Phycologia Volume 54 (5), 545–552 Published 6 October 2015

The phylogeny of the genus *Nannochloropsis* (Monodopsidaceae, Eustigmatophyceae), with descriptions of *N. australis sp. nov.* and *Microchloropsis gen. nov.*

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Abstract: Strains of the genus *Nannochloropsis* (Eustigmatophyceae) from the Australian National Algae Culture Collection were evaluated by DNA sequence analysis. Strains were first assessed by sequence analysis of a 900-base-pair region of the plastid *rbc*L gene (bar-code analysis). This analysis allowed us to identify most of the strains at specific rank. However, the strains CS-416 and CS-759 produced a unique *rbc*L DNA sequence and were sister to *N. oceanica* in the phylogenetic analysis. Complete *rbc*L sequences and nuclear 18S ribosomal (r)DNA sequences were produced for these two strains. The results of phylogenetic analyses of an alignment of the concatenated 18S rDNA and *rbc*L sequences for *Nannochloropsis* taxa revealed that strains CS-416 and CS-759 represent a new species, *N. australis*. In addition, the analysis provided strong support for erecting a new genus, *Microchloropsis*, comprising the species *M. salina* and *M. gaditana*.

Key Words: DNA bar code, Eustigmatophyceae, *Microchloropsis*, *Nannochloropsis*, *rbc*L, rDNA

INTRODUCTION

Species of the tiny unicellular eustigmatophycean alga *Nannochloropsis* have been used for many years as a source of unsaturated fatty acids in the diet of aquaculture-raised marine invertebrates (Brown *et al.* 1997; Apt & Behrens 1999). Several strains of *Nannochloropsis* species are now available commercially for culture or as prepared formulations for aquaculture. More recently, *Nannochloropsis* species have been under intense investigation as possible biofuel organisms because of their high neutral lipid content (Khozin-Goldberg & Boussiba 2011). The potential of *Nannochloropsis* species for biofuel production has led to a rapid increase in the number of studies on the complete genome sequences of *Nannochloropsis* species (Pan *et al.* 2011; Radakovits *et al.* 2012; Vieler *et al.* 2012; Jinkerson *et al.* 2013; Carpinelli *et al.* 2014; Wang *et al.* 2014), comparative genomics of the plastid and mitochondrial genomes (Wei *et al.* 2013; Starkenburg *et al.* 2014), studies of genome-wide gene expression responses to growth conditions (Liang *et al.* 2013; Zheng *et al.* 2013; Carpinelli *et al.* 2014; Wang *et al.* 2014), and methods for genetic transformation (Kilian *et al.* 2011; Radakovits *et al.* 2012). The combination of high lipid content, ease of growth on a large scale, new genomic information, and genetic transformation techniques has led to the suggestion that *Nannochloropsis* has high potential as a model for photons-to-fuel commercial systems (Jinkerson *et al.* 2013).

The genus *Nannochloropsis* was erected by Hibberd (1981) with the type species *N. oculata* (Droop) D.J. Hibberd and the additional species, *N. salina* D.J. Hibberd, both marine species. Later authors have added three additional marine species [*N. gaditana* L.M. Lubián (Lubián 1982), *N. oceanica* Suda & Miyashita (Suda *et al.* 2002) and *N. granulata* B. Karlson & D. Potter (Karlson *et al.* 1996)] and one freshwater species [*N. limnetica* L. Krienitz, D. Hepperle, H.-B. Stich & W. Weiler (Krienitz *et al.* 2000)]. These species are primarily characterized by the nuclear 18S ribosomal RNA gene (18S rDNA) and plastid *rbc*L sequences. Although some morphological variation is present, no consistent morphological features support the existing species-level taxonomy (Andersen *et al.* 1998; Fawley & Fawley 2007).

The increased interest in *Nannochloropsis* has led to an abundance of strains of the genus now available from many algal culture collections or held in private collections. These strains have often been characterized only by the 18S rDNA sequence. As a result, there are (as of 7 January 2015) 150 18S rDNA sequences of over 1 kb listed for *Nannochloropsis* available in GenBank. Many researchers are apparently using the 18S rDNA sequences as a bar-coding region for the identification of species; however, 18S rDNA is a highly conserved locus that is not generally considered appropriate for identification at the species level (Leliaert *et al.* 2014). Indeed, several species and varieties of *Nannochloropsis* were initially characterized by the much more variable plastid *rbc*L gene (Suda *et al.* 2002; Fawley & Fawley 2007; Cao *et al.* 2013). Recent studies have shown that strains with identical 18S rDNA sequences can have considerable variation at the genome level (Wei *et al.* 2013; Carpinelli *et al.* 2014).

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DOI: 10.2216/15-60.1

Table 1. New *rbc*L and 18S rDNA DNA sequence data for strains of *Nannochloropsis* and *Microchloropsis* from the Australian National Culture Collection of Algae investigated in this study with their source locations and GenBank accession numbers. Most *rbc*L sequences were produced using the bar-coding primers; more complete *rbc*L sequences are indicated in bold. A full version of the complete data set of strains used in this study, including georeferenced isolation localities, is presented in the supplementary data Table S1.

Strain number	Original identification	Revised identification	Source location	*rbc*L	18S rDNA
CS-179	*N. oculata*	*N. oceanica*	Japan	**KT149177**	KT031995
CS-246	*Nannochloropsis* sp.	*N. oceanica*	Deception Bay, Queensland, Australia	**KT149178**	KT031996
CS-416	*Nannochloropsis* sp.	*N. australis*	Tasmania, Australia	**KT149179**	KT031997
CS-446	*N. salina*	*M. salina*	Scarborough, Rhode Island., USA	KT149180	
CS-448	*N. gaditana*	*M. gaditana*	Tortola, British Virgin Islands	KT149181	
CS-699	Marine *Chlorella*	*N. oceanica*	Japan	KT149182	
CS-700	Marine *Chlorella*	*N. oceanica*	Japan	KT149183	
CS-701	*N. gaditana*	*M. gaditana*	Comacchio Lagoon, Ferrara, Italy	KT149184	
CS-702	*N. oceanica*	*N. oceanica*	Qingdao, China	KT149185	
CS-703	*N. oculata*	*N. oceanica*	Provenance uncertain	KT149186	
CS-759	Unidentified	*N. australis*	Orford, Tasmania, Australia	**KT149187**	KT031998

The commercial interest in *Nannochloropsis* has also led to numerous identical strains of this alga that are held in multiple culture collections. Sometimes the provenance and original strain names are not clear. To clarify this situation we undertook a systematic survey of strains with 18S or *rbc*L sequence data to document issues of correct strain identification. We also georeferenced source localities when possible. These data are listed in supplementary data Table S1. Table S1 shows that of 77 unique strains that can be georeferenced, only seven were isolated from the Southern Hemisphere. The Southern Hemisphere could harbour additional diversity for the genus that should be explored. The major algal collection in the Southern Hemisphere is the Australian National Algae Culture Collection (ANACC), which includes 19 strains of *Nannochloropsis*, of which four are Southern Hemisphere isolates.

In this study, we examined the marine strains of *Nannochloropsis* from the ANACC using a new *rbc*L bar-coding region for initial identification of the strains. We also examined the phylogeny of the genus with a combined analysis of 18S rDNA and *rbc*L sequences. The results of our analyses clearly indicate the division of *Nannochloropsis* into two major lineages. On the basis of this result, which has already been shown by several phylogenetic analyses of the genus (e.g. Krienitz *et al*. 2000; Suda *et al*. 2002; Fawley & Fawley 2007), we propose the new genus *Microchloropsis* comprising the species *M. salina comb. nov*. and *M. gaditana comb. nov*. We also describe a new species of *Nannochloropsis*, *N. australis*, from ANACC strains that were isolated from Australian waters.

MATERIAL AND METHODS

Strains of *Nannochloropsis* from the ANACC (Table 1) were cultured in either f/2 (Guillard 1975) or GSe (Blackburn *et al*. 2001) media at 20°C with cool-white fluorescent illumination at about 25 μmol photons m^{-2} s^{-1} with a 14:10 light:dark photoperiod. Batch cultures grown in 125-ml flasks were used for DNA isolation and microscopy after approximately 1 week of growth. Light microscopy was performed with a Nikon NiU microscope (Nikon, Melville, New York, USA) equipped with differential interference contrast optics and a Plan Achromat ×100 objective, numerical aperture 1.45. Digital images were captured with a Nikon DS-Fi2 digital camera and cell measurements were performed using Nikon Elements BR image analysis software.

DNA was isolated from ANACC *Nannochloropsis* strains according to Fawley & Fawley (2004). For initial sequence analysis, a 1037-base-pair (bp) portion of the *rbc*L coding region was amplified using the primers Eustig*rbc*L-FB (5′GATCCRATTGAAGCTGC3′, corresponding to bases 91103 to 91119 of the *N. oceanica* strain CCMP531 chloroplast genome, GenBank accession KC598090) and Eustig*rbc*L-RB (5′TTAAGTAATTGGTGCATTTGT3′, bases 92130 to 92110 of KC598090) with annealing at 53°C. The polymerase chain reaction (PCR) products were sequenced using the primers that were used for PCR. The *Nannochloropsis* sp. strains CS-416 and CS-759 and the additional *N. oceanica* strains CS-179 and CS-246 were selected for further sequencing. The complete *rbc*L coding region was PCR amplified from these strains using the primers Eu-*rbc*L-F1 (M. Eliáš, unpublished, ATGTTTCAATCTGTAGAAGAAAG, bases 90947 to 90969 of KC598090) and Eu-*rbc*L-R1 (M. Eliáš, unpublished, CCTTGTGTTAATCTCACTCTTC, bases 92479 to 92458 of KC598090) with annealing at 50°C. The resulting PCR products were sequenced with the same primers used for PCR and two newly designed internal primers, Eustig F900 (TGGATGCGTATGGCWGGTGT, bases 91904 to 91923 of KC598090) and Eustig R900 (CACCWGCCATACGCATCC, bases 91922 to 91905 of KC598090). These four primers were designed to be useful for a wide range of Eustigmatophyceae. The primers NS1-X and 18L-X (Fawley & Fawley 2004) were used to PCR amplify the nuclear 18S rDNA from the four selected strains, with annealing at 53°C. The primers used for PCR and the additional primers NS5 (White *et al*. 1990) and 18JX (Fawley *et al*. 2014) were used for sequencing. The DNA Resource Center at the University of Arkansas at Fayetteville performed the sequencing using ABI automated sequencers. The GenBank accession numbers for newly generated sequences are given in Table 1.

The *rbc*L sequence data for all named marine strains of *Nannochloropsis* and representative freshwater strains (*N. limnetica*) were downloaded from GenBank using MEGA

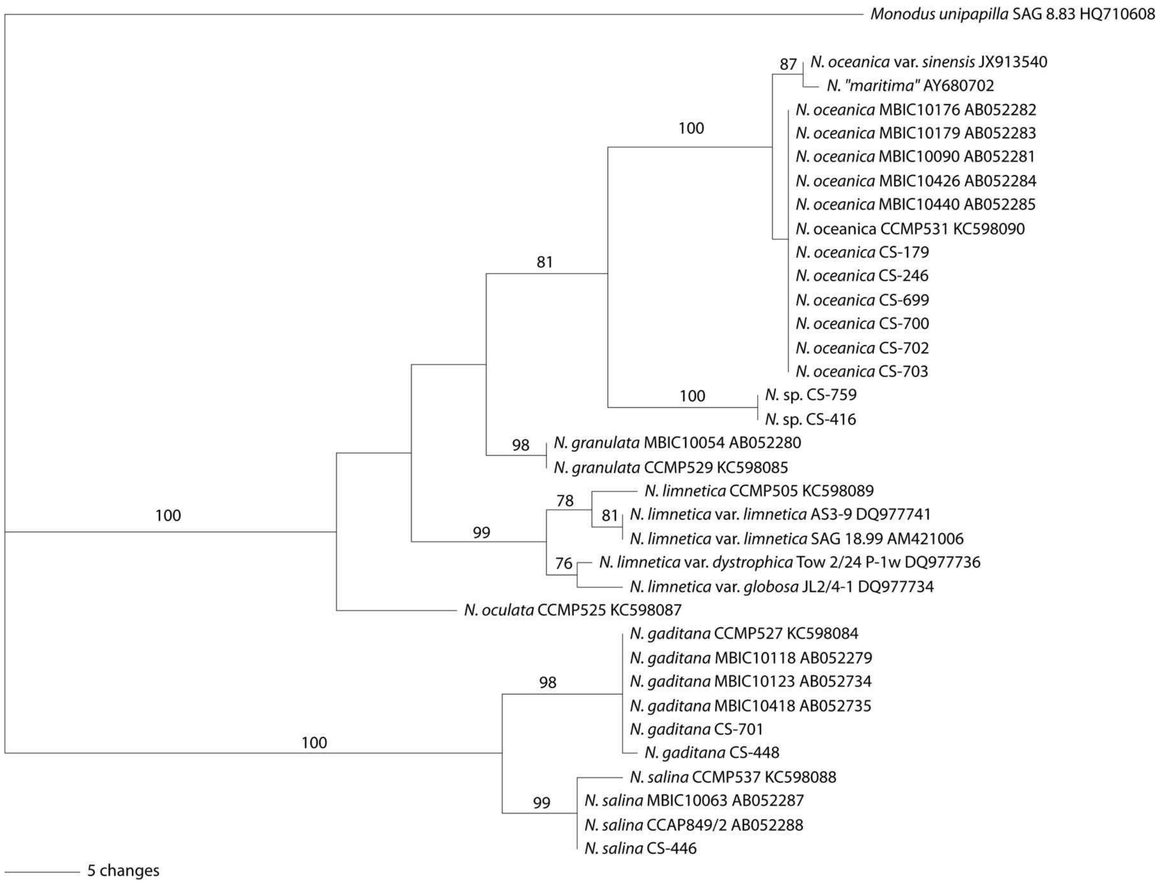


Fig. 1. Phylogram inferred from maximum-parsimony analysis of the *rbc*L bar-coding region of strains of *Nannochloropsis* and *Microchloropsis*. One of eight most parsimonious trees shown. Bootstrap values over 70 are indicated.

5.10 (Tamura *et al.* 2011). Sequences from *Monodopsis* (including *Monodus*, see Fawley *et al.* 2014) were included as an outgroup. *Monodopsis* and *Pseudotetraëdriella* are sister taxa to *Nannochloropsis* in the Monodopsidaceae (Fawley *et al.* 2014). The sequences from the bar-coding region of *rbc*L generated for all ANACC *Nannochloropsis* strains were added to the data set and an alignment was made with the Muscle program (Edgar 2004) incorporated in MEGA 5.10. The resulting alignment was analyzed using maximum parsimony (MP) in PAUP* 2.0b (Swofford 2002), with bootstrap analysis based on 1000 random resamplings of the data.

A data set of concatenated 18S rDNA and *rbc*L sequence data was constructed using MacClade 4.08. Only *Nannochloropsis* strains with sequence data for both loci were included in this alignment. All marine strains with data for both loci were included in the alignment along with data for select strains from the freshwater species, *N. limnetica*. Sequences from *Monodopsis* were included as an outgroup (Fawley *et al.* 2014). MP analyses were performed with PAUP* 2.0b (Swofford 2002) and maximum likelihood (ML) analyses used the Garli 0.96 software (Zwickl 2006). For ML analysis, the data set was divided into four partitions, one for the 18S rDNA data and one for each of the three codon positions of the *rbc*L data. The GTR+I+Γ model of DNA substitution (Tavaré 1986) was used for ML analysis, with parameters determined by the Garli software. The ML analysis used 20 independent random starting trees. Bootstrap analyses used 1000 resamplings (with fast search) of the data for MP analysis and 500 resamplings for ML analysis, each with two random starting trees.

RESULTS

A 1037-bp portion of the *rbc*L coding regions of 11 ANACC strains of *Nannochloropsis* was amplified and sequenced. The results of a MP phylogenetic analysis of these sequences and published *rbc*L sequences from *Nannochloropsis* species are shown in Fig. 1. The results of the analysis showed the separation of *Nannochloropsis* into two major lineages and bootstrap support for all described species. Monophyly of *Nannochloropsis* was not supported in this analysis of the partial *rbc*L sequences. Our analysis indicated that the majority of the ANACC strains can be placed in existing species (Table 1). However, some strains varied slightly from published sequences for type specimens. In particular, two strains, CS-416 and CS-759, which possessed identical sequences for this region, were distinct from all recognized taxa and vary from the type strains of the most similar species, *N. oceanica*, by 22 substitutions.

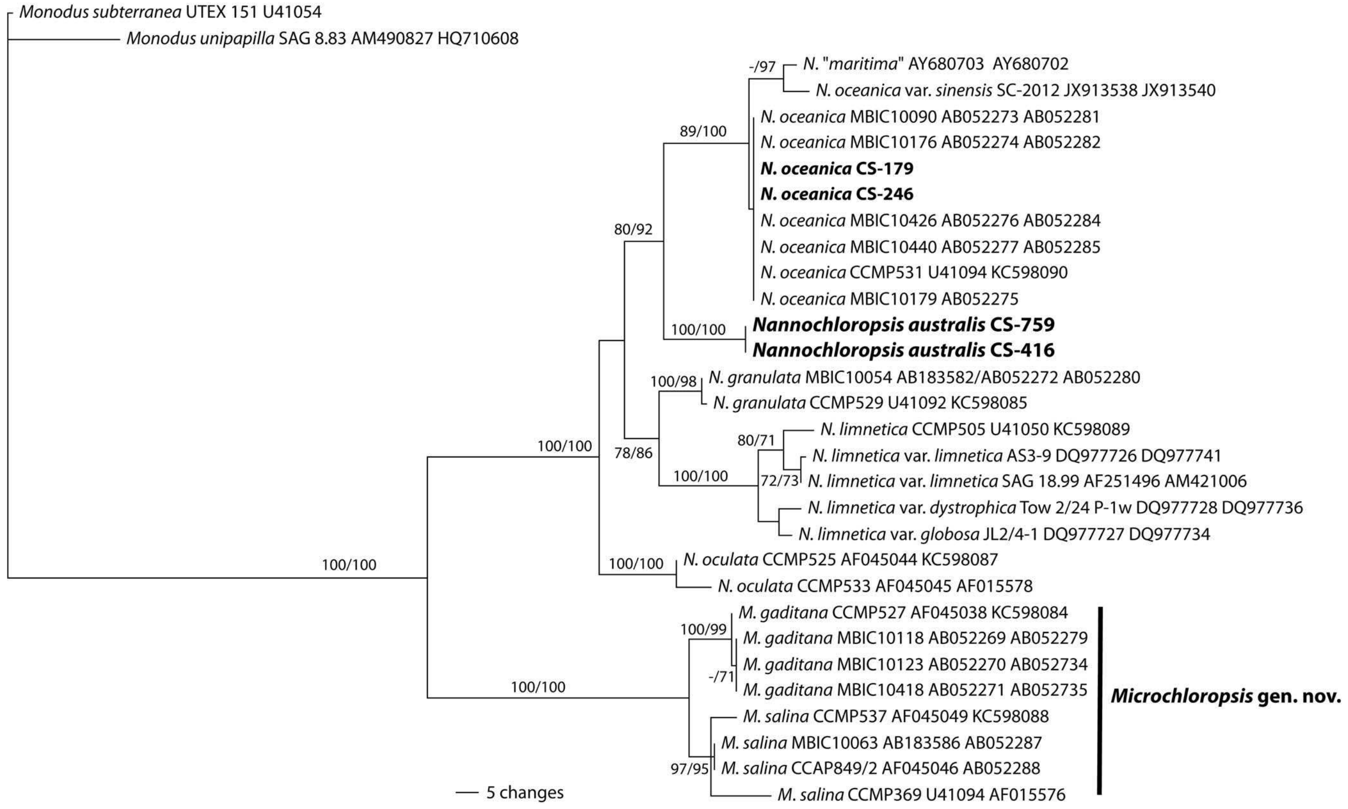


Fig. 2. Phylogram inferred from maximum-likelihood analysis of the combined *rbc*L and 18S rDNA sequence data for *Nannochloropsis* and *Microchloropsis*. The analysis included 3183 characters. Bootstrap values over 70 for maximum parsimony and maximum likelihood are shown (MP/ML). Sequences generated for this study are shown in boldface.

A combined 18S rDNA and *rbc*L sequence data set was produced that comprised 29 strains of *Nannochloropsis*, including the type strains of all described species. Results of phylogenetic analyses of these sequences (Fig. 2) supported the same two major lineages within the genus found with the analysis of partial *rbc*L sequences (Fig. 1). All described species were supported by bootstrap analysis. The Australian strains CS-416 and CS-759 had identical sequences for both loci and formed a separate lineage that was sister to *N. oceanica*. The sequences of strains CS-416 and CS-759 differed from the most similar established species, *N. oceanica*, by four substitutions for 18S rDNA (1713 total characters) and 36 substitutions for *rbc*L (1395 total characters). Our results also revealed some diversity within *Nannochloropsis* species. The strain referred to in GenBank as '*N. maritima*' and the recently described *N. oceanica* var. *sinensis* Cao (Cao *et al.* 2013) were sister taxa in the *N. oceanica* lineage with weak bootstrap support. The two strains of *N. oculata* included in the analysis have identical 18S rDNA sequences; however, their *rbc*L sequences differed by eight substitutions. The MBIC100054 and CCMP529 strains of *N. granulata* had identical *rbc*L sequences, whereas their 18S sequences differed by a single substitution. Although all strains of *N. gaditana* from the MBIC collection possessed identical 18S and *rbc*L sequences, the *rbc*L sequence of *N. gaditana* CCMP527 differed from the sequences of the MBIC strain by a single substitution. Finally, strains of *N. salina* that had no variability in the 18S rDNA sequences had three distinct *rbc*L sequences.

The two strains CS-416 and CS-759 have morphologies typical for marine *Nannochloropsis*, with approximately spherical mature cells and hemispherical or angular young cells (Figs 3, 4). The mature cells of CS-416 tend to be more spherical, whereas those of CS-759 were mostly elliptical. Young cells of CS-416 were strongly angular, whereas those of CS-759 quickly become elliptical.

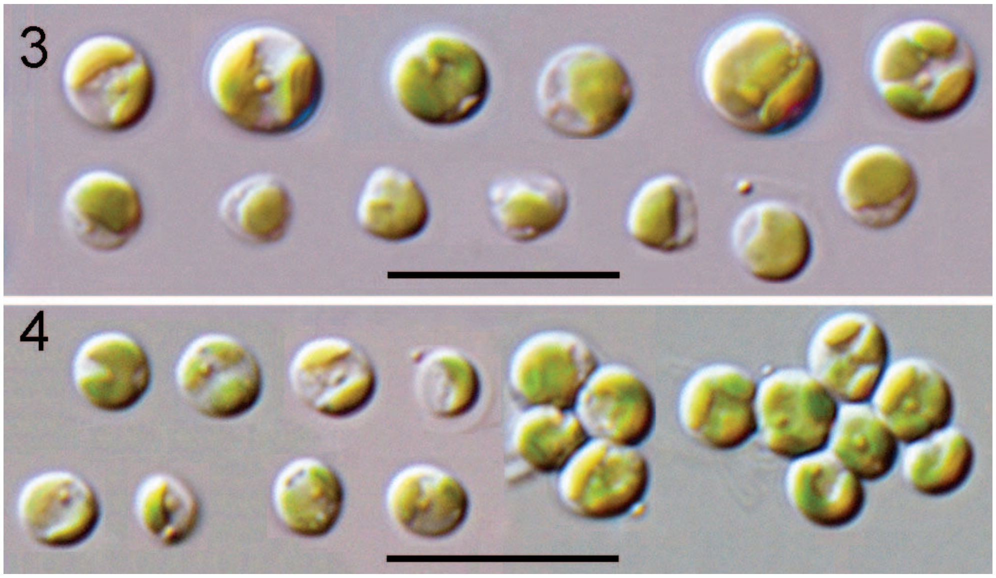
Taxonomic treatments

Microchloropsis M.W. Fawley, I. Jameson & K.P. Fawley *gen. nov.*

DESCRIPTION: Cells small (2–8 μm), cylindrical or rarely spherical with a single parietal chloroplast. Pyrenoids absent. Some cells with an orange or red lipid body free in the cytoplasm. Refractive granules or short rods usually present in the cytoplasm. Reproduction by autospore production. Young autospores were usually hemispherical but became cylindrical or spherical as they matured. Monophyletic lineage in analyses of the nuclear 18S rDNA and plastid *rbc*L.

TYPE SPECIES: *Microchloropsis salina* (D.J.Hibberd) M.W. Fawley, I. Jameson & K.P. Fawley *comb. nov.*

ETYMOLOGY: The modifier *micro* is chosen to replace *nanno* that is used for the closely related genus *Nannochloropsis*. *Nanno* suggests that the organism is very small, whereas *micro* is typically considered to be very small, but larger than *nanno*. This term describes the known taxa of *Microchloropsis*, which are generally slightly larger



Figs 3, 4. Light microscopy of *Nannochloropsis australis, sp. nov*. Scale bars = 10 µm.

Fig. 3. Strain CS-416. Montage of representative cells. Mature cells (top row) are typically spherical or nearly so, whereas autospores (bottom row) are typically angular.

Fig. 4. Strain CS-759. Montage of representative cells. The mature and immature cells are spherical or elliptical and are either solitary (left) or in small clumps (right).

than typical marine members of *Nannochloropsis*. We retain the '*chloropsis*' derived from *Nannochloropsis* for our new genus to emphasize the close relationship between *Nannochloropsis* and *Microchloropsis*.

Microchloropsis salina (D.J. Hibberd) M.W. Fawley, I. Jameson & K.P. Fawley *comb. nov*.

BASIONYM: *Nannochloropsis salina* D.J. Hibberd 1981. *Journal of the Linnean Society of London, Botany* 82: 93.

SYNONYM: *Monallantus salina(us)* Bourrelly 1958. *Bulletin du Laboratoire Maritime de Dinard* 43: 111, fig. 5, *nomen nudum*.

Microchloropsis gaditana (L.M. Lubián) M.W. Fawley, I. Jameson & K.P. Fawley *comb. nov*.

BASIONYM: *Nannochloropsis gaditana* L.M. Lubián 1982. *Lazaroa* 4: 287, fig. 1.

Nannochloropsis australis M.W. Fawley, I. Jameson & K.P. Fawley *sp. nov*.

Figs 3, 4

DESCRIPTION: Cells typical for marine species of the genus *Nannochloropsis*. Older cells, nearly spherical (diameter 4.0–5.1 µm) or elliptical (about 3.4 × 4.1 µm), and typically possess the orange body in the cytoplasm typical for the Eustigmatophyceae. Two or four autospores produced; autosporulation the only means of reproduction observed. Young cells typically angular or flattened on one side (typically about 2.0 × 3.3 µm). A few much larger spherical cells often observed in old (more than 1 month) cultures (not shown). Cells either solitary or in irregular clumps. Distinguished from other species of *Nannochloropsis* by the DNA sequences of nuclear 18S rDNA (GenBank accession KT031997) and the plastid *rbc*L (GenBank accession KT149179).

ETYMOLOGY: The specific epithet *australis* refers to either the type locality of Australia or that the organism is found in a southern ocean.

HOLOTYPE: Permanent slide of strain CS-416 deposited in the herbarium of the University of Arkansas at Monticello, designated M. Fawley 2015-1.

TYPE STRAIN: Strain CS-416 held in the ANACC, collected and isolated by Jeannie-Marie Leroi, 1993.

TYPE LOCALITY: Coastal marine waters off St. Helens, east coast of Tasmania, Australia.

ISOTYPE: Strain CS-759 held in the ANACC, collected and isolated by J.-M. Leroi, from a lagoon near Orford, east coast of Tasmania, Australia, 1993.

DISCUSSION

A major split of the genus *Nannochloropsis sensu lato* has been observed in phylogenetic analyses of the genus since the first such analysis of 18S rDNA sequences by Andersen *et al.* (1998). Their results (Andersen *et al.* 1998 fig. 3) clearly show two lineages; one lineage comprised *N. salina* and *N. gaditana* and the other lineage comprised the marine species *N. granulata* and *N. oculata* along with additional unnamed strains now assigned to *N. oceanica* (CCMP531, CCAP211/46, CCAP211/78) and the freshwater species, *N. limnetica* (CCMP505). All subsequent analyses, including those utilizing the more variable *rbc*L gene sequences, have also supported the presence of these two lineages within *Nannochloropsis* (e.g. Suda *et al.* 2002, Fawley & Fawley 2007; Cao *et al.* 2013). Although most strains of *N. salina* are larger than other marine *Nannochloropsis* species (Andersen *et al.* 1998), one strain of *N. salina* (CCMP538) is as small as *N. oceanica* or *N. granulata*. Andersen *et al.* (1998) also indicated that all strains of *N. salina* were cylindrical, whereas strains of *Nannochloropsis* species tended to be spherical. However, this feature cannot define the lineage comprising *N. salina* and *N. gaditana* because *N. gaditana* has small, spherical cells (Lubián 1982; Andersen *et al.* 1998). Therefore, no known morphological feature can be used to define these two lineages.

However, recent evidence from genome sequencing does provide further evidence for the distinction between these two lineages and supports the erection of the new genus. The most significant differences between the two genera are found in the organelle genomes. Plastid genomes of *Microchloropsis salina* CCMP537 and *M. gaditana* CCMP527 have internal repeat regions (IR) that are missing the *clp*C-I, *pub*V and *pet*J loci that are present in the IR regions of all sequenced plastid genomes of *Nannochloropsis* (Wei *et al.* 2013). *Microchloropsis salina* CCMP537 and *M. gaditana* CCMP527 also have a duplication of the mitochondrial *cox*I gene not found in species of *Nannochloropsis* (Wei *et al.* 2013). Therefore, the erection of the new genus *Microchloropsis* emphasizes the genetic differences among these organisms. The designation of new genera without morphological variation has precedence in other algae. The best-known example is the green algal genus *Chlorella*, which has a simple morphology so similar to *Nannochloropsis* and *Microchloropsis* that some strains of *Nannochloropsis*, such as CCAP211/78, were originally identified as *Chlorella* sp. *Chlorella* has now been subdivided into multiple genera on the basis of features other than morphology, such as rDNA and *rbc*L sequences (see Neustupa *et al.* 2013).

The separation of *Nannochloropsis australis* from other congeners is well supported by monophyly as shown by the analysis of the combined 18S rDNA and *rbc*L data set and by analysis of a partial *rbc*L data set alone. The *rbc*L sequence (1395 characters) of *N. australis* differs from that of *N. oceanica* by 36 substitutions; thus, the *rbc*L gene sequence can be used to distinguish *N. australis* from other *Nannochloropsis* and *Microchloropsis* species. The new species cannot be separated from other *Nannochloropsis* species by morphology, a problem already well known for this genus (e.g. Krienitz *et al.* 2000; Suda *et al.* 2002), although the cells of strain CS-416 are slightly larger than typical marine *Nannochloropsis*. The tendency of *N. australis* strain CS-759 to form clumps of cells is unusual for the genus; however, strain CS-416 grows as solitary cells and thus this growth form is variable in the species. *Nannochloropsis australis* (as *Nannochloropsis* sp. CS-416) has already been used in a study of algal lipids and hydrocarbons (Brown *et al.* 2014). Although constituent pigments and lipids are chemotaxonomic markers for the Eustigmatophyceae and are complementary to DNA sequence analysis for discriminating *Nannochloropsis* from Chlorophyta taxa, there are no known profile variations that differentiate among species of *Nannochloropsis*. Some species of *Nannochloropsis*, such as *N. oceanica*, are found in both northern and southern oceans. Bongiovani *et al.* (2014) characterized a new strain of *N. oceanica* (CCALA 978, Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic) from coastal waters of Argentina. This strain has the same 18S DNA sequence as authentic strains of *N. oceanica*. Although Bongiovani *et al.* (2014) state that the *rbc*L sequence of their strain is distinct from the sequences of other strains of *N. oceanica*, the differences are two indels and one base indicated as 'N.' Thus, the differences in *rbc*L sequences may be attributable to sequencing errors. However, the discovery of *N. australis* from the Southern Hemisphere indicates the need for more thorough sampling of southern marine waters for very small coccoid algae. These waters may hold many unnamed species of these organisms that cannot be distinguished by light microscopy.

The utility of bar coding for species identification as opposed to species delineation is an open debate in more than just the protist community (Taylor & Harris 2012), but the reality is that studies attempting to inform species biogeography need to draw on a large strain data set with a common set of characters, be they phenotypic or genetic. PhytoREF, a new reference database of the plastid 16SrRNA gene (Decelle *et al.* 2015), is one such data set. PhytoREF includes data for only a few strains of *Nannochloropsis* and *Microchloropsis*, specifically those strains studied by Wei *et al.* (2013), and is intended as a reference for environmental metabar coding rather than species discrimination. Our results have shown the utility of the plastid *rbc*L locus for bar coding both *Nannochloropsis* and *Microchloropsis*. The primer pair used in this study produces robust amplification of a portion of the *rbc*L gene. This amplicon discriminates among all known species, while also providing some information on intraspecific variability that could contribute to new understanding of the diversity and biogeography of these important organisms. Additional plastid or mitochondrial loci, such as those suggested by Wei *et al.* (2013), should provide even more resolution than does *rbc*L. The nuclear ribosomal RNA internal transcribed spacer (ITS) region has been commonly used for species delimitation in some algal groups, especially for green algae, and can also be used as a bar-coding locus (Pawlowski *et al.* 2012). However, the *rbc*L locus provides some advantages compared with ITS. As a plastid gene, *rbc*L can often be sequenced cleanly even when cultures are contaminated by fungi or non-photosynthetic protists. Primers used for ITS sequencing are typically 'universal' and can amplify the ITS region from contaminating eukaryotes. The *rbc*L primers that we developed for this study are specific for a subset of the Eustigmatophyceae, including *Nannochloropsis* and *Microchloropsis*. Therefore, our *rbc*L primers can be used to identify these organisms from established cultures or in the early stages of purification if other organisms may be present.

Finally, a short cautionary tale on the difficulties of working with widely distributed cultures. Some strains of *Nannochloropsis* have become extensively shared between bioproduct researchers with deposits into several collections under different collection codes. Preserving strain identification is critically important in tracing strain provenance and movements. CS-703 was received into Commonwealth Scientific and Industrial Research Organisation (CSIRO) as *N. oculata* under the strain name Nanno PES during collaborative eco physiology research with the University of Firenze, and it was subsequently accessioned into ANACC.

It later became evident after *rbc*L typing by us that the strain held by ANACC was *N. oceanica*. Tracing the import movements revealed that an original CCAP849/1 strain (*N. oculata*) was imported from CCAP to an aquaculture hatchery in Grosseto, Italy, then moved to the University of Firenze and then to CSIRO. It is likely that somewhere along this chain, including perhaps at CSIRO, a breakdown in strain handling protocols led to a mixing of strains of *N. oculata* and *N. oceanica*.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/15-060.1.s1.

ACKNOWLEDGEMENTS

This research was supported by National Science Foundation grant DEB1248291 to K.P.F. and M.W.F. Additional equipment support was provided by grants from Arkansas INBRE (funded by the National Center for Research Resources (grant number 5P20RR016460-11) and the National Institute of General Medical Sciences (grant number 8P20GM103429-11) of the National Institutes of Health) to M.W.F. We thank Bobbie Okimoto at the University of Arkansas DNA Resource Center for sequencing and Marek Eliáš, University of Ostrava, for sharing the new *rbc*L primers Eu-*rbc*L-F1 and Eu-*rbc*L-R1. We thank Editor David Garbary, Associate Editor Antonio Jose Calado and two anonymous reviewers for helpful comments and careful editing.

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Received 10 June 2015; accepted 5 August 2015
Associate Editor: Antonio Jose Calado