A new species *Jenufa aeroterrestrica* (*Chlorophyceae incertae sedis*, *Viridiplantae*), described from Europe

Nový druh *Jenufa aeroterrestrica* (*Chlorophyceae incertae sedis*, *Viridiplantae*), popsaný z Evropy

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The chlorophycean genus *Jenufa* includes chlorelloid green microalgae with an irregularly spherical cell outline and a parietal perforated chloroplast with numerous lobes. Two species of the genus are known from tropical microhabitats. However, sequences recently obtained from various temperate subaerial biofilms indicate that members of the *Jenufa* lineage do not only occur in the tropics. In this paper, we describe and characterize a new species of the genus *Jenufa*, *J. aeroterrestrica*, which was identified in five samples of corticolous microalgal biofilms collected in Europe. These strains shared the general morphological and ultrastructural features of the genus *Jenufa*, but differed in having a larger average cell size and higher numbers of autospores. Phylogenetic analyses showed that the strains clustered in a sister position to two previously described tropical species, together with previously published European 18S rDNA sequences. This pattern was also supported by the ITS2 rDNA sequences of the genus *Jenufa*. Our data and previously published sequences indicate that the newly described species *J. aeroterrestrica* frequently occurs in temperate and sub-Mediterranean European subaerial biofilms, such as those occurring on tree bark or surfaces of stone buildings.

Keywords: 18S rDNA, *Chlorophyceae*, ITS2 rDNA, green algae, morphology, phylogeny, subaerial biofilms, taxonomy

Introduction

The diversity of unicellular green microalgae is generally underestimated by traditional morphological taxonomy compared with data provided by molecular analyses. Because of their overall morphological uniformity (Ettl & Gärtner 1995), this is especially conspicuous in taxa occurring in various terrestrial microhabitats, such as soil crusts, tree bark, or surfaces of walls (Lewis & Lewis 2005, Hallmann et al. 2013, Kulichová et al. 2014). Recently, a number of previously unknown trebouxiophycean and chlorophycean genera inhabiting terrestrial microhabitats were described in taxonomic studies using molecular phylogenetics combined with light and electron microscopy. A number of genus-level lineages of *Trebouxiophyceae* with typical chlorelloid morphology, such as *Heveochlorella*, *Kalinella*, *Leptochlorella* and *Parachloroidium*, are reported to occur frequently in microhabitats on tree bark (Zhang et al. 2008, Ma et al. 2013, Neustupa et al. 2013a, b). The newly described coccoid genera *Chloropyrula*, *Desertella*, *Eremochloris*, and *Xerochlorella*, which thrive in soil microhabitats and desert crusts, also belong to the *Trebouxiophyceae* (Gaysina et al. 2013, Fučíková et al. 2014b). An unexpected phylogenetic diversity of terrestrial coccoid taxa was reported recently within the *Chlorophyceae*. Fučíková et al. (2014a) report that unicellular coccoid microalgae isolated from

desert soil crusts and bearing the morphological characteristics of traditionally defined genera such as *Bracteacoccus*, *Dictyococcus* and *Muriella*, actually belong to at least 10 independent families of the order *Sphaeropleales*. These family-level lineages are at the base of this order's phylogenetic tree, indicating a possible ancestral character of the terrestrial life strategy within the *Sphaeropleales*.

The chlorophycean genus *Jenufa*, which contains the two species *J. perforata* and *J. minuta*, was identified by Němcová et al. (2011) in corticolous biofilms collected from tropical forest habitats in Singapore. The *Jenufa* lineage was phylogenetically isolated within the CS-clade (i.e., the clade containing *Sphaeropleales* and *Chlamydomonadales*) of the *Chlorophyceae*. Phylogenetic analyses of 18S rDNA sequences placed *Jenufa* in a moderately supported sister position to the freshwater coccoid genus *Golenkinia* and this clade was recovered in an unsupported position sister to the *Sphaeropleales* and *Chlamydomonadales* (Němcová et al. 2011) or as a sister lineage to the *Chlamydomonadales* (Hodač et al. 2012, Hallmann et al. 2013). The genus has a typical chlorelloid morphology; thus, cells possess a distinct parietal chloroplast perforated by minute apertures and bearing numerous conspicuous lobes but lacking any pyrenoids. These morphological features of *Jenufa* plastids enable relatively confident identification of this genus in natural samples or in biofilms cultured on Petri dishes using light microscopy.

Before tropical strains of *Jenufa* were isolated and taxonomic descriptions of the genus were formalized sequences of an unidentified chlorophycean alga from an endolithic microcommunity thriving beneath the surface of dolomite rocks at Val Piora, Switzerland were reported (Horath & Bachofen 2009). These sequences proved to be closely related to *J. perforata* and *J. minuta*. They form a monophyletic lineage with these two *Jenufa* species, but probably are those of a different, previously undescribed species of *Jenufa* (Němcová et al. 2011). Hodač et al. (2012) report environmental clones and strains of the *Jenufa* lineage in several samples of temperate and tropical subaerial biofilms. A single tropical clone from a tree bark sample collected in Panama by Suutari et al. (2010) proved to be closely related to *J. perforata*, which was originally described from a tropical forest in Singapore. The 18S rDNA *Jenufa* sequences collected by Hodač et al. (2012) from various central-European subaerial microhabitats, such as tree bark biofilms, endolithic microcommunities and soil, invariably clustered with those collected by Horath & Bachofen (2009) from the Alps. Recently, Hallmann et al. (2013) reported additional sequences of this undescribed *Jenufa* lineage from epilithic biofilms growing on a stone building of the ruined medieval castle Gleichen in Thuringia, Germany.

In this study, we investigated nine new *Jenufa* strains from tree bark biofilms thriving in temperate and sub-Mediterranean European forest habitats. Cell morphology of these isolates was studied using light microscopy and transmission and scanning electron microscopy. We obtained 18S rDNA and ITS2 sequences of these isolates. Using the 18S rDNA data we determined their phylogenetic position within the *Jenufa* lineage. These strains provide a basis for the taxonomic description of a third *Jenufa* species, which occurs predominantly in subaerial biofilms in temperate Europe.

Material and methods

Sampling, strain isolation and cultivation

The biofilm samples were collected in 2012 and 2013 from the bark of four tree species at temperate and sub-Mediterranean localities in Slovenia and north-eastern Italy (Electronic Appendix 1). The samples were taken from the shaded northern sides of tree trunks 120–150 cm above the soil surface. Each sample consisted of the biofilm scraped off from approximately 3 cm^2 of bark, which was immediately placed in a sterile bag. Microhabitats covered by macrolichens were avoided and any lichen thalli were carefully removed from the sample before further analysis. The samples were cultured for 3 weeks on 9 cm Petri dishes containing agar-solidified Bold's basal medium (BBM) (Andersen et al. 2005). Then, microcolonies were isolated in tubes containing agar-solidified BBM and cultured at 24 °C under 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ illumination provided by 18-W cool fluorescent tubes.

Light and electron microscopy

Photomicrographs of the isolated strains were taken using an Olympus Z5060 camera and an Olympus BX51 light microscope at a 1000× magnification and using differential interference contrast. For transmission electron microscopy (TEM), samples were fixed for 2 h at 5 °C in 2% glutaraldehyde in 0.05 M phosphate buffer. Samples were post fixed for 2 h in 1% OsO_4 in 0.05 M phosphate buffer and for 12 h at 5 °C in a 1% uranyl acetate solution. Then, samples were dehydrated in a graded ethanol series, transferred to butanol and finally embedded in Spurr's resin medium. Ultrathin sections were cut with a diamond knife, post-stained with lead citrate and examined using a JEOL 1011 transmission electron microscope. For scanning electron microscopy (SEM), samples were fixed for 2 h in 2% glutaraldehyde in 0.1 M phosphate buffer. Cells were washed repeatedly in 0.1 M phosphate buffer and distilled water and collected by centrifugation. Subsequently, cells were allowed to sediment onto poly-L-lysine-treated glass coverslips for 1 h at room temperature. Aliquots of cell suspensions were added regularly to prevent samples from drying. Then, glass coverslips with attached cells were dehydrated in a graded acetone series. Cells were dried to a critical point using liquid CO_2. The glass coverslips were mounted onto SEM stubs with double-sided adhesive carbon tape, coated with gold for 5 min (to produce a 3 nm layer) with a Bal-Tec SCD 050 sputter coater and viewed using a JEOL JSM-740 1F FESEM scanning electron microscope.

DNA isolation, amplification and sequencing

Total genomic DNA was extracted using the protocol described by Kulichová et al. (2014). Two nuclear molecular markers (18S rDNA and ITS rDNA) were amplified using PCR. The 18S rDNA marker was amplified using 18S-F and 18S-R primers as reported in Katana et al. (2001), or using a combination of forward primer 402-23F (5´-GCTACCACATCCAAGGAAGGCA-3´; Katana et al. 2001) or 34F (5´-GTCTCAAAGATTAAGCCATGC-3´; Thüs et al. 2011) and reverse primer 18L (5´-CACCTACGGAAACCTTGTTACGACTT-3´; Hamby et al. 1988). The ITS rDNA marker was amplified using primers that preferentially amplify the algal ITS rDNA region, including AL1700F (5´-CCCACCTAGAGGAAGGAG-3´; Helms et al. 2001)

and ITS4T (5´-GGTTCGCTCGCCGCTACTA-3´; Kroken & Taylor 2000). All PCR reactions were performed in a total volume of 20 µl. The PCR mix for 18S rDNA amplifications contained 14.1 µl of sterile Milli-Q water, 2 µl of 10 × AmpliTaq Gold® 360 buffer, 1.2 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTP mix (10 mM), 0.25 µl of each primer (25 pmol), 0.6 µl of 360 GC enhancer, 0.2 µl of AmpliTaq Gold® 360 DNA polymerase (1 U) and 1 µl of DNA (5–10 ng). The PCR mix for the ITS rDNA amplifications contained 13.8 µl of sterile Milli-Q water, 4 µl of 5 × MyTaq™ reaction buffer, 0.5 µl of each primer, 0.2 µl of MyTaq™ HS DNA polymerase (1 U; Bioline) and 1 µl of DNA (5–10 ng). PCR amplification of the 18S rDNA/ITS rDNA began with an initial denaturation at 94/95 °C for 4/5 min, followed by 35 cycles of denaturing at 94/95 °C for 1/1 min, annealing at 52/56 °C for 1/1 min, and elongation at 72/72 °C for 2.5/1 min, with a final extension at 72/72 °C for 10/7 min. Amplifications were done using a XP thermal cycler (Bioer, Japan) or a Touchgene gradient cycler (Techne, UK). PCR products were quantified on a 1% agarose gel stained with ethidium bromide and purified using a GenElute PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's protocol. The purified PCR products of 18S rDNA were sequenced using the amplification primers, but purified PCR products of ITS rDNA were sequenced with ITS3N forward primer (5´-GATGAAGAACGCAGCGA-3´; Beck et al. 1998) using an automated sequencer (ABI 3730xl, Applied Biosystems) at Macrogen Inc. in Seoul, South Korea. Sequencing reads were assembled and manually edited using SeqAssem (version 09/2004; Hepperle 2004). Occasional ambiguous positions (indicating an intraclonal sequence variation) were recorded by signal peaks with higher intensity. The sequences are available in the GenBank database under the accession numbers KR869869-KR869879 (Electronic Appendix 1).

Phylogenetic analyses

The BLASTN searches and preliminary ML analyses of the newly determined 18S rDNA sequences indicated that the best hits invariably represented sequences of the chlorophycean strains or clones previously attributed to the genus *Jenufa*. Therefore, newly obtained sequences were manually aligned with existing *Jenufa* sequences from the GenBank database, together with members of all the major chlorophycean lineages and representatives of the *Trebouxiophyceae*, *Ulvophyceae* and *Chlorodendrophyceae* (51 taxa in total) using MEGA, ver. 6 (Tamura et al. 2013). Three sequences (AB257662, AB257663, and AB257666), which were identified by Němcová et al. (2011) as PCR artifacts, were examined using Bellerophon software (Huber et al. 2004). These sequences were once again identified to be chimeric; consequently, they were excluded from further analyses together with two additional very short 18S rDNA sequences belonging to the *Jenufa* lineage (JQ988923 and JQ988924). In order to obtain a reliable alignment for further analyses, two short highly variable regions with multiple indels in most of the taxa representing individual chlorophycean lineages (24 bp and 32 bp beginning at positions 1337 and 1649 of the resulting dataset) were excluded from the final alignment as their dubious homology would distort the resulting phylogenetic reconstruction (Lewis & Lewis 2005, Thüs et al. 2011, Fučíková et al. 2014a). Resulting alignment comprised 1705 base positions (Electronic Appendix 2).

The ITS2 rDNA sequences of members of the *Jenufa* lineage were very variable (accession numbers KR869871–KR869879, Electronic Appendix 1), which made it difficult to construct a reasonable alignment. The BLASTN searches did not suggest any related sequences that could be used as templates for the alignment of the primary sequence data or folding of the secondary structure.

The most appropriate evolutionary models for the phylogenetic analyses of the 18S rDNA were determined by the modelTest function in the package phangorn ver. 1.7-4 (Schliep 2011), which estimates the specified models on the basis of Bayesian information criteria (Posada 2008) in R ver. 2.15.3 (R Development Core Team 2013). The phylogenetic trees were constructed with Bayesian inference (BI) using the K80+G+I model in MrBayes ver. 3.2.2 (Ronquist et al. 2012). Two parallel Markov chain Monte Carlo (MCMC) runs were run for 3 million generations, each with one cold and three heated chains. Parameters and trees were sampled every 100 generations. After visual inspection of log-likelihood values of sampled trees, the initial 7501 trees of each run were discarded. The posterior probabilities of tree bipartitions were calculated on the basis of the consensus of the remaining 45,000 (22,500 × 2) trees. The bootstrap supports for individual phylogenetic lineages in the18S rDNA dataset were calculated using maximum likelihood (ML) and maximum parsimony (MP) analyses, ML and weighted MP, respectively. ML bootstrapping was performed using the phangorn package. The function bootstrap.pml was used for non-parametric bootstrapping of the individual tree nodes recovered by the ML analysis (with 1000 replicates). The wMP analysis was based on the pratchet function of the phangorn package and the bootstrapping (1000 replications) was performed using the bootstrap.phydat function in the phangorn package. Phylogenetic trees were displayed and graphically adjusted in FigTree ver. 1.3.1 and Adobe Illustrator ver. CS4.

Results

Phylogenetic analyses

Partial sequences of the 18S rRNA gene were established for eight new *Jenufa* strains isolated from samples of biofilms collected from tree bark. Seven of the isolates had identical 18S rRNA gene sequences (Electronic Appendix 1). The similarity level among the available *Jenufa* sequences was 97–99% for identical positions in the 18S rDNA gene sequence, whereas other members of the *Chlorophyceae* had identity levels ≤ 93%. An analysis based on the 18S rDNA sequences of the major chlorophycean lineages, together with representatives of *Trebouxiophyceae*, *Ulvophyceae* and *Chlorodendrophyceae*, indicated that our isolates were unambiguously clustered within a monophyletic *Jenufa* clade (1.00 Bayesian posterior probability / 100 ML bootstrap support / 100 wMP bootstrap support) (Fig. 1). This clade was placed in a sister position to the genus *Golenkinia* with moderate to low statistical support (0.90/60/65). The *Jenufa+Golenkinia* lineage was placed in an unsupported sister position to *Chlamydomonadales*, which was relatively well supported by the statistical analyses (1.00/94/80).

The *Jenufa* lineage contained three well-supported infrageneric clades (Fig. 1). A highly supported clade (1.00/100/100) included sequences of *Jenufa perforata* strain CAUP H8101 (the type strain of this species) and the *Jenufa* clone from tree bark in

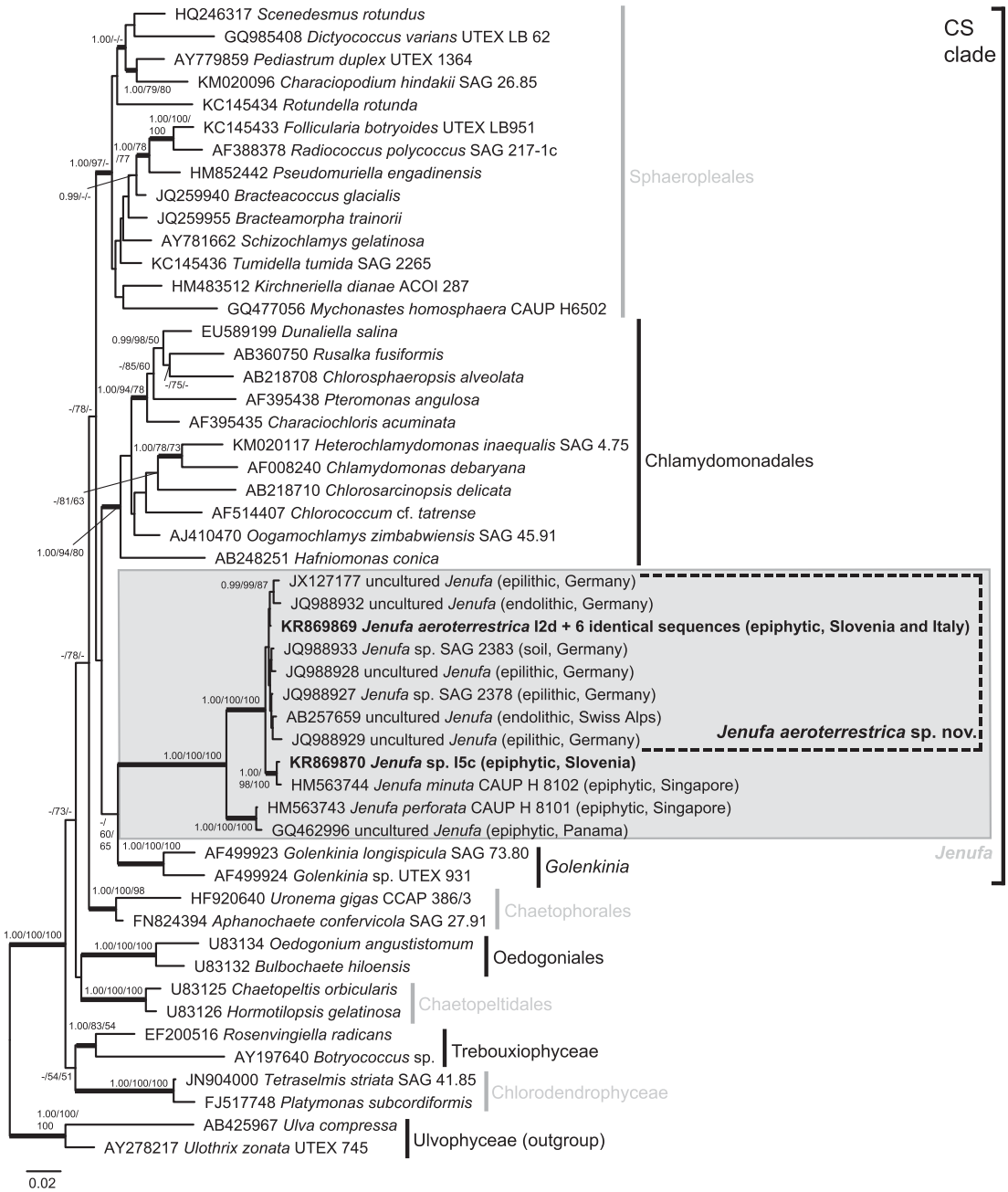


Fig. 1. – Bayesian unrooted tree based on the 18S rDNA gene sequences of the genus *Jenufa*. Bayesian posterior probabilities (BPP)/maximum likelihood (ML)/maximum parsimony (MP) bootstrap values are indicated for nodes where they were greater than 0.95/50%/50%, respectively. Thick branches represent nodes with the highest BPP support (1.00). The sequences newly collected in this study are shown in bold. The scale bar shows the estimated number of substitutions per site.

Panama (GQ462996) published by Suutari et al. (2010). In this clade, the 18S rDNA sequence of the type strain of *Jenufa minuta* (CAUP H8102) formed a well-supported lineage (1.00/98/100) with our isolate I5c collected from bark of *Carpinus orientalis* in southwest Slovenia. All other sequences formed a single highly supported lineage (1.00/100/100) containing the strains isolated in this study from biofilms collected from bark of trees in Europe and sequences from previously published studies that report members of the genus *Jenufa* from various European subaerial microhabitats (Horath & Bachofen 2009, Hodač et al. 2012, Hallmann et al. 2013).

The BLAST searches of the ITS2 region obtained from *Jenufa* isolates did not yield any similar GenBank sequences. The bulk of the European ITS2 sequences from bark biofilms were 573 bp long and very similar to each other. The strain I6g differed from the remaining strains by six substitutions; strains I2d and I5b shared one substitution change. Conversely, the type strains of *J. perforata* (CAUP H8101), *J. minuta* (CAUP H8102) and the isolate I5c had manifestly divergent ITS2 sequences. The overall length of the ITS2 region of I5c was 601 bp and 522 bp for *J. minuta* (CAUP H8102), whereas it was just 358 bp for *J. perforata* (CAUP H8101).

Morphology

The *Jenufa* strains isolated from bark biofilms (except for isolate I5c) were unicellular, with cells (3.3–) 4.5–8.8 (–9.5) µm in diameter. They had spherical, irregularly ellipsoidal, or ovoid shapes (Figs 2A–I, 3A). Occasionally, cells with irregular or tetrahedral outlines were observed (Fig. 2B, E, I). The cells reproduced entirely by asexual autospores (Fig. 2A, B, D). Typically, eight identical autospores were produced within a single spherical autosporangium; occasionally, autosporangia producing four autospores were observed (Fig. 3B). The autosporangia were 8.0–9.5 µm in diameter. The autospores had spherical to irregular shapes 3.0–4.5 µm in diameter. The cell wall of vegetative cells and autosporangia was generally smooth (Fig. 3A, B), but wrinkled shreds of maternal cell walls occasionally remained attached to the daughter cells (Fig. 3A). The cell wall contained two main layers: a granulo-fibrillar or amorphous inner layer (approximately 120–150 nm; Fig. 3C) and an outer trilaminar layer (approximately 30 nm; Fig. 3C, D). The vegetative cell was always uninucleate (Fig. 3C, D). The nucleus was located in the central part of the cell, usually close to a mitochondrion and small vacuoles. The freshly released autospores possessed a single parietal chloroplast (Fig. 2C, F, H, I). The chloroplast was usually divided into two to four lobes and typically had a wide opening on one side of the cell (Fig. 2H). The mature cell chloroplast was parietal, but it was localized beneath the plasma membrane with no conspicuous opening (Figs 2B–D, G, 3C, D). The mature chloroplast had multiple lobes and incisions (Figs 2B, 3C). Transformation of vegetative cells into autosporangia was accompanied by division of the chloroplast into several smaller parietal plastids, which were subsequently distributed into the newly developing autospores. The chloroplast consistently lacked any pyrenoids; however, spherical electron-dense pyrenoglobuli (up to 100 nm diameter) were regularly observed in TEM sections (Fig. 3C, D). Secondary carotenoids were not produced, not even in old and nutrient-starved cells. Sexual reproduction or production of zoospores was not observed.

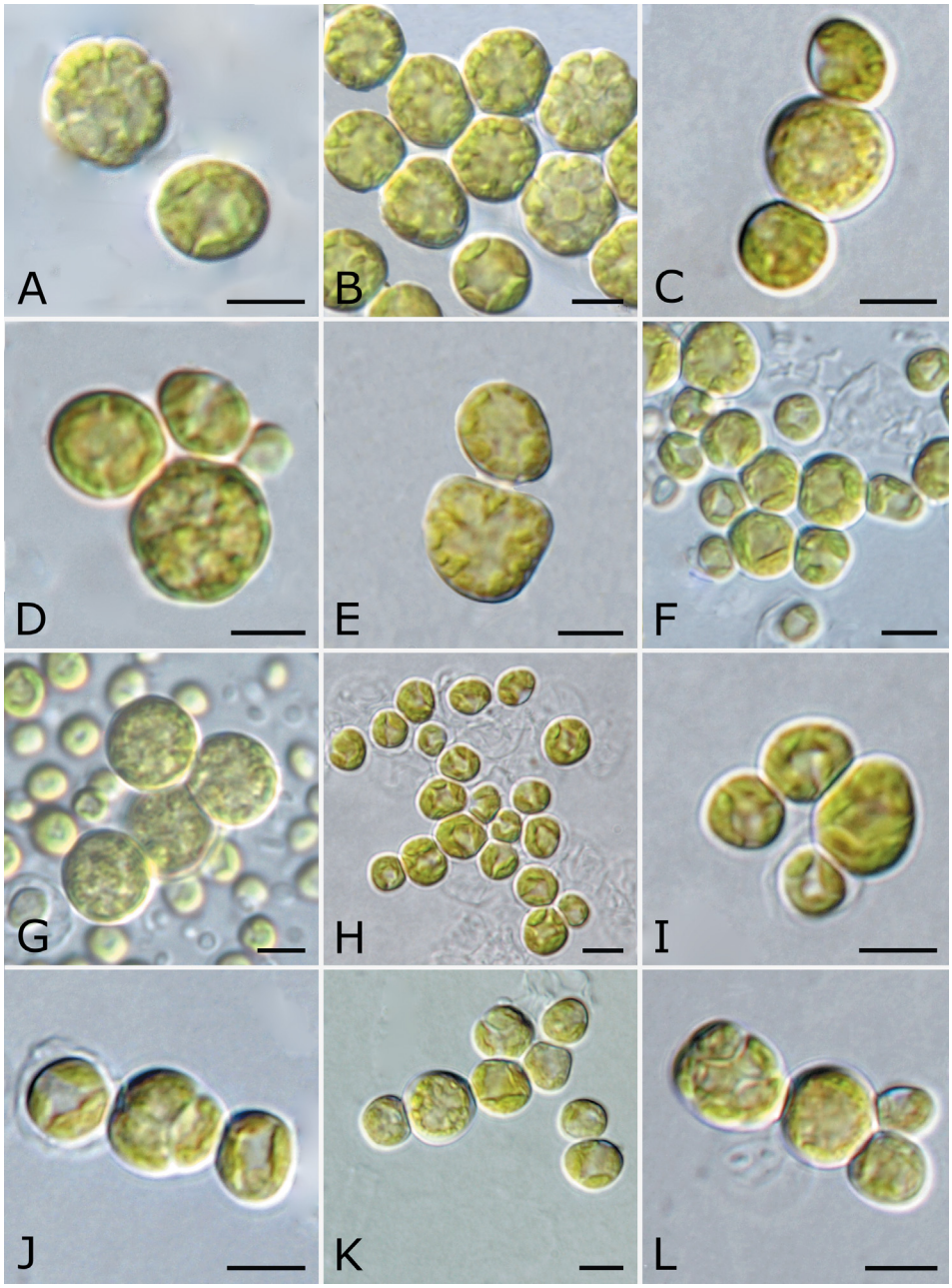


Fig. 2. – Morphology of *Jenufa aeroterrestrica* sp. nov. (A–I) and *J.* cf. *minuta* (J–L). (A) Vegetative cells and eight-celled autosporangium, strain I1d. (B) Vegetative cells and autosporangia, strain I1d. (C) Vegetative cells, strain I2d. (D) Autosporangium and vegetative cells, strain I2d. (E) Irregularly shaped vegetative cells with a parietal chloroplast, strain I5b. (F) Mature and young vegetative cells, strain I5b. (G) Detail of mature vegetative cells, strain I6g. (H) Young vegetative cells with a parietal chloroplast, strain S1a. (I) Detail of young vegetative cells, strain S1a. (J) Detail of vegetative cells and four-celled autosporangium, strain I5c. (K) Vegetative cells and autosporangium, strain I5c. (L) Vegetative cells and autosporangium, strain I5c. Scale bars = 10 μm.

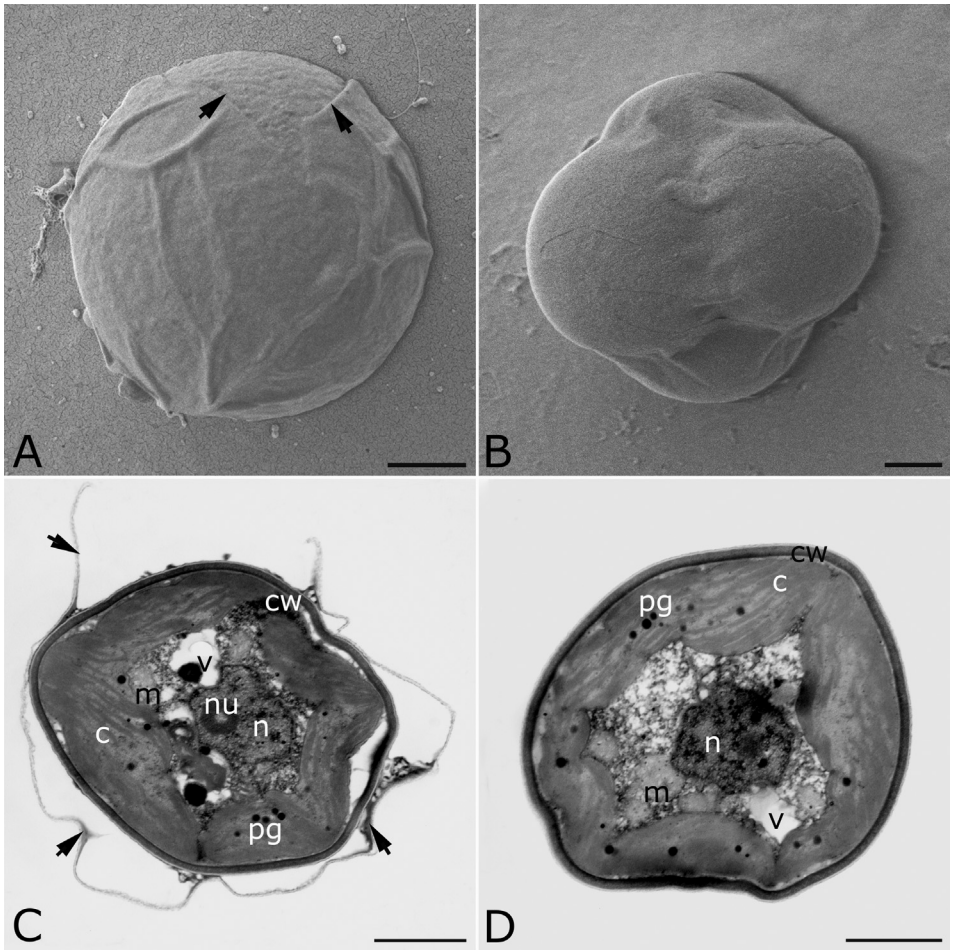


Fig. 3. – Scanning and transmission electron microscopy of *Jenufa aeroterrestrica*. (A) Young vegetative cell surrounded by the remnants of the wall of the mother cell (arrows), strain S1a. (B) Four newly formed autospores still enclosed within the wall of the mother cell, strain S1b. (C, D) Vegetative cells with single nuclei, plastid and mitochondria. Shreds of the wall of the mother cell (arrows) attached to the newly released autospores. Abbreviations: n, nucleus; nu, nucleolus; c, chloroplast; m, mitochondria; pg, plastoglobuli; v, vacuole. Scale bars = 1 μm.

The strain I5c had spherical to irregular cells (2.5–) 3.5–6.0 (–6.5) μm in diameter. Typically, four autospores (2.0–3.5 μm diameter) were produced within a single autosporangium (5.0–6.5 μm diameter). The vegetative cells were uninucleate. The autospores and young vegetative cells had single parietal chloroplasts (Fig. 2J–L) with two to four lobes and an opening (Fig. 2J, K), whereas the mature cells had parietal chloroplasts stretching more or less continuously beneath the plasma membrane (Fig. 2L). Pyrenoids were lacking and secondary carotenoids were not produced. Sexual reproduction or production of zoospores was not observed.

Formal taxonomic description

Jenufa aeroterrestrica K. Procházková et J. Neustupa, **spec. nova**

D e s c r i p t i o n: Vegetative cells coccoid, solitary, uninucleate, with irregular or spherical outline, (3.3–) 4.5–8.8 (–9.5) μm in diameter. The single chloroplast is perforated and lobed, without a pyrenoid. In mature cells it is stretched beneath the cell wall. Asexual reproduction is via 4–8 autospores, 3.0–4.5 μm in diameter. Cell size of the autospores is identical within a single autosporangium. Sexual reproduction and production of zoospores not observed. The species also differs from other taxa of the genus *Jenufa* in the 18S rDNA and ITS2 sequences.

H o l o t y p e: The strain CAUP C-H8103, based on the isolate S1c, has been permanently cryopreserved in the Culture Collection of algae at Charles University in Prague, Czech Republic (CAUP; http://botany.natur.cuni.cz/algo/caup.html). CAUP C-H8103 also is maintained as an active culture (CAUP H8103) from which the holotype was derived. This strain is also conserved in the form of a permanent slide (CAUP P-H8103).

T y p e l o c a l i t y: The bark of *Ostrya carpinifolia* in a broad-leaved deciduous temperate forest in the Nanos Mts., Slovenia (45°50'41.0"N, 13°57'53.2"E, altitude 125 m a.s.l.).

E t y m o l o g y: The specific epithet refers to the solely subaerial distribution of this species.

Discussion

The two regions of the rDNA operon (18S and ITS2) were congruent in illustrating phylogenetic distances within the genus *Jenufa*. As expected, the non-coding ITS2 region was considerably more variable than the 18S region, but most of the isolates collected from bark biofilms turned out to have rather homogeneous ITS2 sequences. In general, the ITS2 region of the *Jenufa* strains could not be homologized with any previously published ITS2 sequences either by GenBank BLAST searches or by homology prediction based on the secondary structures available in the ITS2 database (Koetschan et al. 2012). This pattern indirectly supports the hypothesis of the relatively isolated phylogenetic position of the *Jenufa* lineage among the *Chlorophyceae*, previously suggested based on analyses of SSU rDNA (Němcová et al. 2011, Hodač et al. 2012, Hallmann et al. 2013). Most of the ITS2 sequences of *Jenufa* species were also unusually long compared with those of other *Chlorophyceae*. The ITS2 regions of *J. aeroterrestrica* (573 bp) and *J. minuta* (601 bp) were longer than most known chlorophytan ITS2 sequences (Keller et al. 2008, Caisová et al. 2013) and comparable with the longest known ITS2 regions among the Viridiplantae (Yao et al. 2010). Interestingly, an unusually long ITS2 region is reported in the family *Mychonastaceae*, which also forms an isolated lineage among the *Chlorophyceae*, namely, at the base of the *Sphaeropleales* (Krienitz et al. 2011, Fučíková et al. 2014a). However, the present study and other phylogenetic reconstructions do not indicate a closer relationship between these two lineages of the chlorophycean CS-clade (Němcová et al. 2011, Hallmann et al. 2013).

Our 18S rDNA based phylogeny placed the *Jenufa* lineage into a sister position with the freshwater coccoid genus *Golenkinia*. While this relation was not unambiguously supported by our statistical analyses, it was congruent with previously published

phylogenies of the genus *Jenufa* (Němcová et al. 2011, Hodač et al. 2012, Hallmann et al. 2013). The *Jenufa*+*Golenkinia* clade was recovered in a weakly supported sister position to the *Chlamydomonadales* and this pattern, once again, matches previously published 18S rDNA based phylogenetic trees that included the *Jenufa* sequences (Hodač et al. 2012, Hallmann et al. 2013). This evolutionary scenario, placing *Jenufa* at the base of the chlamydomonadalean clade, should apparently be evaluated by multigenic phylogenetic analyses. Given the fact that most members of the *Chlamydomonadales* thrive in freshwater, the basal position of the *Jenufa* lineage with taxa known solely from terrestrial habitats would be intriguing. Interestingly, a similar phylogenetic pattern is reported in the sister order *Sphaeropleales*, where a number of terrestrial lineages with basal positions were recently described (Fučíková et al. 2014a).

The molecular analyses of the *Jenufa* isolates and strains indicated that the genus is formed by three well-delimited lineages. Two lineages correspond to the previously described tropical taxa *J. perforata* and *J. minuta*. The third lineage, formed by multiple sequences from various European subaerial microhabitats, corresponds to a species described here as *J. aeroterrestrica*. This lineage includes all but one of the isolates collected from tree bark biofilms in this study. It also contained sequences from an endolithic high-altitude biofilm community in Switzerland (Horath & Bachofen 2009). The sequences from epilithic, endolithic and soil microbiota from Germany, which are reported by Hodač et al. (2012) and Hallmann et al. (2013), also clustered here. Therefore, it seems that the new species may be relatively frequently distributed in various temperate subaerial microhabitats in Europe. This species is not reported from any other continent or climatic zone. However, there is little information on the distribution of terrestrial microalgae. Additional studies based on the cultivation of unialgal isolates or sequencing may soon increase the known distribution of *J. aeroterrestrica*.

The subaerial coccoid green microalgae are typical in being morphologically uniform and members of the genus *Jenufa* are no exception to this rule. The genus-level morphological features differentiating *Jenufa* cells from other coccoid microalgae are relatively robust, whereas there are few differences among the three species-level lineages. Němcová et al. (2011) argue that *J. minuta* differs from *J. perforata* (the type species of the genus) in its plastid morphology and cell wall ultrastructure. *Jenufa minuta* cells typically have unperforated multilobed plastids and their cell walls include an amorphous electron-dense inner layer (see Fig. 3C in Němcová et al. 2011). *Jenufa aeroterrestrica* differs from both tropical species in having a larger average cell size and greater number of autospores, a feature generally correlated with the diameter of mature vegetative cells (Ettl 1980). Mature vegetative cells of *J. aeroterrestrica* are typically greater than 8.0 µm in diameter, whereas they are generally less than 6.5 µm in *J. minuta* and *J. perforata*. *Jenufa aeroterrestrica* characteristically produces 4 or 8 autospores within a single autosporangium, whereas 8-celled autosporangia almost never occur in type strains of *J. minuta* and *J. perforata*.

The plastid morphology of *J. aeroterrestrica* is characterized by numerous perforations and lobes and is very similar to that of *J. perforata*. Conversely, the inner-most cell wall layer is characterized by electron-dense amorphous matter and corresponds to a similar structure observed in *J. minuta*. The overall variability in shape of vegetative cells, which ranges from spherical to ovoid to irregularly tetrahedral, is common to all three species. In conclusion, cell morphology of the three *Jenufa* species described is very

similar, but individual taxa cannot be considered entirely cryptic because they differ in microscopic or ultrastructural features. However, these features could not be used for unambiguous morphological species identification. Although the generic affiliation of cultured cells to *Jenufa* can be ascertained by light microscopy, species identification must rely on molecular methods.

The phylogenetic and taxonomic structure of the genus *Jenufa* is well reflected in the distribution of individual taxa. *Jenufa aeroterrestrica* occurs relatively frequently in temperate European microhabitats. Conversely, available molecular data on the distribution of *Jenufa* microalgae from tropical ecosystems invariably recover *J. perforata* or *J. minuta*, whereas *J. aeroterrestrica* is not reported (Suutari et al. 2010, Němcová et al. 2011, Hodač et al. 2012). Thus, *J. aeroterrestrica* is probably a dominant species of the genus *Jenufa* in colder climatic regions, whereas the distribution of *J. minuta* and *J. perforata* may predominate in humid and warm tropical microhabitats. However, our isolate I5c, which was collected from a biofilm on bark in sub-Mediterranean Slovenia, clustered together with *J. minuta* with high support, and its morphology was also very similar to the *J. minuta* type strain CAUP H8102. However, the sequences of the I5c clone were not identical with tropical CAUP H8102 and their divergence might warrant future description of this lineage as a separate species. However, this should be dependent on collecting more data on the distribution and morphology of *J. minuta* and its close relatives.

The species concept suggested here for the genus *Jenufa* reflects its phylogenetic structure. Individual species form monophyletic lineages that correspond to discernible morphological differences and the distribution of their members in natural habitats. However, we are aware that additional data on the molecular and morphological diversity of *Jenufa* populations from microhabitats world-wide may lead to the description of additional species or revision of the presently described taxa.

See www.preslia.cz for Electronic Appendices 1–2

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Souhrn

Diverzita jednobuněčných kokálních řas, jež rostou v terestrických mikrobiotopech, je v posledních letech zkoumána zejména s pomocí molekulárně fylogenetických metod. Tento typ výzkumu vedl v nedávné době k celé řadě objevů relativně evolučně izolovaných a taxonomicky nepopsaných linií z tříd *Chlorophyceae* a *Trebouxiophyceae*. Rod *Jenufa*, který byl popsán jako izolovaná linie v třídě *Chlorophyceae*, dosud zahrnoval pouze tropické zástupce. Jedná se o jednobuněčné kokální organismy s nástěnným perforovaným chloroplastem, který má většinou několik drobných laloků. V poslední době se ovšem objevilo několik studií, které ukázaly, že sekvence příbuzné popsaným tropickým druhům rodu *Jenufa*, se zřejmě poměrně hojně vyskytují i v subaerických biofilmech v Evropě. V této naší studii se nám podařilo tyto organismy izolovat z kortikolních nárostů na dřevinách ve Slovinsku a severovýchodní Itálii. Na základě fylogenetických analýz 18S rDNA ukazujeme jejich pozici v rodu *Jenufa,* kde tvoří monofyletickou větev. Morfologicky se tyto evropské populace

od tropických druhů odlišují zejména větší velikostí buněk a tvorbou většího počtu autospor. Nový druh popisujeme jako *Jenufa aeroterrestrica* a ukazujeme, že se v Evropě zřejmě jedná o poměrně velmi hojný organismus, který byl nicméně pro nedostatek jasných morfologických diskriminačních znaků dosud přehlížen. *Jenufa aeroterrestrica* roste na kůře dřevin i v biofilmech, které se vytvářejí na skalách či stavbách.

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