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JENUFA GEN. NOV.: A NEW GENUS OF COCCOID GREEN ALGAE (CHLOROPHYCEAE, INCERTAE SEDIS) PREVIOUSLY RECORDED BY ENVIRONMENTAL SEQUENCING[1]

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The diversity of eukaryotic microorganisms is far from fully described, as indicated by the vast number of unassigned genotypes retrieved by environmental sequencing or metagenomics. We isolated several strains of unicellular green algae from algal biofilms growing on tree bark in a Southeast Asian tropical rainforest and determined them to be relatives of an unidentified lineage of environmental 18S rDNA sequences, thus uncovering its cellular identity. Light, confocal, and electron microscope observations and sequencing the 18S rRNA gene revealed that the strains represent two different species within an apparently new genus, described here as *Jenufa* gen. nov. Both species formed minute coccoid cells with an irregular globular outline, a smooth cell wall, and a single parietal chloroplast without a pyrenoid. The two species, described herein as *J. perforata* and *J. minuta*, differed in chloroplast morphology and cell wall structure. Phylogenetic analyses of 18S rRNA gene sequences showed a firm relationship between the two species and placed the *Jenufa* lineage in an unresolved position within the CS clade (Chlamydomonadales + Sphaeropleales) of the class Chlorophyceae, although possible affinities to the genus *Golenkinia* were suggested both by maximum-likelihood (ML) and Bayesian methods. Furthermore, two almost identical environmental 18S rDNA sequences from an endolithic microbial community occurring in dolomite rock in the central Alps turned out to be specifically related to, yet apparently distinct from, the sequence of *J. minuta*, indicating the existence of an undescribed *Jenufa* species occurring in the temperate zone.

Key index words: **18S rRNA; Chlorophyceae; Chlorophyta; *Jenufa*; phylogeny; taxonomy; ultrastructure**

Abbreviations: **AU, approximately unbiased; BBM, Bold basal medium; CAUP, Culture Collection of algae at Charles University in Prague; CIPRES, Cyberinfrastructure for Phylogenetic Research; COV, covarion; CS, Chlamydomonadales + Sphaeropleales; DDBJ, DNA Data Bank of Japan; DO, directly opposed; EMBL, European Molecular Biology Laboratory; GTR, general time reversible; MCMC, Markov chain Monte Carlo; ML, maximum likelihood; NCBI, National Center for Biotechnology Information; OCC, Oedogoniales + Chaetophorales + Chaetopeltidales**

The actual phylogenetic diversity of eukaryotic microorganisms could not be grasped before the advent of molecular phylogenetics. This claim is especially pertinent to the case of the traditional green algal order Chlorococcales, comprising unicellular algae with coccoid vegetative cells mostly assigned into the trivial category of ''green balls'' (Komárek and Fott 1983). Beginning in the late 1980s, analyses of molecular data, primarily of 18S rDNA sequences, became instrumental in revising the notion of a single coherent group of coccoid green algae (Huss and Sogin 1990). The molecular perspective has necessitated reevaluation of traditional families, genera, and species of coccoid green algae, revealing extensive polyphyly at all levels (e.g., Wilcox et al. 1992, Friedl and Zeltner 1994, Huss et al. 1999, Friedl and O'Kelly 2002). As a result, Chlorococcales is now held as an artificial assemblage of indirectly related phylogenetic lineages that have developed similar morphological appearances via convergent evolution (Krienitz et al. 2003).

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Indeed, representatives of the former order Chlorococcales are actually distributed across at least three classes of ''core'' chlorophytes (Trebouxiophyceae, Ulvophyceae, Chlorophyceae) intermingled with a plethora of lineages of other morphological types (monadoid, capsal, trichal, siphonal, etc.) (Pröschold and Leliaert 2007). Reassessment of the historical legacy in the study of coccoid green algae remains a challenge for our generation.

Besides this line of research into the systematics of coccoid green algae, it is imperative to keep searching for previously undescribed taxa, since there are many indications that a vast yet hitherto unnoticed diversity remains hidden in natural habitats. One of the richest sources of novel phylogenetic diversity of coccoid green algae may be terrestrial habitats in the tropics. Indeed, a number of new lineages at the generic level, including *Spongiochrysis* Rindi, López-Bautista, A. R. Sherwood et Guiry (Rindi et al. 2006); *Heveochlorella* J. Zhang, V. Huss, X. Sun, K. Chang et D. Pang (Zhang et al. 2008); *Kalinella* Neustupa, Němcová, M. Eliáš et Škaloud (Neustupa et al. 2009); *Pseudomarvania* M. Eliáš et Neustupa (Eliáš and Neustupa 2009); *Hylodesmus* M. Eliáš, Němcová, Škaloud et Neustupa (Eliáš et al. 2010); and *Xylochloris* Neustupa, M. Eliáš et Škaloud (Neustupa et al. 2011), were described from such habitats in the past 5 years. It is beyond any doubt that further entries can be added to this list with additional sampling efforts.

Our recent survey of the diversity of subaerial algae on tree bark in tropical mountain habitats in West Java (Neustupa and Škaloud 2008) and on bark and wood in lowland tropical forests in Singapore (Neustupa and Škaloud 2010) revealed a number of morphotypes potentially representing new taxa, including a morphotype provisionally denoted as *Chlorella* sp. 2 (Neustupa and Škaloud 2008) or *Chlorella* sp. 5 (Neustupa and Škaloud 2010). We succeeded in establishing cultures of this morphotype from five different localities in Singapore and Indonesia, and of a similar morphotype from Singapore. To learn more about these organisms, we undertook a more detailed characterization employing light, confocal, and electron microscopy, and 18S rRNA gene sequencing. In this work, we report the results of our investigations, which indicate that these algal isolates represent two distinct species in a novel lineage of the class Chlorophyceae described herein as a new genus, *Jenufa*. Strikingly, analyses of previously published environmental sequences revealed that a third probable species of the genus *Jenufa* thrives in endolithic communities in the Alps, suggesting that *Jenufa* is not restricted to tropical regions.

MATERIALS AND METHODS

Collection, culturing, and microscopic analyses. Algal strains were isolated from samples of subaerial algal biofilms growing on the bark of trees in tropical forest habitats (Table S1 in the supplementary material). The strains were cultivated on Bold basal medium (BBM) agar medium (Bischoff and Bold 1963) at 23°C with illumination of 40 μmol · m^{-2} · s^{-1} provided by 18W cool fluorescent tubes (Philips TLD 18W/33) using a 12:12 light:dark (L:D) cycle. The pure algal samples were examined using an Olympus BX51 light microscope (Olympus Corp., Tokyo, Japan) with differential contrast and Olympus Z5060 microphotographic equipment. Chloroplast morphology was investigated using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Argon-Krypton laser. We used a 488 nm excitation line and an AOBS filter-free system (Leica Microsystems) collecting emitted light between 498 and 700 nm. A Leica 63 × /1.4 N.A. oil immersion objective fitted on a Leica DM IRE2 inverted microscope (Leica Microsystems) was used. A series of optical sections through the chloroplasts was captured and used for three-dimensional (3-D) morphology reconstruction. Chl autofluorescence was exploited for visualization of chloroplast structure. Chloroplast 3-D morphology reconstructions were produced using the program ImageJ 1.34p (Abramoff et al. 2004) and the ''Volume viewer'' plugin.

EM. For TEM observations, samples were fixed for 2 h at 5°C in a 2% solution of glutaraldehyde in 0.05 M phosphate buffer, postfixed for 2 h at 5°C in 1% osmium tetroxide in 0.05 M phosphate buffer, and overnight at 5°C in 1% uranyl acetate in methanol. After dehydration through an ethanol series, cells were embedded in Spurr's medium via isobutanol. Ultrathin sections, cut with a diamond knife on an Ultracut E (Reichert-Jung, Wien, Austria) were poststained with lead citrate and examined using a JEOL 1011 TEM (JEOL Ltd., Tokyo, Japan).

DNA extraction, PCR, and 18S rRNA gene sequencing. The DNA extraction, PCR amplification of partial 18S rRNA gene sequences, and sequencing were done essentially as previously described (Neustupa et al. 2009). Primers used for PCR were as follows: E26a strain, forward (F) and reverse (R) primers according to Katana et al. (2001); E14c and E19a, 34F (Pažoutová et al. 2010) and 18L (Hamby et al. 1988). For the F2g, F5s, and F7b strains, two overlapping fragments were PCR amplified using the forward (F) primer in combination with a green-algal-specific reverse primer vivi1650R (Pažoutová et al. 2010), and a green-algal-specific forward primer 1500af (Helms et al. 2001) in combination with the reverse primer ITS4 (White et al. 1990). GenBank accession numbers of newly obtained sequences are provided in Table S1.

Phylogenetic analyses. The BLASTN searches (National Center for Biotechnology Information [NCBI], http://blast.ncbi.nlm.nih.gov/) and preliminary ML analyses of the newly determined sequences indicated that the strains included in this study are nested within the Chlorophyceae class of green algae. The sequences were therefore added to a comprehensive alignment of chlorophycean 18S rDNA sequences (>680 taxa) from the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank database build using the ClustalX alignment program (Thompson et al. 1997) along with manual editing using GeneDoc (K. B. Nicholas and H. B. Nicholas, http://www.nrbsc.org/gfx/genedoc/) and guided by the secondary structure model of 18S rRNA from *Chlamydomonas reinhardtii* available from the European Ribosomal RNA Database (http://www.psb.ugent.be/rRNA/secmodel/Crei_SSU.html, Wuyts et al. 2000). Following an ML phylogenetic analysis of the comprehensive sequence alignment using RAxML 7.0.3 (Stamatakis et al. 2008), a set of chlorophycean sequences representing all major lineages plus sequences from representatives of Trebouxiophyceae, Ulvophyceae, and Chlorodendrales (''Prasinophyceae'') were selected for final examination (103 taxa, 1,740 positions). This sequence alignment is available upon request. A Bayesian inference was then performed using the program MrBayes 3.1 (Huelsenbeck

and Ronquist 2001). Two parallel Markov chain Monte Carlo (MCMC) runs were carried out for 3 million generations each with one cold and three heated chains employing the general time reversible (GTR)+Γ+I+COV evolutionary model (with parameters estimated from the data) selected because it had been suggested to capture the actual evolutionary process operating on the 18S rRNA gene better than simpler models (Galtier 2001, Huelsenbeck 2002). Trees were sampled every 100 generations. The initial 5,001 trees from each run were discarded as ''burn-in'' based on plotting log likelihood values and posterior probabilities of tree bipartitions were calculated on the basis of the consensus of the remaining 50,000 trees. A ML tree was inferred using RAxML 7.0.4 accessed through the CIPRES Portal (http://www.phylo.org/sub_sections/portal/). We employed a tree inference strategy that has been recently demonstrated to be especially effective in finding trees with the highest likelihood. This strategy combines a novel rapid bootstrap algorithm to infer 100 bootstrap trees followed by thorough ML inference on the original data set utilizing the bootstrap trees for further optimization (Stamatakis et al. 2008). Standard ML bootstrapping (100 replicates) was also performed using RAxML 7.0.3 and bootstrap values from this analysis rather than from the rapid bootstrapping procedure described above are shown in Figure 4. These ML analyses employed a GTR+Γ+I substitution model as the most general model implemented in the RAxML program (following also the recommendation on the use of evolutionary models provided in the manual of the program by its author).

RESULTS

Jenufa Němcová, M. Eliáš, Škaloud et Neustupa, **gen. nov.**

Diagnosis: Cellulae vegetativae solitariae, sphaericae vel irregulares. Nucleus singularis, in media cellulae positus. Paries cellularis levis et distinctus. Chloroplastus unicus, perforatus vel multi-lobatus, parietalis, sine pyrenoide. Propagatio asexualis per 2 aut 8 autosporis. Reproductio sexualis et reproductio per zoosporas non observata. Substantiae carotenoides secundae absentes. A generibus ceteris familiae ordine nucleotidorum in18S rRNA differt.

Vegetative cells are solitary. The cells have spherical or irregular outline. The nucleus single, positioned centrally in the cell. The cell wall is smooth and distinct. The single chloroplast perforated or with numerous lobes is parietal, without a pyrenoid. Asexual reproduction is via formation of 2–8 autospores. Sexual reproduction and production of zoospores was not observed. Secondary carotenoids are absent. The genus differs from other members of Chlorophyceae by the 18S rRNA sequence.

Type species: Jenufa perforata Němcová, M. Eliáš, Škaloud et Neustupa.

Etymology: The genus name *Jenufa* (feminine noun) was selected to honor one of the most exquisite works of 20th-century music: the opera Jenůfa by the Moravian composer Leoš Janáček.

Jenufa perforata Němcová, M. Eliáš, Škaloud et Neustupa, **sp. nov.**

Diagnosis: Cellulae vegetativae solitariae, sphaericae vel irregulares (2.5–) 3.5–6.5 (–7.5) μm in diametro, uninucleatae. Chloroplastus unicus, perforatus, parietalis, sine pyrenoide. Propagatio asexualis per (2) 4 (8) autosporis. Reproductio sexualis et reproductio per zoosporas non observata.

Vegetative cells are solitary, uninucleate with spherical or irregular outline, (2.5–) 3.5–6.5 (–7.5) μm in diameter. The single chloroplast is perforated, parietal and without a pyrenoid. Asexual reproduction via (2) 4 (8) autospores. Sexual reproduction and production of zoospores was not observed.

Holotype: The CAUP C-H8101 strain has been permanently cryopreserved in Culture Collection of algae at Charles University in Prague, Czech Republic (CAUP; http://botany.natur.cuni.cz/algo/caup.html). CAUP C-H8101 is also maintained as an active culture (CAUP H 8101), from which the holotype was derived. The strain is also conserved in the form of a permanent slide (CAUP P- H8101).

Type locality: The bark of an unidentified tree in a lowland tropical forest in the Central Catchment Nature Reserve, Singapore (1°21′27″ N and 103°48′32″ E; altitude 50 m a.s.l.)

Etymology: The specific epithet refers to perforations observed in the single chloroplast.

Jenufa minuta Němcová, M. Eliáš, Škaloud et Neustupa, **sp. nov.**

Diagnosis: Cellulae vegetativae solitariae, sphaericae vel ellipsoideae vel irregulares (2.3–) 3.3–5.8 (–6.5) μm in diametro, uninucleatae. Chloroplastus unicus, multi-lobatus vel perforatus, parietalis, sine pyrenoide. Propagatio asexualis 2 vel 4 autosporis. Reproductio sexualis et reproductio per zoosporas non observata.

Vegetative cells are solitary, uninucleate with spherical, irregular or elliptical outline, (2.3–) 3.3–5.8 (–6.5) μm in diameter. The single chloroplast is perforated or with numerous lobes, parietal and without a pyrenoid. Asexual reproduction is via 2 or 4 autospores. Sexual reproduction and production of zoospores was not observed.

Holotype: The CAUP C-H8102 strain has been permanently cryopreserved in CAUP. CAUP C-H8102 is also maintained as an active culture (CAUP H 8102). The strain is also conserved in a form of a permanent slide (CAUP P- H8102).

Type locality: The bark of an unidentified tree in a lowland tropical forest in the Central Catchment Nature Reserve, Singapore (1°21′27 N and 103°48′32 E; altitude 50 m a.s.l.)

Etymology: The specific epithet reflects the small size of the cells.

Light and confocal microscopy. The tropical subaerial strains described herein as the new genus *Jenufa* formed minute coccoid cells with an irregularly globular outline. They had a smooth cell wall, a single parietal perforated chloroplast, and reproduced exclusively by autospores. Old cultures did not produce secondary carotenoids. *J. perforata* was characterized by mostly spherical cells, (2.5–) 3.5–6.5 (–7.5) μm in diameter (Fig. 1A). In some cases, cells

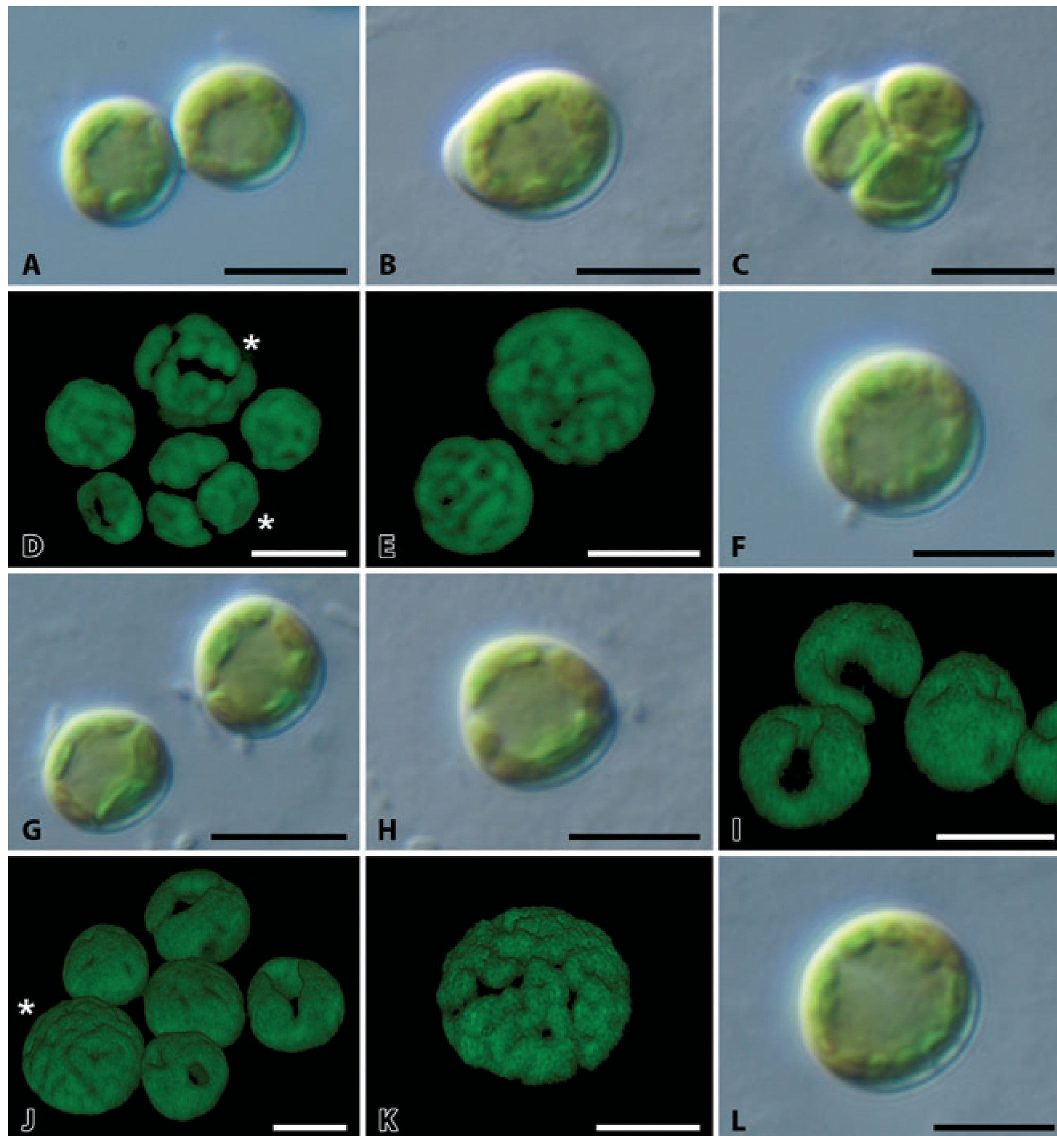

Fig. 1. Light and confocal microscopy of *Jenufa* species. (A–F) *Jenufa perforata.* (A) Young, spherical cells. (B) An irregularly shaped cell with a flat local thickening of the cell wall. (C) Tetrads of cells. (D) Spatial reconstruction of chloroplasts in mature autosporangia (asterisk) and young autospores. (E) Spatial reconstruction of chloroplasts in mature cells, showing their dense perforation by numerous incisions and holes. (F) A mature vegetative cell with a parietal chloroplast. (G–L) *Jenufa minuta.* (G) Young, spherical cells. (H) A cell with irregularly tetrahedrical outline. (I) Spatial reconstruction of chloroplasts in young autospores. (J) Spatial reconstruction of chloroplasts in young cells, showing an incompletely closed chloroplast with distinct lobes. One mature cell with a closed chloroplast (a hollow sphere stage) is marked by an asterisk. (K) Spatial reconstruction of a chloroplast in a mature cell, showing its dense perforation by numerous incisions and holes. (L) A mature vegetative cell with a parietal chloroplast. Scale bars, 5 μm.

had irregular to tetrahedrical outlines, and occasionally a flat local thickening of the wall could be detected (Fig. 1B). Asexual reproduction took place by means of four (or rarely by two or eight) autospores. The vegetative cells were either solitary or were found to form regular tetrads (Fig. 1C). The chloroplast of the autospore was very simple, pot shaped, without any visible perforations (Fig. 1D). In young cells, the chloroplast spread below the plasma membrane and soon formed a hollow sphere (Fig. 1D). In mature cells, this spherical chloroplast was densely perforated by numerous incisions and holes (Fig. 1, E and F). Pyrenoids were absent. The cell wall was smooth. Neither zoospore formation, nor sexual reproduction was observed.

J. minuta had irregularly spherical, solitary cells, (2.3–) 3.3–5.8 (–6.5) μm in diameter (Fig. 1G). Cells typically had irregularly tetrahedrical to elliptical outlines (Fig. 1H). Asexual reproduction took place by means of two to four autospores. Each cell contained a single parietal chloroplast. Similar to *J. perforata*, autospores contained a chloroplast that in young cells spread below the plasma membrane (Fig. 1I). However, in contrast to *J. perforata*, the

chloroplast of young *J. minuta* cells did not close up into a hollow sphere stage but instead formed distinct lobes (Fig. 1J). Merging of chloroplast lobes into a spherical, perforated chloroplast did not occur until cells reached 6.5–7 μm in diameter (Fig. 1, K and L). Pyrenoids were not observed. The cell wall was smooth and thin. Neither zoospore formation, nor sexual reproduction was detected.

Ultrastructure. As typical for small coccoid ''green balls,'' cells of both species possessed a minimal set of organelles. The single nucleus was located centrally, surrounded by a parietal chloroplast. The chloroplast contained starch granules, which in fast-growing cells were reduced (Figs. 2A and 3A). The mitochondrion occupied the space between the nucleus and the chloroplast (Figs. 2B and 3A). The dictyosome was active shortly after cell division (Fig. 2B). One to several large vacuoles with electron-transparent content and a few smaller vacuoles with oil-like content were visible in cross-sections (Fig. 2A). The cell wall was composed of two main layers: a granulo-fibrillar or amorphous inner layer and an outer trilaminar one. The two species differed in their cell wall structure. *J. perforata* possessed a thick inner cell wall layer (up to 300 nm) composed of several discernible microfibrillar sublayers (Fig. 2C). The outer layer (ca. 20 nm) had a trilaminar appearance, although the darker lamina was not so distinct. Shreds of the mother cell wall were often observed to remain attached to newly released autospores. The granulo-fibrillar inner layer of the mother cell wall was reduced and filled with electron-dense inclusions (Fig. 2C). In contrast, *J. minuta* had an amorphous electron-dense inner cell wall layer (ca. 150 nm; Fig. 3D, arrow). The outer layer seemed to be bilaminar (ca. 50 nm; Fig. 3D, arrowhead); a trilaminar substructure was visible in the mother cell wall, after an amorphous inner layer was degraded (Fig. 3E, enlarged box). During autosporulation the nucleus divided into two to eight daughter nuclei followed by cytoplasmic cleavage (Fig. 3B) within the mother cell wall; subsequently, the daughter cell wall was built up (Fig. 3, C and D).

Molecular phylogenetics. Partial 18S rDNA sequences determined for five strains of *J. perforata* (E14c, E19a, F2g, F5s, and F7b) proved identical over the shared region of 1,717 bp. The partial 18S rDNA sequence from *J. minuta* (strain E26a) was 95% identical to the other species tested and differed by seven single-nucleotide indels and 65 substitutions. The portion of the 18S rRNA gene sequenced did not contain any putative introns. BLASTN searches against the nonredundant nucleotide sequence database (NCBI) revealed that the sequences of both *Jenufa* species are most similar to 18S rDNA sequences from uncultured organisms determined in the course of environmental sequencing of an endolithic microbial community in dolomite rock from the central Alps in Switzerland (Horath and Bachofen 2009). For *J. perforata*, these sequences corresponded to four top hits (accession numbers AB257660.1, AB257659.1, AB257662.1, and AB257663.1), whereas for *J. minuta* an additional environmental sequence (AB257666.1) was retrieved. Sequence identity with these environmental sequences was 94%–96% (*J. perforata*) and 92%–98% (*J. minuta*). Among cultured species represented in the nucleotide database, the best match to the sequence of *J. perforata* was *Planktosphaeria gelatinosa* G. M. Sm., strain SAG 262-1b (AY044648.1) with 92% identity, while the best match to the sequence of *J. minuta* was *Bracteacoccus* sp. CNP1VF2 (AF513378.1), also with 92% identity. Both of these taxa and most of the other top hits retrieved by BLASTN searches (not shown) have been previously assigned to the green-algal class Chlorophyceae (Lewis and Flechtner 2002, Wolf et al. 2003b), suggesting that our *Jenufa* species might also belong to this group. This hypothesis was

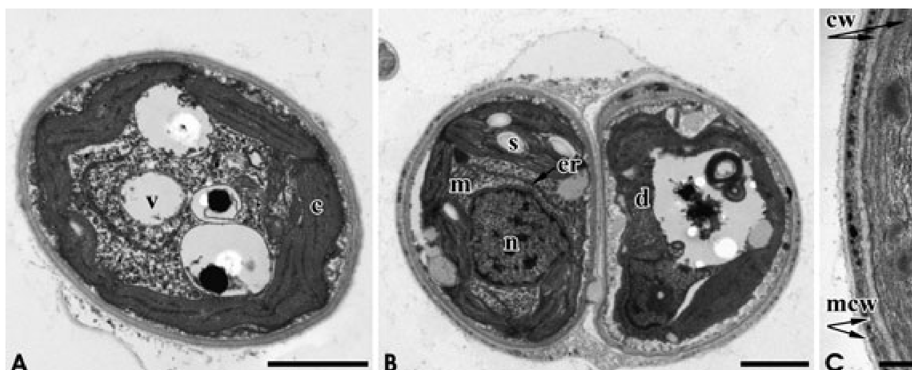

Fig. 2. TEM of *Jenufa perforata*. (A) A young vegetative cell with a parietal chloroplast and numerous vacuoles. Chloroplast (c), vacuole (v). Scale bar, 1 μm. (B) Two newly formed autospores still enclosed within a mother cell wall. Note the starch granules (s) located within a chloroplast. Dictyosome (d), endoplasmic reticulum (er) bearing ribosomes, mitochondrion (m), nucleus (n). Scale bar, 1 μm. (C) A close-up from (B) showing the cell wall ultrastructure. The daughter cell wall (cw) composed of a granulo-fibrillar inner layer and an outer trilaminar layer, the mother cell wall (mcw) composed of a reduced granulo-fibrillar inner layer with electron-dense inclusions and an outer trilaminar layer. Scale bar, 200 nm.

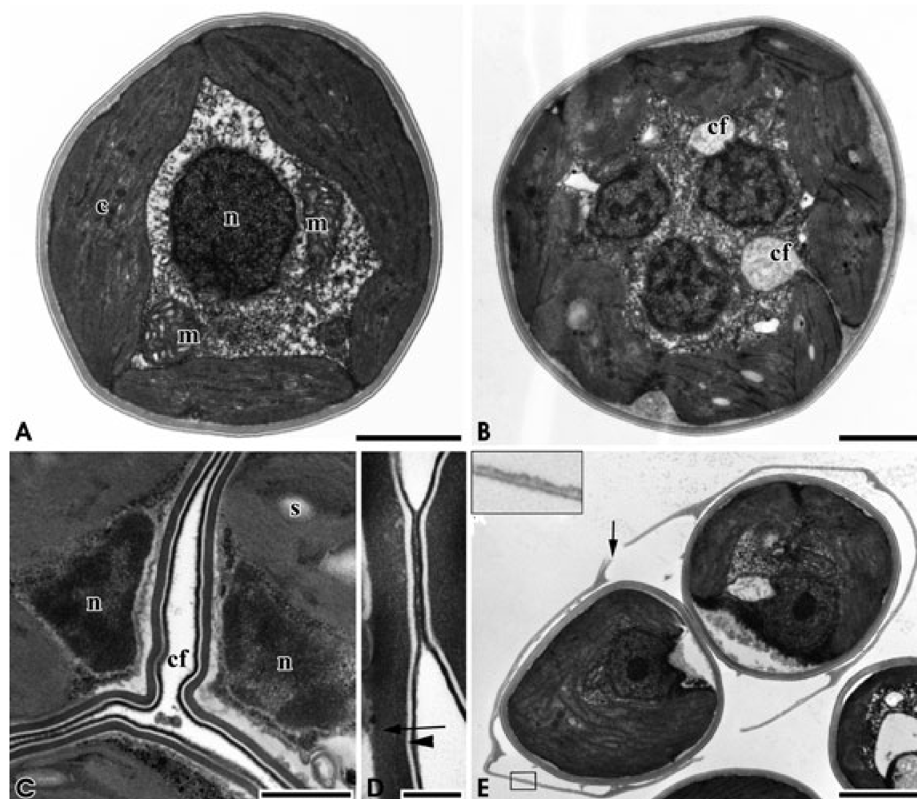

Fig. 3. TEM of *Jenufa minuta*. (A) A young autospore. Chloroplast (c), mitochondrion (m), nucleus (n). Scale bar, 1 μm. (B) An early stage of protoplast division. Cleavage furrows (cf). Scale bar, 1 μm. (C) Newly formed autospores surrounded by a cell wall. Cleavage furrow (cf), nucleus (n), starch granules (s). Scale bar, 0.5 μm. (D) A detailed view of the cell wall in adjacent autospores. Note the amorphous electron-dense inner layer (arrow) followed by an outer trilaminar layer (arrowhead). Scale bar, 200 nm. (E) A mother cell wall with two autospores. Note that the inner layer of the mother cell wall is partly disintegrated while the outer trilaminar layer persists (arrow; inserted figure). Scale bar, 1 μm.

confirmed by preliminary phylogenetic analyses based on a broader selection of chlorophyte taxa (results not shown).

To establish the phylogenetic position of *Jenufa* more precisely, we conducted a detailed phylogenetic analysis employing a comprehensive selection of 18S rDNA sequences from Chlorophyceae along with suitable sequences from Trebouxiophyceae, Ulvophyceae, and Chlorodendrales. The resulting tree (Fig. 4) indicated that *J. minuta* is closely related (with maximal support) to uncultured organisms represented by the sequences AB257660.1 and AB257659.1, and this clade is in turn sister (again with maximal support) to *J. perforata*. Together these sequences formed a clade interpreted herein as the genus *Jenufa*. In both Bayesian and ML analyses, the *Jenufa* clade was recovered in a sister position to sequences of the genus *Golenkinia* Chodat, although this relationship received only marginal support (posterior probability of 0.91, ML bootstrap value of 40%). The *Jenufa/Golenkinia* clade was nested in an unresolved position within the higher-order CS clade comprising two other unsupported clades, which could be interpreted as the putative orders Chlamydomonadales (= Volvocales) and Sphaeropleales. The whole CS clade, with strong support from the Bayesian analysis only (posterior probability of 0.99), was found to be sister to the OCC clade (Oedogoniales, Chaetophorales, Chaetopeltidales), also strongly supported (0.99) by the Bayesian analysis only. In addition, monophyly of the whole class Chlorophyceae (CS+OCC) was supported strongly by the Bayesian analysis (1.00) and moderately by the ML analysis (81%).

With the exception of AB257659.1 and AB257660.1, we omitted from phylogenetic analyses the other environmental sequences retrieved as top BLASTN hits with *Jenufa* spp. sequences (i.e., AB257662.1, AB257663.1, AB257666.1), because careful examination revealed their probable chimeric nature. Specifically, regions 1523–1775 of AB257662.1, 456–966 of AB257663.1, and 281–1772 of AB257666.1 appear to be derived from organisms closely related to or even representing the genus *Stichococcus* Nägeli in the class Trebouxiophyceae (data not shown).

DISCUSSION

Although the morphological characteristics of all the strains investigated in this study do not seem to fit into any previously described species of coccoid

green algae, they would fall into the polyphyletic traditional genus *Chlorella* Beij. (Ettl and Gärtner 1995, Andreyeva 1998). In particular, their very small cell size, the absence of pyrenoids, and their irregularly shaped young cells may indicate specific affinities to *Chlorella zofingiensis* Dönz, which has also been repeatedly reported from subaerial microhabitats (Kalina and Punčochářová 1987, Ettl and Gärtner 1995). The 18S rRNA gene sequence of an authentic strain of *C. zofingiensis* (SAG 211-14a) reported by Huss et al. (1999) does place this species into the class Chlorophyceae and specifically into the CS clade; it is still rather far from the *Jenufa* lineage (Fig. 4). Nevertheless, an alternative topology of the phylogenetic tree with the *Jenufa* sequences and the *C. zofingiensis* sequence constrained to form a clade cannot be excluded as significantly worse when tested by the Shimodaira-Hasegawa test ($P = 0.158$) or the AU test ($P = 0.164$), raising the possibility that the morphological similarities between *Jenufa* spp. and *C. zofingiensis* actually reflect a closer relationship perhaps yet to be demonstrated by multigene phylogenetic analyses. However, it seems appropriate to treat our strains and *C. zofingiensis* as representatives of separate genera as generic assignment of the latter species has yet to be resolved. While it is obvious that *C. zofingiensis* cannot remain in the ultimately redefined genus *Chlorella*, two alternative classifications have been proposed: *Muriella zofingiensis* (Dönz) Hindák (Hindák 1982) and *Mychonastes zofingiensis* (Dönz) Kalina et Punčoch. (Kalina and Punčochářová 1987). However, according to the 18S rDNA sequence from a strain identified as *Muriella terrestris* J. B. Petersen (a type species of the genus *Muriella* J. B. Petersen), this genus belongs to Trebouxiophyceae and is thus unrelated to *C. zofingiensis* (Hanagata 1998). The taxonomy of the genus *Mychonastes* P. D. Simpson et Van Valk. was recently reassessed by molecular approaches, and *Mych. zofingiensis* was not accepted as a genuine member of the genus (Krienitz et al. 2011), although a final decision awaits molecular characterization of the type species of the genus *Mychonastes*, *Mych. ruminatus* P. D. Simpson et Van Valk. *Mychonastes,* in contrast to *Jenufa,* has a discoid mantle or cup-shaped chloroplast(s) without perforations.

The five strains assigned to *J. perforata* do not differ in their morphological characteristics. In fact, upon careful examination, this morphotype was readily recognizable microscopically in different bark samples analyzed by Neustupa and Škaloud (2008, 2010) and determined as *Chlorella* sp. 2 and *Chlorella* sp. 5, respectively. Therefore, we believe that *J. perforata* may actually be a frequently occurring, and occasionally even dominant, species of microalgal growths on the tree bark in humid tropical habitats. In contrast, the second newly described species, *J. minuta,* was isolated only once, and we have not found other populations from our previous investigations of tropical subaerial samples that could be considered morphologically identical to this strain. The two species described herein, *J. perforata* and *J. minuta*, differ in cell wall ultrastructure and chloroplast morphology. The inner cell wall of *J. perforata* is composed of several microfibrillar sublayers, and the prevalent ontogenetic stadium of the chloroplast resembles a hollow perforated sphere. In contrast, *J. minuta* possesses an amorphous electron-dense inner cell wall, and its chloroplast during ontogeny is mostly multilobed, without perforations. The spherical, perforated chloroplast of *J. minuta* was fully developed only in the mature mother cells.

Our analyses of 18S rDNA sequences corroborate insights obtained from observations of the morphology and ultrastructure of the six strains studied herein. First, the apparent morphological identity of the five *J. perforata* strains is reflected by their identical 18S rDNA sequences, suggesting that all these five strains may represent the same species. Admittedly, delimitation of species in unicellular eukaryotes with cryptic or entirely lacking sexual reproduction is somewhat arbitrary. It is possible that sequencing of generally more variable loci, such as the internal transcribed spacer region, would unveil hidden genetic diversity among the five strains. However, considering the present state of knowledge, we believe that treating the E14c, E19a, F2g, F5s, and F7b strains as representatives of the same species is the best working hypothesis. On the other hand, the E26a strain is undoubtedly a different biological entity, as evidenced by the large number of differences (both substitutions and indels) in its 18S rDNA sequence as compared to the *J. perforata* strains. Together with the readily discernible specific morphological features (see above), E26a is best considered to be a separate species (*J. minuta*). In support of this, the extent of sequence divergence between the two species is actually higher than between some green algal species assigned to different genera (compare *Characiochloris acuminata* UTEX 2095 and *Characiosiphon rivularis* UTEX LB 1763 in the *Characiosiphonia* clade or *Ankyra judayi* SAG B 17.84 and *Sphaeroplea annulina* SAG 377-1a in Sphaeropleaceae in the tree in Fig. 4). However, the robustly supported common descent of the two putative species to the exclusion of all other taxa with 18S rDNA sequences available, excepting uncultured organism(s) recorded by environmental sequencing (see below), combined with the suite of the shared morphological features led us to propose a single genus, *Jenufa*, embracing both species.

Second, our molecular phylogenetic analyses support the notion of chlorophycean provenance of *Jenufa* inferred from both morphological and ultrastructural observations. Both phylogenetic methods employed to infer a tree from 18S rDNA sequences (Bayesian and ML analysis) agree on placing *Jenufa* within the chlorophycean CS clade defined on the basis of phylogenomic analyses of chloroplast

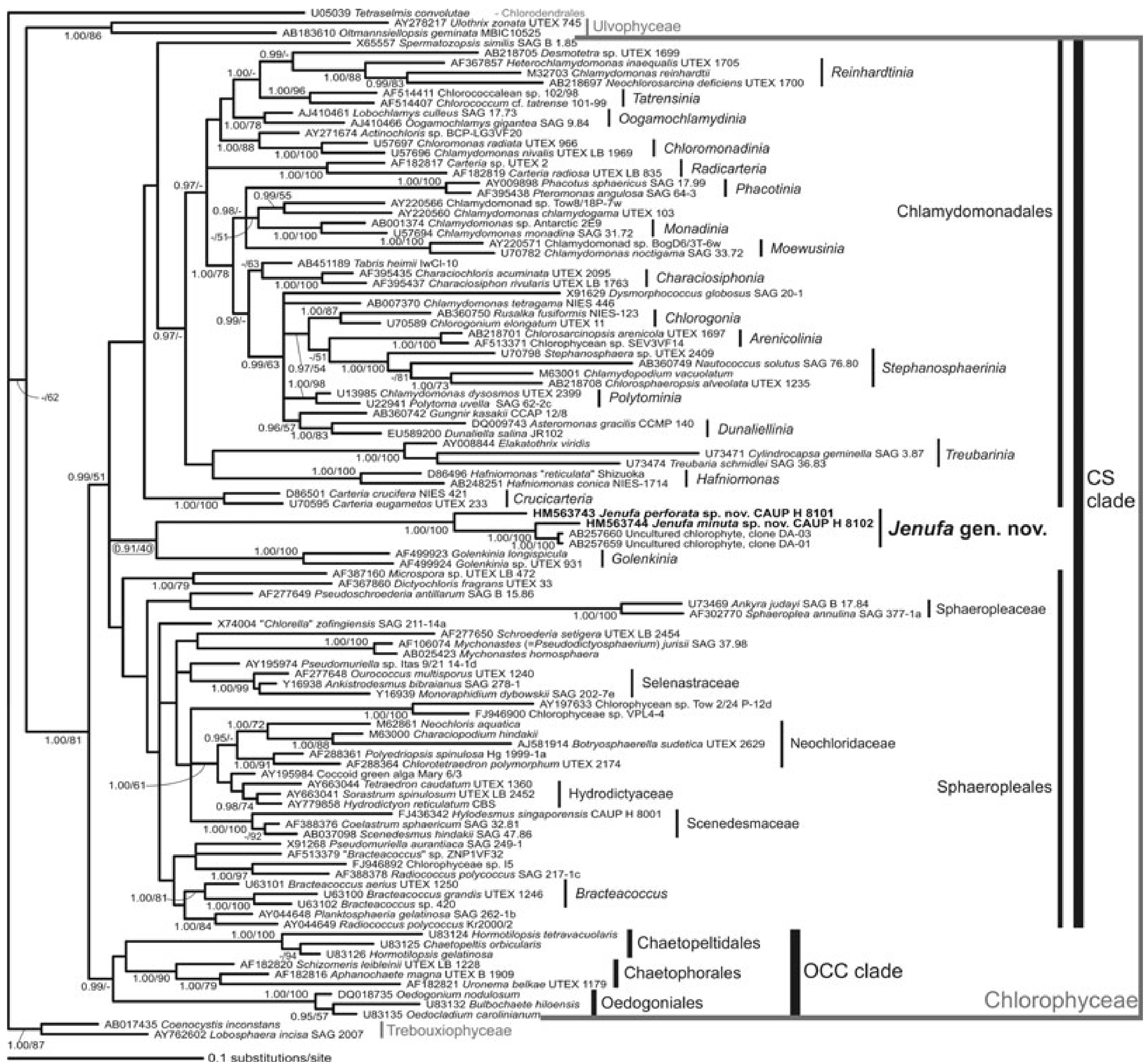

Fig. 4. Phylogenetic position of the genus *Jenufa* within the class Chlorophyceae. The tree was inferred using MrBayes and employing the GTR+Γ+I+COV evolutionary model (see Materials and Methods for details). The tree is arbitrarily rooted by the chlorodendralean ''prasinophyte'' *Tetraselmis convolutae*. Numbers at branches correspond to MrBayes posterior probabilities/maximum-likelihood bootstrap values; only values of at least 0.95/> 50% are shown (except the branch subtending the *Jenufa* and *Golenkinia* lineages). Important previously named clades are annotated on the right. The CS and OCC clades constituting the class Chlorophyceae were defined by Turmel et al. (2008). The various clades annotated within the order Chlamydomonadales were proposed by Nakada et al. (2008) following the rules of PhyloCode.

sequences and uniting the putative orders Chlamydomonadales and Sphaeropleales (Turmel et al. 2008, Brouard et al. 2010). Unfortunately, the 18S rDNA sequences do not provide enough phylogenetic signals to resolve the deepest branches within the CS clade. The Bayesian analysis does recover a clade corresponding to the order Sphaeropleales (characterized by the DO configuration of basal bodies), but with insignificant statistical support. The ML analysis places the very divergent sequences of the family Sphaeropleaceae within the clade otherwise corresponding to the order Chlamydomonadales (not shown). An unstable position of Sphaeropleaceae and problems with recovering monophyly of the putative order Sphaeropleales were encountered in other 18S rDNA-based phylogenies as well (Buchheim et al. 2001, Wolf et al. 2002). The putative order Chlamydomonadales (excluding the genus *Golenkinia*, see below) is in our analyses monophyletic only with the Bayesian

method, again without significant statistical support, whereas with ML methods it is disrupted by intruding sequences of Sphaeropleaceae. Both methods place the *Treubarinia* clade, treated as a volvocalean (chlamydomonadalean) lineage by Nakada et al. (2008), into Chlamydomonadales. However, the actual phylogenetic position of this clade remains an open issue given conflicting results even in combined 18S+26S rDNA phylogenetic analyses, which place this clade to Chlamydomonadales, Sphaeropleales, or as a separate lineage depending on the tree inference method used (Bucheim et al. 2001, Shoup and Lewis 2003). Based on 18S rDNA phylogenies, chlamydomonadalean affiliations have also been suggested for the genus *Golenkinia* (Wolf et al. 2003a, Nakada et al. 2008), but this has never been demonstrated with significant statistical support. Thus, our tree showing *Golenkinia* outside Chlamydomonadales is not in real conflict with previously reported results. In this context, it is not surprising that our analyses failed to define the precise position of the *Jenufa* lineage relative to other lineages of the CS clade. The sisterhood of *Jenufa* to the genus *Golenkinia*, suggested without significant support by the Bayesian as well as the ML method, is consistent with the coccoid vegetative cells shared by both genera, but this tie is too weak to put any weight on it. Well-sampled multigene analyses are apparently critical for obtaining a robustly resolved phylogenetic tree of the CS clade.

Interestingly, our tropical strains were found phylogenetically closely related to previously reported environmental sequences from an endolithic microbial community in dolomite rock from the central Alps in Switzerland. Sequencing of clone libraries made by PCR amplification of specific regions of DNA isolated directly from mixed environmental samples has revolutionized the whole field of microbial ecology and has led to the identification of a plethora of new phylogenetic lineages, especially of bacteria and protists (Epstein and López-García 2008, López-García and Moreira 2008). For green algae, a large number of novel deep phylogenetic lineages have been detected among the paraphyletic ''prasinophytes'' (Viprey et al. 2008). On the other hand, the extent of environmental sequence diversity for the ''core'' chlorophytes (classes Trebouxiophyceae, Ulvophyceae, and Chlorophyceae) may not be as spectacular. For example, a recent systematic analysis of database sequences attributed to the chlorophycean order Volvocales (= Chlamydomonadales) revealed a number of environmental sequences that could not be assigned to sequenced species or even genera, all of which were, however, affiliated with named taxa within higher-order, strongly supported clades (Nakada et al. 2008). In addition, given the fact that a majority of species previously described in Volvocales remains to be surveyed by molecular approaches, it is a question how many of the unassigned volvocalean environmental sequences belong to genuinely new taxa. Our finding that a previously unassigned lineage of environmental sequences (AB257659.1 and AB257660.1) belongs to a clade representing a new green algal taxon at the generic, or even higher, level is therefore of a general interest. The two environmental sequences are (with maximal statistical support) specifically related to *J. minuta*, which means that the respective organisms are phylogenetically nested within the genus *Jenufa* and must be considered as members of this genus. AB257659.1 and AB257660.1 come from rock samples from a single locality and differ by substitutions of only two nucleotides, one of them actually corresponding to a degenerate position in a reverse primer used for PCR amplification (see Horath and Bachofen 2009), suggesting that they may both correspond to the same species. Given the 27–28 substitutions and five indels between the *J. minuta* sequence on one hand and AB257659.1 and AB257660.1 on the other hand, and taking into account the geographic distance and habitat differences between the localities where *J. minuta* and the uncultured *Jenufa* representative were sampled, the endolithic alpine organism most likely represents a separate, presently unknown *Jenufa* species.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. List of algal strains characterized in this study.

This material is available as part of the online article.

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