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Phylogenetic analysis of the '*Nannochloris*-like' algae and diagnoses of *Picochlorum oklahomensis* gen. et sp. nov. (Trebouxiophyceae, Chlorophyta)

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A broadly halotolerant new isolate of a small asexual coccoid chlorophyte and six new, related freshwater isolates provided the impetus for a phylogenetic analysis of the so-called '*Nannochloris*-like' algae within the Trebouxiophyceae. Previous taxonomic disagreements concerning this group had not been rigorously tested with molecular phylogenetic analyses. We show with 18S ribosomal DNA (rDNA) sequence phylogeny that 19 of 22 isolates previously assigned to either *Nannochloris* or *Nanochlorum* fall within a diverse sister clade to a clade including the four 'true' *Chlorella* species *sensu lato*. In addition, *Marvania geminata, Gloeotila contorta, Chlorella* sp. Yanaqocha RA1, *Koliella spiculiformis*, '*Chlorella minutissima*' C-1.1.9, and new *Koliella, Gloeotila* and *Marvania* isolates were included in the *Nannochloris*-like clade. Distinct freshwater and marine or saline lineages comprise at least three major subclades, generally corresponding to cell division pattern. Seven of 14 marine or saline isolates are known (and the others presumed) to divide by autosporulation. Eight freshwater isolates divide by binary fission, including two *Koliella,* two *Gloeotila, N. bacillaris, Chlorella* sp. Yanaqocha RA1, and two new unassigned isolates. Four freshwater isolates divide by budding or autosporulation (three *Marvania,* including CCAP 251/1b, previously assigned to *N. coccoides*). The autosporic taxa *N. eucaryotum* UTEX 2502 (marine) and *C. minutissima* C-1.1.9 (freshwater), which have nearly identical 18S rDNA sequences, are deeper-branching than the freshwater and marine or saline lineages. We propose including the 13 marine or saline, autosporic taxa (excluding *N. eucaryotum* UTEX 2502) in the new genus *Picochlorum* until distinctive morphological or biochemical characters are identified that would indicate multiple genera corresponding to subclades. Such characters exist in the freshwater lineages, supporting retention of *Koliella, Gloeotila, Marvania* and *Nannochloris* as distinct genera, although each is currently represented by few isolates. *Nannochloris* at this time may be restricted to *N. bacillaris* and *Chlorella* sp. Yanaqocha RA1. We also describe halotolerant *P. oklahomensis* Hironaka *sp. nov.* Based on 18S rDNA sequence and lack of chlorophyll *b, Nannochloris* sp. UTEX 2379 should be reassigned to the Eustigmatophyceae.

INTRODUCTION

The taxonomy of the Chlorophyta is rapidly changing at all levels on the basis of DNA sequence data and its phylogenetic analysis, particularly the small subunit of the ribosomal DNA (18S rDNA) gene. Taxonomic assignment of asexual small coccoid chlorophytes is particularly problematic due to the limited number of morphological characters. Sluiman & Reymond (1987) state 'for the establishment of a more stable and "natural" classification of green micro-algae . . . it is essential to de-emphasize gross morphological and reproductive features'. Biochemical characters and molecular phylogeny now indicate that the autosporic coccoid genus *Chlorella* Beijerinck, for example, is polyphyletic (Friedl 1995), leading to a major revision and splitting among the classes Chlorophyceae and Trebouxiophyceae (Huss *et al.* 1999). Hepperle & Krienitz (2001) state 'the so-called *Chlorella*- and *Nannochloris*-like algae . . . are difficult to determine and it is questionable what a "real *Chlorella*" and a "real *Nannochloris*" is'.

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The genus *Nannochloris* Naumann (Naumann 1921) includes some of the smallest and ultrastructurally simplest phototrophic eukaryotes, with genomes as small as 12.6 Mb but with possible genome duplication in some strains (Yamamoto *et al.* 2001). However, the genome of *Ostreococcus tauri* Courties & Chrétiennot-Dinet is smaller at 9.7 Mb (Derelle *et al.* 2002). Circumscription of *Nannochloris* is controversial, with the debate focusing on its original restriction to division by binary fission, vs autosporulation in *Chlorella* and other genera. Naumann (1921) originally described the genus with two binary fission species, *N. bacillaris* Naumann and *N. coccoides* Naumann, but apparently there are no holotype or lectotype specimens and thus no molecular information for type strains. Several species subsequently assigned to *Nannochloris* on the basis of morphology were shown to divide by autosporulation (Sarokin & Carpenter 1982; Brown & Elfman 1983; Menzel & Wild 1989; Krienitz *et al.* 1996). Yamamoto *et al.* (2001) grouped several *Nannochloris* species into three types of cell division: binary fission, budding and autosporulation into two or multiple daughter cells. Based on an initial actin gene sequence phylogeny, *Nannochloris* appeared to be a monophyletic deep branch within the Chlorophyta (Yama-

moto *et al.* 2001). However, no actin sequences were included for other Trebouxiophyceae, so no within-class context was provided. Not surprisingly, subsequent inclusion of only a few other Trebouxiophyceae suggested that *Nannochloris* is polyphyletic (Yamamoto *et al.* 2003).

A much larger database exists for the 18S rDNA gene sequence. Krienitz *et al.* (1996) used 18S rDNA sequences to address what they consider the invalid grouping of '*Nannochloris*-like' algae. They discounted previous more inclusive definitions of *Nannochloris* (Sarokin & Carpenter 1982; Brown & Elfman 1983), and argued for restricting *Nannochloris* to binary fission, and placing all autospore-forming taxa in one or more other genera, e.g. *Choricystis* (Skuja) Fott. However, they included the sequences of only two putative *Nannochloris* strains: *N. coccoides* SAG 251-1, which they found identical to *C. minor* (Skuja) Fott, and *N. eucaryotum* (Wilhelm, Eisenbeis, Wild & Zahn) Menzel & Wild (basionym *Nanochlorum eucaryotum* Wilhelm, Eisenbeis, Wild & Zahn), which they argue should be maintained separate from *Nannochloris*. Moreover, the earlier insistence of Krienitz *et al.* (1996) that *Nannochloris* is in the Ulotrichales is inconsistent with the current placement of this order in the class Ulvophyceae, whereas the published 18S rDNA sequences of '*Nannochloris*' and '*Nanochlorum*' Wilhelm, Eisenbeis, Wild & Zahn isolates fall clearly within the new class Trebouxiophyceae (Friedl 1995), regardless of mode of cell division. A recent 18S phylogeny of the Trebouxiophyceae provided a better context for the resolution of a subset of *Nannochloris* taxa (Yamamoto *et al.* 2003). However, the phylogeny included a limited selection of trebouxiophycean sequences, and the authors concluded that many more taxa must be included to provide a definitive stable phylogeny. Based on existing and new 18S rDNA sequences of multiple *Nannochloris–Nanochlorum* isolates, including a new broadly halotolerant isolate, and numerous other Trebouxiophyceae, we present a finer resolution of the class, particularly the *Nannochloris* clade, and propose a taxonomic solution for the marine or saline members of this controversial group.

MATERIAL AND METHODS

Site description

The Salt Plains National Wildlife Refuge (SPNWR) in northwestern Oklahoma, USA (approximately 36°44′N, 98°16′W), is a minimally studied semiaquatic soil habitat. Perpetually moist salt flats cover approximately 65 km^2. The salts and chronic moisture come from a Permian brine aquifer, typically 150–250 ppt salinity. Occasional heavy rain washes the salts off the surface into a reservoir and leaves scattered low-salinity pools that gradually evaporate and rise in salinity. A few larger pools persist for many weeks in the absence of precipitation. Variable freshwater input from streams provides locally reduced salinity and a potential inoculum of freshwater microbes. In rare cases, heavy rain may fully submerge most of the flats with stream or reservoir water. As an initial effort to document the algae of this environment, we describe here a new species of unicellular chlorophyte.

Algal isolation and culture

A clonal coccoid chlorophyte (980625-4A) was isolated from a small ephemeral saline pool at the SPNWR on 25 June 1998. Isolation involved a combination of streaking on agar plates, where it forms discrete colonies, and culturing in liquid medium. Liquid and solid 'SP' media included salts from the SPNWR redissolved in Nanopure water at 50 ppt salinity, as determined with a refractometer. Both media were enriched with f/2 nutrients (Guillard & Ryther 1962), minus Cu and Si. However, we have demonstrated (Henley *et al.* 2002) that this isolate grows from 0 to > 100 ppt and tolerates up to approximately 150 ppt in SP medium and in the defined medium AS-100 (Starr & Zeikus 1993). Maintenance cultures in liquid media were grown at 20–25°C and 50–200 μmol photons m^{-2} s^{-1} of cool white fluorescent light on a 14 : 10 h light–dark cycle.

Six freshwater strains were isolated from lakes in Arrowwood National Wildlife Refuge, ND, USA, as previously described (Phillips & Fawley 2000). Strain AS 2-10 was isolated from a sample collected 3 February 1995 from Arrowwood Lake; ANR-9 was collected 24 February 1995 from Arrowwood Lake; MDL 5-3 was from Mud Lake, collected 26 May 1995; JL 4-6 was from Jim Lake, collected 30 April 1995; and JL 11-10 and JL 11-11 were from Jim Lake, collected 22 November 1995. Descriptions of the sites and conditions for the growth of these isolates are given in Phillips & Fawley (2000). Sequences of 18S rDNA genes for all were submitted to GenBank provisionally as *Nannochloris,* although some were subsequently changed.

Light microscopy

Cells grown as described above were mounted on glass slides coated with poly-L-lysine. Slides were prepared by coating with a 0.1% solution of poly-L-lysine (P-1522; Sigma-Aldrich, St Louis, MO, USA) in deionized water followed by drying in a 38°C oven for 30 min. An E-600 microscope (Nikon USA, Melville, NY, USA) was used, equipped with differential interference contrast optics and a 150ES (Pixera, Los Gatos, CA, USA) digital camera.

Transmission electron microscopy

Transmission electron microscopy (TEM) of strain RCC 115 (Roscoff Culture Collection, http://www.sb-roscoff.fr/Phyto/RCC/index.php) was conducted as described in Guillou *et al.* (1999). Cells of other strains were collected by gentle centrifugation, fixed for 2 h in 2% glutaraldehyde buffered with 0.15 M phosphate (pH 7.2), washed twice with 0.1 M phosphate buffer, postfixed for 2 h in 1% osmium tetroxide, washed twice with 0.1 M phosphate buffer and dehydrated in an incremented acetone series. Dehydrated cells were imbedded in 1 : 1 acetone–polybed (Polysciences, Warrington, PA, USA) or Spurr's resin (Spurr 1969). After 24 h, fresh polybed or Spurr's resin was added and cured at 60°C for two days prior to ultramicrotomy. Thin sections were stained using uranyl acetate and lead citrate. Cells were examined using either an H7000 (Hitachi High Technologies America, Schaumburg, IL, USA) or a JEM 100 CX II (JEOL USA, Peabody, MA, USA) TEM.

Pigment and osmolyte analyses

Photosynthetic pigments were extracted from pelleted cells in *N,N*-dimethylformamide (DMF) at 4°C for 24 h, then vacuum dried in darkness. Dried pigments were dissolved in 80% methanol–20% 0.5 M aqueous ammonium acetate (solvent A) and separated in a three solvent gradient by reverse phase high-performance liquid chromatography (HPLC) according to Wright & Jeffrey (1997), modified slightly for the specific column (Hironaka 2000). Chlorophyll *a*, β-carotene and xanthophyll standards (1 μg ml^{-1} in solvent A) were purchased from Sigma-Aldrich.

Osmolytes were determined in vacuum-dried aliquots (approximately equal cell densities) of 0 and 40 ppt salinity late-exponential phase cultures of *Nannochloris* sp. Dried cells were ground in liquid N_2 in a mortar and pestle, then sonicated for 5–10 s in 1 ml of 5% perchloric acid and extracted at 4°C for 1 h. After centrifuging, the supernatant was adjusted to pH 6 with K_2CO_3, recentrifuged, and the osmolytes were analysed by HPLC mass spectrometry (see Hironaka 2000 for details).

DNA extraction

Genomic DNA was extracted from the SPNWR isolate as described previously for other green algae (Buchheim & Chapman 1992). Genomic DNA was extracted from new freshwater isolates as previously described (Fawley & Fawley 2004). A Mini-Beadbeater (BioSpec, Bartlesville, OK, USA) was used to break open cells of all *Nannochloris* taxa. Double-stranded DNA sequencing templates were obtained by symmetrically amplifying genomic DNA using the polymerase chain reaction. The flanking primers used to amplify the 18S rRNA gene are described by White *et al.* (1990). Products from two or more independent amplifications were pooled to increase template concentration and to allow for the detection of heterogeneity in the 18S rDNA.

Automated sequencing

New sequence data were obtained using either an ABI-373 or a Beckman CEQ-2000 automated sequencer (Beckman–Coulter, Fullerton, CA, USA), according to the manufacturers' protocols. Primers used for sequencing have been described previously (Hamby *et al.* 1988; Buchheim *et al.* 1997; Fawley *et al.* 2000).

Sequence alignments

No introns were discovered in any of the new 18S rDNA sequences. Previous work (Buchheim *et al.* 2001) served as the starting point for all alignments. Published 18S rRNA gene data from each of the green algal classes (Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Prasinophyceae) were included in the preliminary alignments designed to identify the broad affinity of the unidentified coccoid. MacClade 4.0 (Maddison & Maddison 2000) was used to align the data manually. A total of 122 sites were excluded from phylogenetic analyses of the 18S rDNA data because they exhibit questionable homology in expansion regions that vary in length and exhibit base changes between taxa. A total of 1638 sites were compared. The data set has been deposited in TreeBase and all of the new sequences have been deposited in GenBank.

Phylogenetic analysis

Phylogenetic analysis was conducted using maximum likelihood (ML), maximum parsimony (MP) and Bayesian (B) approaches. All analyses were conducted using PAUP* version 4.0b10 (Swofford 2002) or MrBayes version 3.0B4 (Huelsenbeck & Ronquist 2001). Modeltest 3.06 (Posada & Crandall 1998) and PAUP* 4.0b10 (Swofford 2002) were used in tandem to test the goodness-of-fit of DNA substitution models against the 18S rDNA data for use in ML analyses. Tree searches for ML analysis were conducted heuristically using the tree-bisection–reconnection (TBR) option and the initial tree was generated by the neighbour-joining method. Bootstrap values (Felsenstein 1985) were calculated from 100 resamplings using heuristic nearest neighbour interchange searches with initial trees obtained by neighbour-joining. Tree searches for MP analysis were conducted heuristically using the TBR option with 50 random taxon addition replicates. Bootstrap values (Felsenstein 1985) were calculated from 1000 resamplings using heuristic TBR searches with simple taxon addition. All Bayesian analyses were conducted with MrBayes 3.0B4, using four chains with 500,000 generations.

Initial phylogenetic analyses revealed an alliance of SPNWR 980625-4A with the trebouxiophyte *Nannochloris* (data not shown). Consequently, we sequenced additional *Nannochloris* isolates (*Nannochloris* sp. UTEX 2491, 2378 and 2379, *N. eucaryotum* UTEX 2502, and *N. oculata* Droop UTEX 1998) for comparison with 980625-4A. Six new related freshwater isolates and the previously unpublished sequence for RCC 115 were subsequently added to the analysis. Furthermore, numerous additional trebouxiophycean sequences from the published database were added to the alignment to provide a rich taxonomic context for assessment of the phylogenetic position of 980625-4A.

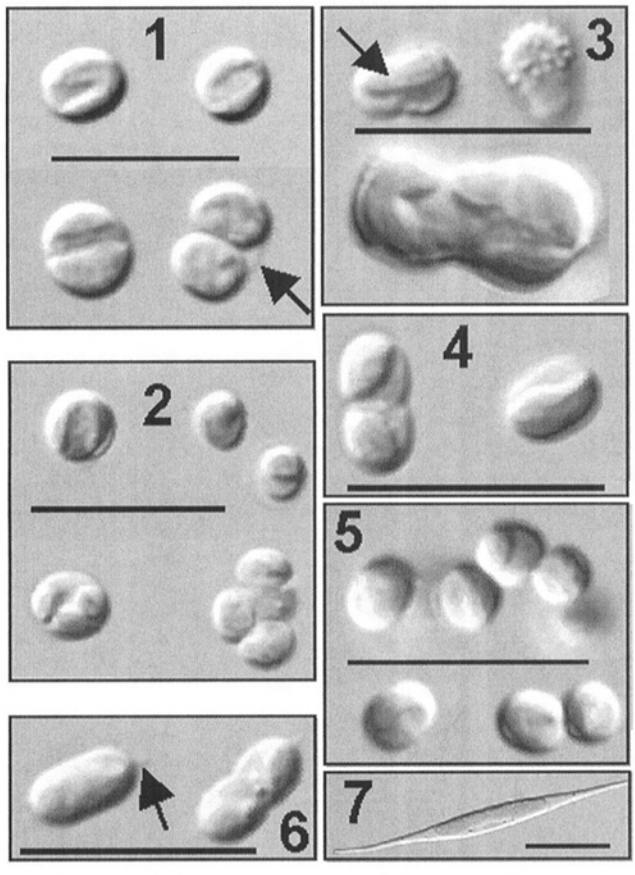
Rooting

The outgroup method was used to root all trees. Sequence data from *Cyanophora paradoxa* Korshikov (X68483) and *Glaucocystis nostochinearum* Itzigsohn (X70803) were used to root the initial trees. These glaucocystophyte taxa have been resolved as a sister group to the green plant lineage in previous studies of 18S rDNA data (Bhattacharya *et al.* 1995). Subsequent Trebouxiophyceae analysis was rooted using data from four chlorophycean taxa (*Oedogonium* Link, *Bulbochaete* C. Agardh, *Aphanochaete* A. Braun and *Chaetophora* F. Schrank). The Chlorophyceae is regarded as the sister group to the Trebouxiophyceae (Friedl 1995) and the Oedogoniales and Chaetophorales have been resolved as basal members of the Chlorophyceae (Buchheim *et al.* 2001). Phylogenetic reanalysis (ML, MP and B) of only the *Nannochloris–Nanochlorum* clade used *Chlorella vulgaris* Beijerinck, *Micractinium pusillum* Fresenius and *Closteriopsis acicularis* (G.M. Smith) J.H. Belcher & Swale as the outgroup.

RESULTS

Morphology, ultrastructure and reproduction

SPNWR 980625-4A has oblong coccoid cells with an approximate mean size of 2 μm and a length:width ratio of 1.15–



Figs 1–7. Differential interference contrast light micrographs of vegetative and/or dividing cells of new algal isolates. Scale bar = 5 μm (Fig. 1) or 10 μm (Figs 2–7).

Fig. 1. *Picochlorum oklahomensis* SPNWR 980625-4A; the arrow indicates the mother cell wall of a two-celled autospore.

Fig. 2. JL 4-6 showing four-celled autosporulation.

Fig. 3. *Marvania* sp. JL 11-11 showing budding-type autosporulation; the arrow indicates the chloroplast extending into the bud.

Fig. 4. '*Nannochloris*' sp. AS 2-10 showing binary fission.

Fig. 5. *Gloeotila* sp. JL 11-10 showing binary fission and a tendency to form short chains.

Fig. 6. '*Nannochloris*' ANR-9 showing binary fission; the arrow indicates terminal spine-like projections.

Fig. 7. *Koliella* MDL 5-3 vegetative cell.

1.2, both decreasing slightly with increasing salinity from 0 to 120 ppt (Hironaka 2000). A single chloroplast that occupies well over half of the cell volume, and a mother cell wall indicative of autosporulation, are clearly visible in light microscopy (LM) (Fig. 1). However, the mother cell walls apparently gelatinize quickly after autosporulation so that remnants are not often observed. Only two autospores were observed per sporangium. Two of the new freshwater isolates, JL 11-11 and JL 4-6, likewise exhibit autosporulation (Figs 2, 3). JL 4-6 produces four autospores, whereas JL 11-11 exhibits a budding-like form of autosporulation and a granular cell wall resembling *Marvania geminata* Hindák. Although regular vegetative reproduction for JL 11-11 is budding, there is occasional formation of aplanospores with division by autosporulation (Fig. 3). In contrast, four of the new freshwater isolates tentatively appear to divide by binary fission: AS 2-10, JL 11-10, ANR-9, and MDL 5-3 (Figs 4–7), the latter resembling *Koliella spiculiformis* (Vischer) Hindák. ANR-9 often exhibits polar spine-like projections as in *Catena* Chodat (Hindák 1977). JL 11-10 tends to form short chains as in *Gloeotila* Kützing (John 2002).

Analysis of SPNWR 980625-4A by TEM reveals a single mitochondrion, nucleus and chloroplast, and a trilaminate cell wall (Figs 8, 9). Pyrenoids and vacuoles have not been observed. Autosporulation is confirmed by a clearly retained mother cell wall (Fig. 10), which was observed in cells grown at 0–120 ppt salinity and 25 and 40°C. Several starch grains are prominent in the chloroplast of old, stationary phase cells (Fig. 11). TEM of freshwater isolate ANR-9 confirms the observation of binary fission from LM (Fig. 12, cf. Fig. 6). In contrast, preliminary TEM of AS 2-10 and JL 11-10 (not shown) were inconclusive regarding division mode. Marine RCC 115 clearly exhibits autosporulation (Fig. 13).

Pigments, osmolytes and cell wall

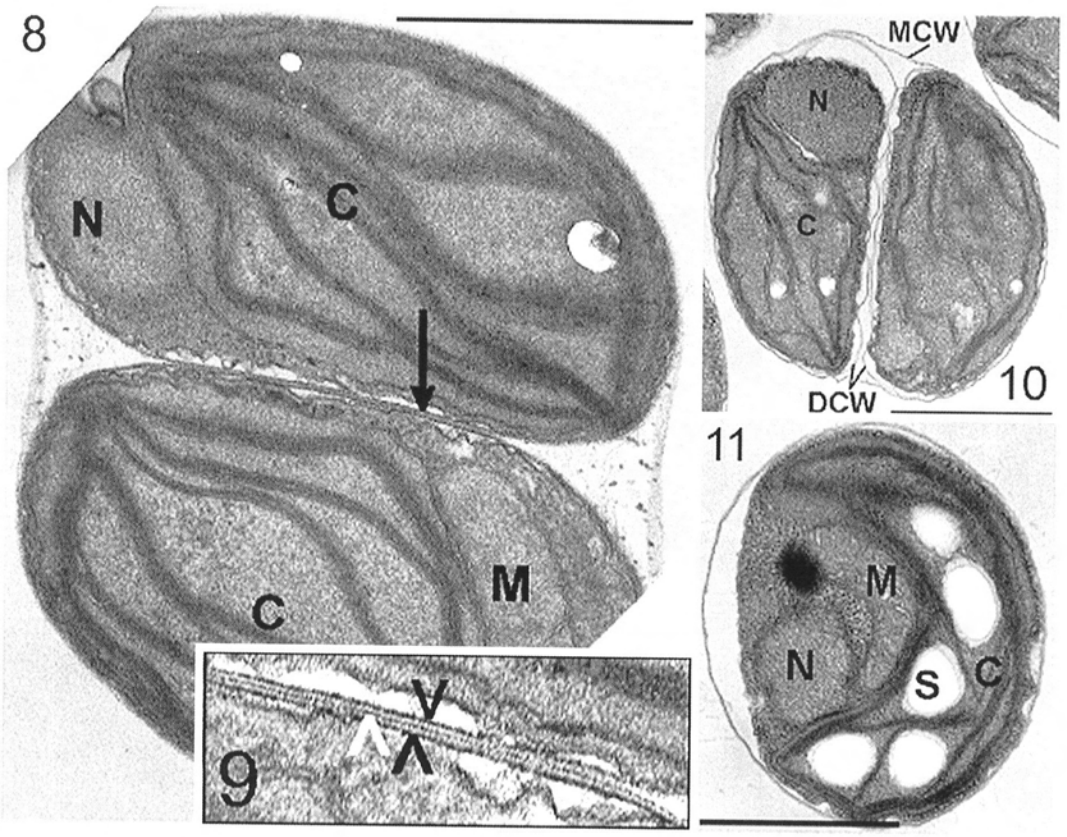
SPNWR 980625-4A contains typical chlorophyte pigments, including chlorophylls *a* and *b*, and lutein as the dominant carotenoid, with lesser amounts of violaxanthin, neoxanthin, and β-carotene (Hironaka 2000). We also detected trace amounts of astaxanthin and vaucheriaxanthin ester, plus several unidentified xanthophylls. We previously reported a molar chlorophyll b : a ratio of 0.25–0.35 over a range of salinities and irradiances (Henley *et al.* 2002). In contrast, *Nannochloris* sp. UTEX 2379 lacks chlorophyll *b* and its dominant xanthophyll is violaxanthin rather than lutein (Hironaka 2000). Proline, glycerol, hexoses (assumed to be mainly glucose) and glucosylglycerol all were present at 1–20 μg ml^{-1} of culture, and increased in concentration in cells grown at 40 ppt compared to 0 ppt salinity. Proline in particular increased 14-fold per cell, whereas the others increased by only 40–140%. Proline thus is a possible major compatible osmolyte in this species. Trace amounts of the known osmolytes glycine betaine and ectoine were also detected. The cell wall did not retain ruthenium red, indicating an absence of acidic polymers in the cell wall (Takeda 1991; Hironaka 2000).

Phylogenetic analysis

A broad phylogenetic context places SPNWR 980625-4A and nearly all other '*Nannochloris–Nanochlorum*' taxa in one diverse *Nannochloris*-like branch (bootstrap = 82) of the Trebouxiophyceae (Fig. 14). Only *Nannochloris* sp. UTEX 2379 (not shown) is in the eustigmatophyte lineage as a close relative of *Nannochloropsis salina* D.J. Hibberd (GenBank AB052278), consistent with its lack of chlorophyll *b* (Hironaka 2000), hence it should be reassigned. *Nannochloris atomus* Butcher SAG 14.87 falls outside this clade as sister taxa to several *Choricystis* isolates. SAG 251-2, which has been independently submitted to GenBank as *Choricystis* sp. and *Nannochloris* sp., is also clearly allied with *Choricystis*.

Phylogenetic reanalysis (ML, MP and B) of only the *Nannochloris–Nanochlorum* clade, using *Chlorella vulgaris, Micractinium pusillum* and *Closteriopsis acicularis* as the outgroup (Fig. 15), reveals distinct freshwater and marine or saline lineages comprising at least three major subclades, corresponding more or less to cell division pattern.

Among freshwater isolates, the *Marvania* Hindák subclade (bootstrap = 100) includes three isolates dividing by autosporulation or budding. The other subclade (bootstrap < 70)



Figs 8–11. *Picochlorum oklahomensis* SPNWR 980625-4A, TEM. Scale bars = 1 μm. M, mitochondrion; C, chloroplast; S, starch grain; N, nucleus; MCW, mother cell wall; DCW, daughter cell walls.

Fig. 8. Cell grown in AS-100 medium at 25°C and 50 ppt salinity; the arrow indicates the trilaminate cell wall.

Fig. 9. Close-up of trilaminate cell wall from Fig. 8; black arrowheads, inner electron-dense cell wall layer of daughter cells; white arrowhead, shared outer electron-dense cell wall layer.

Fig. 10. Cells clearly showing autosporulation in SPNWR 980625-4A grown in AS-100 medium at 25°C and 80 ppt salinity.

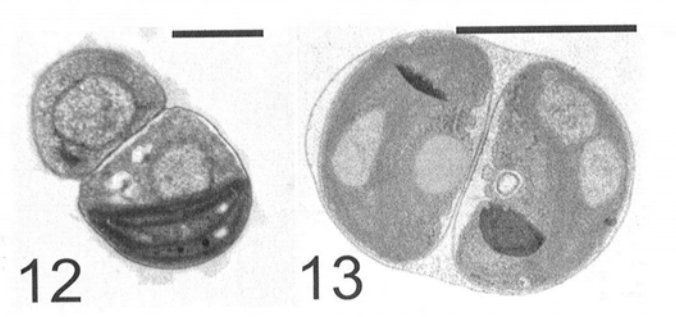
Fig. 11. Old, stationary phase cell grown in AS-100 medium at 25°C and 0 ppt salinity.

includes *Chlorella* sp. Yanaqocha RA1, *Koliella spiculiformis*, *Gloeotila contorta* Chodat SAG 41.84, the new isolates *Koliella* Hindák sp. MDL 5-3, *Gloeotila* sp. JL 11-10 and the *Nannochloris* isolates AS 2-10, ANR-9 and JL 4-6. Of these, JL 4-6 is autosporic, and all others except *Chlorella* sp. Yanaqocha RA1 (unavailable for study) are suspected (based on phylogenetic affiliation or LM only) to divide by binary fission (Table 1). Naumann's original species, *N. coccoides* and *N. bacillaris*, respectively, are in the autosporic *Marvania* and binary fission subclades of the freshwater lineage. The identical 18S rDNA sequences of *N. coccoides* CCAP 251/1b and new isolate JL 11-11 indicate an alliance with *M. geminata* (ML and MP bootstrap = 100, Bayesian posterior probability = 1.00).

The marine or saline lineage (ML bootstrap = 77; Bayesian posterior probability = 0.99) contains 13 isolates, six of which are known (and the others presumed) to divide by autosporulation (see Table 1). Broadly halotolerant SPNWR 980625-4A is resolved as the sister taxon to *Nannochloris* sp. UTEX 2491. Sequence comparison reveals that UTEX 2491 and SPNWR 980625-4A are identical at the 18S rDNA level. Subclades within the marine or saline lineage have similar branch lengths to morphological genera in the freshwater lineage. However, at this time there are no apparent characters on which to split the marine or saline subclades into separate genera. The autosporic taxa *N. eucaryotum* UTEX 2502 (marine) and '*Chlorella minutissima*' Fott & Nováková C-1.1.9 (freshwater), which have nearly identical 18S rDNA sequences, form a separate lineage basal to the major freshwater and marine or saline lineages with high bootstrap support (ML and MP = 100, B = 1.00).

DISCUSSION

SPNWR 980625-4A features a trilaminate cell wall with an outer electron-dense layer indicative of sporopollenin, consistent with other trebouxiophyte taxa (Krienitz *et al.* 1996, 1999). The apparent presence of proline as a major compatible osmolyte also is consistent with other Trebouxiophyceae, e.g. a putative *N. bacillaris* (Brown 1982) and *Stichococcus bacillaris* (Brown & Hellebust 1978). Given the 18S rDNA sequence identity of our isolate and *Nannochloris* sp. UTEX 2491, we tentatively consider them conspecific, and provide the new genus and species diagnoses *Picochlorum oklahomensis* Hironaka at the end of the discussion. Notably, both are from hypersaline environments; UTEX 2491 was isolated from the Salton Sea. Likewise, Pacific isolates *Nannochloris* sp. UTEX 2378 and *Nannochloris* sp. MBIC 10208 have identical 18S rDNA sequences, and may be considered a conspe-



Figs 12–13. Dividing cells of new freshwater algal isolate '*Nannochloris*' ANR-9 and marine *Picochlorum* sp. RCC 115, TEM. Scale bars = 1 μm.
Fig. 12. Binary fission in '*Nannochloris*' ANR-9.
Fig. 13. Autosporulation in *Picochlorum* sp. RCC 115.

cific sister species to *P. oklahomensis*. Both pairs of isolates appear to be relatively recently diverging species based on the shallow branch lengths.

Nannochloris coccoides CCAP 251/1b and new isolate *Marvania* sp. JL 11-11 may also be tentatively considered conspecific, but should be renamed *M. coccoides* comb. nov., consistent with Tschermak-Woess (1999). Naumann's original species *N. coccoides* lacks a holotype and CCAP 251/1b is a distinct isolate that may not be closely related to Naumann's isolate (Tschermak-Woess 1999). Significantly, whereas Yamamoto *et al.* (2003) reported *N. bacillaris* and *N. coccoides* CCAP 251/1b as monophyletic, our inclusion of numerous related taxa reveals genus-level divergence of these two species. The variably granular cell wall, budding-like autosporulation and occasional aplanospore formation in *Marvania* sp. JL 11-11 (Fig. 3) fit very well with descriptions of *M. geminata* (Hindák 1976; Reymond *et al.* 1986).

As in several previous studies (Sarokin & Carpenter 1982; Brown & Elfman 1983; Menzel & Wild 1989; Yamamoto *et al.* 2001, 2003), autosporulation in SPNWR 980625-4A and other nominal *Nannochloris* species conflicts with Naumann's (1921) restriction of *Nannochloris* to species dividing only by binary fission. Yamamoto *et al.* (2003) concluded that autosporulation is an ancestral character and binary fission or budding is derived in *Nannochloris* and relatives. Our results are consistent with this observation. The robust 18S rDNA phylogeny indicates a diverse *Nannochloris*-like clade (about 3.75% 18S rDNA sequence change from the base of the clade to *Nanochlorum* sp. MBIC 10096) containing most, but not all species originally named *Nannochloris* or *Nanochlorum* (Fig. 14). These taxa fall into a freshwater clade with a variety of cell division patterns and a marine or saline, autosporic clade (Fig. 15).

Retention of Naumann's description, which restricts *Nannochloris* to binary fission, would necessitate moving all putative *Nannochloris* taxa exhibiting autosporulation to one or more new genera (Krienitz *et al.* 1996). In the case of 13 marine or saline isolates with a Bayesian posterior probability of 0.99, such a move is strongly supported by our 18S rDNA phylogeny. In the absence of known morphological or biochemical characters to justify splitting the subclades into separate genera, we recommend that all taxa falling in this group be reassigned to *Picochlorum* gen. nov. We formally transfer previously named species at the end of the discussion. We reject the previously used genus name *Nanochlorum* (Wilhelm *et al.* 1982) because it represents only a grammatical variant of *Nannochloris*, was subsequently incorporated into *Nannochloris* by one of the same authors (Menzel & Wild 1989) and is confusing given that the similar names are often used interchangeably. Based on partial 18S rDNA sequences (D. Vaulot, personal communication), RCC isolates 236, 237, 289 (= clonal RCC 011), 475, 484, 490 and MBIC 10059, all marine, are also likely to align with the *Picochlorum* clade.

The freshwater lineage is more complex, with at least four existing morphologically recognized genera supported by 18S rDNA phylogeny (Bayesian posterior probabilities of 0.99–1.00 for *Nannochloris*, *Koliella*, *Marvania* and *Gloeotila*) plus three new isolates of unclear affiliation (*Nannochloris* AS 2-10, ANR-9 and JL 4-6). Relationships among the latter three isolates have low bootstrap support and branch positions readily change with addition of new taxa. Numerous additional sequences in this region will be necessary to clarify phylogenies. *Nannochloris coccoides* CCAP 251/1b clearly should be reassigned to *Marvania*; the budding type of cell division in the former (Yamamoto *et al.* 2003) resembles that in *M. geminata* (Hindák 1976; Reymond *et al.* 1986; Sluiman & Reymond 1987) and *Marvania* sp. JL 11-11 (Fig. 3). *Nannochloris* then would include only Naumann's original *N. bacillaris* and *Chlorella* sp. Yanaqocha RA1. Unfortunately, the latter strain is apparently unavailable for characterization, so it would not be a meaningful reassignment. *Nannochloris coccoides* would presumably remain a valid taxon for existing or new freshwater isolates exhibiting binary fission as in Naumann's original diagnosis, particularly if the 18S rDNA sequence aligns it with *N. bacillaris*. Katana *et al.* (2001) showed that *Koliella* species are polyphyletic within the Trebouxiophyceae, which we confirm here with the distant placement of *K. spiculiformis* and *K. sempervirens*. The latter authors concluded that the *Klebsormidium* Silva, Mattox & Blackwell type (Type VII) cell division used to define this genus is homoplastic, thus not a reliable taxonomic character. Because *Koliella spiculiformis* is the type species of the genus (Hindák 1963), *K. sempervirens* and other apparently unrelated species will need to be reassigned to a new genus.

Sluiman & Reymond (1987) and Krienitz *et al.* (2003) assert that gross morphology or binary fission vs autosporulation have little phylogenetic value within this group of algae. We have already shown that 18S rDNA lineages are generally consistent with cell division pattern and habitat (*Nannochloris* JL 4-6 is a possible exception). Similarly, there is some indication of morphologically consistent 18S rDNA phylogenetic lineages. *Koliella* sp. MDL 5-3 and *K. spiculiformis* are needle shaped, *Gloeotila* sp. JL 11-10 closely resembles *G. contorta* with respect to cell division and tendency to form short chains, and *Marvania* sp. JL 11-11 and *M. geminata* are similar morphologically.

The taxa *N. eucaryotum* UTEX 2502 and *C. minutissima* C-1.1.9, with indistinguishable 18S rDNA sequences except for a possible insertion or deletion in a hypervariable region, form a problematic sister group to the rest of the *Nannochloris*-like taxa. At this time, neither can be definitively assigned to any existing genus (although the apparent absence of C-1.1.9 in any current culture collection may render its taxonomic position meaningless). Huss *et al.* (1999) previously showed that *C. minutissima* C-1.1.9 is only distantly related to other *Chlorella* spp., and distinct from a different

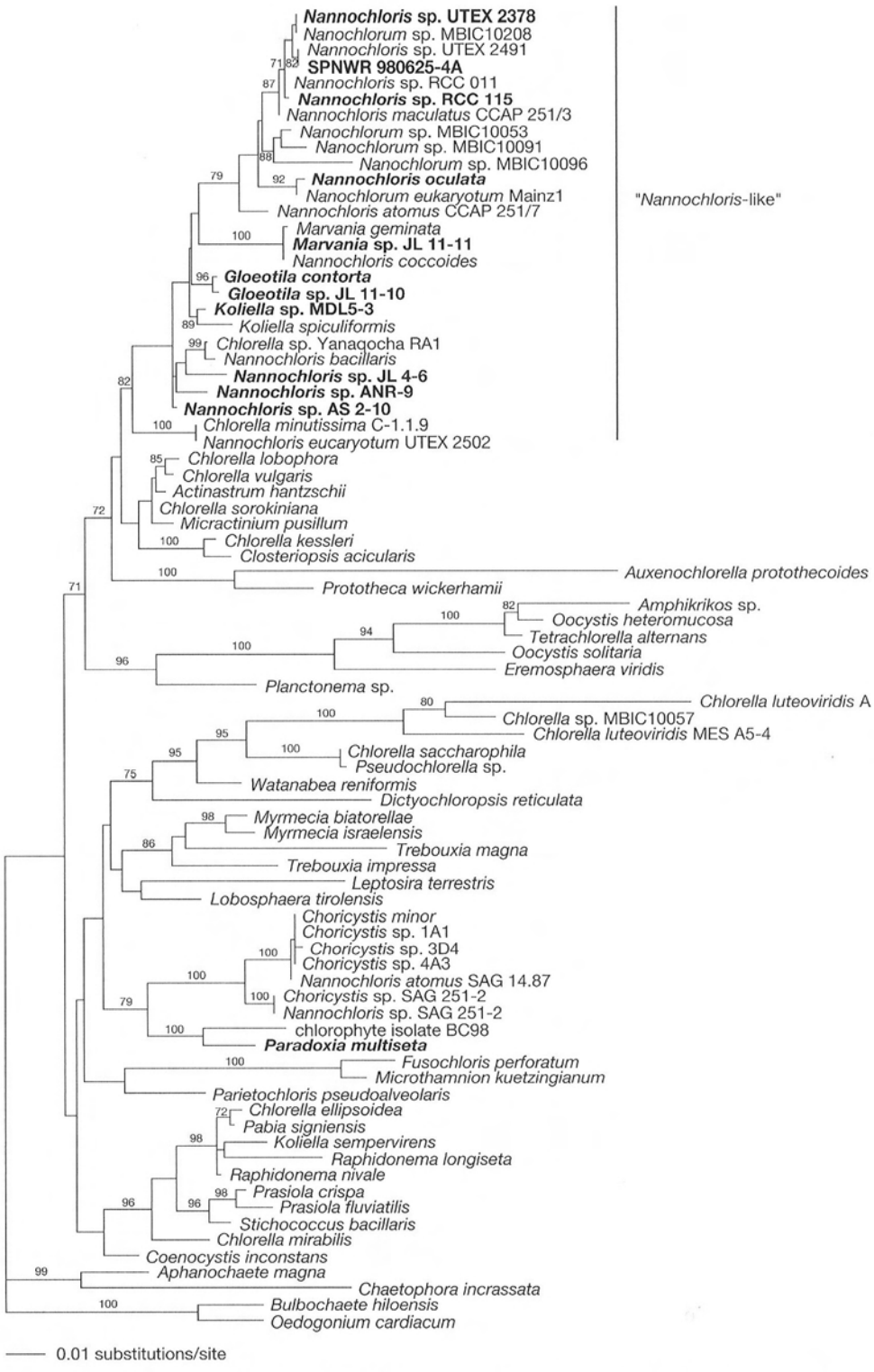


Fig. 14. Maximum likelihood tree of 18S rDNA data from trebouxiophycean taxa rooted with four Chlorophyceae as the outgroup (−Ln likelihood = 14111.67490). Likelihood settings from best-fit model (TrNef + I + G) selected by AIC in Modeltest version 3.06 (Posada & Crandall 1998): Base = equal; Nst = 6; Rmat = (1.0000 2.4020 1.0000 1.0000 5.0277); Rates = gamma; Shape = 0.5135; Pinvar = 0.5038. Bootstrap values (based on 100 replicates) greater than 70 are indicated above the branches. Branch lengths are drawn proportional to evolutionary change (see scale). Taxa sequenced in this study are in boldface.

Table 1. Algal isolates included in the phylogenetic analyses, using names as they currently appear in culture collections and/or GenBank. F, freshwater or terrestrial; M, marine; E, estuarine; H, hypersaline; S, symbiont; A, autosporulation; Bd, budding; BF, binary fission; BF?, apparent binary fission based on LM only. 18S rDNA sequences first reported in this study are in boldface.

Isolate	Accession no.	Habitat	Cell division	Cell division reference
Trebouxiophyceae (ingroup)				
Actinastrum hantzschii Lagerheim SAG 2015	AF288365	F	A	Wolf *et al.* (2002)
Auxenochlorella protothecoides (Krüger) Kalina & Punocháóvá SAG 211-7a	X56101	F		
Chlorella ellipsoidea Gerneck SAG 211-1a	X63520	F		
Chlorella kessleri Fott & Nováková SAG 211-11g	X56105	F	A	Yamamoto *et al.* (2001, 2003)
Chlorella lobophora Andreyeva 750-I	X63504	F		
Chlorella luteoviridis Chodat SAG 211-1a, clone A	X73997	F		
Chlorella luteoviridis MES A5-4	AB006045	F		
'*Chlorella minutissima*' Fott & Nováková C-1.1.9	X56102	F		
Chlorella mirabilis Andreyeva 748-I	X74000	F		
Chlorella saccharophila (Krüger) Migula SAG 211-9a	X63505	F		
Chlorella sorokiniana Shihira & Krauss Prag A14	X74001	F	A	Yamamoto *et al.* (2003)
Chlorella vulgaris Beijerinck SAG 211-11b	X13688	F	A	Yamamoto *et al.* (2003)
Chlorella Beijerinck sp. MBIC10057	AB058305	M		
Chlorella sp. Yanaqocha RA1	Y14950	F	?	
Chlorophyte Isolate BC98 (endosymbiont of Ginkgo)	AJ302940	S		
Choricystis minor (Skuja) Fott SAG 251-1	X89012	F	A	Krienitz *et al.* (1996)
Choricystis Skuja sp. 1A1	AF357147/55	F		
Choricystis sp. 3D4	AF357148/56	F		
Choricystis sp. 4A3	AF357149	F		
Choricystis sp. SAG 251-2	X81965	F	A	Yamamoto *et al.* (2001)
Closteriopsis acicularis (G.M. Smith) J.H. Belcher & Swale SAG 11.86	Y17470	F		
Coenocystis inconstans Hanagata & Chihara	AB017435	F		
Dictyochloropsis reticulata (Tschermak-Woess) Tschermak-Woess CCHU 5616	Z47207	F/S		
Eremosphaera viridis de Bary UTEX 34	AF387154	F		
Fusochloris perforata (Lee & Bold) Floyd, Watanabe & Floyd UTEX 2104 (as *Characium perforatum* Lee & Bold)	M62999	F		
***Gloeotila contorta* Chodat SAG 41.84**	**AY422074**	**F**	**?**	
***Gloeotila* Kützing sp. JL 11-10**	**AY195976**	**F**	**BF?**	**this study**
Koliella sempervirens (Chodat) Hindák	AF278747	F	BF	Hindák (1963)
Koliella spiculiformis (Chodat) Hindák	AF278746	F	BF	Hindák (1963)
***Koliella* Hindák sp. MDL 5-3**	**AY352046**	**F**	**BF?**	**this study**
Leptosira terrestris (Fritz & John) Friedl SAG 463-3 (= *Pleurastrum terrestris* Fritz & John SAG 463-3)	Z28973	F		
Lobosphaera tirolensis Reisigl ASIB S234	AB006051	F?		
Marvania geminata Hindák SAG 12.88	AF124336	F	Bd/A	Hindák (1976); Reymond *et al.* (1986); Sluiman & Reymond (1987)
***Marvania* Hindák sp. JL 11-11**	**AY195977**	**F**	**Bd/A**	**this study**
Micractinium pusillum Fresenius SAG 13.81	AF364101	F		
Microthamnion kuetzingianum Nägeli UTEX 1914	Z28974	F		
Myrmecia biatorellae (Tschermak-Woess & Plessl) Peterson UTEX 907	Z28971	F		
Myrmecia israelensis (Chantanachat & Bold) Friedl UTEX 1181 (= *Friedmannia israeliensis* Chantanachat & Bold UTEX 1181)	M62995	F		
Nannochloris atomus Butcher SAG 14.87	AB080305	M	A	Yamamoto *et al.* (2001, 2003)
Nannochloris atomus CCAP 251/7	AB080303	M	A	Yamamoto *et al.* (2001, 2003)
Nannochloris bacillaris Naumann	AB080300	F	BF	Yamamoto *et al.* (2001, 2003)
Nannochloris coccoides Naumann CCAP 251/1b	AB080301	F	Bd/A	Menzel & Wild (1989); Yamamoto *et al.* (2001, 2003)
Nannochloris eucaryotum (Wilhelm *et al.*) Menzel & Wild UTEX 2502	AB080304	M	A	Yamamoto *et al.* (2001, 2003)
Nannochloris maculatus Butcher CCAP 251/3	AB080302	M	A	Brown & Elman (1983); Menzel & Wild (1989); Yamamoto *et al.* (2001, 2003)
***Nannochloris oculata* Droop UTEX 1998** (= *N. atomus* CCAP 251/6?)	**AY422075**	**E**	**A**	Menzel & Wild (1989)
***Nannochloris* Naumann sp. AS 2–10**	**AY195968**	**F**	**BF?**	**this study**
***Nannochloris* sp. ANR-9**	**AY220081**	**F**	**BF**	**this study**
***Nannochloris* sp. JL 4–6**	**AY195983**	**F**	**A**	**this study**

Table 1. Continued.

Isolate	Accession no.	Habitat	Cell division	Cell division reference
Nannochloris sp. RCC 011	AJ131691	M	?	
Nannochloris sp. SAG 251-2	AB080306	F	A	Yamamoto *et al.* (2001, 2003)
***Nannochloris* sp. UTEX 2378**	**AY422076**	**M**	**?**	
***Nannochloris* sp. UTEX 2379**	**AY560119**	**M**	**?**	
***Nannochloris* sp. UTEX 2491**	**AY422077**	**H**	**?**	
Nanochlorum eucaryotum Wilhelm *et al.* Mainz1	X06425	M	A	Menzel & Wild (1989)
Nanochlorum Wilhelm *et al.* sp. MBIC 10053	AB058304	M	?	
Nanochlorum sp. MBIC 10091	AB058309	M	?	
Nanochlorum sp. MBIC 10096	AB058312	M	?	
Nanochlorum sp. MBIC 10208	AB058331	M	?	
Oocystis heteromucosa Hegewald SAG 1.99	AF228689	F		
Oocystis solitaria Wittrock SAG 83.80	AF228686	F		
Pabia signiensis Friedl & O'Kelly SAG 7.90	AJ416108	F?		
***Paradoxia multiseta* Svirenko UTEX 2460**	**AY422078**	**F**		
Parietochloris pseudoalveolaris (Deason & Bold) Watanabe & Floyd	M63002	F		
***Picochlorum oklahomensis* Hironaka UTEX 2795**	**AY422073**	**H**	**A**	**Hironaka (2000); this study**
***Picochlorum* Henley *et al.* sp. RCC 115**	**AY526738**	**M**	**A**	**this study**
Planctonema Schmidle sp. M110-1	AF387148	F		
Prasiola crispa (Lightfoot) Meneghini SAG 43.96	AJ416106	F		
Prasiola fluviatilis (Summerfelt) Areschoug	AF189072	F		
Prototheca wickerhamii Tubaki & Soneda SAG 263-11	X74003	F		
Pseudochlorella Lund sp. CCAP 264-2	AB006049	F		
Raphidonema longiseta Vischer	U18520	F		
Raphidonema nivale Lagerheim CCAP 470-4	AF448477	F		
Stichococcus bacillaris Nägeli K4-4	AB055866	F		
Tetrachlorella alternans Korsikov SAG B42.81	AF228687	F		
Trebouxia impressa Ahmadjian UTEX 892	Z21551	F		
Trebouxia magna Ahmadjian UTEX 902	Z21552	F		
Watanabea reniformis Hanagata *et al.* SAG 211-9b	X73991	F		
Chlorophyceae (outgroup)				
Aphanochaete magna Godward UTEX B 1909	AF182816	F		
Bulbochaete hiloensis (Nordstedt) Tiffany UTEX 952	U83132	F		
Chaetophora incrassata (Hudson) Hazen UTEX 1289	D86499	F		
Oedogonium cardiacum Wittrock UTEX 40	U83133	F		

nominal *C. minutissima* isolate that was reassigned to *Mychonastes* Simpson & Van Valkenburg in the Chlorophyceae. *Nannochloris eucaryotum* UTEX 2502 is listed in the UTEX catalogue as being a relative of SAG 55.87, and the SAG catalogue claims that the two isolates are identical. It is unclear whether the 'Mainz1' isolate (GenBank X06425) is the same organism as SAG 55.87, as implied by Huss *et al.* (1999) and Yamamoto *et al.* (2003). Sequencing of SAG 55.87 will be necessary to resolve this uncertainty. We obtained the identical sequence for UTEX 2502 as Yamamoto *et al.* (2003), who noted that the culture was heterogeneous.

Nannochloris atomus CCAP 251/7 falls within the marine or saline clade, whereas *N. atomus* SAG 14.87 is only distantly related and clusters within a minimally varying *Choricystis* clade. These two strains, which have > twofold different genome sizes (Yamamoto *et al.* 2001), are clearly different species, and we advise that only CCAP 251/7 be reassigned as *Picochlorum atomus* comb. nov. Note that *N. atomus* CCAP 251/6 (not studied here) is supposedly equivalent to *N. oculata* UTEX 1998. The taxonomic placement of *N. atomus* SAG 14.87 is uncertain at this time, because of the discrepancy between phylogenies based on 18S rDNA, which places it with *Choricystis*, and an actin gene, which places it with other *Nannochloris* taxa (Yamamoto *et al.* 2003). In contrast, *Nannochloris* sp. SAG 251-2 is clearly *Choricystis* rather than *Nannochloris*, because both the 18S rDNA and actin phylogenies place it far from other *Nannochloris* taxa (Yamamoto *et al.* 2003).

Given the broad halotolerance of *P. oklahomensis* (growth from 0 to at least 100 ppt; Henley *et al.* 2002) and preference for low salinity (10 ppt) by marine *Nannochloris* spp. UTEX 1998 and 2055 (W.J. Henley, unpublished observations) and *N. eucaryota* UTEX 2502 (Tschermak-Woess 1999), habitat does not appear likely to be an obligatory correlate of cell division pattern in this group, despite the apparent divergence of the freshwater and marine or saline lineages. This observation is further corroborated by the 18S rDNA sequence near-identity of freshwater *Chlorella minutissima* C-1.1.9 and marine *N. eucaryotum* UTEX 2502, the near-identity of marine *N. atomus* SAG 14.87 and freshwater *Choricystis* isolates, and the tentative alignment of autosporic *Nannochloris* sp. JL 4-6 with other freshwater strains exhibiting binary fission. Moreover, several nominally freshwater '*Chlorella*' species are halotolerant up to 20–50 ppt, depending on species (Kessler 1974), and a marine *Nanochlorum eucaryotum* isolate grows from 0 to 120 ppt (Zahn 1984). Thus, it was presum-

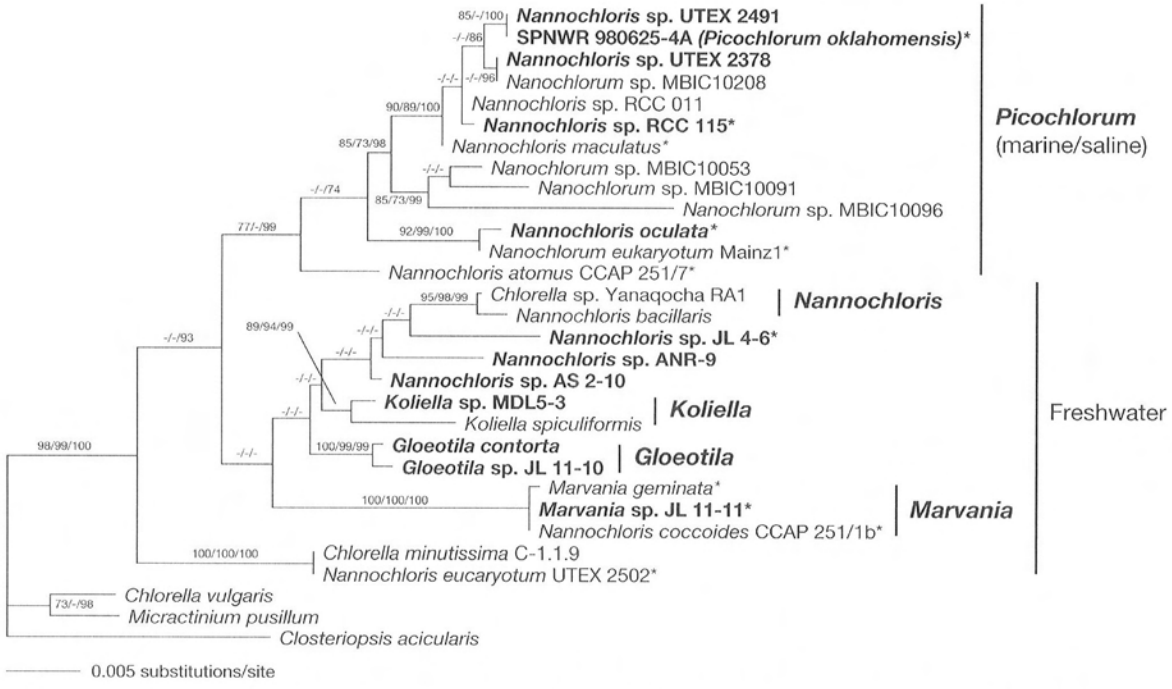


Fig. 15. Maximum likelihood tree of 18S rDNA data from '*Nannochloris*-like' taxa rooted with *Chlorella vulgaris*, *Micractinium pusillum* and *Closteriopsis acicularis* as the outgroup (−Ln likelihood = 4362.85024). Likelihood settings are as noted for Fig. 14. Bayesian analysis was conducted using likelihood settings with gamma and invariants. The posterior probabilities are based on four chains and 500,000 generations. Values greater than 70 are indicated above the branches; left-to-right: ML bootstrap (100 replicates), MP bootstrap (1000 replicates), and B posterior probabilities (× 100); dashes or missing values are < 70. Branch lengths are drawn proportional to evolutionary change (see scale). Taxa sequenced in this study are indicated in boldface. Asterisks denote known autosporic strains; all others in the *Picochlorum* clade have unconfirmed division, whereas others in the freshwater group either have unconfirmed division (AS 2-10, JL 11-10, *Chlorella* sp. Yanaqocha RA1) or are known to divide by binary fission.

ably coincidental that the ancestral freshwater *Nannochloris* divided by binary fission whereas the ancestral marine *Picochlorum* and freshwater *Choricystis* reproduced by autosporulation.

DIAGNOSES

Picochlorum Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley & K. Fawley, *gen. nov.*

Cellulae virides, rotundae vel ovales, 1.5–3 μm diametro, in terra madida vel aqua, aut salina aut dulci. Nucleus unicus, mitochondrius unicus, chloroplastus unicus lateraliter positus sine pyrenoide. Flagella nulla. Inter pigmenta chloroplasti chlorophylla *a*, *b*. Reproductio asexualis autosporis in partes duas vel plus; reproductio sexualis ignota. 18S rRNA sequentia genetica demonstrant differentias a speciebus ceteris Trebouxiophyceearum.

Cells green, spherical or oval, with a diameter of 1.5–3 μm, growing in moist soil or water, either saline or fresh. One nucleus, one mitochondrion, one lateral chloroplast, pyrenoid absent. Flagella absent. Chloroplast pigments include chlorophylls *a*, *b*. Reproduction by autosporulation, leading to two or more daughter cells. Sexual reproduction unknown. Analyses of 18S rRNA sequences show differences from those of other Trebouxiophyceae.

TYPE OF GENUS: *P. oklahomensis* Hironaka, *sp. nov.*, designated here.

Picochlorum oklahomensis Hironaka, *sp. nov.*

Cellulae virides, rotundae vel ovales, 2 μm diametro, in terra madida vel aqua, aut salina aut dulci. Nucleus unicus, mitochondrius unicus, chloroplastus unicus lateraliter positus sine pyrenoide, granulis amyli interdum praesentibus. Flagella nulla. Inter pigmenta chloroplasti chlorophylla *a*, *b*, lutein, violaxanthin, neoxanthin et β-carotene. Paries cellulae tristratus, componens acidum nullum. Reproductio asexualis autosporis in partes duas; reproductio sexualis ignota. 18S rRNA sequentia genetica (GenBank AY422073) demonstrant differentias a speciebus ceteris generis.

Cells green, spherical or oval, with a diameter of 2 μm, growing in moist soil or water from 0 to at least 100 ppt. One nucleus, one mitochondrion, one lateral chloroplast lacking a pyrenoide, starch grains sometimes present. Flagella absent. Chloroplast pigments include chlorophylls *a*, *b*, lutein, violaxanthin, neoxanthin, and β-carotene. Cell wall trilaminate, lacking acidic residues. Reproduction by autosporulation into two daughter cells. Sexual reproduction unknown. Analysis of 18S rRNA gene sequence (GenBank accession AY422073) shows differences from sequences of other species in the genus.

HOLOTYPE (designated here): A sample of cultured cells was collected on a GF/F filter and attached to a herbarium sheet and deposited in the Oklahoma State University Botany Department herbarium. Live cultures have been submitted to the culture collection of the University of Texas at Austin (UTEX 2795) and Culture Collection of Marine Phytoplankton (CCMP 2329), Bigelow Laboratories (Booth Bay Harbor, Maine, USA). Also, we tentatively consider strain UTEX 2491 conspecific based on 18S rDNA sequence.

TYPE LOCALITY: Ephemeral variably saline pool or soil at the Salt Plains National Wildlife Refuge, Oklahoma, USA.

Picochlorum atomus (Butcher) Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley & K. Fawley, *comb. nov.*

BASIONYM: *Nannochloris atomus* Butcher (1952, pp. 181–182).

SYNONYM: *Nannochloris atomus sensu* CCAP 251/7 *non* SAG 14.87.

Note that strain SAG 14.87 is definitely a distinct entity, with apparent affiliation with *Choricystis*, not synonymous with CCAP 251/7.

Picochlorum eukaryotum (Wilhelm, Eisenbeis, Wild & Zahn) Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley & K. Fawley, *comb. nov.*

BASIONYM: *Nanochlorum eukaryotum* Wilhelm, Eisenbeis, Wild & Zahn (1982, p. 107).

SYNONYM: *Nanochlorum eukaryotum sensu* Mainz1; *Nannochloris eucaryotum* (Wilhelm, Eisenbeis, Wild & Zahn) Menzel & Wild (1989, p. 157). Note that *N. eucaryotum* UTEX 2502, which is reportedly the type culture associated with Menzel & Wild (1989), has a markedly different 18S rDNA sequence from the Mainz1 strain.

Picochlorum maculatus (Butcher) Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley & K. Fawley, *comb. nov.*

BASIONYM: *Nannochloris maculatus* Butcher (1952, p. 181).

SYNONYM: *Nannochloris maculatus sensu* CCAP 251/3.

Picochlorum oculata (Droop) Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley & K. Fawley, *comb. nov.*

BASIONYM: *Nannochloris oculata* Droop (1955, p. 235).

SYNONYM: *Nannochloris oculata sensu* UTEX 1998; tenatively *Nannochloris atomus* Butcher (1952) *sensu* CCAP 251/6, pending 18S rDNA sequence confirmation of its identity with UTEX 1998, as claimed in the catalogues of both culture collections.

Marvania coccoides (Naumann) Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley & K. Fawley, *comb. nov.*

BASIONYM: *Nannochloris coccoides* Naumann (1921, p. 18). Note that Naumann's original diagnosis apparently lacks a type specimen, so we cannot verify its synonymy with the distinct isolate CCAP 251/1b.

SYNONYM: *Nannochloris coccoides sensu* CCAP 251/1b.

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