l., other similar species and the classical taxonomical system based on morphological criteria.

MATERIALS AND METHODS

A total of fifty five Hydrodictyaceae strains originating from various ecosystems all over the world (Europe – 34 strains, North America – 8 strains, Australia – 6 strains, Africa – 2

strains, and Asia – 1 strain; 4 strains without known locality) were subjected to morphological and/or phylogenetic analysis (Figs 1–6, Tables 1, S1). Among them were forty one strains representing *P. boryanum*, *P. duplex* s.l. varieties and other morphologically similar taxa, as well as fourteen strains representing other taxa of the family Hydrodictyaceae, including *Hydrodictyon* spp., *Monactinus* spp., *Pediastrum angulosum*, *P. braunii*, *Parapediastrium biradiatum*, *Pseudopediastrium kawraiskyi*, *Sorastrum* spp. and *Stauridium tetras*.

Morphological analysis. Altogether, thirty nine strains exhibiting morphology typical of, or close to, *Pediastrum boryanum* s.l. and *P. duplex* s.l. were subjected to morphological analysis (Table 1). Two strains, ACOI 893 and ACOI 1009 included in the phylogenetic analysis (Fig. 1) represented the varieties of the species of interest (*P. duplex* var. *duplex* and *Pseudopediastrium boryanum* var. *cornutum*, respectively), but their morphology could not be analysed because of the lack of iconographic documentation in the literature.

Among thirty nine strains subjected to morphological analysis were fifteen strains examined by the authors using light and scanning electron microscopes, twenty strains examined based on microphotographs and drawings published elsewhere (PARRA 1979; NIELSEN 2000; BUCHHEIM et al. 2005; McMANUS & LEWIS 2005, 2011; McMANUS et al. 2011; JENA et al. 2014; the website of the Algae Culture Collection COIMBRA: <http://acoi.ci.uc.pt/>), as well as four strains examined both on the basis of our own observations and iconographic documentation in the above mentioned literature (Table 1).

Among the strains subjected to our own microscopic observations were twelve original monoclonal strains isolated from field samples collected from lakes and ponds in Poland and seven strains acquired from European algae culture collections: three from the Culture Collection of Algae at Göttingen University in Germany (SAG 29.81, SAG 87.81, SAG 2440), two from the Algae Culture Collection COIMBRA in Portugal (ACOI 75, ACOI 685) and two from the algae culture collection of the W. Szafer Institute of Botany, Polish Academy of Sciences in Poland (01.021213, 04.080210).

The original strains were started with single coenobia, which were isolated using a Narishige MM 188 micromanipulator (Tokyo, Japan) and JenaVal microscope (Carl Zeiss, Jena, Germany). The strains were maintained in sterile liquid L–S₂T₂ medium (LENARCZYK 2014) at ca 22 °C in a Bolarus G–18–2 (Bochnia, Poland) phytotron or in a sterile liquid modified Waris–H medium (LENARCZYK & McMANUS 2016) at ca 20 °C, 15–20 µmol photons.m⁻².sec⁻¹, a 12:12 light:dark regime in a POL–EKO–APARATURA ST2 COMF/S (Wodzisław Śląski, Poland) phytotron (Table 1). The strains were deposited in the algae culture collection of the W. Szafer Institute of Botany PAS (Kraków, Poland). Strains acquired from European algae culture collections were kept in a modified Waris–H medium under the same culture conditions.

Light microscopy (LM) observations were made using a Nikon DS–Fi1 (Tokyo, Japan) digital camera and Nikon Optiphot–2 (strains cultured in L–S₂T₂ medium) or Nikon Eclipse Ni (strains cultured in a modified Waris–H medium) microscopes (Tokyo, Japan). For scanning electron microscopy (SEM), the samples were prepared and analysed as presented in LENARCZYK & McMANUS (2016).

The strains were morphologically compared paying attention to the most prominent morphological features. These were the (1) size of coenobia, (2) size of perforations in coenobia, (3) marginal cell shape and (4) cell wall surface (Table 1). The size of coenobia was described as small for strains

with up to 32–celled coenobia or as large for strains with up to 128–celled coenobia. The analysed coenobia had small, large or no perforations. To minimize the error in defining shape of the marginal cells resulting from morphological variability and the limited source of available microphotographs, only five general types of marginal cell shape were distinguished: rounded or long U–shape, robust or slender H–shape, and rounded O–shape. The cell wall surface was described as smooth or possessing various types of ornamentation, including wrinkles, ridges, triangular mesh, polygonal mesh, thickenings and granules which were situated loosely, of medium density or as dense on the surface. The nomenclature of the surface ornamentation follows LENARCZYK (2014). The names of the published strains were kept as given by previous authors, whereas the names of original strains were given on the basis of morphological observations and identification according to the taxonomic system of KOMÁREK & JANKOVSKÁ (2001).

Phylogenetic analysis. Two molecular markers, nuclear LSU (26S rDNA) and chloroplast RUBISCO LSU (*rbcL*), were sequenced for each of the twelve original strains originating from Polish water bodies. Both gene regions of forty three other strains of the family Hydrodictyaceae were obtained from the GenBank database (National Centre for Biotechnology Information [NCBI] <http://www.ncbi.nlm.nih.gov/>). These strains were published elsewhere by BUCHHEIM et al. (2005), McMANUS & LEWIS (2005, 2011), JENA et al. (2014) and LENARCZYK & McMANUS (2016) (Table S1).

DNA extraction, amplification and sequencing protocols of the twelve original Polish strains follow LENARCZYK & McMANUS (2016). Primers for amplification and sequencing of the 26S rDNA and *rbcL* cpDNA regions are listed in Table 2. The PCR conditions for the 26S rDNA reactions included 94 °C for 3 min and then 35 cycles of 94 °C for 1 min, 59 °C for 1 min and elongation at 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The PCR conditions for the *rbcL* cpDNA reactions were 94 °C for 3 min and then 30 cycles of 94 °C for 1 min 15 sec, 56 °C for 2 min and elongation at 72 °C for 2 min 15 sec, with a final extension step at 72 °C for 7 min.

The consensus 26S and *rbcL* sequences of the original strains (Tables 1 & S1) were automatically built with Variant Reporter, ver. 1.0 (Applied Biosystems). Phylogenetic analyses were performed jointly on the 26S and *rbcL* sequences. *Neochloris aquatica* Starr, from the closely related family Neochloridaceae, was selected as the outgroup. Examined sequences were edited using Geneious, ver. 10.1.3 (Biomatters, USA), and aligned with the Geneious alignment with Muscle algorithm set to default parameters. Regions of partially incomplete data at the beginning and end of the sequences were identified and manually excluded from subsequent analyses with BioEdit, ver. 7.2.5 (HALL 1999). In case of the 26S alignment, 416 bp long fragment of *Parapediastrium biradiatum* (UTEX 37) was excluded from subsequent analyses because of insertion and deletion (indel) polymorphism. Similarly, two substantial indels, 1782 and 1696 bp long fragments, were eliminated from the middle part of the *rbcL* sequence alignment. Here, we identified indel polymorphism for six different accessions: *Lacunastrum gracillimum* (Hg2004–1), *L. gracillimum* (LN0201NC), *L. gracillimum* (SML0401VA), *Pediastrum duplex* var. *duplex* (ACOI 893), *Pseudopediastrium boryanum* var. *cornutum* (UTEX LB470) and *Ps. kawraiskyi* (SAG 35.81). Consequently, alignments of 26S and *rbcL* sequences were respectively 1940 and 1229 bp long. The aligned sequences were concatenated into a single data matrix (26S

Table 1. Morphology of the analysed *Pediastrum boryanum*-like and *P. duplex*-like strains described on the basis of their original microphotographs or published microphotographs and drawings as given in references. Coenobium size is classified as “small” for strains composed of up to 32-celled coenobia or as “large” for strains composed of up to 128-celled coenobia. Perforations are described as “absent”, “small” or “large”. Marginal cell shape is classified as “rounded U-shape”, “long U-shape”, “robust H-shape”, “slender H-shape” or “slender H-shape”. The wall surface is described as “smooth” or possessing various ornamentation ultrastructures, including “wrinkles”, “ridges”, “triangular mesh”, “polygonal mesh”, “loose granules”, “medium dense granules”, “dense granules”, “thickenings”. Original strains isolated from Polish water bodies are in boldface. Original strains and strains acquired from European algae culture collections were cultured in L-S₂T₂(¹) or a modified Warris-H medium (²). Asterisk (*) indicates morphological characteristics other than observed here with appropriate citations in references.

Taxon	Strain no	Coenobium size	Perforations	Marginal cell shape	Cell wall surface	References
<i>Lacunastrum gracillimum</i>	ACOI 392	no data	large	slender H-shape	smooth	McMANUS et al. (2011, fig. 3)
<i>Lacunastrum gracillimum</i>	Hg2004-1	n/d	large	slender H-shape	smooth	McMANUS et al. (2011, fig. 3)
<i>Lacunastrum gracillimum</i>	LN0201NC	small	large	slender H-shape	smooth	McMANUS & LEWIS (2011, fig. 4f); McMANUS et al. (2011, figs 3 & 4)
<i>Lacunastrum gracillimum</i>	SML0401VA	n/d	large	slender H-shape	smooth	McMANUS et al. (2011, fig. 3)
<i>Pediastrum duplex</i>	CR0501a	small	small	slender H-shape	polygonal mesh, thickenings (?)	McMANUS & LEWIS (2011, fig. 3c)
<i>Pediastrum duplex</i>	LB0503a	small	large	slender H-shape	polygonal mesh, thickenings	McMANUS & LEWIS (2011, fig. 4e)
<i>Pediastrum duplex</i>	PL0501b	small	large	slender H-shape	wrinkles, thickenings (?)	McMANUS & LEWIS (2011, fig. 3f)
<i>Pediastrum duplex</i>	SL0401MN	small	large	slender H-shape	polygonal mesh, thickenings	McMANUS & LEWIS (2011, fig. 3b)
<i>Pediastrum duplex</i>	WR0401	small	large	slender H-shape	polygonal mesh, thickenings	McMANUS & LEWIS (2011, fig. 4b)
<i>Pediastrum duplex</i> var. <i>asperum</i>	ACOI 395	small	large	slender H-shape	polygonal mesh, thickenings	Algae Culture Collection COIMBRA, Portugal (http://acoici.ue.pt/)
<i>Pediastrum duplex</i> var. <i>asperum</i>	UTEX LB1364	small	small	robust H-shape	ridges	PARRA (1979, pl. 34, figs a–l); McMANUS & LEWIS (2011, fig. 3d)
<i>Pediastrum duplex</i> var. <i>cohaerens</i>	ACOI 391	n/d	small	robust H-shape	polygonal mesh, thickenings	Algae Culture Collection COIMBRA, Portugal (http://acoici.ue.pt/)
<i>Pediastrum duplex</i> var. <i>duplex</i>	02.310811	small	small	robust H-shape	smooth	original microphotographs ¹ (Fig. 2g–i)
<i>Pediastrum duplex</i> var. <i>rugulosum</i>	05.310112	small	small	robust H-shape	polygonal mesh, thickenings	original microphotographs ¹ (Fig. 2e–f)
<i>Pediastrum duplex</i> var. <i>rugulosum</i>	14.280112	small	small	robust H-shape	polygonal mesh, thickenings	original microphotographs ¹ (Fig. 2c–d)
<i>Pediastrum duplex</i> var. <i>rugulosum</i>	30.260911a	small	small	robust H-shape	polygonal mesh, thickenings	original microphotographs ¹ (Fig. 2a–b)

Table 1 Cont.

<i>Pediastrum duplex</i> var. <i>rugulosum</i>	ACOI 152	small	large	slender H-shape	n/d	Algae Culture Collection COIMBRA, Portugal (http://acoi.ci.uc.pt/)
<i>Pediastrum</i> cf. <i>subgranulatum</i>	19.300112	small	large	robust H-shape	triangular or polygonal mesh, loose granules	original microphotographs ² (Fig. 4d–f)
<i>Pediastrum</i> sp.	RL0201FR	small	absent	long U-shape	triangular mesh, medium dense granules	McMANUS & LEWIS (2005, figs 4–5); McMANUS & LEWIS (2011, fig. 5d)
<i>Pseudopediastrium alternans</i>	01.021213	large	small	long U-shape	wrinkles, thickenings	original microphotographs ² (Fig. 5a–c), Lenarczyk & McManus (2016, figs 6–17, 24–31)
<i>Pseudopediastrium alternans</i> ?	LWiv0503b	small	small	rounded U-shape	wrinkles	McMANUS & LEWIS (2011, fig. 4c)
<i>Pseudopediastrium</i> cf. <i>alternans</i>	SAG 2440 (MJ2008/83)	small/ * small	absent/ * absent	rounded U-shape/ long U-shape	triangular or polygonal mesh, medium dense granules/ * triangular mesh, medium dense granules	original microphotographs ² (Fig. 2j–l), Lenarczyk & McManus (2016, figs 18–20, 32–33)/ * JENA et al. (2014, fig. 4a–b)
<i>Pseudopediastrium boryanum</i>	EL0203CT	small	absent	rounded U-shape	triangular mesh, loose granules	McMANUS & LEWIS (2011, fig. 5b)
<i>Pseudopediastrium boryanum</i>	IL0402MIN	small	absent	rounded U-shape	triangular mesh (?), granules (?)	McMANUS & LEWIS (2011, fig. 5a)
<i>Pseudopediastrium boryanum</i> var. <i>boryanum</i>	02.310112	large	absent	rounded U-shape	triangular mesh, medium dense granules	original microphotographs ¹ (Fig. 5g–i)
<i>Pseudopediastrium boryanum</i> var. <i>boryanum</i>	08.040414	small	absent	long U-shape	triangular mesh, medium dense granules	original microphotographs ² (Fig. 5d–f)
<i>Pseudopediastrium boryanum</i> var. <i>boryanum</i>	ACOI 75	small	absent	rounded U-shape	triangular, polygonal mesh or wrinkles, loose granules	original microphotographs ² (Fig. 6a–c)
<i>Pseudopediastrium boryanum</i> var. <i>cornutum</i>	19.090508	small	small	rounded U-shape	triangular or polygonal mesh, medium dense granules	original microphotographs ² (Fig. 5j–l)
<i>Pseudopediastrium boryanum</i> var. <i>cornutum</i>	UTEX LB470	small	small	robust H-shape	triangular mesh, medium dense (?) granules	PARRA (1979, pl. 21, figs f–j); McMANUS & LEWIS (2005, no iconographic documentation; small perforations and granules on wall surface, as written in the article)
<i>Pseudopediastrium boryanum</i> var. <i>forcipatum</i>	SAG 87.81	small/ * large	small/ * absent	long U-shape/ * rounded U-shape	triangular, polygonal mesh or wrinkles, dense granules/ * triangular mesh or wrinkles, dense granules or without granules	original microphotographs ² (Fig. 4g–i)/ * NIELSEN (2000, figs 39–44); McMANUS & LEWIS (2011, fig. 5c)

Table 1 Cont.

<i>Pseudopediastrum boryanum</i> cf. var. <i>forcipatum</i>	07.030414	small	absent	rounded U-shape	triangular mesh, dense granules	original microphotographs ² (Fig. 6d–f)
<i>Pseudopediastrum boryanum</i> var. <i>longicorne</i>	02.290811a	large	absent	long U-shape	triangular or polygonal mesh, loose granules	original microphotographs ¹ (Fig. 4a–c)
<i>Pseudopediastrum boryanum</i> var. <i>longicorne</i>	04.080210	large	absent	long U-shape	triangular or polygonal mesh, loose granules	original microphotographs ² (Fig. 3j–l), Lenarczyk & McManus (2016, figs 21–23, 34–35)
<i>Pseudopediastrum boryanum</i> var. <i>longicorne</i>	04.020414	small	absent	long U-shape	polygonal mesh or wrinkles, loose granules	original microphotographs ² (Fig. 3g–i)
<i>Pseudopediastrum brevicorne</i>	ACOI 685	small (?)	small (?)	rounded U-shape	n/d	original microphotographs ² (Fig. 3a–c)
<i>Pseudopediastrum brevicorne</i>	SAG 261–7	small	absent	rounded U-shape	triangular mesh, medium dense granules	PARRA (1979, pls 16–17); NIELSEN (2000, figs 11–15)
<i>Pseudopediastrum brevicorne</i> f. <i>granulatum</i>	ACOI 1466	small	absent	long U-shape	triangular mesh, loose granules	Algae Culture Collection COIMBRA, Portugal (http://acoici.uc.pt/)
<i>Pseudopediastrum integrum</i>	SAG 29.81	small	absent	rounded O-shape	triangular or polygonal mesh, loose granules	original microphotographs ² (Fig. 3d–f)
<i>Pseudopediastrum</i> cf. <i>integrum</i>	11.310112	large	absent	rounded U-shape	triangular mesh, loose granules	original microphotographs ¹ (Fig. 4j–l)

+ *rbcL*) with SeaView, ver. 4.6.1. (GOUY et al. 2010). Finally, the concatenated alignment of the 26S and *rbcL* dataset consisted of 3169 characters.

Phylogenetic analyses were performed using two methods: Maximum Likelihood (ML) and Bayesian inference (BI). ML was performed in RAxML, ver. 8.2.11 via the Geneious, ver. 10.1.3 (Biomatters, USA) (STAMATAKIS et al. 2008; STAMATAKIS 2014). ML branch support was estimated using 1000 rapid ML bootstrap pseudoreplicates. We chose the GTRGAMMA nucleotide model because it includes the parameter G for rate heterogeneity among sites. In RAxML, by default, G has 25 rate categories making the estimation of proportion of invariable sites (I) unnecessary because G mathematically accounts for I (STAMATAKIS 2006; NGUYEN et al. 2015).

Prior to BI analysis, jmodeltest, ver. 2.1.6 (DARRIBA et al. 2012), was used to select a TrN + I + G model for 26S, and a GTR + I + G for *rbcL*. Both identified nucleotide evolution models were selected on the basis of Bayesian Information Criterion (SCHWARZ 1978). BI analysis was performed with MrBayes, ver.3.2.2 (RONQUIST et al. 2012). The concatenated data matrix was divided into two partitions, each corresponding to a continuous DNA region. The MCMC algorithm was run for 10⁶ generations with one cold and three heated chains, and storing one tree every 100 generations. The stationary phase was reached after 847.000 generations. Average standard deviation of split frequencies was 0.009968. The first 25% of trees were discarded as burn-in, and a 50% majority-rule consensus tree was constructed with posterior probabilities (PP) as a measure of clade support.

Consensus topologies of ML and BI trees with maximum likelihood bootstrap percentages and Bayesian branch support values were compiled and drawn using FigTree, ver. 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). No topological conflicts were found between ML and BI analyses resulted in identical phylogenetic trees. Thus, we presented one phylogenetic tree (Fig. 1) elaborated using CorelDRAW X4, ver.14 (Corel Corporation).

RESULTS

A total of 22 strains exhibiting morphology typical of, or close to, *Pediastrum boryanum* s.l. and 17 strains morphologically typical of, or close to, *P. duplex* s.l. were subjected to morphological analysis (Table 1). The *Pediastrum boryanum*-like strains represented 14 taxa, including *Pseudopediastrum boryanum* (Turpin) E. Hegewald (2 strains; formerly *P. boryanum* (Turpin) Meneghini), *Ps. boryanum* var. *boryanum* (3 strains), *Ps. boryanum* var. *cornutum* Sulek (3 strains), *Ps. boryanum* var. *forcipatum* Corda (1 strain), *Ps. boryanum* cf. var. *forcipatum* (1 strain), *Ps. boryanum* var. *longicorne* Reinsch (4 strains, including the synonym *Ps. brevicorne* (A. Braun) Jena et C. Bock f. *granulatum* (Raciborski) Parra), *Ps. brevicorne* (2 strains; formerly *P. boryanum* var. *brevicorne* A. Braun), *Ps. alternans* (Nygaard) Jena et C. Bock (1 strain), *Ps. alternans?* (1 strain), *Ps. cf. alternans* (1 strain), *Ps. integrum* (Nägeli) Jena et C. Bock (1 strain), *Ps. cf. integrum* (1 strain),

Table 2. Primers used in amplifying and sequencing the 26S rDNA and *rbcl* cpDNA numbered from the 5' end. F indicates forward and R indicates reverse primers.

Primers for 26S rDNA	Sequence (5'–3')
ITS4rcR	GCATATCAATAAGCGGAGGA
LS1R	GTACCGTGAGGGAAGA
LS13F	GCTTACCAAAAATGGC
LS14R	GGCCATTTTTGGTAAGC
LS18F	TCCCCTTGTCCTACCAGT
LS20F	TTTCTCCACTTCAGTCTTC
26F	CAGAGCACTGGGCAGAAATCAC
26BF	GGTCCGTGTTTCAAGACG
26BrC	CCCGTCTTGAAACACGGA
Primers for <i>rbcl</i> cpDNA	Sequence (5'–3')
M35F	GGRWTTAAAGCTGGTGTAAGACT
M379F	GGTTTCAAAGCTCTTCGTGC
M633F	CATGCGTTGGAGAGACCGT
M650R	CGGTCTCTCCAACGCATGA
M955F	CGTWTATCTGGTGGAGAYC
M1010R	CCTTCAAGTTTACCTACAAC
M1161R	CATGTGCCATACGTGAATAC
M1338R	AATTCWGGACTCCATTTACAAGC

P. cf. subgranulatum Komárek et Jankovská (1 strain) and *Pediastrum* sp. (1 strain). They usually formed small coenobia without perforations between rounded U-shaped marginal cells and polygonal inner cells, which were covered with triangular mesh and granulations of various density. Only exceptionally did the strains also have large coenobia composed of more than 32 cells, coenobia with perforations, long U-shaped, rounded O-shaped or even robust H-shaped marginal cells, as well as polygonal mesh, wrinkles and thickenings on the cell wall surface.

In contrast, the *P. duplex*-like strains were represented only by small perforated coenobia, usually with large holes between slender or robust H-shaped cells. They were identified as 6 taxa, including *P. duplex* Meyen (5 strains), *P. duplex* var. *duplex* (2 strains), *P. duplex* var. *asperum* (A. Braun) Hansgirg (2 strains), *P. duplex* var. *cohaerens* Bohlin (1 strain), *P. duplex* var. *rugulosum* Raciborski (4 strains) and *Lacunastrum gracillimum* (W. West et G.S. West) H. McManus (4 strains; formerly *P. duplex* var. *gracillimum* W. West et G.S. West) (Tables 1, S1).

In the phylogenetic tree from Bayesian analysis of joint 26S rDNA and *rbcl* cpDNA (Fig. 1), *Pediastrum boryanum*-like and *P. duplex*-like strains, as well as strains representing other morphological types, including

those of *Hydrodictyon*, *Monactinus*, *P. angulosum* and *Ps. kawraiskyi*, form a well supported clade (Bayesian Posterior Probability BPP 0.998, Maximum Likelihood ML 66), which is sister to a clade composed of *Sorastrum* and *Parapediastrum* strains. The strains of *P. braunii* and *Stauridium tetras* form outer branches.

Pediastrum duplex-like strains

All the isolates of *Lacunastrum gracillimum* from Europe and North America are gathered in a well resolved lineage (GROUP I in Fig. 1; BPP 1, ML 100), which, together with two isolates of *Monactinus* spp. gathered in a sister lineage, form a well supported branch (BPP 0.987, ML 56). The *L. gracillimum* strains share similar morphological features; their coenobia have large perforations between slender H-shaped cells and their cell wall is smooth (not ornamented).

The other *Pediastrum duplex*-like strains form one well resolved lineage (BPP 1, ML 98), which is sister to a lineage composed of all *P. boryanum*-like strains. Both lineages form a strongly supported branch (BPP 1, ML 84).

The lineage composed of the other *P. duplex*-like strains is divided into two well resolved clades, the upper and the lower one.

In the upper clade (BPP 0.999, ML 79), a strongly

supported GROUP II (Fig. 1; BPP 1, ML 99) is composed of 6 strains, mainly European *P. duplex* var. *rugulosum* (30.260911a, 14.280112 and 05.310112 from Poland, and ACOI 152 from Portugal), which have slender or robust H-shaped cells and polygonal mesh with thickenings on the cell wall surface (Fig. 2a–f). The described group also contains one European strain identified as *P. duplex* var. *asperum* (ACOI 395 from the Czech Republic), which exhibits the same morphology as the strains identified to the variety *rugulosum*, and one Australian *P. duplex* strain (CR0501a), whose morphology (indistinctly perforated coenobia with slender H-shaped cells covered with polygonal mesh and putative thickenings) is close to the morphology of the strains identified as the variety *rugulosum*.

In the upper clade, two European isolates of *P. duplex* var. *duplex* (ACOI 893 and 02.310811), whose coenobia possess a smooth cell wall surface (Fig. 2g–i), also form a strongly supported monophyletic branch (GROUP IV in Fig. 1; BPP 0.986, ML 94). Similarly, three strains of *Hydrodictyon* spp. are gathered in a well supported branch in the upper clade composed mostly of *P. duplex*-like strains. However, the strains of *P. duplex* var. *duplex* are more closely related to the *P. duplex* var. *rugulosum*-like strains than to the strains of *Hydrodictyon* spp.

In the upper clade, three other strains are also located. One of them, identified as *Pediastrum duplex* var. *cohaerens* (ACOI 391 from Portugal; GROUP III in Fig. 1), exhibiting morphology typical of the *P. duplex* var. *rugulosum* strains from Poland, is most closely related to the strains of GROUP II (Fig. 1) representing this variety. However, the relationship is weakly supported (BPP < 0.95, ML < 50). The second strain, identified as *P. duplex* (SL0401MN; GROUP V in Fig. 1) from North America, which possesses a cell wall surface ornamented as in the *P. duplex* var. *rugulosum* strains, is most closely related to the strains of GROUP IV (Fig. 1) representing the variety *duplex*, but again with weak support. The last strain in the upper clade represents *P. duplex* var. *asperum* (UTEX LB1364; GROUP VI in Fig. 1) from Europe, whose coenobia with robust H-shaped cells are covered with ridges. This strain is basal to a weakly supported branch (BPP < 0.95, ML < 50) containing all the other strains of the upper clade.

The lower strongly supported clade (GROUP VII in Fig. 1; BPP 1, ML 100) contains three Australian *Pediastrum duplex* isolates (LB0503a, WR0401 and PL0501b), three *P. angulosum* strains (HL0201WI, ACOI 1354 and MB0403b) from distant localities (North America, Europe and Australia, respectively) and an Australian strain identified as *Pseudopediastrum alternans?* (LWiv0503b). The *P. duplex* strains have distinctly perforated coenobia with slender H-shaped cells and cell wall ornamentation (polygonal mesh or wrinkles with clearly visible or questionable thickenings) resembling that of *P. duplex* var. *rugulosum*. The strain identified as *Ps. alternans?* has rounded U-shaped marginal cells

which are typical of *P. boryanum*, but its cell wall ornamentation is composed of wrinkles without granules or thickenings. In GROUP VII, only the Australian *Ps. alternans?* strain and another strain from Australia identified as *P. angulosum* form a strongly supported branch (BPP 0.968, ML 85). The relationships between the other isolates of GROUP VII are weakly supported (BPP < 0.95, ML < 70).

***Pediastrum boryanum*-like strains**

The strains which represent morphology typical of, or close to, *Pediastrum boryanum*, except for the Australian *Pseudopediastrum alternans?* strain located in GROUP VII mentioned above and a European strain identified as *Pediastrum* sp. (RL0201FR; GROUP VIII in Fig. 1) forming small compact coenobia with long U-shaped marginal cells and cell walls covered with triangular mesh and medium dense granulation, are gathered in a strongly supported branch (BPP 0.996, ML 85). This branch is divided into two well resolved lineages (both having BPP 1 and ML 100).

One of these lineages (GROUP IX in Fig. 1) contains two strains, one identified as *Pseudopediastrum brevicorne* (ACOI 685, from Europe), and the second identified as *Ps. cf. alternans* (SAG 2440, from Africa). Whereas the morphology of the *Ps. cf. alternans* strain (Fig. 2j–l) is well resolved (small compact coenobia with rounded or long U-shape cells covered with mesh and medium dense elongated granules), the typical morphology of the *Ps. brevicorne* strain is questionable as it often occurs in the form of planar or three-dimensional chaotic arrangements of cells (Fig. 3a–c).

This two-strained clade is sister to a lineage in which a strain identified as *Ps. integrum* (SAG 29.81, no locality) and a strain of *Ps. kawraiskyi* (SAG 35.81, from Europe) form a well supported clade (GROUP X in Fig. 1; BPP 1, ML 99). The coenobia of SAG 29.81 are small and compact, and their marginal cells are rounded O-shaped with one or two processes (Fig. 3d–e). Its cell wall ornamentation is composed of loosely arranged granules on a triangular or polygonal mesh (Fig. 3f).

The *Pseudopediastrum integrum/kawraiskyi* clade (GROUP X in Fig. 1) is basal to all *Ps. boryanum* strains, single strains of *Pediastrum cf. subgranulatum* (19.300112), *Ps. alternans* (01.021213), *Ps. brevicorne* (SAG 261–7) and *Ps. cf. integrum* (11.310112), being gathered in a well supported (BPP 1, ML 100) branch. This branch, composed mostly of *Ps. boryanum* strains, is divided into five more or less supported lineages.

The first lineage is weakly supported (BPP < 0.95, ML < 50). It is divided into two branches. The first weakly supported branch is divided into three clades with two strains in each clade (GROUPS XI–XIII in Fig. 1). The first clade (GROUP XI; BPP 0.991, ML 81) is composed of two Polish lowland strains identified as *Ps. boryanum* var. *longicorne* (04.020414 and 04.080210), sharing similar morphology, including the lack of perforations between cells, long U-shaped marginal cells

and the type of wall ornamentation being a polygonal mesh with loosely situated rounded or distorted granules (Fig. 3g–l). The second clade (GROUP XII in Fig. 1; BPP 0.998, ML 79) is composed of morphologically very similar strains from Europe, including 02.290811a identified as *Ps. boryanum* var. *longicorne* (Fig. 4a–c) and ACOI 1466 identified as *Ps. brevicorne* f. *granulatum* (the synonym of *Ps. boryanum* var. *longicorne*). The strains have small or large coenobia, but share the lack of perforations, the long–U shape of marginal cells and loose granulation situated on a triangular or polygonal mesh. The rounded granules in 02.290811a are of different sizes (Fig. 4c). The third clade (GROUP XIII in Fig. 1; BPP 0.992, ML 50) is composed of two European strains differing in origin and morphology. The strain identified as *Pediastrum* cf. *subgranulatum* (19.300112) from a mountain lake in Poland has small distinctly perforated coenobia, robust H-shaped marginal cells with short processes and loose granulation (Fig. 4d–f). In contrast, the strain identified as *Pseudopediastrum boryanum* var. *forcipatum* (SAG 87.81) from a lowland lake in Germany has small or large coenobia either lacking or with small perforations, long or rounded U-shape marginal cells, sometimes with converging processes, as well as dense or no granulation (Fig. 4g–i).

The second branch of the first lineage is strongly supported (GROUP XIV in Fig. 1; BPP 0.996, ML 92). It includes a strain identified as *Ps. cf. integrum* (11.310112) and a strain identified as *Ps. alternans* (01.021213). Both originate from Polish water bodies, a mountain and a lowland lake, respectively. Although they both possess large coenobia, they clearly differ with respect to the presence of perforations between cells, the shape of marginal cells and the wall ornamentation (Figs 4j–l, 5a–c).

The second lineage (GROUP XV in Fig. 1; BPP 1, ML 99) is composed of two European strains identified as *Ps. boryanum* var. *boryanum* (08.040414 and 02.310112; Fig. 5d–i), a North American strain of *Ps. boryanum* not identified to the variety level (IL0402MN) and a strain identified as *Ps. brevicorne* (SAG 261–7, without locality information). The morphology of these strains is similar, but not identical (small or large compact coenobia with rounded or long U-shaped marginal cells and triangular mesh with medium dense granules on the cell wall surface, not readily visible in the strain IL0402MN). In this lineage, the strain of *Ps. boryanum* var. *boryanum* no. 08.040414 is more closely related to the strain SAG 261–7 of *Ps. brevicorne* than to the other strain of *Ps. boryanum* var. *boryanum* (02.310112).

The third lineage (GROUP XVI in Fig. 1; BPP 0.979, ML 94) is composed of three strains identified as *Ps. boryanum* var. *cornutum* (19.090508, UTEX LB470 and ACOI 1009, from European lowland localities). Among them, the morphology is resolved for 19.090508 (Fig. 5j–l) and UTEX LB470. Their small coenobia with small perforations have a cell wall surface covered with mesh and granules, but their marginal cells are rounded

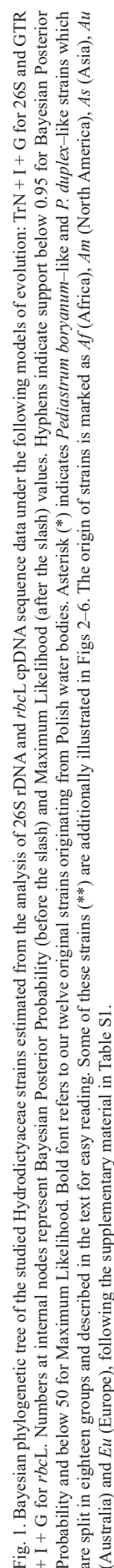
U- or robust H-shaped, respectively. The fourth lineage (GROUP XVII in Fig. 1; BPP 1, ML 98) is composed of two morphologically very similar strains from Europe and North America, including one identified as *Ps. boryanum* var. *boryanum* (ACOI 75; Fig. 6a–c) and the second not identified to the variety level (EL0203CT). The strains have small compact coenobia, rounded U-shaped marginal cells and loose granules on the cell wall surface. The granules in ACOI 75 are small and delicate (Fig. 6c). The last lineage (GROUP XVIII in Fig. 1) contains only one European strain identified as *Ps. boryanum* cf. var. *forcipatum* (07.030414). It has small compact coenobia, rounded U-shape marginal cells and dense granulation and a triangular mesh on the cell wall surface (Fig. 6d–f).

DISCUSSION

In the present study, the phylogenetic relationships among some varieties of *Pediastrum boryanum* and *P. duplex* s.l., morphologically similar taxa and other representatives of the family Hydrodictyaceae, shown on the basis of a joint analysis of two molecular markers, including 26S rDNA and chloroplast *rbcL*, were resolved.

All strains identified as *P. duplex* or its varieties, including *duplex*, *asperum* (A. Braun) Hansgirg, *cohaerens* Bohlin, *gracillimum* W. West et G.S. West and *rugulosum* Raciborski, form seven different groups in the obtained phylogenetic tree. Similarly, in the studies by McMANUS & LEWIS (2011), JENA et al. (2014) and LENARCZYK & McMANUS (2016), *P. duplex* strains were divided in two or three groups, including strains identified as *Lacunastrum gracillimum* (W. West et G.S. West) H. McManus (formerly *P. duplex* var. *gracillimum*) and forming a monophyletic clade which was most closely related to *Monactinus* Corda strains. The latter taxon, formerly known as *P. simplex* Meyen, forms coenobia composed of triangular marginal cells with only one lobe and process, unlike *P. boryanum* and *P. duplex* s.l. (KOMÁREK & JANKOVSKÁ 2001). The present results show the monophyly of *L. gracillimum* (GROUP I) on the basis of strains from distant localities in Europe and North America, confirming the results of a number of previous authors (McMANUS & LEWIS 2011; McMANUS et al. 2011; JENA et al. 2014).

Besides the variety *gracillimum*, having slender H-shaped cells and a smooth cell wall surface, KOMÁREK & JANKOVSKÁ (2001) distinguished three other varieties in *P. duplex*, having robust H-shaped cells and various types of cell wall surface. The surface can be smooth in the variety *duplex* or ornamented in the varieties *asperum* (araneose ornamentation composed of coarse ridges) and *rugulosum* (net-like ornamentation composed of fine wrinkles and thickenings called *verrucae*). One of the strains analyzed in this study, ACOI 391 from Europe (GROUP III), was named *P. duplex* var. *cohaerens* in the literature. KOMÁREK & JANKOVSKÁ (2001) treated this



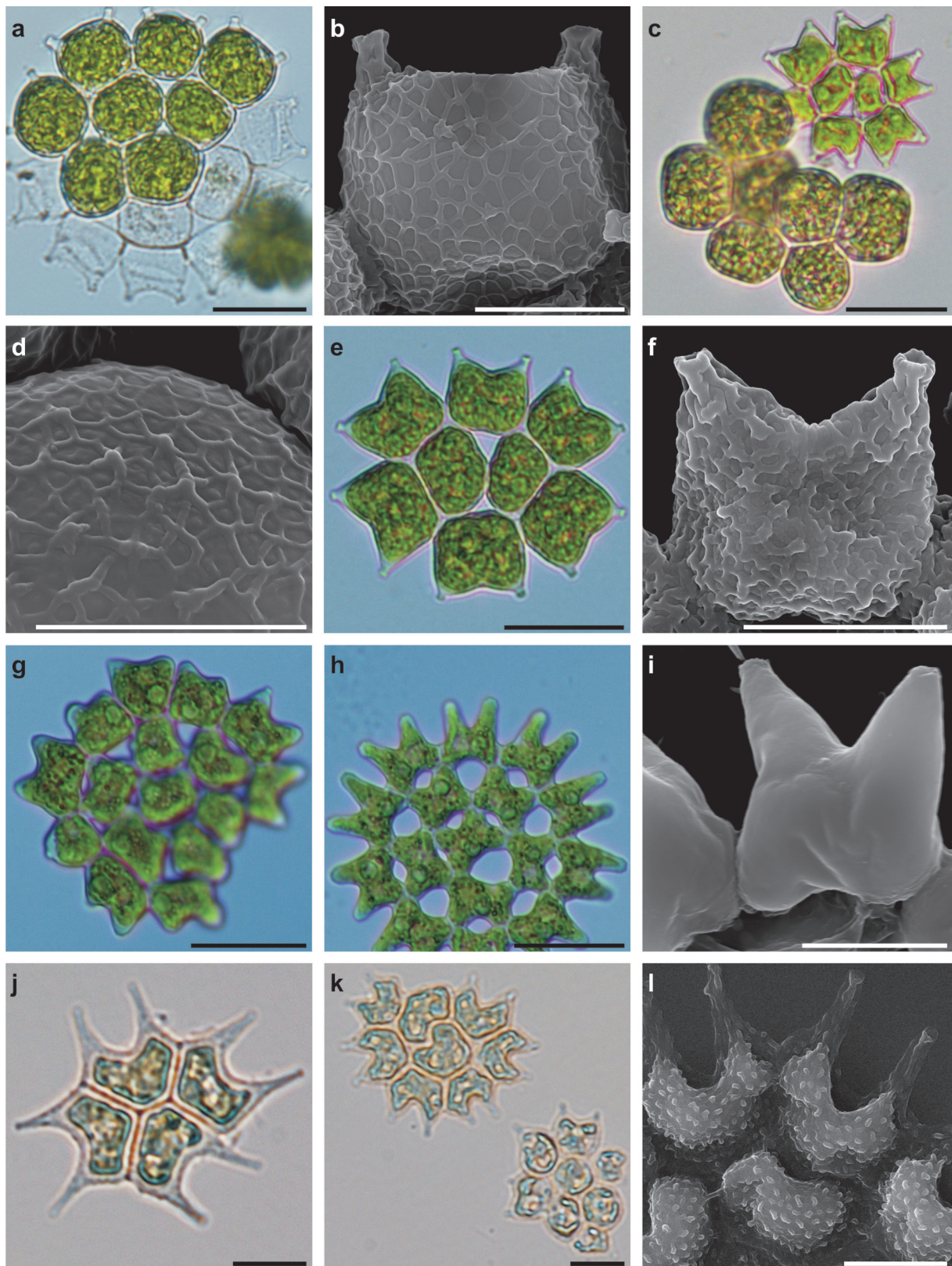


Fig. 2. *Pediastrium* s.l. strains analyzed morphologically using light and scanning electron microscopy. (a–b) *P. duplex* var. *rugulosum* (30.260911a); (c–d) *P. duplex* var. *rugulosum* (14.280112); (e–f) *P. duplex* var. *rugulosum* (05.310112); (g–i) *P. duplex* var. *duplex* (02.310811); (j–l) *Pseudopediastrium* cf. *alternans* (SAG 2440); (a, c, e, g–h, j–k) scale bars 20 μm; (b, d, f, i & l) scale bars 5 μm.

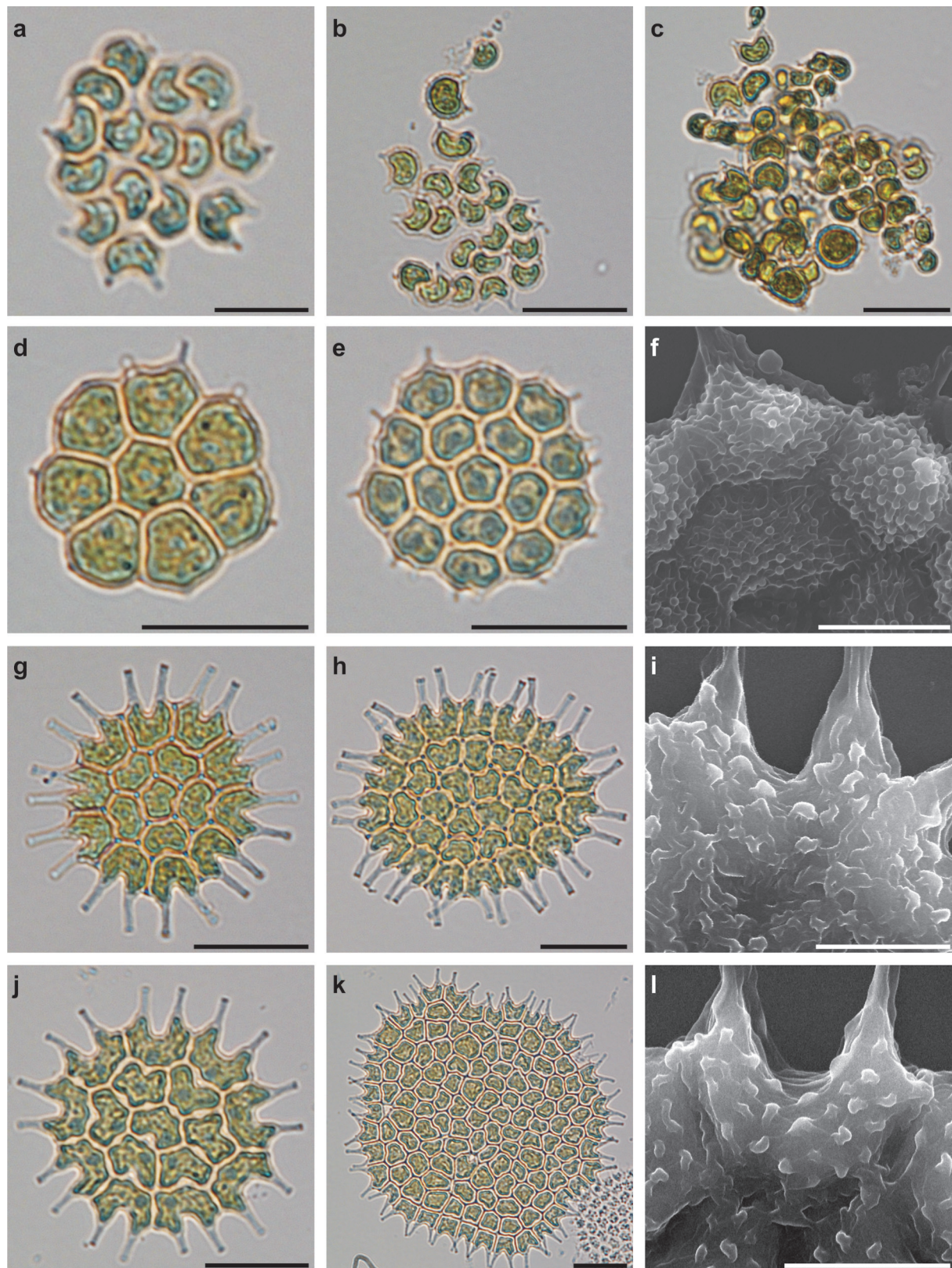


Fig. 3. *Pediastrum* s.l. strains analyzed morphologically using light and scanning electron microscopy. (a–c) *Pseudopediastrum brevicorne* (ACOI 685); (d–f) *Ps. integrum* (SAG 29.81); (g–i) *Ps. boryanum* var. *longicorne* (04.020414); (j–l) *Ps. boryanum* var. *longicorne* (04.080210); (a–b, d–e, g–h, j–k) scale bars 20 µm; (c) scale bar 10 µm; (f, i & l) scale bars 5 µm.

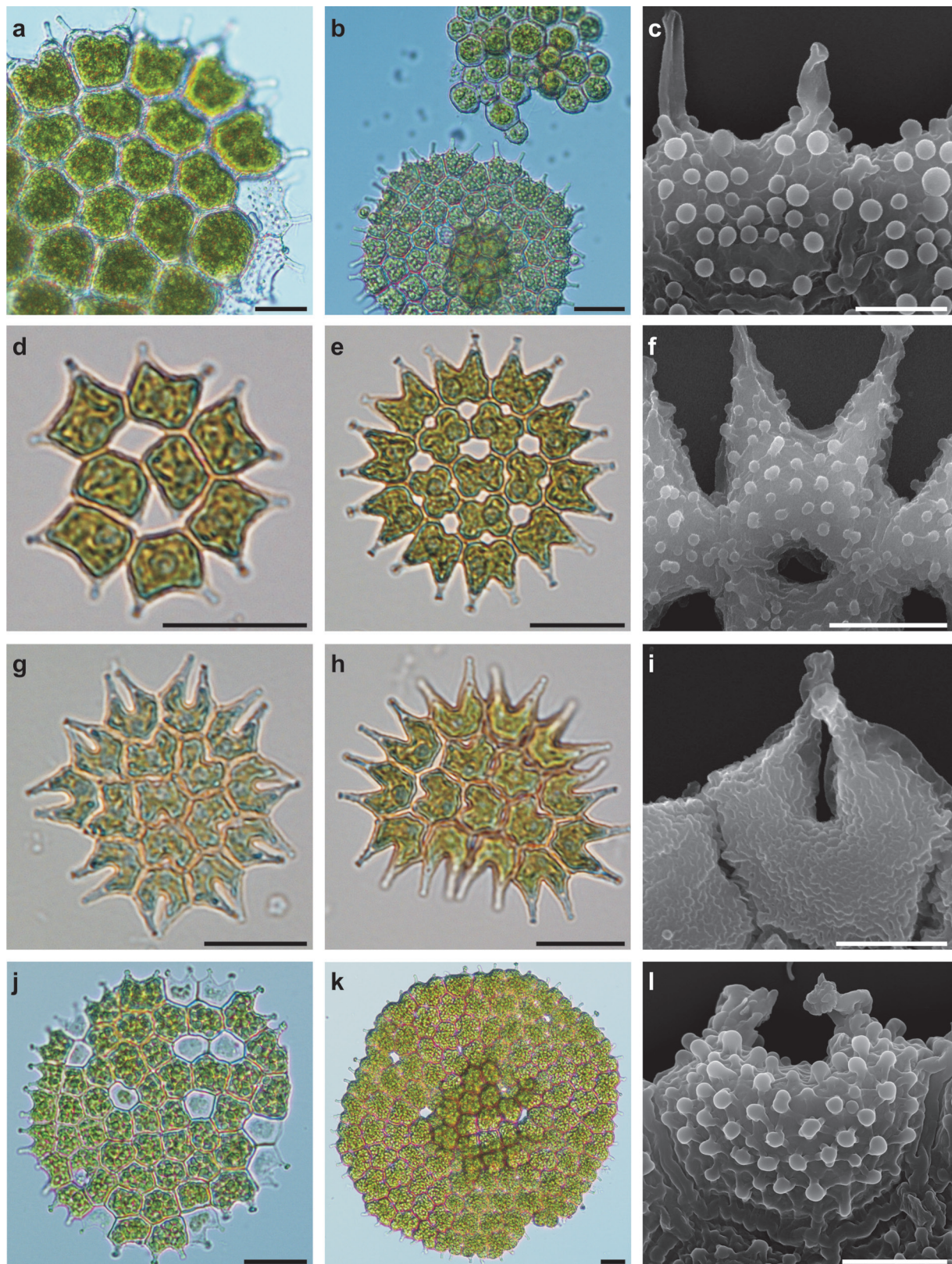


Fig. 4. *Pediastrum* s.l. strains analyzed morphologically using light and scanning electron microscopy. (a–c) *Pseudopediastrum boryanum* var. *longicorne* (02.290811a); (d–f) *P. cf. subgranulatum* (19.300112); (g–i) *Ps. boryanum* var. *forcipatum* (SAG 87.81); (j–l) *Ps. cf. integrum* (11.310112); (a–b, d–e, g–h, j–k) scale bars 20 µm; (c, f, i & l) scale bars 5 µm.

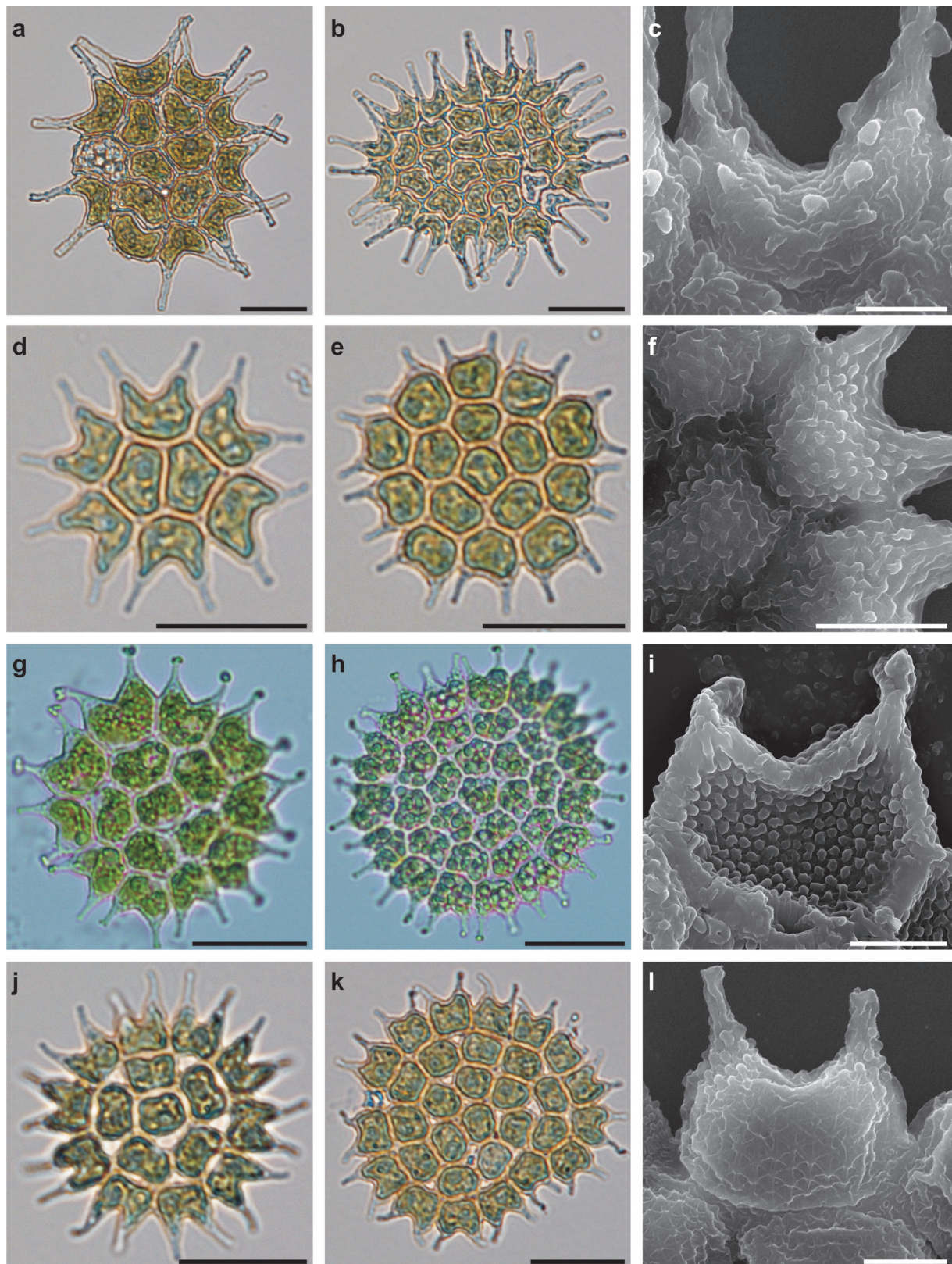


Fig. 5. *Pediastrum* s.l. strains analyzed morphologically using light and scanning electron microscopy. (a–c) *Pseudopediastrum alternans* (01.021213); (d–f) *Ps. boryanum* var. *boryanum* (08.040414); (g–i) *Ps. boryanum* var. *boryanum* (02.310112); (j–l) *Ps. boryanum* var. *cornutum* (19.090508); (a–b, d–e, g–h, j–k) scale bars 20 µm; (c, f, i & l) scale bars 5 µm.

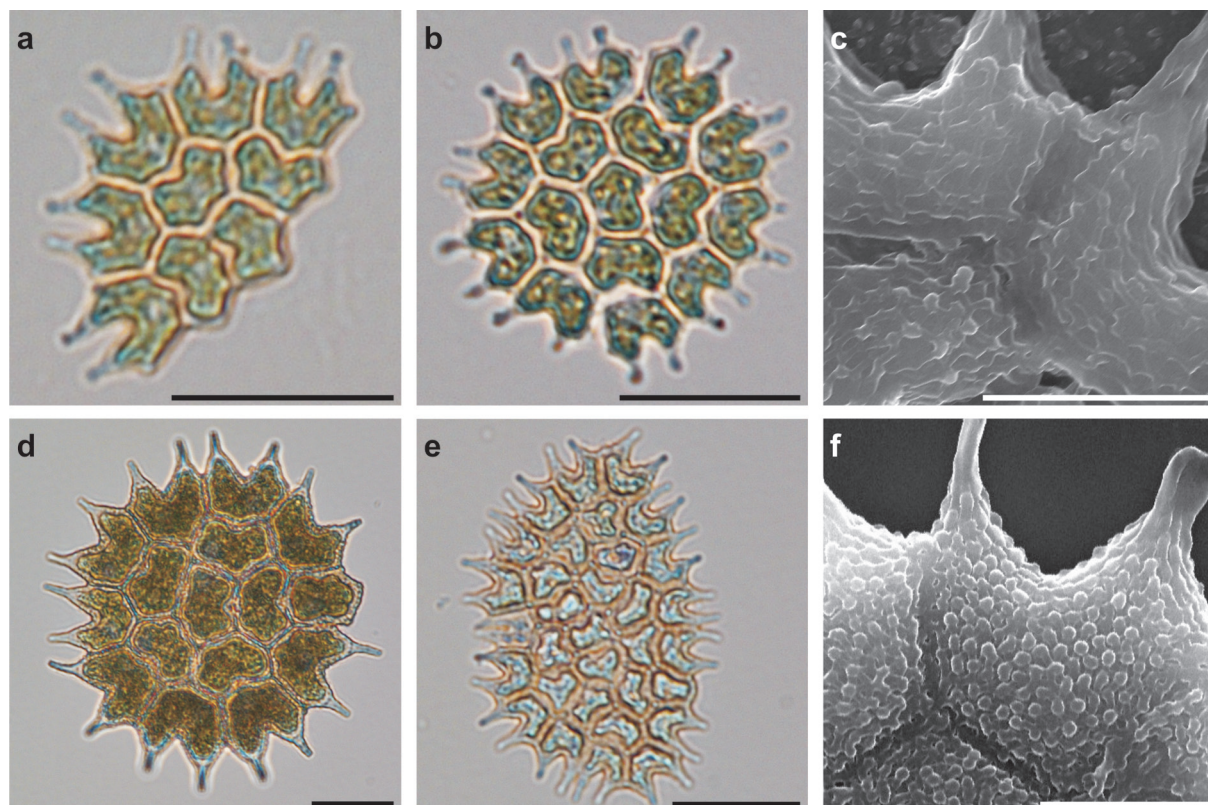


Fig. 6. *Pediastrum* s.l. strains analyzed morphologically using light and scanning electron microscopy. (a–c) *Pseudopediastrum boryanum* var. *boryanum* (ACOI 75); (d–f) *Ps. boryanum* cf. var. *forcipatum* (07.030414); (a–b, d–e) scale bars 20 µm; (c & f) scale bars 5 µm.

variety as a synonym of *P. argentinense* Bourrelly et Tell. However, the morphology of this strain corresponds more to that of other *P. duplex* var. *rugulosum* strains analyzed in this study than to the morphology of *P. argentinense* (Table 1). Morphological differences between *P. duplex* var. *rugulosum* and *P. argentinense* were previously analyzed in detail by LENARCZYK et al. (2015).

In the present study, two strains identified as *P. duplex* var. *duplex* from Europe formed a well supported lineage (GROUP IV). In the study by McMANUS & LEWIS (2011), one of these strains (ACOI 893) with a smooth wall surface (as mentioned by McMANUS & LEWIS 2011) was closely related to a strain identified as *P. duplex* having also a smooth wall surface (SR0201NJ) and representing the morphotype of the variety *duplex*, as well as another strain identified as *P. duplex*, but without information on the cell shape and wall surface (SAG 84.80). If the latter strain also possesses a smooth wall surface, it will make it more plausible that *P. duplex* var. *duplex* is a monophyletic taxon.

Four European (Polish, Portuguese) strains identified as *P. duplex* var. *rugulosum*, as well as one European (Portuguese) strain with the given name *P. duplex* var. *asperum* (ACOI 395) and one Australian strain identified as *P. duplex* (CR0501a), which exhibit the same or similar morphology to *P. duplex* var. *rugulosum*, formed a well supported clade (GROUP II) in the obtained phylogenetic tree. The strains should be regarded as true representatives of *P. duplex* var. *rugulosum*, as their morphology generally

corresponds to the original description of the taxon by RACIBORSKI (1889) from its European *locus classicus* (Poland, Chełmek). Small differences in the wall ornamentation between the European strains (polygonal mesh and thickenings), the Australian strain (polygonal mesh and putative thickenings), the description by KOMÁREK & JANKOVSKÁ (2001) (net-like ornamentation composed of fine wrinkles with thickenings called *verrucae*) and the original description (*membrana rugosa, undulato verruculosa, granulationes dense dispositae, inaequales, irregulares, nonnunquam paullo elongatae*) seem to be connected with different spatial resolution of available microscopes and ontogenetic variability (the change from a mesh to wrinkles as a result of wall stretching) observed by PARRA (1979). Similarly, differences in the H-shaped marginal cells (robust or slender) resulting in small or large perforations in coenobia of the strains in GROUP II can result from culture conditions, as observed for *P. duplex* by NEUSTUPA & HODAČ (2005), and should not be treated as diagnostic characters.

The other strains of *P. duplex* from distant localities (Australia and North America) with cell wall ornamentation typical of, or close to, the variety *rugulosum* (polygonal mesh with thickenings or wrinkles with putative thickenings) are placed in three separate lineages (GROUPS III, V and VII), which seem to represent at least three new taxa. Similarly, *P. duplex* strains possessing wavy and netlike ornamentation patterns were grouped in several separate lineages in the phylogenetic analysis

made by McMANUS & LEWIS (2011). One of the present lineages (GROUP VII) corresponds to Group II in the tree presented by McMANUS & LEWIS (2011). Both groups contain not only strains identified as *P. duplex*, but also representatives of Australian, European and North American *P. angulosum* exhibiting morphology different from *P. boryanum* and *P. duplex*, as well as an Australian strain identified as *Pseudopediastrum alternans*? (LWiv0503b) with U-shaped cells, resembling that of *P. boryanum*. However, detailed morphological analysis, e.g. with the help of geometric morphometric methods previously used for taxonomical delimitation of another *P. duplex*-like morphotype, *L. gracillimum* (McMANUS et al. 2011), are needed to make sound conclusions on the systematics of the *P. duplex* var. *rugulosum*-like morphotypes. McMANUS & LEWIS (2011) stated that not only other forms of morphological information, but also more variable molecular markers are needed to clarify the relationships between *P. duplex* var. *rugulosum*-like, *P. angulosum* and *Ps. alternans*-like morphotypes.

Only one strain of *P. duplex* var. *asperum* (UTEX LB1364) from a European locality, except for the misidentified ACOI 395, has been molecularly analysed so far (McMANUS & LEWIS 2005, 2011; LENARCZYK & McMANUS 2016). It forms a separate lineage (GROUP VI) in the present study. However, it is difficult to infer the phylogenetic position of *P. duplex* var. *asperum* from only one strain. In previous studies, the strain has been mostly related to a strain of *Pediastrum* sp. (MB0404) from Australia (McMANUS & LEWIS 2011) or to several *P. duplex*, *Pediastrum* sp. and *P. angulosum* (EHRENBERG) ex MENEGHINI strains (McMANUS & LEWIS 2005; LENARCZYK & McMANUS 2016). The cell wall ornamentation of *P. angulosum* is also araneose (so far observed only in *P. duplex* var. *asperum* and *P. angulosum*), but the cell shape is different to that of *P. duplex* var. *asperum*. More strains representing the *P. duplex* var. *asperum* morphotype are needed to determine the phylogenetic position of the taxon and its taxonomical homogeneity.

The present study confirms the polyphyletic nature of *P. boryanum* s.l. McMANUS & LEWIS (2005) stated that the species is monophyletic, but sequencing a higher number of strains showed its paraphyletic character (McMANUS & LEWIS 2011; JENA et al. 2014; LENARCZYK & McMANUS 2016).

Altogether, five varieties of *P. boryanum* s.l. were subjected to the present phylogenetic analysis, including *boryanum* (as *Pseudopediastrum boryanum* (Turpin) E. Hegewald var. *boryanum*), *brevicorne* A. Braun (as *Ps. brevicorne* (A. Braun) Jena et C. Bock), *cornutum* Sulek (as *Ps. boryanum* var. *cornutum*), *forcipatum* Parra (as *Ps. boryanum* var. *forcipatum*) and *longicorne* Reinsch (as *Ps. boryanum* var. *longicorne* and *Ps. brevicorne* f. *granulatum* (Raciborski) Parra).

Only the variety *cornutum* formed a well supported monophyletic clade composed of more strains (GROUP XVI). LENARCZYK (2014) and LENARCZYK &

WOŁOWSKI (2016) found that the coenobia of *P. boryanum* var. *cornutum* and *P. boryanum* var. *perforatum* (Raciborski) Nitardy are very similar in cell shape and wall ornamentation. Similarly, NIELSEN (2000) questioned the distinction between these varieties. However, no strains of the variety *perforatum* have been included in molecular studies to date, so the taxonomical problem remains unresolved.

Only one strain representing *Ps. boryanum* var. *forcipatum* was included in the present phylogenetic analysis. A morphologically similar strain, identified as *Ps. boryanum* cf. var. *forcipatum*, slightly differed in the marginal cell shape (processes not converging). These two strains did not form a common lineage (GROUPS XIII and XVIII) in the present study, suggesting that the latter strain represents a new taxon, but more strains with such morphology are needed to confirm its taxonomic position.

Pediastrum boryanum var. *forcipatum* is also morphologically similar to *P. boryanum* var. *pseudoglabrum* PARRA. These two taxa generally differ in cell wall ultrastructure, but some intermediate forms were also observed by LENARCZYK (2014) and LENARCZYK & WOŁOWSKI (2016). Thus, it was suggested that the variety *pseudoglabrum*, usually forming a scabrate wall pattern (composed of tiny *verrucae* and *punctae*), can be a form of the variety *forcipatum*, usually forming a granulate wall pattern (composed of granules situated densely in regular rows, and *punctae*), but with wall ornamentation not well developed. No strains representing the variety *pseudoglabrum* have been molecularly analysed so far, so the relationships between these two taxa remain unclear.

The only strain representing *Ps. boryanum* var. *forcipatum* was closely related to a strain resembling *Pediastrum subgranulatum*, which exhibits a very different morphology (GROUP XIII). The marginal cells of the *P. cf. subgranulatum* strain were a little longer than presented in the pictures of *P. subgranulatum* in the monograph by KOMÁREK & JANKOVSKÁ (2001), who elevated *P. duplex* var. *subgranulatum* RACIBORSKI to species rank as a new combination. *Pediastrum subgranulatum* combines features typical of *P. boryanum*, namely a reticulate wall pattern with regularly disposed granules, as well as including perforated coenobia, H-shaped cells and very short processes (rosettes), typical of *P. duplex*. Previously, *P. subgranulatum* had been classified as a variety of *P. duplex*, with the basionym *P. duplex* var. *subgranulatum* Raciborski, and included the synonyms *P. duplex* var. *typicum* f. *punctatum* Krieger or *P. duplex* var. *punctatum* Parra (KOMÁREK & JANKOVSKÁ 2001). In both *Ps. boryanum* var. *forcipatum* and *P. cf. subgranulatum* large evolutionary changes have occurred in this lineage, which can be explained by adding more strains representing both the *Ps. boryanum* var. *forcipatum*- and *P. subgranulatum*-like morphologies.

Except for *Ps. boryanum* var. *cornutum* and *Ps. boryanum* var. *forcipatum*, the varieties *boryanum*,

brevicorne and *longicorne*, proved to be complex taxa as strains of each of the varieties were dispersed in different clades.

The strains representing the variety *boryanum* are to be found in two separate lineages (GROUPS XV and XVII). The lineages differ in the cell wall granulation, which is of medium density in GROUP XV (not visible in IL0402MN) and loose in GROUP XVII. High variability in *P. boryanum* var. *boryanum* morphology, including cell shape and granule density, was previously observed by LENARCZYK (2014) and LENARCZYK & WOŁOWSKI (2016).

In the present study, two strains identified as *Ps. brevicorne* (formerly *Pediastrum boryanum* var. *brevicorne*) are grouped in distant lineages with different species, with *P. boryanum* var. *boryanum* and *P. boryanum* (GROUP XV), and with *Ps. cf. alternans* (GROUP IX). McMANUS & LEWIS (2011) similarly found two strains identified as *Ps. boryanum* var. *brevicorne* in two separate lineages, with *Ps. boryanum* and *Pediastrum* sp., further confirming the polyphyletic nature of the variety. Based only on morphological studies, NIELSEN (2000) stated that the variety *brevicorne* should be rejected and subsumed into the nominate variety. Molecular studies indicate that the taxon should be divided into two other taxa, maybe one of them representing the variety *boryanum*. However, additional detailed morphometric analyses are required. The variety *longicorne* was divided into two clades (GROUPS XI and XII). Similarly, LENARCZYK & SAŁUGA (2013) observed a division of the variety *longicorne* into lowland and mountainous groups of Polish strains in a phylogenetic tree based on 18S, 26S and ITS rDNA sequences. In the present study, the cell wall surface was shown to be covered with prominently rounded granules in the Polish mountainous strain from GROUP XII, but with more or less rounded granules in the Portuguese strain from the same group and the Polish lowland strains from GROUP XI. The morphological differences shown in Polish strains were pointed out by LENARCZYK & WOŁOWSKI (2016), who also suggested the complexity of *Ps. boryanum* var. *longicorne*. REINSCH (1867), who described the variety *longicorne*, mentioned that its cell wall surface is smooth. It could have been connected with low spatial resolution of available microscopes, as subsequent researchers (e.g. PARRA 1979; KOMÁREK & JANKOVSKÁ 2001; LENARCZYK & WOŁOWSKI 2016) have always observed ornamented cell walls covered with rarely disposed granules.

One Polish strain (01.021213) identified as *Ps. alternans* in the present study has coenobium and cell shape typical of this species, described by NYGAARD (1949) as *Pediastrum alternans* from Danish lakes (LENARCZYK & McMANUS 2016). KOWALSKA & WOŁOWSKI (2010) suggested that the presence of *verrucae*, not observed by NYGAARD (1949), but observed on the cell wall surface of *Ps. alternans* found in the Polish coastal lakes (and on the surface of the 01.021213 strain) indicates a probable new variety. The 01.021213 strain was located within a

clade dominated with *Ps. boryanum* strains. The close relationship of *Ps. alternans* and *Ps. boryanum* was previously stated also by LENARCZYK & McMANUS (2016).

In the present study, the 01.021213 strain (*Ps. alternans*) was most closely related (GROUP XIV) to a strain resembling *Ps. integrum* (11.310112), which exhibited different morphology, i.e., loose cell wall granulation. This is the case of closely related, but morphologically different taxa, similar to that of *Ps. boryanum* var. *forcipatum* and *P. cf. subgranulatum*, where adding more strains representing their morphology could clarify large evolutionary changes occurring in the lineage. The 11.310112 strain identified as *Ps. cf. integrum* may represent a new species, but detailed morphological analyses are necessary to prove its distinctiveness.

Detailed morphological analyses, using geometric morphometric methods, which allow analyzing shape as a whole without reducing it to single measurements, like length or width (ZELDITCH et al. 2004), along with molecular analyses, revealed that the African strain SAG 2440, previously identified as *Ps. alternans* by JENA et al. (2014), is not a representative of this species (LENARCZYK & McMANUS 2016). Thus, it was identified as *Ps. cf. alternans* in the present study. In both the present and previous studies (LENARCZYK & McMANUS 2016), the strain forms a separate lineage (GROUP IX) with the strain ACOI 685, identified as *Ps. brevicorne* or *Ps. boryanum* var. *brevicorne*, respectively. Limited data on appearance of the latter strain do not allow us to make any definite conclusions regarding typical morphology in this lineage.

Pseudopediastrum integrum (formerly *Pediastrum integrum*) formed a clade (GROUP X) with *Ps. kawraiskyi* which exhibits different morphology to that of *Pediastrum boryanum* and *P. duplex*. The clade was sister to a clade composed mostly of *Ps. boryanum* strains. Similar results were obtained by McMANUS & LEWIS (2005, 2011), JENA et al. (2014) and LENARCZYK & McMANUS (2016).

The RL0201FR strain identified as *Pediastrum* sp. was the last strain representing *P. boryanum*-like morphology. In the present study, it was placed close to *Pseudopediastrum* taxa, although the relationship was weakly supported (GROUP VIII). In the study by McMANUS & LEWIS (2011), the strain was grouped together with *Pseudopediastrum* strains based on *rbcL* data or closer to *Pediastrum* strains based on 26S rDNA data. According to McMANUS & LEWIS (2011), the RL0201FR strain forms a common lineage with a morphologically similar strain, SAG 2062, which was named *P. berolinense* by BUCHHEIM et al. (2005).

In the case of two strains, SAG 2440 (*Pseudopediastrum cf. alternans*) and SAG 87.81 (*Ps. boryanum* var. *forcipatum*), there were small differences in their morphology observed by the present authors and shown in the literature (Table 1). The variation could have resulted from differences in culture media and culture conditions. So far, only a few experimental studies on the impact

of environmental factors on morphological variability of *Pediastrum* s.l. have been conducted (e.g., NEUSTUPA & HODAČ 2005; ROJO et al. 2007; LENARCZYK 2016; LENARCZYK & McMANUS 2016), and they need to be continued to make taxonomic conclusions more reliable.

CONCLUSIONS

The gene topologies reveal many discrepancies in the morphological features used to delimit analysed taxa. Adding more strains representing the morphology of *Pediastrum duplex*, as well as representing both the analysed varieties of *Pseudopediastrum boryanum* and other varieties of the species, including vars. *campanulatum* WILLE, *caribeanum* COMAS, *pseudoglabrum* Parra and *perforatum* (Raciborski) Nitardy, is necessary to clarify the relationships in these very diverse groups. Even though two molecular markers with long sequences were used in the present study, the distances between strains in the *Pseudopediastrum* clade were very short. Thus, it is important to add information from more variable molecular markers. In the case of such variable taxa with intricate cell shape as observed in *P. boryanum*, *P. duplex* s.l. and similar species, detailed morphological analyses using modern methods like geometric morphometrics are required. The present study shows that taxonomical changes within both analysed species need to be made.

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Supplementary material

the following supplementary material is available for this article:

Table S1. Origin and the GenBank accession numbers of the studied *Pediastrum* s.l. strains and the outgroup *Neochloris aquatica* STARR (UTEX 138).

This material is available as part of the online article (<http://fottea.czechphycology.cz/contents>)