

Elliptochloris, not Coccomyxa, is the photobiont of three lichenized Multiclavula species collected in Japan

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Abstract

Approximately 20,000 species of lichenized fungi have been described to date; however, the diversity of their photobionts remains poorly understood. In this study, photobionts were isolated and cultured from three *Multiclavula* species (*M. mucida*, *M. petricola*, and *M. vernalis*) collected in Japan, and identified using light microscopy, transmission electron microscopy, and molecular phylogenetic analyses. Previous studies have generally reported that the photobionts of *Multiclavula* belong to the genus *Coccomyxa*. In contrast, our results demonstrate that the photobiont of *M. mucida* is *Elliptochloris subsphaerica*, a conclusion further supported by successful thallus formation in co-culture experiments. The photobionts of *M. petricola* and *M. vernalis* were found to represent a previously undescribed species, which is described herein as *Elliptochloris parasphaerica* sp. nov. This study represents the first report of *Elliptochloris* as a photobiont of lichenized basidiomycetes. Phylogenetic analyses based on the *rbcl* dataset revealed that *E. subsphaerica* comprises several subclades. Notably, *M. mucida* preferentially associates with a particular subclade of *E. subsphaerica*, suggesting a degree of photobiont selectivity.

1 Introduction

Lichens are complex organisms composed of lichenized fungi (mycobionts) and symbiotic algae (photobionts). While about 20,000 species of lichenized fungi are known (Lücking et al. 2017), only about 100–200 species of algae and cyanobacteria are known to serve as lichen photobionts (e.g., Voytsekhovich 2013; Spribille 2019). Green algal genera such as *Trebouxia* and *Trentepohlia* have long been known as lichen photobionts, but our knowledge of the diversity of lichen photobionts has expanded greatly over the last decade. For example, *Apatococcus*, once considered solely a free-living alga, was shown to be a lichen photobiont of *Fuscidea* spp. (Zahradníková et al. 2017). *Jaagichlorella*, phylogenetically placed in Watanabeales and distant from Trebouxiaceae (Li et al. 2021), has emerged as a major unicellular green algal photobiont in foliicolous lichens (Sanders et al. 2016, as *Heveochlorella*). In addition, a recent study of the photobionts of four cyanolichen genera in Lichinales (Lichinomycetes) revealed two new cyanobacterial genera, *Compactococcus* and *Pseudocyanosarcina*, belonging to Chroococcidiopsidales s. lat., both of which have been little studied as lichen photobionts (Jung et al. 2021). For chlorolichens, only 3% have had their photobionts identified, either from the morphology of cultured isolates or from sequences obtained from thalli or cultured strains (Voytsekhovich and Beck 2016); thus, the photobionts of many lichen groups remain unknown.

Multiclavula (Cantharellales, Agaricomycetes, Basidiomycota) is a small fungal genus currently containing 14 described species (Masumoto and Degawa 2020; Reschke et al. 2021), many of which are associated with green algal photobionts: *Multiclavula caput-serpentis* Lotz-Winter & Reschke, *M. clara* (Berk. & M.A. Curtis) R.H. Petersen, *M. constans* (Coker) R.H. Petersen, *M. coronilla* (G.W. Martin) R.H. Petersen, *M. corynoides* (Peck) R.H. Petersen, *M. delicata* (Fr.) R.H. Petersen, *M. hastula* (Corner) R.H. Petersen, *M. ichthyiformis* Nelsen & al., *M. mucida* (Pers.) R.H. Petersen, *M. petricola* H. Masumoto & Degawa, *M. pogonati* (Coker) R.H. Petersen, *M. samuelsii* R.H. Petersen, *M. sharpii* R.H. Petersen, *M. vernalis* (Schwein.) R.H. Petersen. A few authors question whether the alga-associated *Multiclavula* species qualify as lichens because of the lack of a well-differentiated thallus (e.g., Brodo et al. 2001). However, the photobionts clearly occur in distinct groups surrounded by fungal hyphae or even by pseudoparenchyma (Geitler 1955); the fungus thus represents the “exhabitant” according to the lichen concept of Hawksworth (1988), and is therefore generally considered to be truly lichenized (e.g., Ertz et al. 2008; Watling 2009; Lücking et al. 2017; Masumoto and Degawa 2020). The lichenized thalli of *Multiclavula* species are crustose and composed of a gelatinous film with flattened to globular structures, and among these structures, clusters of algal cells are surrounded by fungal hyphae (Oberwinkler 1970; Rico and Barrasa 2011). Geitler (1955) was the first to describe the photobiont of *Multiclavula* (*M. mucida*) in detail and to examine the anatomy of the lichenized thallus using light microscopy, suggesting *Coccomyxa*-like algae as the likely photobiont. Based on this study, the photobionts of *Multiclavula* spp. have long been believed to be members of *Coccomyxa* (e.g., Poelt 1974; Brodo et al. 2001; Watling 2009; Rico and Barrasa 2011; Voitek and Ohenoja 2011; Wirth et al. 2013; Stenroos et al. 2016; McCune 2017; Franchi and Marchetti 2021). However, Geitler (1955) also mentioned that the photobiont of *M. mucida* has a pyrenoid; later, Tschermak-Woess (1988) questioned the identification of the photobiont because *Coccomyxa* lacks pyrenoids (Komárek and Fott 1983; Darienko et al. 2015). She earlier established another lichen-forming algal genus, *Elliptochloris* (Tschermak-Woess 1980), encompassing taxa that share many characteristics with *Coccomyxa*, such as ellipsoidal to spherical cells with a simple parietal chloroplast and trilaminar cell wall layers—characters later shown to occur in both genera (Brunner and Honegger 1985). *Coccomyxa* and *Elliptochloris* are now known to represent distinct but closely related clades, together forming a highly supported monophyletic lineage as sister taxa (Darienko et al. 2016). Since *Elliptochloris* was not recognized until approximately 80 years after the establishment of *Coccomyxa* (Schmidle 1901), and even now the two genera cannot always be reliably distinguished using light microscopy, the identities of taxa traditionally attributed to *Coccomyxa* require reinvestigation in light of recent advances in our understanding of the clade’s biodiversity.

To determine the taxonomic identity of the photobionts associated with *Multiclavula*, lichenized thalli of *M. mucida*, *M. petricola*, and *M. vernalis* were collected in Japan, and algal cells were isolated from each thallus. Both the lichenized thalli and the algal isolates were examined by light microscopy (LM) and transmission electron microscopy (TEM). Sequences of 18S rDNA, ITS2, and *rbcl* were obtained from the algal isolates and subjected to phylogenetic analyses. In addition, co-culture experiments were conducted *in vitro* for *M. mucida* using the algal isolates obtained from its thallus.

2 Materials and methods

2.1 Collection of *Multiclavula* species

Fresh materials of *M. mucida*, *M. petricola*, and *M. vernalis* were collected from 2017 to 2019 from several locations in Japan (Table 1) and identified by the authors. Voucher specimens were deposited in the herbarium of the Osaka Museum of Natural History (OSA; Osaka, Japan) or the Gunma Museum of Natural History (GMNHJ; Tomioka, Gunma, Japan) (Table 1).

2.2 Isolation and cultivation of algae

Photobiont cultures were obtained from the thalli of three *Multiclavula* species (*M. mucida*, *M. petricola*, and *M. vernalis*) using the method of Nakano (1988) with the following modifications. Briefly, small pieces of the thalli were placed in a drop of sterile distilled water and then ground between two sterile glass slides. The resulting suspension of photobiont and mycobiont cells was ultrasonicated for about 30 s and spread with a sterile glass spreader onto sterile 2% (w/v) agar plates containing Bold's Basal Medium (BBM) as modified by Bischoff and Bold (1963). Petri dishes were incubated under the following conditions (20 ± 1 °C, $20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and a 16 h/8 h light/dark cycle). After about two weeks, algal colonies were located on the BBM agar plates using a light microscope (Nikon SE; Nikon Corporation, Tokyo, Japan) with a long-working-distance objective lens. Cells from these colonies were transferred to BBM agar slants. In addition, *Elliptochloris* strains were also isolated from various substrates other than thalli of *Multiclavula*. The unialgal and axenic photobiont cultures were deposited at the National Institute for Environmental Studies (NIES Collection, Tsukuba, Japan). Microscopic observations and micrographs of field specimens were obtained using a differential interference contrast microscope (BX53; Olympus, Tokyo, Japan) equipped with a digital camera (DP73; Olympus, Tokyo, Japan).

2.3. Co-culture experiments

The fungal strain *Multiclavula mucida* NBRC 114396 was isolated from basidiospores from a single basidioma of the specimen OSA-MY-9325, and the algal strain *Elliptochloris subsphaerica* NIES-4525 was isolated from the thallus of the same specimen. The fungal and algal isolates were pre-cultured on potato dextrose agar (PDA; Nissui, Tokyo, Japan) and on 2% (w/v) agar plates supplemented with Bold's Basal Medium (BBM), respectively.

Co-culture experiments between *M. mucida* NBRC 114396 and *E. subsphaerica* NIES-4525 were conducted in plastic Petri dishes (6 cm in diameter). First, a piece of commercial balsa wood (2 mm thick, approximately 3×3 cm) was placed on corn meal agar (CMA; Nissui, Tokyo, Japan). Small mycelial fragments (approximately 2×2 mm) of *M. mucida*, pre-cultured on PDA, were cut with a sterile toothpick and placed near each of the four corners of the balsa wood. An algal suspension of *E. subsphaerica* was prepared in sterile distilled water at a concentration of 1.0×10^4 cells mL^{-1} , and 10 μL of the suspension was pipetted onto each fungal inoculum. Petri dishes were incubated for four months at 20 ± 1 °C under illumination of $20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 16 h/8 h light/dark cycle.

After four months of incubation, the surface of the balsa wood was examined with a light microscope (SW350B, Swift, China), and images were captured with a digital camera (TG-4, Olympus, Tokyo, Japan) mounted on the eyepiece of the microscope. When three-dimensional aggregates of algal cells were observed, they were mounted in distilled water on glass slides and examined with a light microscope (DMLB, Leica, Wetzlar, Germany) equipped with a digital camera (EOS Kiss X5, Canon, Tokyo, Japan).

2.4. Transmission electron microscopy

For TEM observation of the algal isolates, algal cells cultured on BBM agar plates were pre-fixed in 2.5% glutaraldehyde (0.05 M cacodylate buffer, pH 7.2) for 1 h at room temperature with the aid of an automatic vacuum infiltrator described in Machida et al. (1994) and then rinsed three times for 10 min each with 0.05 M cacodylate buffer. The cells were post-fixed in 1% osmium tetroxide for 1 h at room temperature. Fixed cells were pelleted at $16,300 \times g$ for 1 min. After washing with distilled water, the pellet was embedded in 2% (w/v) agarose type VII-A (Sigma-Aldrich, Missouri, USA). Agarose blocks containing algal cells were dehydrated in an ethanol series (10%, 30%, 50%, 70%, 75%, and 90% for 15 min per step, and 95% once and 100% twice for 20 min per step), and embedded in Agar Low Viscosity Resin (Agar Scientific, Stanstead, UK) using the automatic vacuum infiltrator. For TEM observation of the thalli of *Multiclavula mucida* and *M. vernalis* specimens, the thalli were embedded in 2% (w/v) agarose type VII-A before fixation and processed as described above. The thallus of *M. petricola* was not observed by TEM because fixation was not successful. Ultrathin sections (80 nm thick) were prepared with an RMC MT-X ultramicrotome (RMC Products, Arizona, USA). Sections were stained with platinum blue (Inaga et al. 2007) and lead citrate (Venable and Coggeshall 1965). Sections were observed using a Hitachi HT7700 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

2.5. DNA extraction and amplification

Genomic DNA of each algal isolate was extracted from axenic cultures using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR reactions were performed with KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). A fragment of the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcl*), the nuclear small subunit ribosomal RNA gene (18S rDNA), and the internal transcribed spacer 2 (ITS2) were amplified using the primers listed in Appendix S1 (Supporting Information). PCR cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 10 touchdown cycles of 98 °C for 10 s, 55–50 °C (decreasing 0.5 °C per cycle) for 30 s, and 68 °C for 90 s for the mycobiont (or 60 s for ITS2, 90 s for *rbcl*, and 150 s for 18S rDNA in the photobiont); followed by 30 cycles of 98 °C for 10 s, 50 °C for 30 s, 68 °C for 90 s for the mycobiont (or 60 s for ITS2, 90 s for *rbcl*, and 150 s for 18S rDNA in the photobiont), with a final hold at 4 °C. PCR products were purified by polyethylene glycol (PEG) precipitation. Cycle sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with PCR and internal primers (Appendix S1, Supporting Information). DNA sequences were analyzed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). All newly generated sequences have been registered in GenBank (Appendix S2, Supporting Information).

2.6. Phylogenetic analyses of algal isolates

The newly generated DNA sequences were aligned with MAFFT v.7 (Katoh et al. 2019) using the “auto” strategy and manually adjusted in SeaView v.5.0.5 (Gouy et al. 2010). Phylogenetic analyses of the combined 18S rDNA + ITS2 dataset and the *rbcl* dataset were conducted using both maximum-likelihood (ML) and Bayesian inference (BI) approaches. The optimal nucleotide substitution models for each dataset and partition were determined using ModelTest-NG v.0.2.0 (Darriba et al. 2020) based on the Bayesian Information Criterion (BIC). For the ML analyses, RAxML-NG v.1.2.2 (Kozlov et al. 2019) was employed. In the 18S rDNA + ITS2 dataset, TIM2ef+I was applied to the 18S rDNA partition, and TrN+G4 to the ITS2 partition. For the *rbcl* dataset, which was partitioned by codon position, TIM1+I was applied to the first codon position, JC+I to the second codon position, and TrN+G4 to the third codon position. Branch support

was evaluated using 1,000 nonparametric bootstrap replicates. Bayesian analyses were performed using MrBayes v.3.2.7a (Ronquist et al. 2012). Because MrBayes does not implement TIM or TrN models directly, the best-fit models inferred by ModelTest-NG were approximated by more general models: GTR+I was used in place of TIM2ef+I for 18S rDNA; GTR+G in place of TrN+G4 for ITS2; GTR+I in place of TIM1+I for the first codon position of *rbcl*; JC+I for the second codon position; and GTR+G in place of TrN+G4 for the third codon position. Two independent Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run for 3,000,000 generations for the 18S+ITS2 dataset and 2,000,000 generations for the *rbcl* dataset, with trees sampled every 1,000 generations. Convergence of the MCMC runs was assessed by confirming that the average standard deviation of split frequencies was below 0.01 and that all effective sample sizes (ESS) exceeded 200, as assessed using MrBayes and Tracer v.1.7.2 (Rambaut et al. 2018). The first 25% of sampled trees were discarded as burn-in, and the remaining trees were used to construct a majority-rule consensus tree and to estimate posterior probabilities (PP) for each branch. Phylogenetic trees were visualized using FigTree v.1.4.4 and further edited in Inkscape v.1.3.

2.7. ITS2 secondary structure analysis

The secondary structures of the ITS2 sequences were predicted using the RNAfold server (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) and manually refined based on the consensus ITS2 secondary structure of *Elliptochloris* reported by Darienko et al. (2016). To find molecular signatures for species delimitation, compensatory base changes (CBCs) and hemi-CBCs in the ITS2 secondary structure were analyzed using the ITS2/CBC approach described by Darienko et al. (2015) for *Coccomyxa*. The resulting ITS2 barcodes are shown in Fig. 7, and the ITS2 secondary structures drawn with PseudoViewer 3 (Byun and Han 2009) are summarized in Appendix S3 (Supporting Information).

3 Results

3.1. Observation of the thallus of *Multiclavula mucida* and its photobiont cultures

The thallus of *Multiclavula mucida* was often bright green and composed of fungal hyphae and unicellular green algae (photobionts), together forming mainly globular structures that were interspersed with the mycobiont's club-shaped fruiting bodies on wet rotten wood (Fig. 1a, b). In younger stages, one or a few algal cells were surrounded by the fungal hyphae (Fig. 1e, f). In a well-developed stage, dozens of algal cells were enveloped within a sac-like structure composed of fungal hyphae (Fig. 1c, g). The developed globules were 30–50 µm in diameter. The thallus globules were easily crushed by pressing the cover glass, and many algal cells were discharged from the inside (Fig. 1d). Photobiont cells were subglobose, ellipsoidal, or ovoid and 7.0–12.5 × 6.5–10.5 µm in size, containing many oil droplets (Fig. 1h, i). The algal cell possessed a parietal greenish chloroplast; no haustoria or pyrenoids were evident under LM. The lichenized thallus was often intermingled with algal cells other than the photobiont, but these additional algal cells were not closely associated with fungal hyphae.

Transmission electron microscopy (TEM) revealed that the cytoplasm of fungal hyphae usually contained electron-dense granules, and the hyphae not only constituted the periphery of the thallus globule but also penetrated between the photobiont cells inside the thallus globule (Fig. 1k, l). No haustoria were observed under TEM. The parietal chloroplast of the photobiont was filled with developed thylakoid lamellae and a pyrenoid (Fig. 1l, n). Many electron-dense granules (pyrenoglobuli) were found in the pyrenoid matrix (Fig. 1m, o). The pyrenoid matrix was surrounded by many starch grains and was homogeneous except for the presence of pyrenoglobuli (Fig. 1m, o). Thylakoid-derived membranes penetrating the pyrenoid were visible as tubules (Fig. 1o).

Spherical green algae (Fig. 1p) were isolated and cultured from thalli of *M. mucida* collected in six localities in Japan (Table 1). Their morphology matched the characteristics of *Elliptochloris subsphaerica* (Reisigl) Ettl & Gärtner. TEM observation of algal cultures of *E. subsphaerica* revealed that the chloroplast contained developed thylakoid lamellae, and a pyrenoid containing peripheral groups of minute, electron-dense granules (pyrenoglobuli) surrounded by many starch grains (Fig. 1q, r). Thus, the chloroplast ultrastructure of *E. subsphaerica* isolated in culture was consistent with that observed in the algae within thallus globules of *M. mucida*. The characteristics of the algal culture identified as *E. subsphaerica* in this study are summarized below.

Elliptochloris subsphaerica (Reisigl) Ettl & Gärtner

Cells exhibit two different shapes, elongated (E-type) or spherical (S-type); E-type cells 6.0–10.0 × 3.5–5.5 µm; S-type cells 9.0–15.0 µm in diameter. Chloroplast parietal, cup-shaped. Pyrenoid single, surrounded by many starch grains, usually indistinct in younger cells. Oil droplets present in mature cells. Occurs as the photobiont of *Multiclavula mucida*.

Notes. In addition to *E. subsphaerica*, other *Elliptochloris* species known to possess a pyrenoid (or a pyrenoid-like structure) are *E. marina* Letsch and *E. philistinensis* Novis & Visnovsky. *Elliptochloris marina* has been reported as a symbiont of the intertidal sea anemone *Anthopleura* spp. (Letsch et al. 2009; Morelan et al. 2019). However, no isolates were available for comparison in culture. The photobiont of *M. mucida* formed a closely related but distinct lineage separated from *E. marina* in the *rbcl* phylogeny (Fig. 6). *Elliptochloris philistinensis* was distinguished from the photobiont of *M. mucida* by the relatively small size of mature cells, up to 8.5 µm (Novis and Visnovsky 2012).

3.2. Co-culture experiments between *Multiclavula mucida* and *Elliptochloris subsphaerica*

After four months of co-culture, scattered three-dimensional green algal aggregates were observed on the surface of the balsa wood, either directly on or near the fungal inocula of *M. mucida* (Fig. 2a, b). Light microscopic examination revealed that these aggregates consisted of globular or granular structures in which several algal cells were tightly surrounded by fungal hyphae (Fig. 2c–e). The algal cells within the structures generally possessed a well-developed chloroplast and contained one to several relatively large oil droplets (Fig. 2f, g). These morphological characteristics closely resembled those observed in field-collected lichenized thalli of *M. mucida*. The formation of such globular structures under experimental conditions indicates successful resynthesis of lichenized thalli in *M. mucida*.

3.3. Observation of the thallus of *Multiclavula petricola* and its photobiont cultures

The thallus of *Multiclavula petricola* was saxicolous, greenish, globular, and scattered on the substrate. It consisted of several spherical unicellular green algal cells surrounded by fungal hyphae that form a minute sac-like structure, as in *M. mucida* (Fig. 3b–d). The size of the thallus globules was 20–35 µm in diameter, smaller than those of *M. mucida* (30–50 µm in diameter). Photobiont cells in the thallus were globose or subglobose, 7–11 µm in diameter (Fig. 3e, f). The chloroplast was parietal and cup-shaped, and a pyrenoid was usually visible at the center of the chloroplast (Fig. 3e, f). Several algal isolates morphologically similar to the photobiont in the thallus were obtained (Fig. 3g, h). These cultured algal cells were globose, subglobose, or broadly ellipsoid, 8.5–15.5 µm in diameter, and possessed a cup-shaped chloroplast, a pyrenoid, and prominent oil droplets (Fig. 3g, h). These characteristics were similar to those of *E. subsphaerica*, which was found to be the photobiont of *M. mucida* in this study, but different in lacking E-type autospore formation.

3.4. Observation of the thallus of *Multiclavula vernalis* and its photobiont cultures

The thallus of *M. vernalis* was terricolous, greenish, amorphous, granulose, and often more inconspicuous than that of *M. mucida* (Fig. 4a–c). It consisted mainly of spherical unicellular green algae surrounded by fungal hyphae (Fig. 4d), which occasionally form sac-like globular structures, as in *M. mucida*, but contain fewer algal cells (Fig. 4e, f). The size of the thallus globules was 15–25 µm in diameter. The photobiont was mainly globose to subglobose and 6.5–9.5 × 4.5–7.0 µm in size, usually containing many oil droplets (Fig. 4g). The chloroplast was parietal, and no haustoria were observed by LM. The thallus often intermingled with algal cells other than the photobiont, but these additional algal cells were not closely associated with fungal hyphae.

TEM observations of the lichenized thallus revealed that the mycobiont hyphae containing electron-dense granules in the cytoplasm were closely attached to the photobiont cells (Fig. 4h, i, k). No haustoria were found. The chloroplast of the photobiont was parietal and filled with thylakoid lamellae, similar to that of *M. mucida* (Fig. 4i, k). Numerous electron-dense granules (pyrenoglobuli) were present within a homogeneous pyrenoid matrix at the center of the chloroplast, surrounded by starch grains (Fig. 4j, l). The pyrenoid matrix was penetrated by several thylakoids in the form of tubules (Fig. 4j).

Photobiont cells from the thallus of *M. vernalis* were isolated and incubated on BBM agar. Only spherical green cells were obtained in culture (Fig. 4m–p). In the culture on BBM agar, the autospores were usually spherical; very rarely, elongate autospores were produced (Fig. 4p). TEM observation of the photobiont cultures showed a parietal chloroplast filled with developed thylakoid lamellae. A pyrenoid containing pyrenoglobuli was often located at the center of the chloroplast (Fig. 4q, r). The pyrenoid was surrounded by starch grains and penetrated by thylakoid stacks in the form of tubules (Fig. 4q, r). In the cytoplasm, lipid bodies and electron-dense bodies were present (Fig. 4r). The ultrastructure of the cultured alga was consistent with that of the alga within the thallus of *M. vernalis*. LM observations of the cultured algal isolates were also consistent with those observed from the thallus of *M. petricola*.

3.5. Molecular analyses of the algal isolates

In the phylogenetic analyses based on the concatenated 18S rDNA and ITS2 dataset, an alignment consisting of 1692 nucleotides from 18S rDNA and 283 nucleotides from ITS2 was used. Of the 1975 characters included in the alignment, 269 were variable, and 1706 were conserved. Figure 5 shows the ML tree with the highest log-likelihood value (-5286.218165) inferred using RAXML-NG. In this tree, the *Elliptochloris* isolates derived from *M. mucida* were placed within the *E. subsphaerica* clade, which received support only in the Bayesian inference (BS/PP = -/0.99). The *Elliptochloris* isolates from *M. petricola* and *M. vernalis* formed a sister clade to *E. subsphaerica* (hereafter described as *E. parasphaerica* sp. nov.); however, this relationship lacked statistical support. Although *E. subsphaerica*, *E. parasphaerica*, and *E. marina* appeared to be closely related in the phylogenetic tree, their monophyly of these taxa was not supported, and their relationships remained unresolved.

In the phylogenetic analyses of the *rbcL* dataset, an aligned dataset of 1104 nucleotides was used. Of these, 325 were variable, and 779 were conserved. Figure 6 depicts the ML tree with the highest log-likelihood value (-3926.049005) generated by RAXML-NG. In this tree, *E. subsphaerica* and *E. marina* formed a well-supported monophyletic clade (BS/PP = 94/1). Within this clade, *E. subsphaerica* was further subdivided into several subclades. All *Elliptochloris* isolates from *M. mucida*, except for NIES-4529, were included in a subclade designated “MM-1.” The isolate NIES-4529 was placed in a closely related subclade designated “MM-2.” In contrast, the *Elliptochloris* isolates from *M. vernalis* and *M. petricola* formed a strongly supported independent lineage (BS/PP = 100/1), which in turn formed a strongly supported monophyletic group (BS/PP = 100/1) together with the clade comprising *E. subsphaerica* and *E. marina*.

The ITS2 secondary structures of the photobionts from *M. petricola* and *M. vernalis* (= *E. parasphaerica*) were compared with those of *E. subsphaerica* and *E. marina*, which together formed a well-supported monophyletic clade in Fig. 6. The comparison revealed that the photobionts of *M. petricola* and *M. vernalis* possessed one hemi-compensatory base change (hemi-CBC) in ITS2 relative to *E. subsphaerica* and *E. marina* (Fig. 7; Appendix S3). Specifically, at a position in Helix III, both photobionts of *M. petricola* and *M. vernalis* exhibited a G·U pair, whereas *E. subsphaerica* and *E. marina* possessed a G–C pair at the corresponding position. Furthermore, the photobionts from *M. vernalis* exhibited one additional hemi-CBC compared with those from *M. petricola* at position 53 of Helix III: a G·U pair in the former and an A·U pair in the latter. Furthermore, in the region near the loop of Helix IV, *E. subsphaerica* and *E. marina* shared the sequence “GCUUG,” whereas the corresponding position in *E. parasphaerica* was characterized by the sequence “AGU” (Appendix S3).

3.6. Taxonomy

As described above, the photobionts of *M. petricola* and *M. vernalis* were morphologically similar to *E. subsphaerica*, but clearly distinguishable from it by their predominantly spherical autospores, distinct molecular signatures, and unique phylogenetic placement. Accordingly, a new species, *E. parasphaerica*, is described here to accommodate this clade.

Elliptochloris parasphaerica H. Masumoto & S. Handa, sp. nov. (Figs. 3g, h; 4m–r)

Description. Cells solitary, two types, spherical (S-type) and ellipsoidal (E-type). Mature cells spherical, subspherical, or elongated, 12.0–17.0 × 10.0–14.0 µm. Young S-type cells spherical to subspherical, 8.0–11.5 × 6.5–9.0 µm. Young E-type cells ellipsoidal, reniform, or cylindrical 5.5–9.0 × 2.5–3.5 µm. Chloroplast single, parietal, saucer-shaped, with a pyrenoid. Cell wall thin, slightly thickened with age. Oil droplets prominent throughout cultivation. Pyrenoid single, surrounded by many starch grains, usually obscure in younger cells. Asexual reproduction usually via 2 or 4 spherical autospores, rarely 8 or 16 narrow ellipsoidal autospores. Autospores released by the rupture of the mother cell wall.

Diagnosis. The species differs from *E. subsphaerica* in the ITS2 and *rbcl* sequences, and possesses a unique hemi-CBC in ITS2 helix III compared to *E. subsphaerica* (Fig. 7; Appendix S3 in the Supporting Information). The ellipsoidal-type autospore formation is much less common than that in *E. subsphaerica*.

Habitat. As the photobiont of *Multiclavula petricola* and *M. vernalis*.

Holotype. Strain NIES-4523 permanently cryopreserved in a metabolically inactive state at NIES, originated from the thallus of basidiolichen *Multiclavula vernalis* (herb. GMNHJ-FF1008191; Table 1).

Type locality. JAPAN. Nagano Prefecture, Ueda, Mt. Nekodake, ca. 1800 m elev.

Etymology. The epithet refers to its resemblance to *Elliptochloris subsphaerica*.

Living culture (ex-holotype). A living culture derived from the holotype is available at the National Institute for Environmental Studies (NIES Collection, Tsukuba, Japan), under the code NIES-4523 (= S-253-PPSC-007).

DNA sequences: LC597449 (*rbcl* gene), LC597448 (18S rDNA), LC597447 (ITS2).

Molecular signature: A Hemi-CBC in helix III of the ITS2 secondary structure (position 44, G•U), compared with *E. subsphaerica* and *E. marina* (position 44, G-C). An “AGT” motif in the region near the loop of Helix IV of the ITS2 secondary structure, corresponding to the “GCUUG” motif of *E. subsphaerica* and *E. marina*.

4 Discussion

Based on LM and TEM observations of the thalli of three *Multiclavula* species (*M. mucida*, *M. petricola*, *M. vernalis*) examined in this study (Figs. 1, 3, 4), species of *Coccomyxa* were excluded as a possible photobiont, because the photobionts observed within their thalli possessed a pyrenoid, which is absent in *Coccomyxa* (Komárek and Fott 1983; Darienko et al. 2015), and the photobionts of all three *Multiclavula* species in Japan were shown to be members of the green algal genus *Elliptochloris*. The photobiont of *M. mucida* was shown to be *E. subsphaerica*, while that of *M. petricola* and *M. vernalis* was shown to be a new species, *E. parasphaerica*. Indeed, the formation of globular thalli in co-culture experiments between *M. mucida* and *E. subsphaerica* further supports the interpretation that *E. subsphaerica* serves as the photobiont of *M. mucida*. Although species of *Elliptochloris* have been reported as photobionts of several groups of lichenized ascomycetes, such as *Baeomyces rufus*, *Catolechia wahlenbergii*, *Micarea* spp., *Sticta* spp., and *Verrucaria sublobulata* (Tschermak-Woess 1980; Trembley et al. 2002; Thüs et al. 2011; Voytsekhovich et al. 2011; Lindgren et al. 2020), the genus has not been previously reported in association with lichenized basidiomycetes. *Elliptochloris subsphaerica* has previously been reported as free-living from soils, bark, stones, and roof tiles (e.g., Reisligl 1964; Watanabe 1977; Handa and Nakano 1988), and in symbiosis with lichenized ascomycetes such as *Micarea* spp. (Voytsekhovich et al. 2011; Yahr et al. 2015). The species *E. parasphaerica*, newly described herein, is so far known only from the lichenized thalli of *M. petricola* and *M. vernalis*. Its ecology outside its role as a lichen photobiont remains unknown.

The 18S rDNA region has been widely used for phylogenetic analyses across the genus *Elliptochloris* (Darienکو et al. 2016). However, in this study, this marker provided insufficient resolution to clarify the relationships of *E. subsphaerica* with its closely related taxa. Therefore, we conducted phylogenetic analyses using a concatenated dataset including the more variable ITS2 region. In the phylogenetic analyses of the concatenated 18S rDNA and ITS2 dataset, *E. subsphaerica*, *E. parasphaerica*, and *E. marina* appeared to be closely related. However, branch support for each species was lacking, and the relationships among these taxa remained unresolved. The ITS2 sequence of *E. parasphaerica* exhibited one hemi-compensatory base change (hemi-CBC) not observed in *E. subsphaerica* or *E. marina*, as well as a characteristic sequence motif in the loop region of Helix IV. These features likely represent molecular signatures useful for species delimitation. Phylogenetic analyses based on the *rbcl* dataset yielded improved resolution. *Elliptochloris subsphaerica* and *E. marina* formed a well-supported monophyletic clade. Within this clade, *E. subsphaerica* was further subdivided into several subclades. Notably, almost all isolates derived from *M. mucida* were consistently recovered in subclade “MM-1,” regardless of collection locality. This pattern suggests that *M. mucida* may preferentially associate with a particular lineage of *E. subsphaerica*. In contrast, all *Elliptochloris* isolates derived from *M. petricola* and *M. vernalis* were assigned to *E. parasphaerica*, which formed an independent lineage sister to the monophyletic group comprising *E. subsphaerica* and *E. marina*. The host species from which *E. parasphaerica* was isolated, *M. petricola* and *M. vernalis*, both occur in cool subalpine environments. These results may indicate that *E. parasphaerica* is adapted to, or preferentially associated with, such cooler habitats.

Geitler (1955) and Oberwinkler (1970) illustrated pseudoparenchyma in the outer layer of thallus globules of *M. mucida* observed with LM. In our examination of *M. mucida*, by contrast, a surrounding pseudoparenchymatous layer was not clearly observed by LM. However, TEM observations showed that the mycobiont tightly surrounded the algal cells without any visible gaps, thereby forming a globule. It is possible that pseudoparenchyma could not be detected in LM due to the very thin hyphae forming the tissue (up to 1.5 µm; Fig. 1k, l). Poelt and Obermayer (1990) observed that *M. vernalis* formed distinctive bulbils 50–70 µm in diameter that consisted of pseudoparenchymatous hyphae and photobiont cells, and may function as vegetative diaspores. However, the thallus of *M. vernalis* examined in this study was mainly amorphous and granulose, lacking a pseudoparenchymatous layer, and the globules were smaller in

diameter (15–25 µm). Additional sampling is required to determine whether the thalli of *M. vernalis* exhibit morphological plasticity in response to environmental conditions.

An algal strain, *Coccomyxa* sp. SAG 49.84, collected in Germany by Wulf Koch in 1983, has been registered as a photobiont (phycobiont) of *M. vernalis* (https://sagdb.uni-goettingen.de/detailedList.php?str_number=49.84, cited on 26 February 2026) and was morphologically identified as *Coccomyxa dispar* Schmidle by Darienko et al. (2015). However, as noted by those authors, *C. dispar* was isolated from *M. vernalis* only in that single instance, and it remains uncertain whether the alga represents a true photobiont or merely an epiphyte on the lichen thallus. It is conceivable that certain *Coccomyxa* species, including *C. dispar* SAG 49.84, preferentially colonize moist microhabitats such as the thalli of *Multiclavula* species. Furthermore, it is plausible that some *Multiclavula* populations may utilize *Coccomyxa* as a secondary photobiont, as documented in ascolichens such as *Micarea* spp. (Voytsekhovich et al. 2011; Yahr et al. 2015). Additional investigation will be required to determine whether such an association occurs within *Multiclavula*.

In addition to the three species examined in this study (*M. mucida*, *M. petricola*, *M. vernalis*), two species have recently been described from Central America: *M. ichthyiformis* Nelsen, Lücking, L. Umaña, Trest & Will-Wolf (Nelsen et al. 2007) and *M. caput-serpentis* Lotz-Winter & Reschke (Reschke et al. 2021). These two species have been shown to form a well-supported monophyletic lineage within *Multiclavula* (Reschke et al. 2021). The thallus of *M. ichthyiformis* is characterized by fungal hyphae enveloping individual algal cells (Nelsen et al. 2007); however, unlike the globular thalli observed in *M. mucida* and *M. petricola* in this study, its thallus is not globular but rather predominantly amorphous, resembling the morphology commonly observed in *M. vernalis*. The photobiont of *M. ichthyiformis* has been reported as *Coccomyxa* (Nelsen et al. 2007); however, a culture of this photobiont has not been established, and its molecular sequences have not yet been analyzed. It would therefore be of considerable interest to determine whether *M. ichthyiformis* truly associates with *Coccomyxa*, or whether its photobiont is in fact *Elliptochloris*, as demonstrated here for the three other lichen-forming species of *Multiclavula* that were previously reported to associate with *Coccomyxa*. In contrast, the thallus of *M. caput-serpentis* has been described as forming “bulbils,” in which fungal hyphae envelop broadly ellipsoidal algal cells (Reschke et al. 2021). This morphology appears similar to the globular thalli observed in *M. mucida* and *M. petricola* in the present study. However, the taxonomic identity of its photobiont and the presence or absence of a pyrenoid have not been addressed. Further sampling and integrative analyses will be necessary to determine whether *Elliptochloris* represents a universal photobiont within *Multiclavula* species.

To date, the ultrastructure of *Elliptochloris* has been observed in only a few cases (e.g., Brunner and Honegger 1985; Novis and Visnovsky 2012), and such ultrastructural information has not been incorporated into the taxonomy of *Elliptochloris*. In a previous TEM study of the thallus of *M. mucida* (figs. 13–14 in Oberwinkler 1984), the ultrastructure of the photobiont cells was not discussed. Yet a pyrenoid is clearly visible in those micrographs. Furthermore, the presence of electron-dense granules (probably pyrenoglobuli) in the pyrenoid matrix surrounded by starch grains, and many electron-dense granules in the hyphae surrounding the photobiont cells are consistent with our observations of the thallus of *M. mucida* (Fig. 11–o). In addition, O'Brien (1978) observed a similar pyrenoid structure in the chloroplast of green algal symbionts in the gastrodermal tissue of the sea anemone *Anthopleura xanthogrammica*. The alga was subsequently described as *E. marina* by Letsch et al. (2009), which was shown to be phylogenetically very close to *E. subsphaerica* (Darienko et al. 2016; this study). *Elliptochloris philistinensis* was also observed with TEM, and pyrenoids surrounded by starch grains were observed in the chloroplast (Novis and Visnovsky 2012). On the other hand, Brunner and Honegger (1985) found no pyrenoids in cultured *Elliptochloris* sp. isolated as the photobiont of *Micarea prasina*. Additional observations on various species are needed before these ultrastructural features can be applied taxonomically.

Declarations

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Competing interests

The authors declare no competing interests.

Compliance with ethical standards

This article does not contain any studies performed on humans or animals.

References

1. Bischoff HW, Bold HC (1963) Phycological studies IV. Some soil algae from Enchanted Rock and related algal species. University of Texas Publications 6318: 1–95
2. Brodo IM, Sharnoff SD, Sharnoff S (2001) Lichens of North America. Yale University Press, New Haven and London.
3. Brunner U, Honegger R (1985) Chemical and ultrastructural studies on the distribution of sporopollenin-like biopolymers in six genera of lichen phycobionts. Can J Bot 63: 2221–2230. <https://doi.org/10.1139/b85-315>

4. Byun Y, Han K (2009) PseudoViewer3: generating planar drawings of large-scale RNA structures with pseudoknots. *Bioinformatics* 25: 1435–1437. <https://doi.org/10.1093/bioinformatics/btp137>
5. Darienko T, Gustavs L, Eggert A, Wolf W, Pröschold T (2015) Evaluating the species boundaries of green microalgae (*Coccomyxa*, Trebouxiophyceae, Chlorophyta) using integrative taxonomy and DNA barcoding with further implications for species identification in environmental samples. *PLoS ONE* 10: e0127838. <https://doi.org/10.1371/journal.pone.0127838>
6. Darienko T, Gustavs L, Pröschold T (2016) Species concept and nomenclatural changes within the genera *Elliptochloris* and *Pseudochlorella* (Trebouxiophyceae) based on an integrative approach. *J Phycol* 52: 1125–1145. <https://doi.org/10.1111/jpy.12462>
7. Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T (2020) ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Mol Biol Evol* 37: 291–294. <https://doi.org/10.1093/molbev/msz192>
8. Ertz D, Lawrey JD, Sikaroodi M, Gillevet PM, Fischer E, Killmann D, Sérusiaux E (2008) A new lineage of lichenized basidiomycetes inferred from a two-gene phylogeny: the Lepidostromataceae with three species from the tropics. *Am J Bot* 95: 1548–1556. <https://doi.org/10.3732/ajb.0800097>
9. Franchi P, Marchetti M (2021) I funghi clavarioidi in Italia, vol 2. Associazione Micologica Bresadola, Fondazione Centro Studi Micologici, Vicenza.
10. Geitler L (1955) *Clavaria mucida*, eine extratropische Basidiolichene. *Biol Zentralbl* 74: 145–159
11. Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 27: 221–224. <https://doi.org/10.1093/molbev/msp259>
12. Handa S, Nakano T (1988) Some corticolous algae from Miyajima Island, western Japan. *Nova Hedwigia* 46: 165–186
13. Hawksworth DL (1988) The variety of fungal-algal symbioses, their evolutionary significance, and the nature of lichens. *Bot J Linn Soc* 96: 3–20. <https://doi.org/10.1111/j.1095-8339.1988.tb01836.x>
14. Inaga S, Katsumoto T, Tanaka K, Kameie T, Nakane H, Naguro T (2007) Platinum blue as an alternative to uranyl acetate for staining in transmission electron microscopy. *Arch Histol Cytol* 70: 43–49. <https://doi.org/10.1679/aohc.70.43>
15. Jung P, Brust K, Schultz M, Büdel B, Donner A, Lakatos M (2021) Opening the gap: rare lichens with rare cyanobionts – unexpected cyanobiont diversity in cyanobacterial lichens of the order Lichinales. *Front Microbiol* 12: 728378. <https://doi.org/10.3389/fmicb.2021.728378>
16. Katoh K, Rozewicki J, Yamada KD (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform* 20: 1160–1166. <https://doi.org/10.1093/bib/bbx108>
17. Komárek J, Fott B (1983) Chlorophyceae (Grünalgen) Ordnung: Chlorococcales. In: Huber-Pestalozzi G (ed) *Das Phytoplankton des Süßwassers*, Band 7, Teil 1. E Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.
18. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A, Wren J (2019) RAXML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* 35: 4453–4455. <https://doi.org/10.1093/bioinformatics/btz305>
19. Letsch MR, Muller-Parker G, Friedl T, Lewis LA (2009) *Elliptochloris marina* sp. nov. (Trebouxiophyceae, Chlorophyta), symbiotic green alga of the temperate Pacific sea anemones *Anthopleura xanthogrammica* and *A. elegantissima*. *J Phycol* 45: 1127–1135. <https://doi.org/10.1111/j.1529-8817.2009.00738.x>
20. Li S, Tan H, Liu B, Zhu H, Hu Z, Liu G (2021) Watanabeales ord. nov. and twelve novel species of Trebouxiophyceae (Chlorophyta). *J Phycol* 57: 1167–1186. <https://doi.org/10.1111/jpy.13116>
21. Lindgren H, Moncada B, Lücking R, Magain N, Simon A, Goffinet B, Sérusiaux E, Nelsen MP, Mercado-Díaz JA, Widholm TJ, Lumbsch HT (2020) Cophylogenetic patterns in algal symbionts correlate with repeated symbiont switches during diversification and geographic expansion of lichen-forming fungi in the genus *Sticta*. *Mol Phylogenet Evol* 150: 106860. <https://doi.org/10.1016/j.ympev.2020.106860>
22. Lücking R, Hodkinson BP, Leavitt SD (2017) The 2016 classification of lichenized fungi in the Ascomycota and Basidiomycota – approaching one thousand genera. *Bryologist* 119: 361–416. <https://doi.org/10.1639/0007-2745-119.4.361>
23. Machida R, Nagashima T, Yokoyama T (1994) Mesoderm segregation of a jumping bristletail, *Pedetontus unimaculatus* (Hexapoda, Microcoryphia), with a note on an automatic vacuum infiltrator [in Japanese]. *Proc Arthropodan Embryol Soc Jpn* 29: 23–24
24. Masumoto H, Degawa Y (2020) *Multiclavula petricola* sp. nov. (Cantharellales, Basidiomycota), a new clavarioid and lichenized fungus growing on rocks. *Mycoscience* 61: 155–159. <https://doi.org/10.1016/j.myc.2020.03.004>
25. McCune B (2017) *Microlichens of the Pacific Northwest*, vols 1–2. Wild Blueberry Media, Corvallis.
26. Morelan IA, Gaulke CA, Sharpton TJ, Thurber RV, Denver DR (2019) Microbiome variation in an intertidal sea anemone across latitudes and symbiotic states. *Front Mar Sci* 6: 1–13. <https://doi.org/10.3389/fmars.2019.00183>
27. Nakano T (1988) Phycobionts of some Japanese species of the Graphidaceae. *Lichenologist* 20: 353–360. <https://doi.org/10.1017/S0024282988000383>
28. Nelsen MP, Lücking R, Umaña L, Trest MT, Will-Wolf S, Chaves JL, Gargas A (2007) *Multiclavula ichthyiformis* (Basidiomycota, Cantharellales), a remarkable new basidiolichen from Costa Rica. *Am J Bot* 94: 1289–1296. <https://doi.org/10.3732/ajb.94.8.1289>
29. Novis PM, Visnovsky G (2012) Novel alpine algae from New Zealand: Chlorophyta. *Phytotaxa* 39: 1–30. <https://doi.org/10.11646/phytotaxa.39.1.1>
30. Oberwinkler F (1970) Die Gattungen der Basidiolichenen. *Ber Dtsch Bot Ges*, N. F. 4: 139–169
31. Oberwinkler F (1984) Fungus-alga interactions in basidiolichens. *Nova Hedwigia Beih* 79: 739–774
32. O'Brien TL (1978) An ultrastructural study of zoochlorellae in a marine coelenterate. *Trans Am Microsc Soc* 97: 320–329. <https://doi.org/10.2307/3225645>
33. Poelt J (1974) *Bestimmungsschlüssel europäischer Flechten*. Cramer, Vaduz.

34. Poelt J, Obermayer W (1990) Lichenisierte Bulbillen als Diasporen bei der Basidiolichene *Multiclavula vernalis* spec. coll. *Herzogia* 8: 289–294
35. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol* 67: 901–904. <https://doi.org/10.1093/sysbio/syy032>
36. Reisl H (1964) Zur Systematik und Ökologie alpiner Bodenalgae. *Österr Bot Z* 111: 402–499
37. Reschke K, Lotz-Winter H, Fischer CW, Hofmann TA, Piepenbring M (2021) New and interesting species of Agaricomycetes from Panama. *Phytotaxa* 529(1): 1–26. <https://doi.org/10.11646/phytotaxa.529.1.1>
38. Rico VJ, Barrasa JM (2011) Basidiomycota liquenizados y liquenícolas: Agaricales y Cantharellales. *Flora Liquenológica Ibérica* 9. Sociedad Española de Lichenología, Madrid.
39. Ronquist F, Teslenko M, Van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 61: 539–542. <https://doi.org/10.1093/sysbio/sys029>
40. Sanders WB, Pérez-Ortega S, Nelsen MP, Lücking R, de los Ríos A (2016) *Heveochlorella* (Trebouxiophyceae): a little-known genus of unicellular green algae outside the Trebouxiales emerges unexpectedly as a major clade of lichen photobionts in foliicolous communities. *J Phycol* 52: 840–853. <https://doi.org/10.1111/jpy.12495>
41. Schmidle W (1901) Über drei Algengenera. *Ber Dtsch Bot Ges* 19: 10–24
42. Spridille T (2019) Lichen symbionts outside of symbiosis: how do they find their match? *Ann Bot* 124: vi–vii. <https://doi.org/10.1093/aob/mcz045>
43. Stenroos S, Velmala S, Pykälä J, Ahti T (2016) Lichens of Finland. *Norrinia* 30. Finnish Museum of Natural History LUOMUS, University of Helsinki, Helsinki.
44. Thüs H, Muggia L, Pérez-Ortega S, Favero-Longo SE, Joneson S, O'Brien H, Nelsen MP, Duque-Thüs R, Grube M, Friedl T, Brodie J, Andrew CJ, Lücking R, Lutzoni F, Gueidan C (2011) Revisiting photobiont diversity in the lichen family Verrucariaceae. *Eur J Phycol* 46: 399–415. <https://doi.org/10.1080/09670262.2011.582106>
45. Trembley ML, Ringli C, Honegger R (2002) Morphological and molecular analysis of early stages in the resynthesis of the lichen *Baeomyces rufus*. *Mycol Res* 106: 768–776. <https://doi.org/10.1017/S0953756202006159>
46. Tschermak-Woess E (1980) *Elliptochloris bilobata*, gen. et sp. nov., der Phycobiont von *Catolechia wahlenbergii*. *Plant Syst Evol* 136: 63–72. <https://doi.org/10.1007/BF00937958>
47. Tschermak-Woess E (1988) The algal partner. In: Galun M (ed) *CRC handbook of lichenology*, vol 1. CRC Press, Boca Raton, pp 39–92
48. Venable JH, Coggeshall R (1965) A simplified lead citrate stain for use in electron microscopy. *J Cell Biol* 25: 407–408. <https://doi.org/10.1083/jcb.25.2.407>
49. Voitek A, Ohenoja E (2011) Genus *Multiclavula* in Newfoundland and Labrador. *Fungi* 2: 26–31. <https://doi.org/10.3390/fungi2020026>
50. Voytsekhovich A, Dymytrova L, Nadyeina O (2011) Photobiont composition of some taxa of the genera *Micarea* and *Placynthiella* from Ukraine. *Folia Cryptogam Est* 48: 135–148
51. Voytsekhovich A (2013) Lichen photobionts: the origin, diversity and relationships with mycobiont [in Russian]. Lambert Academic Publishing, Saarbrücken.
52. Voytsekhovich A, Beck A (2016) Lichen photobionts of the rocky outcrops of Karadag massif (Crimean Peninsula). *Symbiosis* 68: 9–24. <https://doi.org/10.1007/s13199-016-0419-6>
53. Watanabe S (1977) The genus *Chlorella* (Chlorococcales) from Japanese soils (2). *J Jpn Bot* 52: 129–136. https://doi.org/10.5103/jjapbot.52_5_6742
54. Watling R (2009) *Multiclavula*. In: Smith CW, Aptroot A, Coppins BJ, Fletcher A, Gilbert OL, James PW, Wolseley PA (eds) *The lichens of Great Britain and Ireland*. British Lichen Society, London, pp 612–613
55. Wirth V, Hauck M, Schultz M (2013) *Die Flechten Deutschlands*, Band 2. Ulmer, Stuttgart.
56. Yahr R, Florence A, Škaloud P, Voytsekhovich A (2015) Molecular and morphological diversity in photobionts associated with *Micarea* s. str. (Lecanorales, Ascomycota). *Lichenologist* 47: 403–414. <https://doi.org/10.1017/S0024282915000062>
57. Zahradníková M, Andersen HL, Tønsberg T, Beck A (2017) Molecular evidence of *Apatococcus*, including *A. fuscideae* sp. nov., as photobiont in the genus *Fuscidea*. *Protist* 168: 425–438. <https://doi.org/10.1016/j.protis.2017.09.003>

Table

Table 1 Specimens of *Multiclavula* species collected for the photobiont isolation.

Taxon	Herbarium or collector nos.	Collection Date	Locality	Collector	Strain nos the isolate
<i>Multiclavula mucida</i>	OSA-MY-9325	31-Oct-2017	Japan: Mt. Daimonji, Sakyo Ward, Kyoto. Elev. 212 m.	H. Masumoto	NIES-4525 S-200-PL-1
	GMNHJ-FF1008189	1-Nov-2017	Japan: Mt. Arikasa, Nakanojo, Gunma. Elev. 558 m.	Y. Degawa	S-208-PL-1 S-208-PL-1 S-208-PL-1
	GMNHJ-FF1008190	4-Dec-2017	Japan: Lake Sugawa, Ueda, Nagano. Elev. 700 m.	H. Masumoto	S-216-PL-1 S-216-PL-1
	GMNHJ-FF1008193	3-Oct-2018	Japan: Otari, Nagano.	T. Matsuzaki	S-278-PL-1 S-278-PL-1
	GMNHJ-FF1008194	11-Oct-2018	Japan: Kumorizawa, Naganohara, Gunma.	Y. Degawa	S-279-PL-1
	GMNHJ-FF1008195	10-Oct-2018	Japan: Tambarakogen, Numata, Gunma.	Y. Degawa	S-280-PL-1
	H. Masumoto 283	24-Oct-2018	Japan: Mt. Daimonji, Sakyo Ward, Kyoto.	H. Masumoto	NIES-4525
<i>Multiclavula petricola</i>	GMNHJ-FF1008198	29-Sep-2019	Japan: Katashima, Gunma. Elev. 1757 m.	H. Masumoto	NIES-4524 S-356-PL-1 S-356-PL-1 S-356-PL-1
<i>Multiclavula vernalis</i>	GMNHJ-FF1008191	9-Jun-2018	Japan: Mt. Nekodake, Ueda, Nagano. Elev 1819 m.	H. Masumoto	NIES-4523 S-253-PP5 S-253-PP5 S-253-PP5

Figures

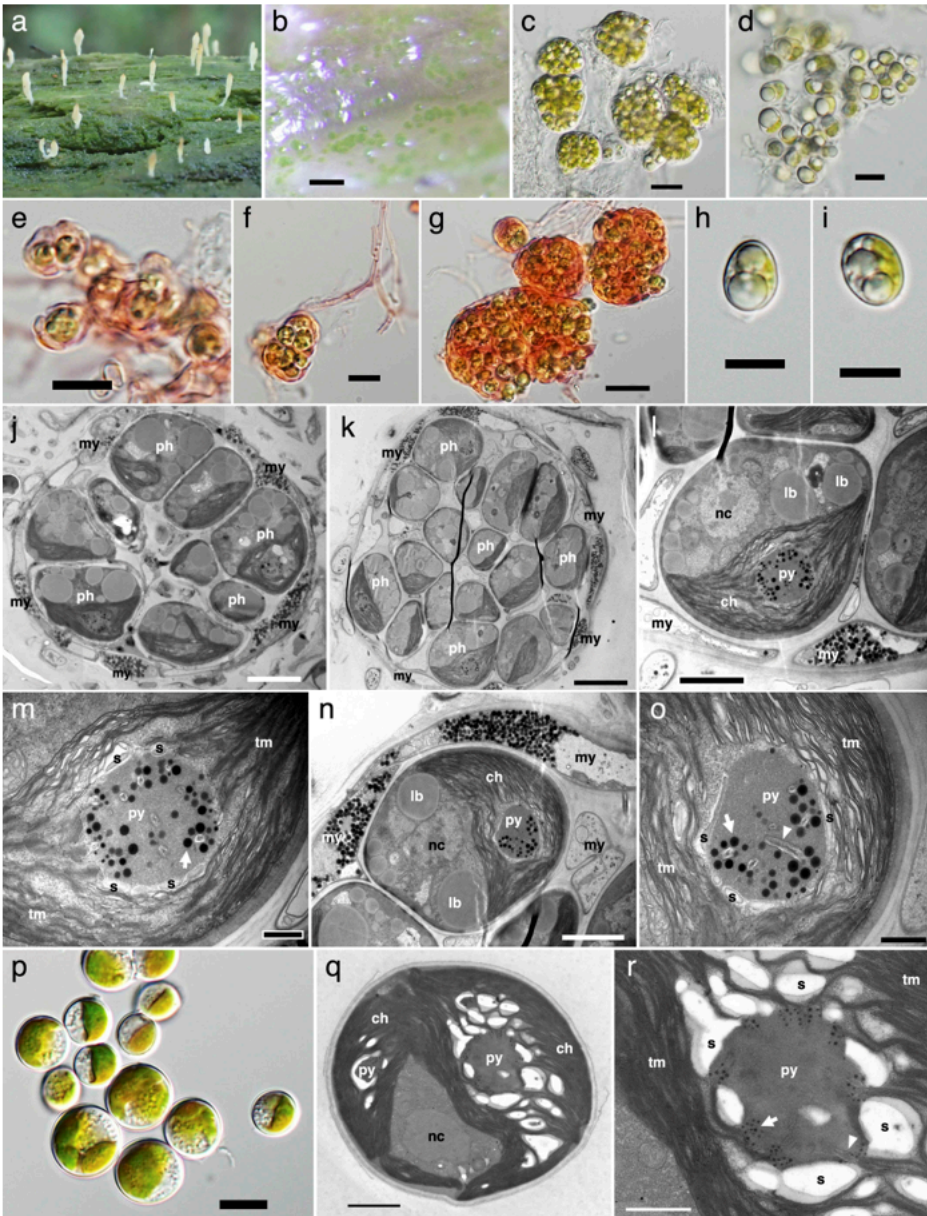


Figure 1

Multiclavula mucida and its photobiont *Elliptochloris subsphaerica*. (a) Basidiomata of *M. mucida*. (b) Thallus globules of *M. mucida* on wet rotten wood. (c) Light micrograph of the lichenized thalli. (d) Crushed thalli. (e, f) Young thalli mounted in 3% KOH with 1% Congo Red. Mycobiont hyphae are stained red. (g) Mature thalli mounted in 3% KOH with 1% Congo Red. (h, i) Photobiont cells derived from the thallus. (j, k) TEM micrograph of a thallus globule. (l, n) Photobiont cell within the thallus. (m) High magnification of Fig. 1l. (o) High magnification of Fig. 1n. (p) *E. subsphaerica* NIES-4525 isolated from the thallus of *M. mucida*. (q) TEM image of *E. subsphaerica* NIES-4525. (r) Details of a pyrenoid in Fig. 1q. ch = chloroplast, lb = lipid bodies, my = mycobiont hyphae, nc = nucleus, ph = photobiont cells, py = pyrenoid, s = starch grains, tm = thylakoid membrane. Arrows and arrowheads indicate pyrenoglobuli and thylakoid membranes entering the pyrenoid as tubules, respectively. Scale bars: b 100 µm; c, g 20 µm; d-f, h, i, p 10 µm; k 5 µm; j 3 µm; l, n, q 2 µm; r 1 µm; m, o 500 nm.

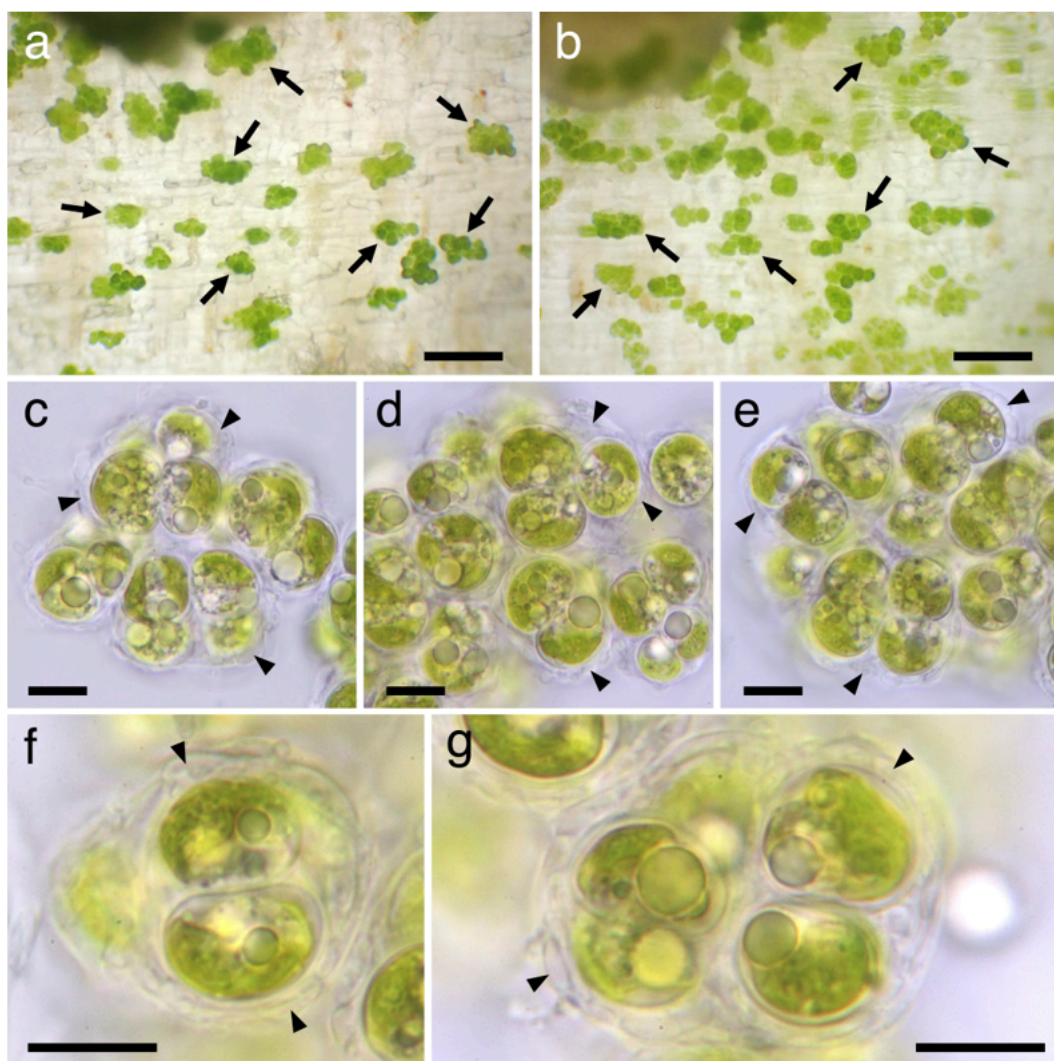


Figure 2

Resynthesized thalli of *Multiclavula mucida* in co-culture experiments. (a, b) Granular thalli of *M. mucida* scattered on the surface of the balsa wood. (c–e) Light micrographs of aggregates of granular thalli mounted in water. Note that fungal hyphae tightly intertwine with multiple algal cells, linking them to form larger structures. (f, g) Higher magnification of individual thalli. Arrows indicate resynthesized thalli of *M. mucida*; arrowheads indicate fungal hyphae closely attached to cells of *E. subsphaerica*. Scale bars: a, b 300 µm; c–g 10 µm

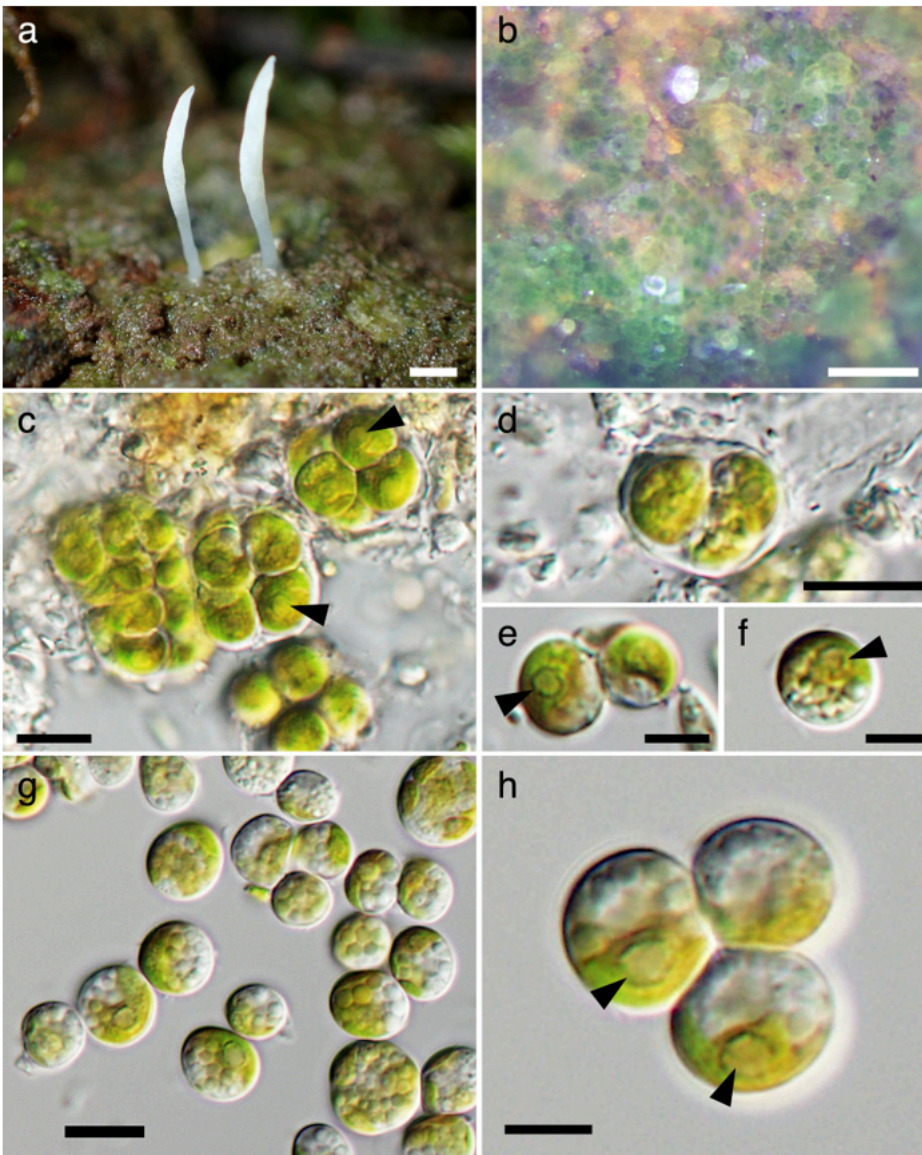


Figure 3

Multiclavula petricola and its photobiont *Elliptochloris parasphaerica* sp. nov. (a) Basidiomata of *M. petricola* growing on rock. (b) Thallus globules on rock. (c, d) Light micrographs of thallus globules. (e, f) Photobiont cells derived from the thallus. (g, h) The photobiont culture of *E. parasphaerica* NIES-4524 isolated from *M. petricola*. Arrowheads indicate pyrenoids. Scale bars: a 1 mm; b 100 µm; c, d, g 10 µm; e, f, h 5 µm.

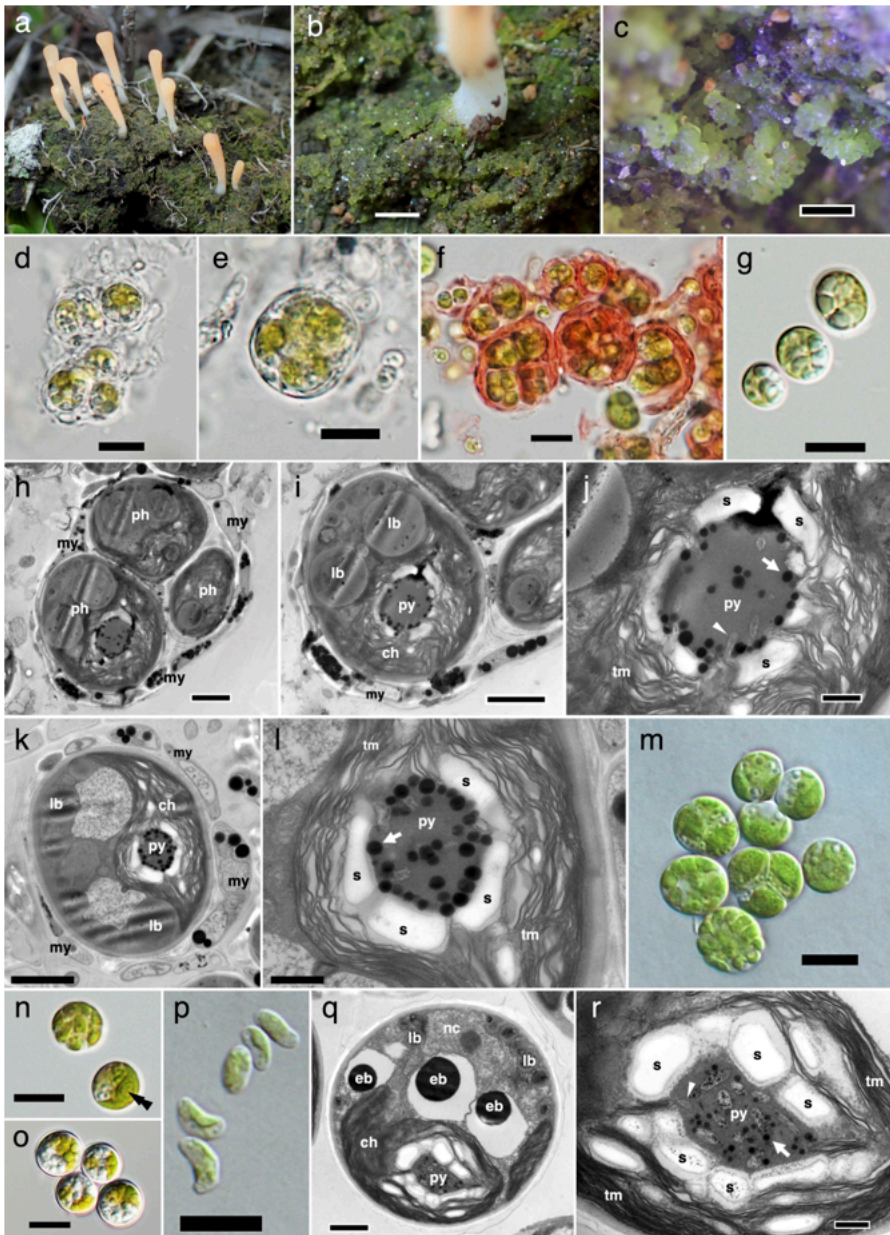


Figure 4

Multiclavula vernalis and its photobiont *Elliptochloris parasphaerica* sp. nov. (a) Basidiomata of *M. vernalis*. (b) Membranous lichenized thallus covering the soil around a basidioma. (c) Amorphous thallus of *M. vernalis* on soil. (d, e) Thalli of *M. vernalis* mounted in water. Note that one to several algal cells are surrounded by the hyphae of *M. vernalis*. (f) Thalli of *M. vernalis* mounted in 3% KOH with 1% Congo Red. Mycobiont hyphae are stained red. (g) Algae from the thalli. (h) TEM micrograph of a thallus globule. (i) Photobiont cell within the globule. (j) High magnification of Fig. 4i. (k) Photobiont cell surrounded by the mycobiont hyphae. (l) High magnification of Fig. 4k. (m–p) *E. parasphaerica* NIES-4523 isolated from the thallus. (q) TEM picture of *E. parasphaerica* NIES-4523. (r) Details of a pyrenoid of Fig. 4q. ch = chloroplast, eb = electron dense bodies, lb = lipid bodies, my = mycobiont hyphae, nc = nucleus, ph = photobiont cells, py = pyrenoid, s = starch grains, tm = thylakoid membrane. Arrows and arrowheads indicate pyrenoglobuli and thylakoid membranes entering the pyrenoid as tubules, respectively. Scale bars: b 1 mm; c 500 μ m; d–g, m–p 10 μ m; h, i, k, q 2 μ m; j, l 500 nm; r 300 nm.

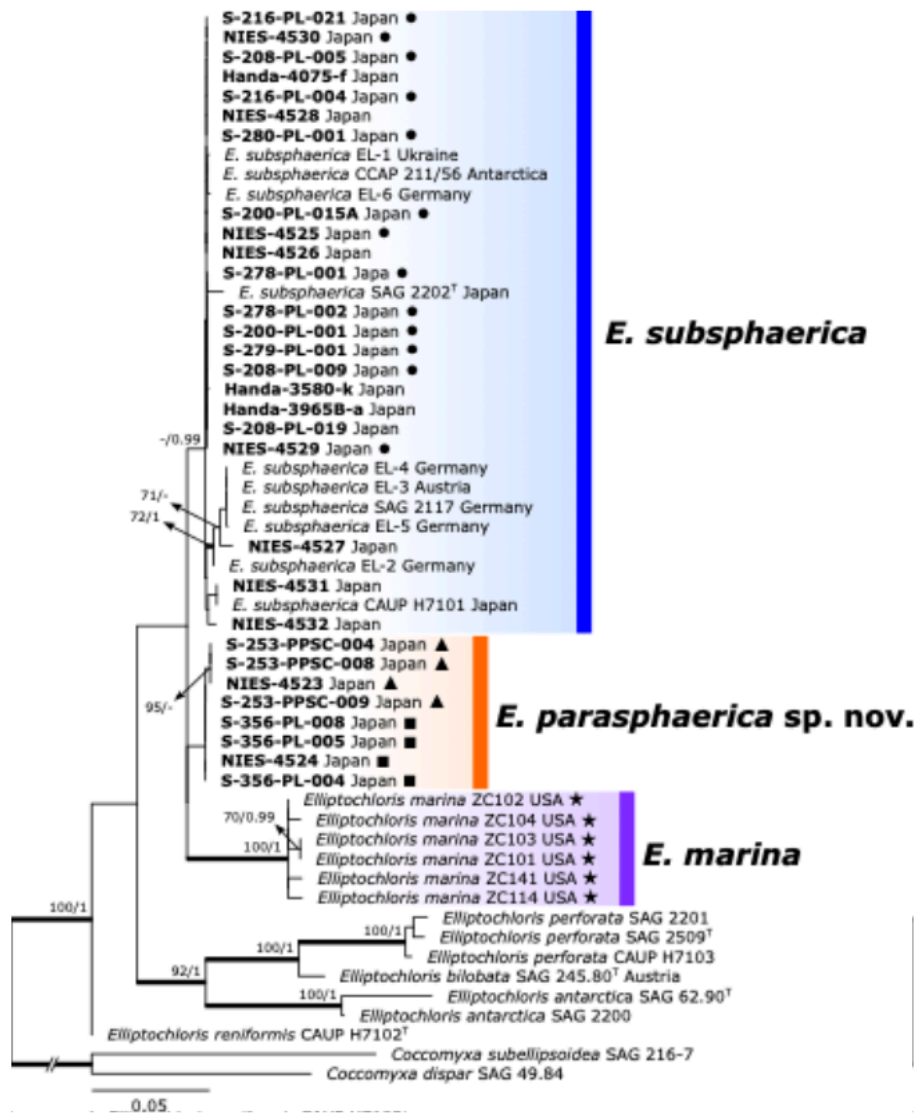


Figure 5

Molecular phylogenetic tree of the genus *Elliptochloris* inferred from maximum likelihood (ML) analysis of the concatenated 18S rDNA (1692 bp) + ITS2 (283 bp) dataset using RAXML-NG. The tree is rooted with *Coccomyxa dispar* SAG 49.84 and *C. subellipsoidea* SAG 216-7. ML bootstrap values (BS) and Bayesian posterior probabilities (PP) are indicated above or below branches, before and after the slash, respectively. Branches shown in bold indicate strong support (BS ≥ 70% and PP ≥ 0.95). The branch marked with a double slash is shortened. Sequences generated in this study are shown in bold. Sequences derived from algal isolates of the basidiolichen *Multiclavula mucida* are indicated by circles; those from *M. petricola* by squares; those from *M. vernalis* by triangles; and previously published sequences from the sea anemone *Anthopleura* spp. by stars. The scale bar represents the expected number of nucleotide substitutions per site.

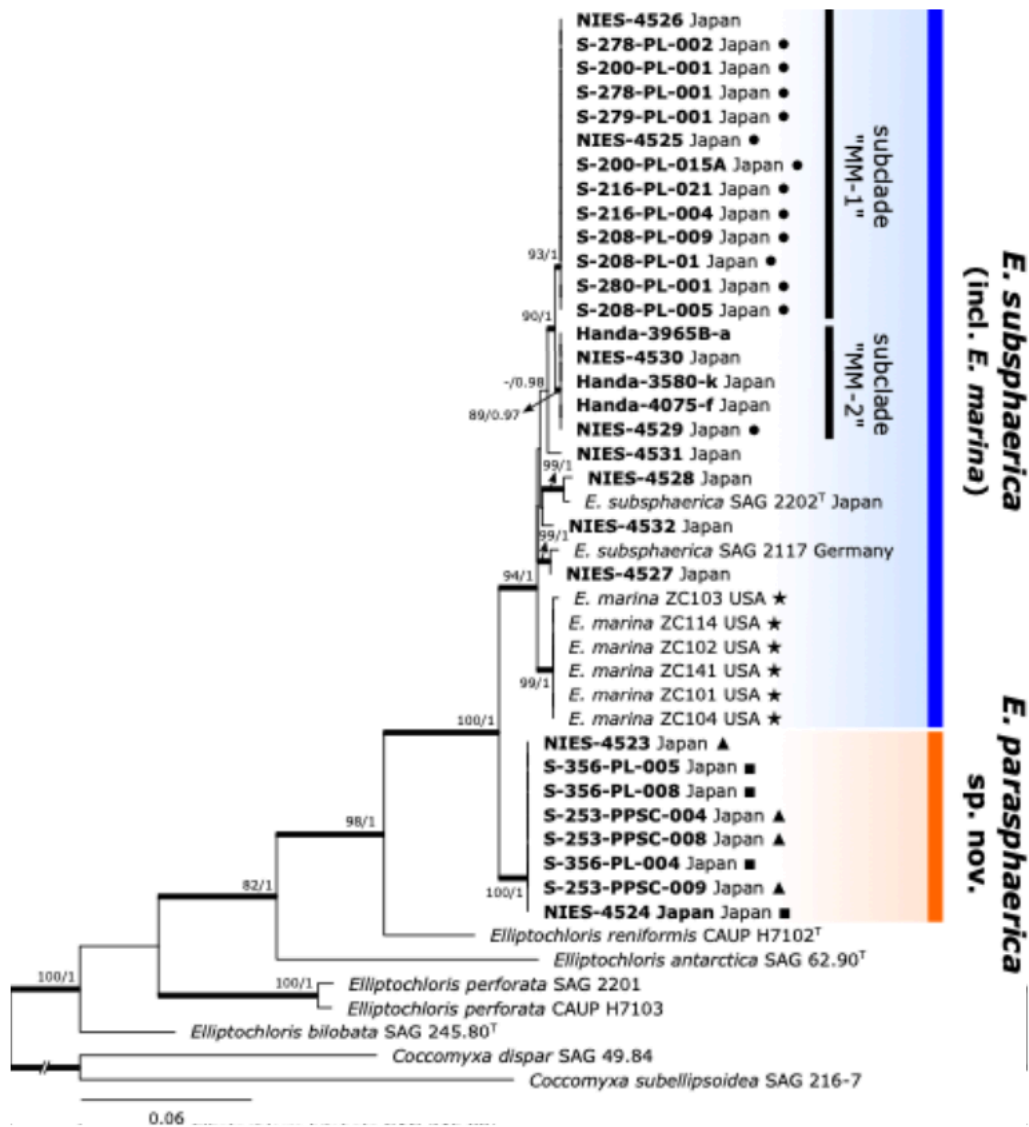


Figure 6

Molecular phylogenetic tree of the genus *Elliptochloris* inferred from maximum likelihood (ML) analysis of the *rbcL* (1104 bp) dataset using RAxML-NG. The tree is rooted with *Coccomyxa dispar* SAG 49.84 and *C. subellipsoidea* SAG 216-7. ML bootstrap values (BS) and Bayesian posterior probabilities (PP) are indicated above or below branches, before and after the slash, respectively. Branches shown in bold indicate strong support (BS $\geq 70\%$ and PP ≥ 0.95). The branch with the double slash is shortened. Sequences generated in this study are shown in bold. Sequences derived from algal isolates of the basidiolichen *Multiclavula mucida* are indicated by circles; those from *M. petricola* by squares; those from *M. vernalis* by triangles; and previously published sequences from the sea anemone *Anthopleura* spp. by stars. The scale bar represents the expected number of nucleotide substitutions per site.

	5.8S/LSU stem	Helix I	Helix II	Helix III	
	00000000011111	11111	22222222223	33333333344444444455555555566666666	
	12345678901234	56789	01234567890	12345678901234567890123456789012345678	
Barcode					
SAG 2202	23442154245326	64444	63367774643	33424388134133444453341413633613431442	
SAG 2117				1.....	8
NIES-4526					8
NIES-4525					8
S-200-PL-015A					8
Handa-3580-k					8
NIES-4530				5.....	8
CAUP H7101					8
CCAP 211/56					8
NIES-4532				7.....	8
E. subsphaerica					
E. marina ZC103		6.....			8
NIES-4523				5.....5.....	8
S-253-PPSC-008				5.....5.....	8
NIES-4524				5.....	8
S-356-PL-004				5.....	8
E. parasphaerica					
CAUP H7102		2.....	433.....	1.....	4
E. reniformis					
SAG 2200	8.....	2.....	33.....	688344..2888..3.....	888
SAG 62.90	8.....	2.....	33.....	688344..2888..3.....	888
E. antarctica					
CAUP H7103	..6..8.....	1.....	4.....1.....	..488.....888..3.....	888
SAG 2201	..6..8.....	1.....	4.....1.....	..488.....888..3.....	888
SAG 2509	..6..8.....8..	1.....	4.....1.....	..488.....888..3.....	888
E. perforata					
SAG 245.80	8.....	1.....	33.....1.....	..488.....3..8..3.....	888
E. bilobata					

Figure 7

The ITS2 barcodes of *Elliptochloris* species based on the ITS2/CBC approach described in Darienko et al. (2016). Newly determined barcodes in this study are indicated in bold. The aligned conserved region of ITS2 was translated into a base pair alignment using a numeric code for each base pair (1 = A-U, 2 = U-A, 3 = G-C, 4 = C-G, 5 = G-U, 6 = U-G, 7 = mismatch, 8 = deletion, unpaired or single bases). Codes listed for newly obtained strains are given in Fig. S1. Base pairs compared with those of *E. subsphaerica* SAG 2202 are marked with a dot.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AppendixS1Primers.pdf](#)
- [AppendixS2GenBankv260312.pdf](#)
- [AppendixS3ITS2secondarystructure.pdf](#)