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Dispora speciosa, a new addition to the genus *Parallela* and the first coccoid member of the family Microsporaceae

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Abstract

The clade that currently represents the green algal family Microsporaceae is one of the few filament-forming groups of Chlorophyceae. Molecular phylogenies show this clade containing the genus *Microspora* and the more recently circumscribed *Parallela*, whose filaments are loosely arranged and often multiseriate. We initially investigated the enigmatic bog-loving *Dispora speciosa* as a commonly accepted member of the mucilage-forming Radiococcaceae or a putative member of crucigenoid chlorophytes (a non-monophyletic group formerly placed in Scenedesmaceae) based on its two-dimensional colony formation. However, our plastid and nuclear ribosomal phylogenies confidently placed *Dispora* within the genus *Parallela* instead, and therefore distantly related to both Radiococcaceae and crucigenoids. Upon further examination of the cell morphology and ultrastructure, we found several corresponding features between *Dispora* and *Parallela*, despite *Dispora*'s apparent coccoid-colonial gross morphology. Both genera have cells with a parietal plastid positioned around a large central nucleus. The loose, multiseriate filament formation in *Parallela* can be interpreted as similar to *Dispora*'s flat colony formation in its natural state. Because we only present data from one non-type species and strain of *Dispora*, we cannot merge the entire genus with *Parallela*. We do however argue that D. *speciosa*, of which this strain is the sole available, morphologically and ecologically faithful representative, should be transferred into Parallela, and the specimen prepared from strain ACOI 1508 be designated as type. Our study also impacts the current view on evolution of multicellular (colonial and filamentous) forms in Chlorophyceae.

Key words: 18S rRNA, *atpB*, *epitype*, *phylogeny*, *psaB*, TEM, *rbcL*

Introduction

The ancient common ancestor of all green plants and algae likely was a single-celled flagellate (Leliaert *et al.* 2012 and references within). Within the different green algal classes, complex morphologies are thought to have evolved independently multiple times including colonial, coenobial, filamentous, thalloid and other forms. Coccal (single-celled, vegetatively non-motile) forms are common for example in the classes Chlorophyceae and Trebouxiophyceae (e.g., Fučíková *et al.* 2014a,b), and in some cases may represent repeated evolutionary reductions from more complex ancestors. In other cases coccoid forms may be ancestral. An accurate understanding of the diversity within these algal groups and a robust assessment of their phylogenetic relationships are critical to answering fundamental evolutionary questions about the evolution of complex body forms.

The green algal phyla Chlorophyta and Streptophyta contain numerous ancient lineages, the biodiversity of which is likely drastically underestimated. Recent studies have demonstrated time and again that the morphological diversity of microscopic green algae does not reflect their phylogenetic diversity. Similar, putatively convergent morphologies are common across distantly related groups (e.g., Fučíková *et al.* 2014a,b). Cases of morphological crypsis uncovered by molecular data are especially common in coccoid microalgae, but have been documented even in more complex taxa, such as the filamentous *Klebsormidium* P.C. Silva, K.R. Mattox & W.H. Blackwell (1972: 643) (Škaloud & Rindi 2013). In some cases, morphological, ultrastructural, ecological, or other species-delimiting features are discovered post-hoc, in light of a molecular phylogeny (e.g., Škaloud & Rindi 2013).

The fairly rare, peat pond inhabiting green coccal alga *Dispora speciosa* Korshikov (1953: 324) is characterized by its flat, four-celled coenobial organisation and a wide mucilage cover (Korshikov 1953). Cell organisation and presence of mucilage covers had been considered crucial morphological characters for categorization of green algae

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for the last two centuries (Lemmermann 1915, Smith 1950, Korshikov 1953, Fott 1959, Komárek & Fott 1983, Ettl & Gärtner 1988, Kostikov *et al.* 2002). The genus *Dispora* Printz (1914: 32) was originally described in the family Pleurococcaceae (Printz 1914), and subsequently went through several different taxonomic placements (e.g. Bourrelly 1966, Fott 1974). Nevertheless, the latest complex morphological studies (Komárek 1979, Komárek & Fott 1983, Ettl & Gärtner 1988, Kostikov *et al.* 2002) all placed *Dispora* in the family Radiococcaceae, highlighting especially the presence of mucilage covers. The few available insights to the phylogeny of Radicococcaceae all uncovered that the family is considerably polyphyletic (Wolf *et al* 2003, Pažoutová 2008, Pažoutová *et al.* 2010, Fučíková 2014a, Zhang *et al.* 2018). Former Radicococcaceae members appeared scattered in the class Trebouxiophyceae (Hanagata & Chihara 1999, Wolf *et al.* 2003, Pažoutová 2008, Pažoutová *et al.* 2010) and in the class Chlorophyceae (Wolf *et al.* 2003, Pažoutová 2008, Fučíková 2014a, Zhang *et al.* 2018) in various lineages, which proves that extracellular mucilage is a rather common and circumstantial trait and thus offers limited taxonomic information.

Radiococcaceae taxa (including *Dispora*) with cells organized in flat tabelar coenobia have been grouped in the subfamily Disporoideae (Komárek & Fott 1983). The phylogenetic placement of some radiococcacean genera is now known, but not for any of the Disporoideae as yet. The flat four-celled coenobia of *Dispora speciosa* remarkably resemble the coenobia of algae assigned to the scenedesmacean subfamily Crucigenoideae *sensu* Komárek (1974) and Komárek & Fott (1983). A typical trait defining crucigenoid algae is the propagation by autospores, which have not been reported in *Dispora* spp. Further, much like the Radiococcaceae, crucigenoid algae also are demonstrably polyphyletic, and their members are distributed throughout the green algal phylogeny and inside both the classes Trebouxiophyceae (Hepperle *et al.* 2000, Bock *et al.* 2013, Štenclová *et al.* 2017) and Chlorophyceae (Hegewald *et al.* 2010, Bock *et al.* 2013). The relationship of the genus *Dispora* to radiococcacean and crucigenoid lineages is suspected but remains unexplored.

In recent years, there have been efforts to reconcile the traditional, morphology-based taxonomy with molecular approaches to describe biodiversity. By combining the two approaches, researchers strive to classify traditional and newly discovered taxa in a way that reflects their evolutionary history and relatedness. One of the challenges is typification—the standards of type designation have changed over time, and many species described in the 19th and early 20th century are not accompanied with detailed (or any) illustrations, precise morphological descriptions, preserved specimens, and almost never with a living culture available for further examination and experimentation. Occasionally, modern phycologists have attempted to revisit type localities and establish new types for old species and genus names that would otherwise be taxonomically questionable or ambiguous (e.g., Fučíková *et al.* 2013). In some cases, an existing isolate is selected to serve as new type, ideally one collected near the type locality (e.g., Allewaert *et al.* 2015)—often this is the most practical solution, especially when the locality information is insufficient in the original species description, and it is thus impossible to find and revisit it. The description of the type locality of *Dispora speciosa* (North part of the European part of the former USSR (Korshikov 1953)) is very broad and thus collecting material from the original site is not possible.

Given these limitations, we examined the only publicly available strain of *Dispora speciosa* (ACOI 1508) in order to determine the higher classification of this taxon. This strain originated from a locality distant to the original (Abrantes, Capo Militar de Sta Margarida, lake North of Lagoa da Murta in Portugal), but morphologically corresponded well with Korshikov´s description. The gross morphology of the species is rather unusual in Chlorophyta, and therefore an array of methods was used to pinpoint the species' taxonomic placement. Our assessment included morphological, ultrastructural, and molecular data analyses, exemplifying a modern polyphasic approach to taxonomy.

Materials & Methods

Strain information & culture conditions

The green algal strain ACOI 1508 *Dispora speciosa* was acquired from the public culture collection Coimbra Collection of Algae (ACOI), Portugal. The strain was cultivated on both solid and liquid medium LM-7 (prepared following the instructions of ACOI) and kept under the standard conditions: irradiance 22 μmol · m−2 · s−1 and constant temperature 16°C.

Light microscopy (LM)

Basic morphology was observed using an Olympus BX light microscope equipped with an Olympus DP71 camera and DP software (Olympus, Center Valley, PA, USA) under 1000x magnification using immersion oil. Methylene blue staining was used to detect the gelatinous covers around the cells.

Autofluorescence

Observations of chlorophyll autofluorescence were carried out on an Olympus BH-2 photomicroscope equipped with a mercury lamp at a 1000x magnification and micrographs were captured using an AmScope MU1000 digital camera (AmScope, Irvine, CA, USA).

Transmission electron microscopy (TEM)

For ultrastructural observation, ultrathin sections of the cell culture were prepared. Samples were processed by staff at the Electron Microscopy Laboratory, Institute of Parasitology, Academy of Sciences, Czech Republic. Samples were treated with 0.05 M phosphate buffer, postfixed with 2% osmium tetroxide in 0.05 M phosphate buffer at room temperature for 2 h and then repeatedly washed with 0.05 M phosphate buffer. Washed cells were dehydrated serially in isopropanol concentration gradient, dissolved in propylene oxide and finally embedded in Spurr's resin (Spurr 1969). Thin sections were prepared and stained with uranyl acetate and lead citrate. Specimens were observed using a Jeol JEN 1010 transmission electron microscope (JEOL, Peabody, MA, USA) at an accelerating voltage of 80 kV.

Picture plates documenting microscopic methods were constructed using CorelDraw 2018 (Corel Corporation, Ottawa, Canada).

Molecular data & analyses

Biomass was manually ground with sterile sand and DNA was subsequently isolated using the DNeasy PowerPlant Pro kit (Qiagen Inc., Germantown, MD, USA). The chloroplast genes *atpB*, *psaB* and *rbcL* were selected because of their availability for a wide sampling of Chlorophyceae, including various *incertae sedis* taxa (Fučíková *et al.* 2019). The 18S nuclear ribosomal gene was also selected because of its common usage for phylogenetic systematics in green algae. Polymerase chain reaction (PCR) was run as described in McManus & Lewis (2011) for *rbcL*, according to Novis *et al.* (2010) for *atpB* and *psaB*, and according to Shoup & Lewis (2003) for 18S. Initially, after obtaining partial *rbcL* data, we used BLAST (Altschul 1990) to determine the approximate phylogenetic placement of *Dispora*. Based on this information, we refined the *atpB* and *psaB* primers of Novis *et al.* (2010) to be more taxon-specific and less degenerate, and also designed a new taxon-specific *atpB* primer based on alignments of *Parallela* E.A. Flint (1974: 358) and *Microspora* Thuret (1850: 222) sequences. Based on alignments we also selected 18S primers to fit the Microsporaceae clade and simultaneously circumvent amoebal contamination in the *Dispora* culture, which was otherwise preferentially amplified with most standard algal 18S primers. A nested PCR was necessary to obtain at least partial 18S data, initially using the primer pair SSU1 (Shoup & Lewis 2003) and 1650R and re-amplifying from the resulting product using the pair 1170F and 1650R—the only successful 18S amplification. Cycle sequencing and Sanger sequence analysis was done at Macrogen USA (Boston, MA, USA). Primers successfully used for PCR and sequencing are listed in TABLE 1. Genbank accession numbers of sequences used in all analyses are provided in TABLE 2; alignments and analysis specifications are available in Supplements.

TABLE 1. Primers used to amplify plastid genes and 18S of *Dispora speciosa* and their sources. Taxon-specific primers designed for this study are highlighted in boldface font. Tm is as determined by Oligo Analyzer 3.1: Integrated DNA Technologies. * indicates modifications from published primer.

Gene	Name	F/R	Sequence	Position (bp)	Tm (°C)	Citation
18S	1170F	F	CTGTGGCTTAATTTGACTCAACACG	1170	56.6	Pažoutová *et al.* 2010
18S	1650R	R	TCACCAGCACACCCAAT	1650	54.2	Kipp 2004
AtpB	Pa2b	F	ATYTTTGAAACAGGWATTAAAGT	411	46–53	*Novis *et al.* 2010
AtpB	D_atpB_1345	R	GCTAAACTTACATATTTTCCAGG	1345	49.0	Present study
PsaB	Pp1b	F	TTCCAYGTAGCWTGGCAAGG	195	55–61	*Novis *et al.* 2010
PsaB	Pp3b	R	AAGAAAATRGCWCCRTGRGCAAA	1158	52–62	*Novis *et al.* 2010
RbcL	28F	F	GGTGTTGGATTWAAAGCTGGTGT	28	55.9	McManus & Lewis 2011
RbcL	650R	R	CGGTCTCTCCAACGCATGA	650	57.3	McManus & Lewis 2011

TABLE 2. Algal strains used in phylogenetic analyses and GenBank accession numbers for their 18S, *atpB*, *psaB*, and *rbcL* sequences. Strains are ordered to reflect their phylogenetic groupings. Newly obtained sequences highlighted in boldface font. In cases where information from multiple strains of the same species was used, both/all strain numbers are given. In species where two different names have recently been used in literature, both names are shown for easier comparison to other studies.

Strain	Name	18S	*atpB*	*psaB*	*rbcL*
Microsporaceae					
ACOI 1508	*Dispora speciosa*	**MG991819**	**MG991818**	**MG991820**	**MG991817**
Liffey	*Parallela novae-zelandiae*	N/A	GQ423922.1	GQ423927.1	GQ423930.1
SAG 27.83	*Parallela transversalis*	N/A	GU270868.1	GU270869.1	GU270870.1
UTEX LB 1252	*Parallela transversalis*	AF387161.1	EF113533.1	MG786420.1	EF113468.1
UTEX LB 472	*Microspora sp.*	AF387160.1	EF113517.1	KT693221.1	KT693222.1
Sphaeropleales *sensu lato*					
SAG 34.88	*Crucigenia pulchra*	KF673376.1	N/A	N/A	N/A
BCP SEV3VF49	*Flechtneria rotunda*	HQ246317.1	N/A	KC145475.1	HQ246350.1
UTEX 393	*Tetradesmus obliquus*	AJ249515.1	NC008101	NC008101	NC008101
UTEX 1450					
UTEX LB1365	*Hariotina reticulata*	AH012395.2	KY792693.1	JN630546.1	JQ394815.1
SAG 8.81					
UTEX 2979	*Rotundella rotunda*	KC145434.1	KT369368.1	KT369353.1	KT369354.1
UTEX 138	*Neochloris aquatica*	M62861.1	KT199248.1	KT199248.1	KT199248.1
UTEX LB1364	*Pediastrum duplex* var. *asperum*	AY779859.1	MF536520.1	MF536515.1	MF536514.1
SAG 43.81	*Chlorotetraedron incus*	AF288363.1	KT199252.1	KT199252.1	KT199252.1
SAG 2137	*Pseudomuriella schumacherensis*	HQ292768.1	KT199256.1	KT199256.1	KT199256.1
UTEX LB62	*Dictyococcus varians*	GQ985408.1	N/A	KC145487.1	GQ985404.1
UTEX LB 951	*Follicularia botryoides*	KC145433.1	MG778401.1	KC145485.1	JQ259910.1
SAG 217-1c	*Radiococcus polycoccus*	AF388378.1	N/A	KC145490.1	HM852437.1
SAG 66.94	*Schizochlamys gelatinosa*	AY781662.1	N/A	KC145483.1	KC145516.1
UTEX 1250	*Bracteacoccus aerius*	U63101.1	KT199254.1	KT199254.1	KT199254.1
UTEX 1251	*Bracteacoccus giganteus*	U63099.1	KT625421.1	KT625421.1	KT625421.1
UTEX 66	*Bracteacoccus minor*	U63097.1	KT199253.1	KT199253.1	KT199253.1
UTEX 56	*Chromochloris zofingiensis*	HQ902933.1	KT199251.1	KT199251.1	KT199251.1
SAG 2004	*Kirchneriella aperta*	AJ271859.1	KT199250.1	KT199250.1	KT199250.1
UTEX 1240	*Ourococcus multisporus*	AF277648.1	JN630550.1	KT369443.1	KT369475.1
CAUP 6502	*Mychonastes homosphaera*	GQ477056.1	KT199249.1	KT199249.1	KT199249.1
SAG 37.98	*Mychonastes jurisii*	AF106074.1	KT625411.1	KT625411.1	KT625411.1
UTEX 127	*Dictyochloris fragrans*	AF367861.1	MG778236.1	KC145480.1	KC145513.1
UTEX LB 606	*Trochiscia hystrix*	AF277651.1	EF113543.1	MG778511.1	EF113480.1
SAG 38.83	*Treubaria triappendiculata*	LC192143.1	KT625410.1	KT625410.1	KT625410.1
NIES 394					
SAG 3.87	*Cylindrocapsa geminella*	U73471.1	EF119849.1	MG778196.1	MG778214.1
SAG 9.94	*Elakatothrix viridis*	AY008844.1	MG778344.1	MG778308.1	MG778310.1
SAG 73.80	*Golenkinia longispicula*	AF499923.1	KT625129.1	KT625105.1	KT625127.1
CAUP H8102	*Jenufa minuta*	HM563744.1	KT625414.1	KT625414.1	KT625414.1
CAUP H8101	*Jenufa perforata*	HM563743.1	KT625413.1	KT625413.1	KT625413.1
SAG 17.84	*Ankyra judayi*	U73469.1	KT369399.1	KT369399.1	KT369399.1
UTEX 2309	*Atractomorpha echinata*	U73470.1	EF113487.1	JN630539.1	EF113412.1
SAG B 1.85	*Spermatozopsis similis*	X65557.1	EF113535.1	MG778500.1	MG778500.1
Volvocales					
UTEX 2227	*Chlorococcum tatrense*	MG991815.1	MG778173.1	MG778173.1	MG778173.1
SAG 11–43	*Chloromonas perforata*	U70794.1	KT625416.1	KT625416.1	KT625416.1

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TABLE 2. (Continued)

Strain	Name	18S	*atpB*	*psaB*	*rbcL*
UTEX B99	*Protosiphon botryoides*	JN880460.1	KT693220.1	JN630554.1	JN880463.1
SAG 78-1a	*Stephanosphaera pluvialis*	LC066326.1	KT625300.1	KT625323.1	KT625343.1
UTEX 1186	*Chlorosarcinopsis eremi*	AB218706.1	MG778185.1	MG778185.1	HQ246342.1
UTEX 11 CCAP 12/2a	*Chlorogonium capillatum*	AB278612.1	KT625087.1	KT625086.1	KT625086.1
SAG 34-1b	*Haematococcus lacustris*	AF159369.1	KT625206.1	KT625227.1	KT625244.1
CCAP 19/18	*Dunaliella salina*	EF473745.1	GQ250046.1	GQ250046.1	GQ250046.1
SAG 11–9	*Chlamydomonas applanata*	FR865616.1	KT625417.1	KT625417.1	KT625417.1
SAG 31.95 UTEX 2095	*Characiochloris acuminata*	AF395435.1	KT625418.1	KT625418.1	KT625418.1
SAG 61-1 KR 91/1	*Phacotus lenticularis*	X91628.1	KT625422.1	KT625422.1	KT625422.1
UTEX 1593 SAG 17.95	*Borodinellopsis texensis*	KM020129.1	MG778120.1	MG778121.1	MG778126.1
SAG 31.72	*Microglena monadina*	JN903976.1	KT624718.1	KT624742.1	KT624766.1
SAG 19.72 SAG 18.72	*Lobochlamys culleus*	U70594.1	KT625172.1	KT625186.1	KT625162.1
SAG 9.83	*Lobochlamys segnis*	U70593.1	KT624821.1	KT624809.1	KT624842.1
SAG 44.91	*Oogamochlamys gigantea*	AJ410465.1	KT625412.1	KT625412.1	KT625412.1
UTEX 1708	*Palmellopsis texensis*	MG991816.1	MG778453.1	MG778482.1	MG778476.1
UTEX LB 1969	*Chloromonas nivalis/typhlos*	U57696.1	KT624652.1	KT624641.1	KT624639.1
UTEX 1337	*Chloromonas rosae*	U70796.1	AB084315.1	AB084350.1 AB084351.1	AB022536.2
UTEX 966	*Chloromonas radiata*	U57697.1	KT625014.1	KT625021.1	KT625036.1
NIES 1363 NIES 1362	*Pleodorina starrii*	LC086359.1	JX977846.1	JX977846.1	JX977846.1
UTEX 2908 UTEX 1885	*Volvox carteri* f. *nagariensis*	X53904.1	GU084820.1	GU084820.1	GU084820.1
K3-F3-4 NIES 569	*Gonium pectorale*	LC066324.1	AP012494.1	AP012494.1	AP012494.1
CC-503 cw92 UTEX 90	*Chlamydomonas reinhardtii*	AB511834.1	FJ423446.1	FJ423446.1	FJ423446.1
SAG 70.72	*Chlamydomonas peterfii/asymmetrica*	U70788.1	KT624943.1	KT624953.1	KT624961.1
UTEX 962	*Desmotetra stigmatica*	AB218711.1	MG778232.1	MG778231.1	MG778232.1
NIES 425	*Carteria cerasiformis*	AB688624.1	KT625420.1	KT625420.1	KT625420.1
UTEX 432	*Carteria crucifera*	D86501.1	KT624917.1	KT624903.1	KT624910.1
NIES 257	*Hafniomonas laevis*	AB101517.1	KT625415.1	KT625415.1	KT625415.1
SAG 8-5 UTEX 2	*Carteria* sp.	AF182817.1	KT625419.1	KT625419.1	KT625419.1
OCC clade (Oedogoniales, Chaetophorales, Chaetopeltidales)					
UTEX LB 422	*Chaetopeltis orbicularis*	U83125.1	KT693210.1	KT693211.1	KT693212.1 KT693224.1
UTEX 1709	*Floydiella terrestris*	D86498.1	NC014346.1	NC014346.1	NC014346.1
NIES 3575	*Koshicola spirodelophila*	KT693223.1	KT713390.1	KT713392.1	KT713390.1 KT713391.1 KT713392.1
CCAP 334/1	*Uronema sp.*	FN824391.1	MG778533.1	MG778533.1	MG778533.1
UTEX LB1228	*Schizomeris leibleinii*	AF182820.1	NC015645.1	NC015645.1	NC015645.1
UTEX 441	*Stigeoclonium helveticum*	U83131.1	NC008372.1	NC008372.1	NC008372.1
UTEX LB1686	*Oedocladium carolinianum*	U83135.1	NC031510.1	NC031510.1	NC031510.1
UTEX 1557	*Oedogonium angustistomum*	U83134.1	KT693216.1	KT693217.1	KT693218.1
UTEX LB40	*Oedogonium cardiacum*	U83133.1	NC011031.1	NC011031.1	NC011031.1

Plastid gene sequences of *Dispora speciosa* were manually added to existing alignments (Fučíková *et al.* 2019) and ambiguously aligned codons were manually removed prior to analyses. The Supplements contain the full untrimmed alignments, with asterisks designating nucleotide positions to be removed, as well as ready-to-analyze trimmed alignments and the resulting trees for full transparency and reproducibility. 18S sequences were aligned using ClustalW (Larkin *et al.* 2007) in MEGA v.4 (Tamura *et al.* 2007). Fast-evolving, unalignable 18S positions were eliminated using GBlocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with default settings.

The four gene alignments were concatenated and analyzed using MrBayes v3.2 (Ronquist *et al.* 2012), implementing the nucleotide GTR+I+Γ model and partitioning by codon position, with 18S as a separate partition. Two MCMC chains were run for 5,000,000 iterations, sampling every 500, and discarding the first 20% of the trees as burn-in. Analogously, a Maximum Likelihood (ML) analysis was carried out using RAxML (Stamatakis 2014) with 100 rapid bootstrap pseudoreplicates. An analysis of each single-gene alignment was also carried out as described above. The single-gene analyses are available in the Supplements, including the consensus trees and their underlying alignments.

Results

Morphology

Multiple microscopical methods were combined to fully assess the morphology and ultrastructure of *Dispora speciosa*. The strain's cells are arranged in multiples of 2 or, more commonly, of 4 in flat, *Crucigeni*a-like (Morren 1830: 426) coenobia. Tetrads are arranged rather irregularly in the algal culture. Staining by methylene blue shows wide gelatinous covers around the cell agglomerations (FIGURE 1: C). Cells are spherical or oval to elliptical, usually slightly asymmetric or flattened where adjacent to another cell. Cell wall is considerably robust. Individual cells or tetrads enclosed in wide mucilage cover. Inside the cell, one to two large cup-shaped parietal chloroplasts are visible. Chloroplasts along with the large nucleus fill most of the cell (FIGURE 1). The chloroplast shape appears indistinct under light microscope but is confirmed using both fluorescent and transmission electron microscopy as parietal and bowl- or cup-shaped (FIGURE 1). TEM also shows that individual chloroplasts contain numerous starch grains but no pyrenoid. Numerous granules or inclusions are present in the cell, likely outside the chloroplast (FIGURE 1). No process of propagation was observed in the present study. Cell dimensions (6–7µm × 9–11 µm) also fit in the dimension range reported in the original description of the species (Korshikov 1953).

Molecular analyses

Concatenated analyses as well as analyses of individual plastid genes (the latter only shown in Supplements) all strongly supported *Dispora* inside the clade containing the genera *Parallela* and *Microspora* (Microsporaceae from here on after). *Dispora* was nested inside *Parallela* (FIGURE 2), with *Microspora* being sister to *Parallela* + *Dispora*. Only *atpB* supported *Dispora* as sister to *P. novae-zelandiae* E.A. Flint (1974: 359) (0.99 BPP, not shown). The remaining data sets containing both *Parallela* species supported *Dispora* as sister to *P. transversalis* (Brébisson) Novis, M. Lorenz, Broady & E.A. Flint (2010: 382) (0.93 BPP in *rbcL*, 0.78 BPP in *psaB*; trees available in Supplements). Concatenation of all four genes yielded low BPP for the *P. novae-zelandiae* + *D. speciosa* relationship (FIGURE 2) and low ML BS support of 45 for the *P. transversalis* + *D. speciosa* relationship, which nevertheless appeared in the best ML tree (Supplements). The 18S data set only contained *P. transversalis* (data for *P. novae-zelandiae* are not available), and therefore did not contribute to the resolution of the placement of *D. speciosa*. The uncertainty in placement can likely be attributed to the apparent signal conflict between *atpB* and the remaining two plastid genes.

Taxonomic changes

Though the exact position among other *Parallela* species received poor support, the placement into the genus is obvious. Therefore, the following taxonomic change is proposed, including the establishment of an epitype according to article 9.9 of the Shenzhen Code (Turland *et al.* 2018). We argue that Korshikov's (1953) illustration is detailed enough to confidently match to our live and preserved material, but due to the cryptic, simple-bodied nature of most microalgae, and the rampant polyphyly of many morphotypes, any figure is ultimately ambiguous (the main criterion for establishing epitypes) and attaching names to physical material and live cultures is therefore of great importance.

Parallela speciosa comb. nov. **(Korshikov) Štenclová & Fučíková**

Basionym and heterotypic synonym: *Dispora speciosa* Korshikov 1953: 334, Fig. 308 a, b. Epitype: Formaldehyde-fixed specimen kept at University of South Bohemia in České Budějovice, Czech Republic, found under the serial number CBFS A-107-1.

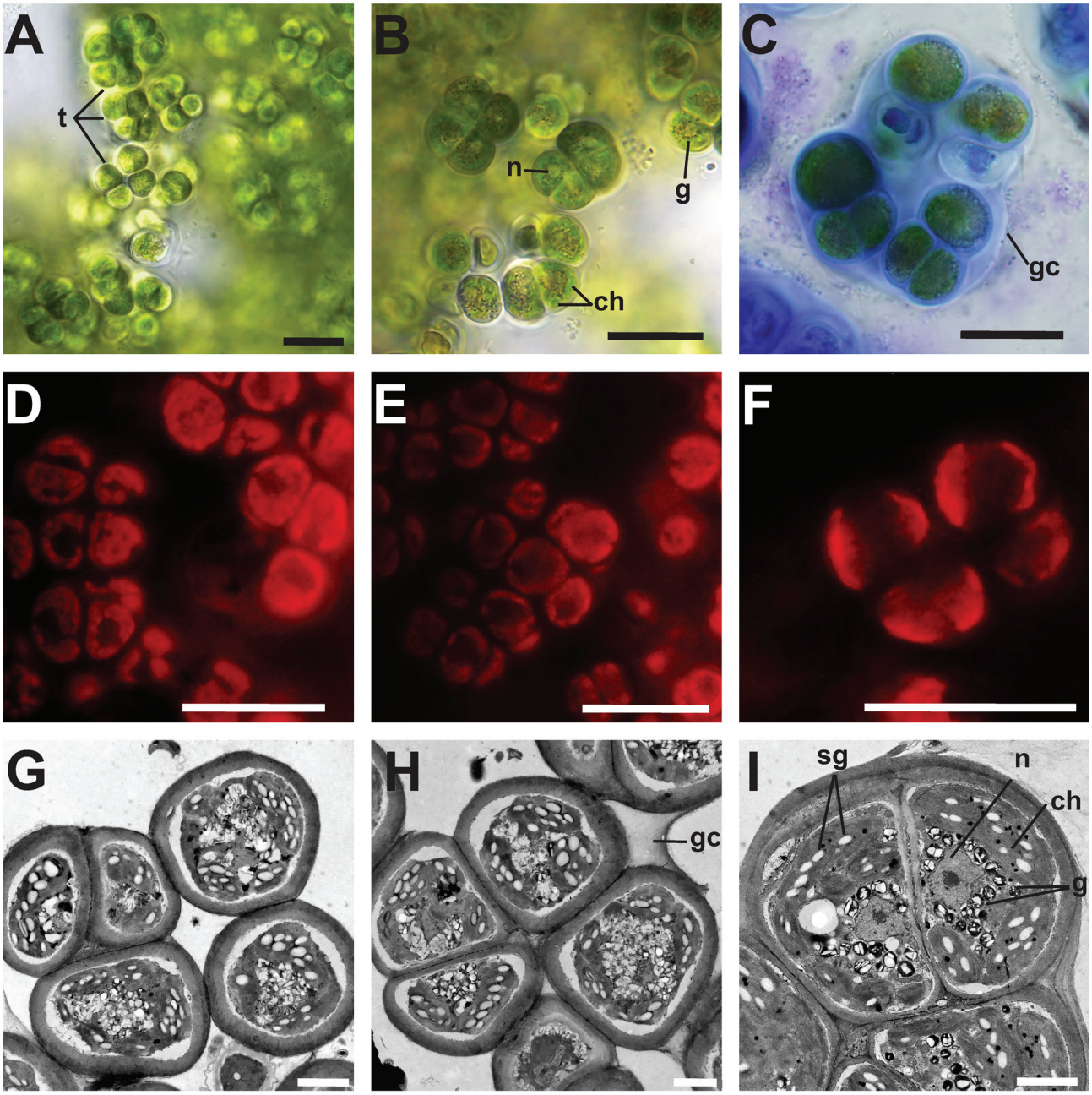

FIGURE 1. Gross morphology of *Dispora speciosa* strain ACOI 1508. Microscopical observations were carried out using: light microscopy (A–C), fluorescence microscopy: observing autofluorescence of chlorophyll (D–F) and transmission electron microscopy (G–I). A: arrangement of the cells into tetrads in the culture, B: distribution of individual organelles inside cells, C: gelatinous cover around the cell aggregation highlighted by methylene blue, D–F: shape of autofluorescent chloroplasts inside cells, G: dividing cells in a tetrade, H: tetrade conjoined to others by the mucilage cover, I: detailed content of the cell. Description: ch=chloroplast, g=granules, gc=gelatinous cover, n=nucleus, sg=starch grain, t=tetrads of cells. The scale bars indicate 20µm (A–F) or 2 µm (G–I).

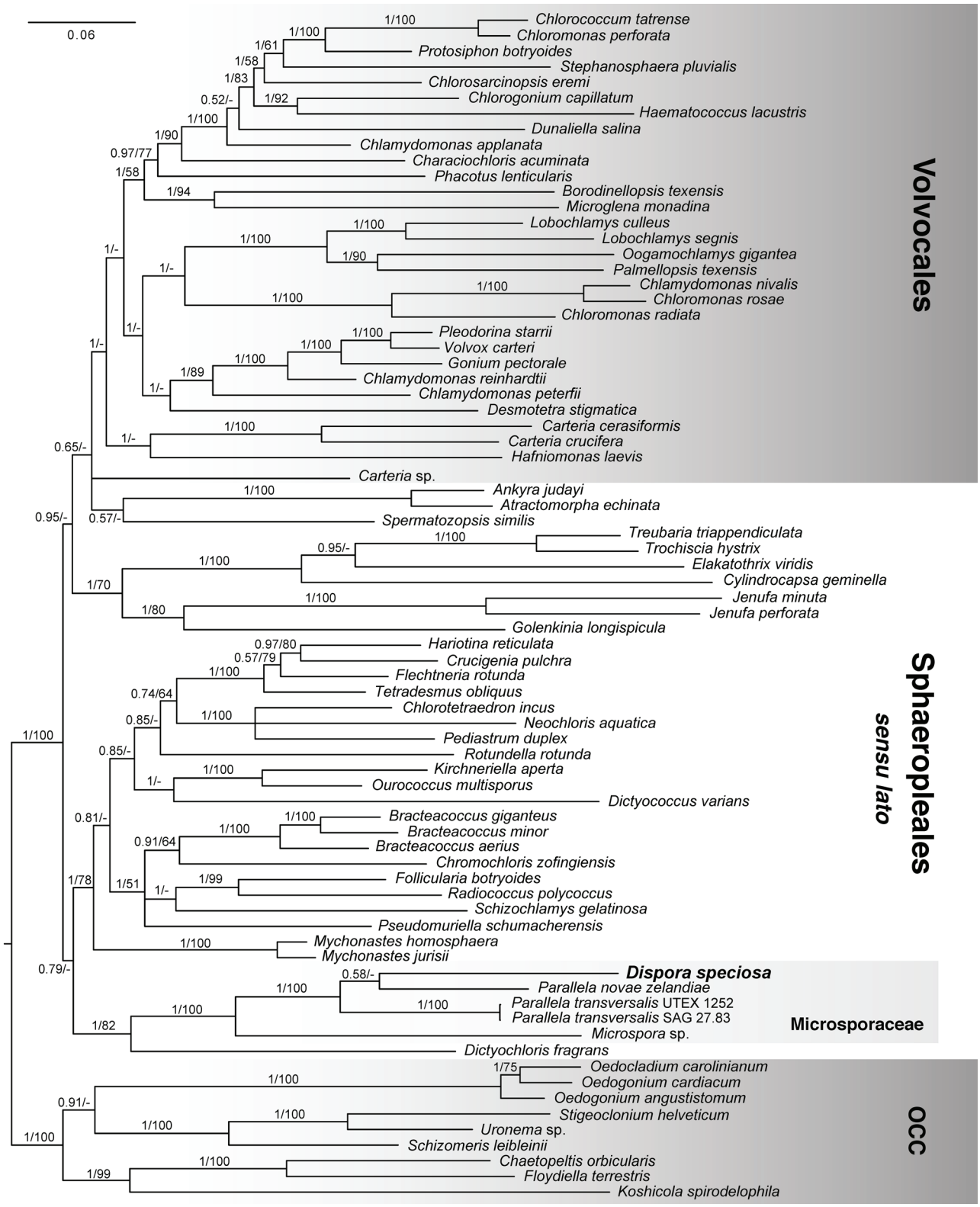

FIGURE 2. Bayesian consensus tree resulting from analysis of concatenated 18S, *atpB*, *psaB* and *rbcL* nucleotide sequences. The species of interest, *Dispora speciosa*, is highlighted in boldface and major taxonomic groups of Chlorophyceae are shown in shaded boxes. Numbers on branches indicate Bayesian posterior probability (BPP) and Maximum Likelihood bootstrap (BS) support, respectively. Only BPP > 0.5 and BS > 50 are shown. Scale bar represents the number of expected substitutions/site as estimated by MrBayes.

Discussion

Dispora in historical context

One clear conclusion from our analyses is that the strain ACOI 1508, from here on referred to as *Parallela speciosa* (unless historical context dictates otherwise), is phylogenetically distant from all previously analyzed lineages of the former, morphologically-defined Radiococcaceae. This is not surprising, considering the previously demonstrated polyphyly of Radiococcaceae (Pažoutová 2008, Pažoutová *et al.* 2010, Fučíková 2014a, Zhang *et al.* 2018). In light of the phylogeny, *Dispora's* mucilage could possibly be referred to as ´gelatinous matrix´ as it is called in *Parallela* in one case (Novis *et al.* 2010) rather than mucilage envelopes/covers of Radiococcaceae, to reinforce the taxonomic distinction. However, it is not currently known whether the two types of extracellular secretions are fundamentally different from each other, either chemically or developmentally.

Despite the similarity in coenobial shape and structure, our analyses also show ACOI 1508 as distant from all available lineages of the former Crucigenoideae, now known to be polyphyletic (Hepperle *et al.* 2000, Hegewald *et al.* 2010, Bock *et al.* 2013, Štenclová *et al.* 2017). Our own analyses only show *Crucigenia pulchra* West & G.S. West (1902: 63) (Scenedesmaceae, Sphaeropleales) (FIGURE 2), because it is the most likely candidate to represent the true *Crucigenia* lineage (*Crucigenia* itself being polyphyletic according to Bock *et al.* 2013), but also because the other crucigenoids are outside Chlorophyceae.

In terms of gross morphology, in ACOI 1508 we find noticeable similarity in coenobium shape and arrangement of the cells especially with the genus *Willea* Schmidle (1900: 157). The cup-shaped chloroplast also occurs in both taxa. Fott (1933) noticed this resemblance and proposed *Willea vilhelmii* (Fott) Komárek (1974: 42) to be placed in the genus *Dispora*, but Komárek (1974) and Komárek & Fott (1983) rejected this idea and recognized both genera as distinct again. Our microscopical assessment confirmed the differences between *Willea* and *P. speciosa*—their individual cells are shaped differently (elongated in *Willea*) and their internal structures differ. Molecular phylogenies support the distinction unambiguously.

Willea belongs in the trebouxiophyte family Oocystaceae (Štenclová *et al.* 2017), and is therefore unrelated to *Parallella*. Consistently with this placement, the pyrenoid with a prominent starch sheath is often clearly visible in *Willea*, whereas in *Parallela* species it is not detectable. Even though presence or absence of pyrenoid likely supports our phylogenetic data, it should be noted that pyrenoids are a taxonomically problematic trait. Their visibility depends on the microscopic technique to some extent, may depend on sample preparation (e.g., staining), and the starch sheath around the pyrenoid may increase or decrease in robustness during a cell's life depending on conditions (e.g., Ramazanov *et al.* 1994).

In the original description of *Dispora* (*D. crucigenioides, D. cuneiformis* (Schmidle) Printz 1914: 33), Printz (1914) noted the absence of pyrenoid ("chromatophoro unico campanulato pyrenoide carente"—single bell-shaped chromatophore lacking a pyrenoid). Later, Korshikov (1953) noted in his circumscription of *D. speciosa*, "без піреноіда"—without a pyrenoid. Komárek & Fott (1983) interestingly mention "Pyrenoid fehlt (oder auch vorkommend?)."—pyrenoid lacking (or also occurring?). This note refers to the South American species *D. globosa* C.E.M. Bicudo & R.M.T. Bicudo (1970: 8), which however bears several features that sharply separate it from other *Dispora* species—spherical, rather than planar, colonies and the presence of pyrenoid, which could place it in the problematic Radiococcaceae according to Komárek & Fott (1983), further emphasizing the complicated nature of the taxonomy in these families and genera. The placement of *D. globosa* has not been resolved, but the taxon likely is not to be placed with the other species of the genus.

Dispora in modern phylogenetic context:

Our phylogenetic analyses confidently placed *Parallela speciosa* in Chlorophyceae, and in the phylogenetic proximity of the order Sphaeropleales. Nevertheless, its family-level classification remains somewhat uncertain due to taxonomic problems outside the scope of our study. Although the ACOI strain belongs to the genus *Parallela* in the family Microsporaceae, as pointed out in previous studies, Microsporaceae itself is a questionable taxon, as no type strain of *Microspora* exists (e.g., Fučíková *et al.* 2019). *Microspora* sp. strain UTEX LB472 has been used in various studies to exemplify the cellular structure of the genus (Pickett-Heaps 1973) and to represent the genus in molecular phylogenies (Buchheim & Buchheim 2001, Watanabe *et al.* 2016), even though it is not an authentic culture and does not even have a species-level identification in culture collections.

We did not observe motile cells in *P. speciosa*. However, the placement of Microsporaceae in the phylogenetic vicinity of Sphaeropleales is corroborated by the slightly uneven flagella and parallel flagellar basal body orientation in *Parallela* and *Microspora* respectively, and is also consistent with the sister placement to *Dictyochloris* (Novis *et al.* 2010, Lokhorst & Star 1999, Shoup & Lewis 2003).

Within the genus *Parallela*, the position of *P. speciosa* depends on which gene is used for phylogenetic inference. *AtpB* lends strong support to the sister relationship of *P. speciosa* and *P. novae-zelandiae*, which also makes the most morphological sense: the multiseriate filaments of *P. novae-zelandiae* shown in Novis *et al.* (2010) and Flint (1974) can be interpreted as similar to the planar colonies that *P. speciosa* forms in nature. The planar thalli were, however, not as obviously formed in our cultured sample, similar to Flint's (1974) observation that under culture conditions *P. novae-zelandiae* produces cell clusters but not the ribbon-like forms. Further, Flint (1974) describes "numerous, unidentified, oscillating granules" in *P. novae-zelandiae*, which are consistent with our observations in live cells of *P. speciosa*. Other cellular features, such as the large, centrally positioned nucleus, a single cup-shaped chloroplast, and the absence of pyrenoid (demonstrated via Lugol staining in *P. transversalis* by Novis *et al.* 2010) are also consistent with our assessment of *P. speciosa*. Interestingly, Flint (1974) brings up the superficial similarity of *P. novae-zelandiae* to *Disporopsis* Korshikov (1953: 202), noting the important differences such as the presence/absence of pyrenoid. For some reason *Dispora* is not mentioned, even though it appears in the same publication by Korshikov (1953) and, at least in our opinion, bears greater morphological resemblance to *Parallela*. *Disporopsis* has since been reclassified as *Planochloris* Komárek (1979: 240) but molecular verification has not yet been attempted.

We examined the only available strain of the genus *Dispora* and without examination of additional live cultures and molecular data, we cannot confidently say whether any of the other *Dispora* species belong to the genus *Parallela*, or to the family Microsporaceae. However, based on morphological features such as cell shape and arrangement, the mucilage cover and the chloroplast characteristics of *D. crucigenioides* (Printz 1914) (which is the type species of *Dispora*) it is rather probable that the entire genus should be merged with *Parallela*. Komárek & Fott (1983) also noted that *D. crucigenioides* and *D. speciosa* may in fact be the same species, as the morphological differences between them are slight.

Several other strains of *Dispora speciosa* as well as *Dispora crucigenioides* are or were kept in the ACOI strain collection, but cannot be provided for future research (per ACOI website and correspondence). *Dispora globosa* appears anomalous within the genus, possessing colonies that are globular rather than flat and tabular, and also has distinct pyrenoids in chloroplasts. For this reason, Komárek & Fott (1983) suggested that this species may be better referred to as *Coenocystis* than *Dispora*. Moreover, the poorly known *Dispora cuneiformis* remains a questionable taxon in clear need of revision because of its incomplete original description (Komárek & Fott 1983). Another taxonomic problem would arise if *Dispora* and *Parallela* were merged, or even if just *D. crucigenioides* were shown as closely related to *P. speciosa*, because *Dispora* is the older name and thus takes priority. However, this cannot happen until a new generitype is established and sequenced. Until then, we believe that our re-classification of *P. speciosa* is an improvement on the current taxonomic situation in *Dispora*, and better reflects evolutionary relationships in Chlorophyceae. Sinking the genus *Parallela* into the ill-defined *Dispora* would not be wise with the limited data that our study presents.

Insights into morphological evolution in Chlorophyceae:

We show that *Parallela speciosa* is a member of an otherwise filamentous clade representing the family Microsporaceae (FIGURE 2). However, filament formation in this group is not easy to interpret in evolutionary terms, though a careful look at the cellular structure and development helps find common features. The peculiar two-part cell wall structure of *Microspora* is initiated during cytokinesis (Ramanathan 1964) and superficially appears quite different from *Parallela*'s bipartite walls (Novis *et al.* 2010), but both are consistent with the Sphaeropleales-specific criterion established by Mattox & Stewart (1984) stating that new walls are deposited within the old filament wall during growth. In Microsporaceae *sensu* Mattox & Stewart (1984) the newly formed walls do not surround the entire surface of daughter cells, distinguishing the family from Sphaeropleaceae.

Fascinatingly, Skuja's (1956) illustration of *D. crucigenioides* includes a filament-like morphotype with clear bipartite character of the cell wall, strikingly reminiscent of *Parallela transversalis* in images by Novis *et al.* (2010). However, such bipartite cell wall is evident neither in *P. novae-zelandiae* (Novis *et al.* 2010) nor in *P. speciosa*, indicating that this particular character may have been lost in some *Parallela* lineages. However, even in *P. speciosa* it is clear (e.g., in FIGURE 1) that the daughter cell wall is deposited within the mother wall. Similarly, the filamentous habit appears to have been 'loosened' in *Parallela* compared to its sister genus *Microspora*, and nearly completely disassembled into a coccoid-like colonial form in *P. speciosa*.

Such a reduction towards a coccoid or colonial form from a more complex, filamentous or coenobial ancestor, has been inferred in other green algal groups before. For example, in Chlorellaceae (Bock *et al.* 2010) or within the genus *Scenedesmus* (e.g., phylogeny of Lewis & Flechtner 2004) multiple shifts between unicellular and coenobial forms may have occurred, although comprehensive analyses of trait evolution would be necessary to conclusively determine the directionality of these shifts.

The present study is an example of a small handful of known filament-to-coccoid transitions. On the other hand, the evolution of a complex form within a clade of otherwise simple-bodied, single-celled algae has been documented as well: for example in the recent study by Kaštovský *et al.* (2016), in which the branched filamentous genus *Ekerewekia* Kaštovský, Fučíková, Štenclová & Brewer-Carías (2016: 171) was clearly demonstrated to have arisen within an otherwise coccoid clade. Interestingly, in the broader context of the *Prasiola* (C.Agardh) Meneghini (1838: 360) clade (the group containing *Ekerewekia* and its closest relatives), another example can be found: *Prasiola* and *Rosenvingiella* P.C. Silva (1957: 41) form multiseriate filamentous to thalloid forms, and analogously to *Ekerewekia* are found within a clade comprising numerous coccoid lineages. Another example, recently documented by Štenclová *et al.* (2017), is the sister relationship between the coccoid *Oonephris* Fott (1964: 134) and the filamentous *Cylindrocapsa* Reinsch (1867: 66). In this case, however, it is unclear whether it represents a reduction or independent evolution of complexity, as the phylogenetic relationships in the morphologically diverse clade are problematic and taxon sampling sparse. While our understanding of morphological evolution in green algae is still incomplete, it is clear that switches between simple and complex body forms have been numerous across the green algal evolutionary history.

Conclusion

The genus *Dispora* exemplifies how tangled taxonomic histories can be, and how placing morphologically defined species and genera in a phylogenetic framework can be both enlightening and complicated. The delimitation and higher classification of *Dispora* is interwoven with other taxa - *Parallela* and *Microspora* in particular. Here we transfer one *Dispora* species into *Parallela* based on extensive review of literature, morphological and ultrastructural observations, and a multigene phylogeny. Despite this detailed evaluation of the former *D. speciosa*, without live material of other *Dispora* species, especially the generitype *D. crucigenioides*, we cannot confidently make genus-level adjustments to the current, morphologically based taxonomy.

Our study also shows that molecular phylogenetics needn't be thought of as a replacement for traditional morphological taxonomy. Instead, a DNA-based phylogeny can be a useful tool to complement morphological approaches, and give them more evolutionary meaning. We use a phylogeny to re-evaluate morphological criteria for taxon classification, and re-interpret morphological characters in light of independently derived evolutionary relationships.

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