South African Journal of Botany 125 (2019) 16–23

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Phylogenetic re-evaluation of previously identified *Chlamydomonas* (Chlorophyta, Chlamydomonadaceae) strains from *The Mosonmagyaróvár Algal Culture Collection*, Hungary, using molecular data

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ARTICLE INFO

Article history:
Received 18 July 2018
Received in revised form 27 May 2019
Accepted 15 June 2019
Available online 4 July 2019

Edited by WA Stirk

Keywords:
Phylogenetic affiliation
Chlamydomonas
MACC

ABSTRACT

Systematic studies on 70 MACC isolates previously identified as '*Chlamydomonas*', a unicellular flagellate, were carried out based on partial 18S rRNA. The aim of this study was to determine the phylogenetic affiliations of *Chlamydomonas* strains in the MACC collection. The study found that most of the strains were not *Chlamydomonas*. Nine clusters of phylogenetically similar taxa were identified. The previous determinations were completed with their new phylogenetic affiliations (partly due to changes in green algae classification). Molecular data revealed that 3 of the 70 strains are from Arenicolinia, 14 are members of the phylogroup Stephanosphaerinia, 11 are Oogamochlamydinia, 1 is Chloromonadinia, 19 are Reinhardtinia, 2 are Polytominia, 9 are Scenedesmaceae, 5 are Moewusinia, and 6 are Chlorella. Clades were established by 18S rRNA similarity and p-distances. This study reveals the need to revise established culture collections whose isolates are solely identified with morphology.

1. Introduction

The genus *Chlamydomonas*Ehrenberg (1833) is one of the largest green algal genera consisting of 400–600 species, most of which are solely morphologically described (Nakada and Tomita, 2011). However, as Ettl (1976, 1981) indicated before, the genus is not a natural assemblage. Phylogenetic studies have clearly showed that the genus is very narrow in the phylogenetic sense (Pröschold et al., 2001; Demchenko et al., 2012). Pröschold et al. (2018), after complex comparative studies, found out that currently only three strains, namely *C. incerta*, *C. reinhardtii* and *C. schloesseri*, are considered to be the member of the genus. *Chlamydomonas* is a model organism to study fundamental processes such as photosynthesis, cell motility, assembly and disassembly of cilia, cell cycle, fertilization, and stress responses of green microalgae (Pan, 2008; Harris, 2009; Ettl and Gärtner, 2014). Since *Chlamydomonas* is an essential biological tool, proper species designations and descriptions are needed.

Algal classifications are currently being revised due to expanding molecular data. Pröschold and Leliaert (2007) advocate using a polyphasic approach when revising algae, which combines molecular information, morphology, electron microscopy, life cycle analyses and ecology. The 18S rRNA gene (Němcová et al. 2011; Barsanti et al., 2013) is the preferred phylogenetic marker for the Volvocales, with hundreds of sequences deposited in public databases, such as GenBank (Nakada et al., 2008b). 18S rRNA gene also has variable regions, so this marker can also be used at lower taxonomic levels, including microevolutionary investigations (Gerloff-Elias et al., 2005; Skaloud, 2008). Nakada et al. (2008b) performed comprehensive molecular analyses of Volvocales, including *Chlamydomonas* species, based on 18S rRNA gene sequences and adopted the PhyloCode (International Code of Phylogenetic Nomenclature, Cantino and de Queiroz, 2010) to explicitly define individual clades (Yumoto et al., 2013, 2014). Apart from this, a few plastid genes (e.g. *psaB*, *rbcL*), as well as combined 18S and 28S rRNA

Abbreviations: MACC, the Mosonmagyaróvár Algal Culture Collection; TBE buffer, Tris/Borate/EDTA; BLAST, Basic Local Alignment Search Tool; MUSCLE, MUltiple Sequence Comparison by Log-Expectation, MEGA, Molecular Evolutionary Genetics Analysis; TIM2 + G + I model, Transition model; RAxML, Randomized Axelerated Maximum Likelihood; OTU, Operational Taxonomic Unit.

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https://doi.org/10.1016/j.sajb.2019.06.028
0254-6299/

Table 1
Summary of the taxonomic reassessment of 70 *Chlamydomonas* strains from the MACC collection with NCBI GenBank accession number.

Phylogenetic affiliation	MACC strain code	Origin/Source	Previous morphological assignment by Vörös (Ördög, 2015)	Closest BLAST hit	Similarity score	NCBI accession number
Chlorella	406	Termite house. Brazil	*Chlamydomonas* sp.	*Chlorella sp.*	97.2%	KY864188
	771	Soil. Brazil	*Chlamydomonas* sp.	*Chlorella sorokiniana*	96.2%	KY864216
	787	Soil. Brazil	*Chlamydomonas* sp.	*Chlorella sorokiniana*	99.5%	KY864223
	793	Soil. Brazil	*Chlamydomonas reinhardtii*	*Chlorella sorokiniana*	97.9%	KY864225
	816	Soil. Brazil	*Chlamydomonas* sp.	*Chlorella sorokiniana*	96.7%	KY864231
	823	Pond. Brazil	*Chlamydomonas* sp.	*Chlorella sp.*	99.5%	KY864236
Moewusinia	27	CCALA 238 (Trebon)	*Chlamydomonas dorsoventralis*	*Chlamydomonas noctigama*	97.7%	KY864170
	30	CCALA 239 (Trebon)	*Chlamydomonas geitleri*	*Chlamydomonas noctigama*	100%	KY864171
	77	CCALA 246 (Trebon)	*Chlamydomonas oblonga*	*Chlamydomonas noctigama*	98.9%	KY864175
	120	Tarn. Slovenia	*Chlamydomonas* sp.	*Chlamydomonas pitschmannii*	99.2%	KY864176
	782	Soil. Brazil	*Chlamydomonas* sp.	*Chlamydomonas pitschmannii*	98.9%	KY864218
Reinhardtinia	53	CCALA 236 (Trebon)	*Chlamydomonas debaryana*	*Chlamydomonas debaryana*	98.5%	KY864172
	216	Sewage plant. Hungary	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	97.5%	KY864179
	285	Kiev. Ukraine	*Chlamydomonas* sp.	*Chlamydomonas zebra*	98.9%	KY864180
	327	Soil. Brazil	*Chlamydomonas* sp.	*Chlamydomonas zebra*	97.9%	KY864181
	335	Soil. Brazil	*Chlamydomonas* sp.	*Chlamydomonas debaryana*	98.7%	KY864182
	382	Soil. Brazil	*Chlamydomonas* sp.	*Chlamydomonas debaryana*	98.2%	KY864183
	415	Puddle. Brazil	*Chlamydomonas* sp.	*Tetraspora sp.*	98.5%	KY864189
	530	Soil. Brazil	*Chlamydomonas* sp.	*Chlamydomonas debaryana*	96.2%	KY864201
	531	Soil. Brazil	*Chlamydomonas* sp.	*Chlamydomonas debaryana*	97.7%	KY864202
	544	Soil. Brazil	*Chlamydomonas* sp.	*Gloeococcus maximus*	98.5%	KY864204
	549	Soil. Hungary	*Chlamydomonas intermedia*	*Chlamydomonas zebra*	97.7%	KY864205
	579	Soil. Hungary	*Chlamydomonas* sp.	*Chlamydomonas debaryana*	98.5%	KY864207
	584	Soil. Hungary	*Chlamydomonas* sp.	*Chlamydomonas debaryana*	98.7%	KY864209
	658	CCALA 247 (Trebon)	*Chlamydomonas peterfii*	*Gloeococcus maximus*	97.9%	KY864212
	674	Kiev, Ukraine	*Chlamydomonas callunae*	*Chlamydomonas mexicana*	99.2%	KY864213
	688	Soil. Brazil	*Chlamydomonas* sp.	*Chlamydomonas debaryana*	97.7%	KY864214
	772	Soil. Brazil	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	95.9%	KY864217
	788	Soil. Brazil	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	99.5%	KY864224
	835	Soil. Brazil	*Chlamydomonas* sp.	*Chlamydomonas rapa*	99.2%	KY864238
Arenicolinia	526	Soil. Brazil	*Chlamydomonas* sp.	*Chlorogonium elongatum*	95.9%	KY864200
	581	Soil. Hungary	*Chlamydomonas* sp.	*Chlorogonium elongatum*	98.2%	KY864208
	607	Soil. Hungary	*Chlamydomonas* sp.	*Chlorogonium elongatum*	96.7%	KY864211
Stephanosphaerinia	388	Soil, Brazil	*Chlamydomonas* sp.	*Chlorococcum ellipsoideum*	99.5%	KY864184
	395	Puddle. Brazil	*Chlamydomonas* sp.	*Chlorococcum ellipsoideum*	98.5%	KY864185
	467	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum diplobionticum*	98.7%	KY864193
	476	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum diplobionticum*	98.9%	KY864195
	482	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum ellipsoideum*	98.5%	KY864196
	487	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum ellipsoideum*	98.9%	KY864198
	543	Sewage water. Brazil	*Chlamydomonas* sp.	*Chlorococcum ellipsoideum*	98.9%	KY864203
	601	Mud. Hungary	*Chlamydomonas* sp.	*Chlorococcum diplobionticum*	98.2%	KY864210
	693	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum ellipsoideum*	98.7%	KY864215
	786	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum ellipsoideum*	99.5%	KY864222
	807	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum ellipsoideum*	98.2%	KY864227
	814	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum diplobionticum*	98.3%	KY864230
	819	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum oleofaciens*	99.2%	KY864233
	821	Soil. Brazil	*Chlamydomonas* sp.	*Chlorococcum oleofaciens*	98.5%	KY864234
Chloromonadinia	557	Pine forest. Hungary	*Chlamydomonas* sp.	*Chloromonas clathrata*	96.9%	KY864206
Oogamochlamydinia	10	CCALA 248 (Trebon)	*Chlamydomonas segnis*	*Lobochlamys culleus*	99.5%	KY806553
	74	CCALA 234 (Trebon)	*Chlamydomonas chlorococcoides*	*Chloromonas clathrata*	98.5%	KY864173
	75	CCALA 249 (Trebon)	*Chlamydomonas subtilis*	*Lobochlamys culleus*	96.2%	KY864174
	194	Sunflower soil. Hungary	*Chlamydomonas gloeogama*	*Lobochlamys culleus*	98.7%	KY864177
	398	AL/G-23. Czech Republic	*Chlamydomonas* sp.	*Lobochlamys culleus*	99.5%	KY864186
	402	Sunflower soil. Hungary	*Chlamydomonas* sp.	*Lobochlamys culleus*	98.9%	KY864187
	425	Soil. Brazil	*Chlamydomonas* sp.	*Lobochlamys culleus*	96.2%	KY864191
	460	Puddle. Brazil	*Chlamydomonas* sp.	*Lobochlamys culleus*	97.9%	KY864192
	496	Soil. Hungary	*Chlamydomonas* sp.	*Lobochlamys culleus*	100%	KY864199
	806	Soil. Brazil	*Chlamydomonas* sp.	*Lobochlamys culleus*	99.5%	KY864226
	822	Soil. Brazil	*Chlamydomonas* sp.	*Lobochlamys culleus*	97.9%	KY864235
Polytominia	475	Soil. Brazil	*Chlamydomonas* sp.	*Polytoma uvella*	98.2%	KY864194
	784	Soil. Brazil	*Chlamydomonas reinhardtii*	*Polytoma uvella*	97.2%	KY864220
Scenedesmaceae	215	IPPAS D-292. Russia	*Chlamydomonas reinhardtii*	*Coelastrella rubescens*	99.7%	KY864178
	424	Soil. Brazil	*Chlamydomonas* sp.	*Scenedesmus vacuolatus*	97.4%	KY864190
	485	Pond. Brazil	*Chlamydomonas* sp.	*Desmodesmus communis*	98.5%	KY864197
	783	Soil. Brazil	*Chlamydomonas* sp.	*Desmodesmus communis*	100%	KY864219
	785	Soil. Brazil	*Chlamydomonas* sp.	*Scenedesmus vacuolatus*	98.7%	KY864221
	810	Soil. Brazil	*Chlamydomonas* sp.	*Coelastrella rubescens*	98.9%	KY864228
	811	Soil. Brazil	*Chlamydomonas* sp.	*Coelastrella striolata*	99.5%	KY864229
	818	Soil. Brazil	*Chlamydomonas* sp.	*Scenedesmus vacuolatus*	98.5%	KY864232
	825	Soil. Brazil	*Chlamydomonas* sp.	*Coelastrella striolata*	99.2%	KY864237

genes, have highlighted numerous chlamydomonadalean lineages (Buchheim et al., 1990, Buchheim et al., 1996, Buchheim et al., 1997a, Buchheim et al., 2013; Nozaki et al., 2000, 2003; Matsuzaki et al., 2010; Lemieux et al., 2015). To confirm the genetic results, Pröschold et al. (2018) used cross experiments of sporangium wall autolysins (VLE), because this enzyme is a good biochemical marker for classification of *Chlamydomonas* species.

Our study focuses on the systematic relationships of 70 MACC strains previously identified as *Chlamydomonas*. No molecular phylogenetic data were available for the *Chlamydomonas* strains in the MACC collection, therefore, we sequenced partial 18S rRNA gene to determine their phylogenetic position and assign them to subgroups defined by Nakada et al. (2008b). The aim of this research is to provide a proper platform for future systematic and biodiversity research.

2. Materials and methods

2.1. Strains and cultivation

Seventy *Chlamydomonas* (Table 1) strains were selected from MACC, Széchenyi István University (Mosonmagyaróvár, Hungary). The former nomenclature is based on the morphological identification, which was carried out in the 1990's by the Balaton Limnological Institute team (Ördög, 2015). Forty-six of the 70 MACC *Chlamydomonas* strains were from Brazil, 11 from Hungary, nine from the Czech Republic, 2 from Ukraine, 1 from Slovenia, and another from Russia. Cultures of *Chlamydomonas* were maintained and cultivated in modified Z8 medium (Kótai, 1972; NIVA, 1976) at 24–26 °C under a light intensity of 20 μmol m^{-2} s^{-1} provided by Lumoflor and cool white light (16:8 h light:dark cycle).

2.2. Genomic DNA extraction and PCR

One mL of cell culture was centrifuged at 14,000 rpm for 2 min to spin down the culture. From the spin down, a 10 mg pellet was resuspended in 500 μL of 10% Chelex-100 diluted in ddH_2O. The suspension was vortexed for 30 s and incubated at 95 °C for 10 min. The sample was cooled to 20 °C and vortexed again for 10 s and centrifuged at 14000 rpm for 2 min. One μL of the supernatant was used in each 20 μL PCR reaction. The 18S rRNA gene was amplified with primers EUK528F (5-′CCGCGGTAATTCCAGCTC-3′) (Elwood et al., 1985; Keresztes et al., 2012) and Chlo02R (5′-CTTCGAGCCCCCAACTTTC-3′) (Zhu et al., 2005). The PCR mixture contained 10 μL Phusion Flash High-Fidelity PCR Master Mix, 7 μL dH_2O, 1 μL of each primer (0.5 um final concentration per primer) and 1 μL purified DNA (50–100 ng) to give 20 μL final volume of PCR reaction.

For PCR amplification, an initial denaturation was carried out at 98 °C for 30 s, followed by denaturation at 98 °C for 10 s, annealing at 58 °C for 20 s, extension at 72 °C for 30 s, and the final extension at 72 °C for 1 min over 40 cycles. After DNA amplification, the products were run for 45 min at 120 V in 0.5% TBE buffer and visualized on a 1.5% agarose gel. The PCR products were purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR products were sequenced using a LifeTech 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) capillary sequencer at the Biological Research Centre of the Hungarian Academy of Sciences (BRC) (Szeged, Hungary).

2.3. Sequence alignment and phylogenetic analyses

Reference sequences of Chlamydomonadaceae strains (Lemieux et al., 2015; Nakada et al., 2016; Possmayer et al., 2016; Watanabe and Lewis, 2017) were retrieved from GenBank (NCBI) (Altschul et al., 1997), in addition to closest BLAST hits. The sequences were aligned using MUSCLE (Edgar, 2004) on MEGA 7 (Kumar et al., 2016). The jModelTest 2 confirmed a TIM2 + G + I model of substitution (Darriba et al., 2012). The maximum likelihood (ML) analysis was run on a dataset of 279 sequences using RAxML (Stamatakis, 2014) with 1000 bootstraps and *Ulothrix zonata* UTEX 745 was used to root the tree. The final phylogenetic tree (Fig. 1) was edited using Adobe Illustrator CC version 2014.01. The similarity matrix (percentages) for MACC strains comparing partial sequences of the 18S rRNA gene was calculated by Geneious 10.2.3 (Table S2). Due to lack of space, only a few representatives of each cluster were shown in the table

3. Results

The sequences formed c.a. 400 bp alignments, including our strains, was composed of 279 OTUs with nine phylogroups within three different orders (following Nakada 2008b) (Fig. 1). One phylogroup belonged to the order Chlorellales (*Chlorella* phylogroup with 6 MACC strains), one in the order Sphaeropleales (Scenedesmaceae phylogroup with 9 MACC strains) and seven phylogroups in the order Chlamydomonadales (*Moewusinia* with 5, *Reinhardtinia* with 19 strains, *Arenicolinia* with 3, *Stephanosphaerinia* with 14, *Chloromonadinia* with 1, *Oogamochlamydinia* with 11 and *Polytominia* phylogroup with 2 MACC strains). Results of present phylogenetic analysis are shown in Table 1.

3.1. Arenicolinia phylogroup

The Arenicolinia phylogroup (Fig. 1) formed a well-supported cluster (BS 99%) with two reference sequences (Chlorophycean sp. SEV3VF14 AF513371 and *Chlorosarcinopsis arenicola* UTEX 1697 AB218701) and three MACC strains (MACC 526, 581, 607). The sequence similarity between *Chlorosarcinopsis arenicola* UTEX 1697 AB218701 and MACC 607 was 97.5% (Table S2).

3.2. Stephanosphaerinia phylogroup

The Stephanosphaerinia phylogroup (Fig. 1) included 14 MACC strains (MACC 395, 388, 467, 476, 482, 487, 543, 601, 693, 786, 807, 814, 819, 821) and forms a well-supported clade (B.S. 99%).

Five strains of the first subclade (MACC 482, 487, 543, 819, 821) had 100% B.S. support with *Chlorosarcinopsis aggregata* UTEX 779 AB218695, but their sequence similarity ranged between 90.4 and 93.7%. MACC 543 originated from sewage water (Brazil), while the remaining four strains were terrestrial (Brazil). MACC 819 and 821 are likely to belong to the same *Chlorosarcinopsis* taxon, since they were 100% homologous, and isolated from the same area.

The sequence similarity of the second subcluster groups MACC 388, 395, 693, 786 and 807 together with *Chlorococcum ellipsoideum* UTEX 972U70586 ranged from 97.2 to 98.2%. MACC 388, 395, 693, 786, and 807 (Table 1) are strains with 96% bootstrap support.

MACC 601 was 97.7% similar (Table S2) to *Nautococcus solutes* SAG 76 80 AB360749. This is the only Hungarian strain in this phylogroup and it originated from mud, whereas the other 13 strains were from Brazil (11 from soil, MACC 395 from a puddle and MACC 543 from sewage water).

MACC 467, 476 and 814 fell close to *Deasonia multinucleata* UTEX 2013 U63106. Their 18S rRNA similarity ranged from 95.3% to 95.7%. They were all isolated from soil (Brazil).

3.3. Oogamochlamydinia phylogroup

The Oogamochlamydinia phylogroup (Fig. 1) is a mixture of genera containing *Chlamydomonas*, *Lobochlamys*, *Oogamochlamys*, *Sarcinochlamys* and 11 MACC taxa (MACC 10, 74, 75, 194, 398, 402, 425, 460, 496, 806, 822) (Table 1). The Oogamochlamydinia group was composed of two subclusters: the first subcluster included MACC 74, 75, and 425. MACC 74 and 75 are from CCALA. As for their 18S rRNA similarity, both showed low values (<50%) with *Chlamydomonad sp.* BogD8 18T2w AY220581. MACC 74 and 75 did not group with the reference sequences, thus further analysis is needed to classify these

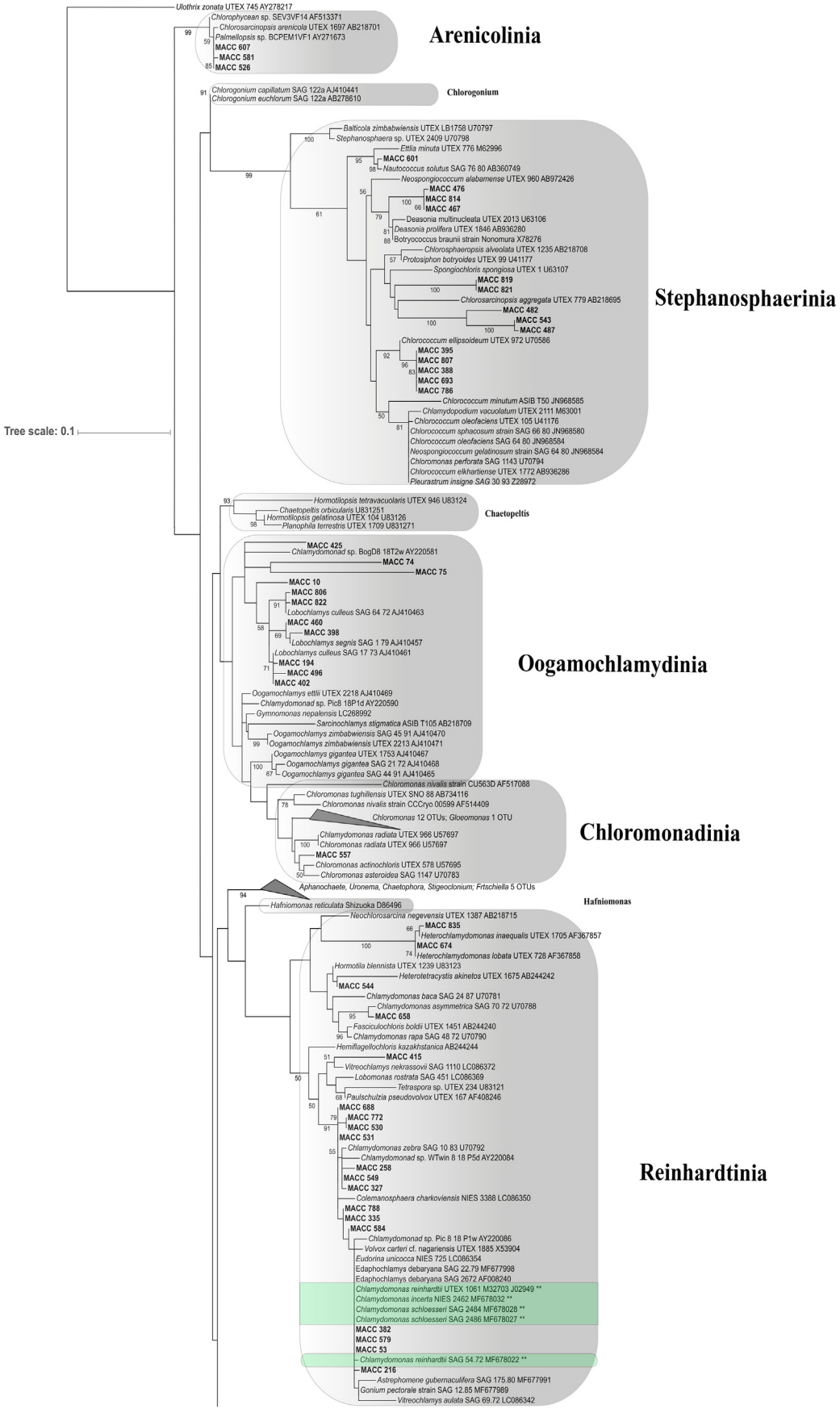

Fig. 1. 18S rRNA gene phylogenetic analysis based on 279 OTUs demonstrating the position of 70 MACC strains. MACC strain names are in bold. The bootstrap support (≥50%) for maximum likelihood (ML) is shown next to the nodes. Ulothrix zonata was used as outgroup.

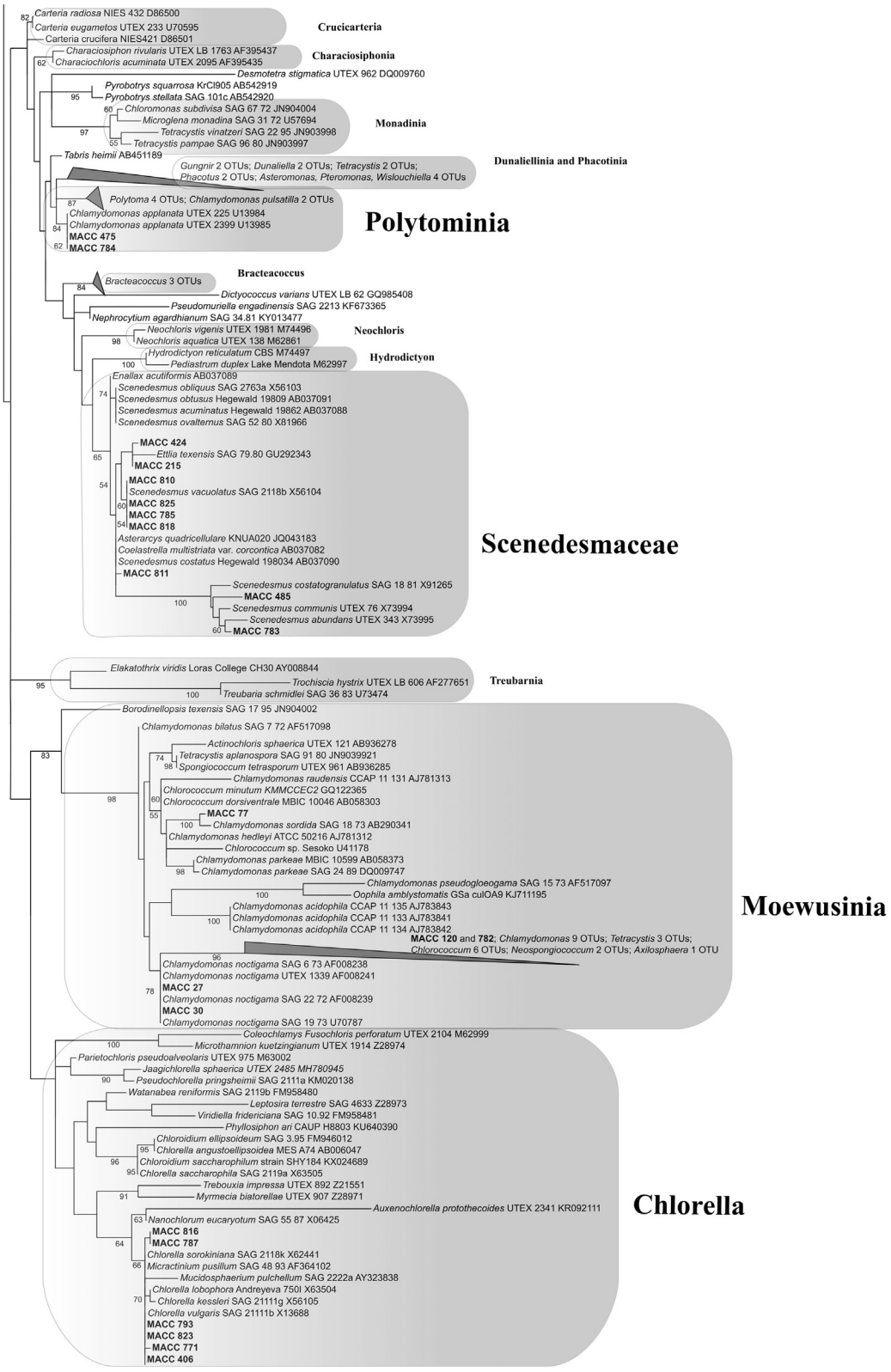

Fig. 1 (*continued*).

strains. MACC 425 originated from soil (Brazil) and grouped with the Chlamydomonad sp. BogD8 18T2w AY220581.

The second subcluster of the Oogamochlamydinia group included MACC 10, 194, 398, 402, 460, 496, 806, 822 and *Lobochlamys*. Their sequence similarity ranged from 96% to 98.7%. MACC 398 and 460 grouped with *Lobochlamys segnis* SAG 1.79 AJ410457, MACC 822 and 806 with *Lobochlamys culleus* SAG 64.72 AJ410463, and MACC 194, 402, and 496 with *Lobochlamys culleus* SAG 17.73 AJ410461. MACC 10 was also part of this subcluster but distant from the other strains. Interestingly, MACC 10 was designated as *Chlamydomonas segnis* and originated from CCALA. MACC 10 neighbored *Lobochlamys segnis,* which was

previously designated as *Chlamydomonas segnis* SAG 1 79 AJ410457. The strains of this subcluster had various places of origin.

3.4. Chloromonadinia phylogroup

The Chloromonadinia phylogroup (Fig. 1) contains mostly *Chloromonas* sequences and the only MACC member of this phylogroup is MACC 557, which is from a pine forest (Hungary) (Table S2). According to the phylogenetic analysis, its closest neighbor is *Chloromonas actinochloris* UTEX 578U57695, but had higher sequence similarity to *Chloromonas asteroidea* SAG 11-47U70783.

3.5. Reinhardtinia phylogroup

The Reinhardtinia phylogroup (Fig. 1) contained 19 MACC strains (MACC 53, 216, 285, 327, 335, 382, 415, 530, 531, 544, 549, 579, 584, 658, 674, 688, 772, 788, 835) and three subclades were identified (Table 1).

The first subclade (100% bootstrap) contained two strains, MACC 674 and 835, along with *Heterochlamydomonas* reference sequences. MACC 674 was 74% similar to *Heterochlamydomonas lobata* UTEX 728 AF367858 (Table S2), whereas MACC 835 was 66% similar to *Heterochlamydomonas inaequalis* UTEX 1705 AF367857. MACC 674 is from Kiev and MACC 835 is from Brazilian soil samples.

The second subclade contained two strains, MACC 544 and 658. MACC 658 originated from Trebon, Czech Republic (*Chlamydomonas peterfii* CCALA 247) and grouped with *Chlamydomonas asymmetrica* SAG 70 72U70788 (97.3% sequence similarity). MACC 544 originated from soil (Brazil), and was placed next to *Heterotetracystis akinetes* UTEX 1675 AB244242. Despite this, MACC 544 showed the greatest sequence similarity (98.3%) to *Hormotila blennista* UTEX 1239U83123, thus further analysis is required to accurately delineate this strain.

The third subclade included 15 MACC strains (MACC 53, 216, 285, 327, 335, 382, 415, 530, 531, 549, 579, 584, 688, 772, 788) from which MACC 415 fell outside and grouped with *Vitreochlamys nekrassovii* SAG 1110 LC086372. MACC 415 was the only strain in the Reinhardtinia phylogroup that originated from a puddle (Brazil), whereas the remaining strains were terrestrial (Brazil and Hungary). The remaining 14 MACC strains (MACC 53, 216, 285, 327, 335, 382, 530, 531, 549, 579, 584, 688, 772, 788) grouped together with such strains as *Edaphochlamys debaryana* SAG 26.72 AF008240, *Chlamydomonas reinhardtii* UTEX 1061M32703J02949, *Chlamydomonas zebra* SAG 10.83U70792 and two other sequences (Chlamydomonad sp. WTwin 8 18 P5d AY220084 and *Volvox carteri* f. *nagariensis* UTEX 1885 X53904). MACC 285, 327 and 549 were most similar to *Chlamydomonas zebra* SAG 10.83 (97–97.5%) whereas MACC 53, 216, 335, 382, 530, 531, 579, 584, 688, 772, and 788 were most similar to *Chlamydomonas reinhardtii* UTEX 1061M32703J02949 and ranged from 96.7% to 98.2% similarity. In a phylogenetic sense, this latter together with *Chlamydomonas incerta* and *Chlamydomonas schloesseri* are considered to be 'real' *Chlamydomonas* genera. They are in a green shaded area on Fig. 1.

3.6. Polytominia phylogroup

The Polytominia cluster (Fig. 1) has two *Chlamydomonas applanata*, two *Chlamydomonas pulsatilla* and four *Polytoma* OTUs as reference sequences. MACC 475 and 784 (Table 1) are 98.5% (Table S2) similar to *Chlamydomonas applanata* UTEX 225U13984 and *C. applanata* UTEX 2399U13985 strains, though their subclade is not well supported (B.S. 84%). Both MACC 475 and 784 were isolated from Brazilian soil samples.

3.7. Scenedesmaceae phylogroup

The Scenedesmaceae phylogroup (Fig. 1) had nine MACC strains (MACC 215, 424, 485, 783, 785, 810, 811, 818, 825) (Table 1) that were isolated from Brazilian soils [except MACC 215 which was from IPPAS D-292 (Russia) and MACC 485 originating from a pond (Brazil)]. MACC 811 fell into the same clade as *Scenedesmus costatus* Hegewald 19862 AB037090 with 98.7% sequence similarity (Table S2). MACC 485 and MACC 783 had the highest bootstrap support of this cluster (100%) and grouped together with *Scenedesmus costato-granulatus* SAG 18 81 X91265, *Scenedesmus communis* UTEX 76 X73994 and *Scenedesmus abundans* UTEX 343 X73995. They showed the highest sequence similarity to *Scenedesmus communis* UTEX 76 X73994 (97.5%). Four MACC strains (785, 810, 818 and 825) were in the same group as *Scenedesmus vacuolatus* SAG 211-8b and their sequence similarity ranged from 97.2% to 98.5%. The remaining two strains (MACC 215 and 424) grouped together with *Ettlia texensis* SAG 79.80 GU292343.

3.8. Moewusinia phylogroup

The Moewusinia phylogroup (Fig. 1) was well-supported [Bootstrap (B.S.) 99%] and included five MACC strains (MACC 27, 30, 77, 120, 782). MACC 120 grouped with *Chlamydomonas moewusii* CCAP 11/16F FR865565 and their 18S rRNA similarity was 98.2%. MACC 782 was only 94.9% similar to *Chlamydomonas raudensis* SAG 49.72 JN903981. Both MACC 27 and 30 were close to *Chlamydomonas noctigama* (also known as *Chlamydomonas pinicola*) UTEX 1339 AF008241, the former with 97.5% similarity, and the latter 99.1% (Table S2). MACC 77 was related to *Chlamydomonas sordida* SAG 18.73 AB290341 and their 18S rRNA similarity was 97%. MACC 27, 30 and 77 were from CCALA (Trebon, Czech Republic) (Table 1). MACC 120 was isolated from a tarn (Slovenia), whereas MACC 782 was terrestrial (Brazil).

3.9. Chlorella phylogroup

The *Chlorella* phylogroup (Fig. 1) contained six MACC strains (MACC 406, 771, 787, 793, 816, 823). MACC 787 and 816 strains were tightly clustered with the reference sequence *Chlorella sorokiniana* SAG 211-8k X62441. Their 18S rRNA similarity was 99% with *Chlorella sorokiniana* SAG 211-8k (Table S2). The subcluster itself was supported by 66% (ML). MACC 406, 771, 793 and 823 were directly grouped with *Chlorella vulgaris* SAG 21111b X13688.

As for the place of origin, MACC 771, 787, 793, 816 are from soil samples (Brazil), whereas MACC 823 was isolated from a pond (Brazil) (Table 1).

4. Discussion

This is the first study using molecular data to determine phylogenetic relationships of MACC green algae strains. The phylogenetic tree, resulting from the analysis of 70 partial 18S rRNA sequences, revealed nine different phylogroups. There is a need for proper identification of culture collections that do not have molecular data. Correcting these descriptions contributes to refining taxonomic models and methods and provides a proper platform for future work.

Phenotypic plasticity combined with constant nomenclatural updates makes it difficult to identify and classify isolates based solely on morphology. Methods of molecular genotyping and subsequent phylogenetic analyses enable more precise determination of the taxonomic position of new isolates (Kravtsova et al., 2013). The circumscription of species in *Chlamydomonas* is problematic because many original descriptions were based on light microscopy of a few specimens from natural samples without considering the plasticity of morphological characters within a population or life history (Pröschold et al., 2001). In addition, several molecular phylogenetic analyses have shown that *Chlamydomonas* is highly polyphyletic (e.g. Buchheim et al., 1997b;

Pröschold et al., 2001; Nakada et al., 2008a; Nakada and Tomita, 2011; Demchenko et al., 2012, Watanabe and Lewis, 2017, Pröschold et al., 2018). Nakada et al. (2008b) adopted the phylogenetic classification system of the order Volvocales based on PhyloCode (Cantino and de Queiroz, 2010), and recognized 21 'primary clades' and we found this code to be the best way to determine the appropriate taxonomic positions of MACC green algae strains.

The MACC strains were distributed among nine clades. Performing molecular analyses (based on partial 18S rRNA gene sequences) allowed the reclassification of 70 strains previously assigned to the *Chlamydomonas* genus into various phylogenetic affiliations. The results showed that most strains previously assigned to *Chlamydomonas*, were not *Chlamydomonas* in the phylogenetic sense. This indicates how molecular approaches to systematics are fundamental for classifying algae. The first explanation for the results could be that the morphological features of *Chlamydomonas* are very variable and overlapping. Therefore, the initial light microscope identification carried out in the 1990's lead to misidentification, and then there is the constantly changing *Chlamydomonas* taxonomy. In addition, 15 of the strains belonged to non-*Chlamydomonas*-like phylogroups, such as Scenedesmacea and Chlorella. Probably the *Chlorella* or *Scenedesmus* species were contaminations in the original Chlamydomonad isolates and later predominated in the culture.

Molecular biologists are currently screening the contents of public strain collections, however, these collections are not representative of the large diversity of taxa in the field and the designations of many taxa in culture collections are doubtful. Many algae are difficult to maintain in cultures and are not available for molecular analysis (Day et al., 2004; Hegewald, 1989; Krienitz and Bock, 2012). Future goals for advanced characterization of the strains within the MACC include fluorescent microscopy and ultrastructural analyses. Watanabe and Lewis (2017) showed that certain ultrastructural traits can be used to diagnose monophyletic lineages and that the investigated traits are informative at different phylogenetic depths, from genera to phylogroups. Morphological characters used for taxonomy (Nakazawa et al., 2001) often disagree with specific lineages or are not diagnostic as originally proposed. Apart from this, using more and longer genes (SSU, ITS rDNA, plastid-coding rbcL) and cross experiments of sporangium wall autolysins (Pröschold et al., 2018), could result more firm phylogenetic separation.

5. Conclusion

This study examined and resolved 70 MACC strains based on molecular methods. This collection offers an unexploited potential as a repository of taxonomic data for algal diversity in relation to unexamined public algal collections. By placing 70 MACC strains on the algal tree of life, we provide references for future systematic and biodiversity research. The re-examination of these strains may contribute to a better understanding of *Chlamydomonas* classification. Based on our findings, we recommend that other culture collections regularly update the status of their strains, thus establishing a more accurate algal taxonomic framework.

Declarations of competing interest

All authors declare that there is no conflict of interest regarding the publication of this paper.

Formatting of funding sources

This research was funded by SABANA (Grant No. 727874) from the EU Horizon 2020 Research and Innovation program and by the NKFI-FK-123899 grant. Gergely Maróti thanks the János Bolyai Research Scholarship of the Hungarian Academy of Sciences for support. Haywood Dail Laughinghouse acknowledges the USDA NIFA, Hatch project # FLA-FTL-005697 for additional support.

Acknowledgements

The authors highly appreciate the valuable contribution of Dr. Wendy Ann Stirk to the improvement of the manuscript. The authors would also like to thank anonymous reviewers for their valuable comments and suggestions leading to the improvement of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sajb.2019.06.028.

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