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Coelastrella laevis sp. nov. (Chlorophyta, Sphaeropleales) from the soils of the Russian Far East

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ABSTRACT

Novel strains of green microalgae were isolated during a study of soil algal diversity in the Russian Far East (Jewish Autonomous Region, Primorsky Territory) and characterized using an integrative taxonomic approach. Phylogenetic analysis based on nuclear (SSU and ITS rDNA) and chloroplast (*tuf*A) DNA sequences resolved the strains as a distinct lineage in the generic clade of *Coelastrella*. Unique ITS2 sequence signatures, five unique substitutions/indels in helices II and III, further differentiated the new alga from congeners. Morphologically, our isolates resembled *C. tenuitheca* and *C. chongqingensis* having smooth cell wall without meridional ribs and polar thickenings (morphotype 3) but differed in smaller dimensions and autospore number (≥8). Our study supports description of a new species, *Coelastrella laevis sp. nov*. expanding the known diversity of *Coelastrella* and contributing to a better understanding of its phylogenetic and morphological variability.

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Green alga; Integrative approach; ITS rDNA secondary structure; New species

INTRODUCTION

The genus *Coelastrella* is a member of the family Scenedesmaceae (Sphaeropleales, Chlorophyta). It was described more than a century ago (Chodat, 1922), and since then, the number of its species has grown mostly due to taxonomic revisions and availability of molecular data (Goecke *et al.*, 2020; Hanagata, 1998; Hegewald & Hanagata, 2000; Hegewald *et al.*, 2010; Kalina & Punčochářová, 1987; Kaufnerová & Eliáš, 2013). Most members of *Coelastrella* were previously assigned to the genera *Graesiella, Chlorella, Cryptodesmus, Scenedesmus, Scotiella*, and *Scotiellopsis* (Hegewald & Hanagata, 2002; Kalina & Punčochářová, 1987; Kaufnerová & Eliáš, 2013; Shihira & Krauss, 1965; Skrebovska & Kostikov, 2012; Wang *et al.*, 2019). In this study, we follow the broad concept of the genus *Coelastrella*, which includes both the so-called 'core *Coelastrella*' clade (*sensu* Kaufnerová & Eliáš, 2013; e.g. *C. aeroterrestrica* Tschaikner, Gärtner & Kofler, *C. oocystiformis* (J.W.G.Lund) Hegewald & Hanagata, *C. striolata* Chodat, and *C. terrestris* (Reisigl) Hegewald & N. Hanagata), and species falling outside this group (e.g. *C. saipanensis* N.Hanagata, *C. thermophila* Q.Wang, H.Song, X.Liu, G.Liu & Z.Hu, and *C. vacuolata* (I.Shihira & R.W. Krauss) Hegewald & N.Hanagata), all of which are retained within *Coelastrella sensu lato*. Non-authentic strains of *Enallax costatus* (Schmidle) Pascher and *Asterarcys quadricellularis* (K.Behre) E.Hegewald & A.W.F.Schmidt also showed phylogenetic affinity to *Coelastrella*. Notably, *E. costatus* (SAG 46.88) was resolved as a member of the 'core *Coelastrella*' clade (Wang *et al.*, 2019).

Coelastrella exhibits considerable morphological heterogeneity. Its species are unicellular or form few-celled aggregations and are characterized by spherical, elliptical, fusiform, or citriform cells. The cell wall is typically decorated with multiple meridional ribs (ranging from 16–40), sometimes accompanied by polar thickenings (Kalina & Punčochářová, 1987; Kaufnerová & Eliáš, 2013; Uzunov *et al.*, 2008). However, some species lack ribs entirely (Wang *et al.*, 2019, 2021). Each cell contains a single cup-shaped chloroplast with a pyrenoid surrounded by starch plates. Asexual reproduction occurs via autospore formation, typically, 2–32 autospores are produced and released through the rupture of the parental cell wall (Tschaikner *et al.*, 2007). These morphological traits have traditionally been used for species delimitation, although molecular data are also essential (Krivina *et al.*, 2024).

Coelastrella species are highly adaptable, colonizing a wide range of habitats from terrestrial to aquatic environments. They are frequently found in aerial habitats such as rock surfaces, tree bark, and artificial structures, including brick walls, as well as soils (Hanagata, 2001; Kawasaki *et al.*, 2020; Mikhailyuk *et al.*, 2019; Novakovskaya *et al.*, 2021). The genus' distribution extends from Arctic regions to tropical climates, highlighting their remarkable ecological flexibility (Boutarfa *et al.*, 2022; Nayana *et al.*, 2022; Skrebovska & Kostikov, 2012). Several *Coelastrella* strains exhibit extreme tolerance to environmental stressors (Hu *et al.*, 2013) and are among the pioneering organisms colonizing anthropogenically disturbed sites (Neofotis *et al.*, 2016).

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Coelastrella species attracted significant attention for their remarkable ability to accumulate high-value compounds, including carotenoids and fatty acids, making them promising for biotechnological applications (Abe *et al.*, 2007; Ali *et al.*, 2022; Hu *et al.*, 2013; Kawasaki *et al.*, 2020; Narayanan *et al.*, 2018). They are also used in environmental biomonitoring, nutrient removal, and the synthesis of nanoparticles (Abe *et al.*, 2007; Luo *et al.*, 2016; MubarakAli *et al.*, 2013). Some species produce secondary carotenoids like astaxanthin and lutein, with antioxidant properties valuable for food, pharmaceutical, and cosmetic industries (Wang *et al.*, 2019), while others accumulate lipids suitable for biodiesel production and demonstrate potential for bioremediation (Aburai *et al.*, 2013; Hu *et al.*, 2013; Maltsev *et al.*, 2021).

A significant number of *Coelastrella* sequences in the GenBank confirm considerable interest in the genus (Wang *et al.*, 2019). Studies on *Scenedesmus*-like microalgae, based on an integrative approach, showed that nuclear markers, such as SSU and ITS rDNA, generally provide lower resolution than the plastid genes *rbc*L and *tuf*A in the family Scenedesmaceae (Wang *et al.*, 2019). Nevertheless, SSU rDNA was widely used in phylogenetic analyses (Kaufnerová & Eliáš, 2013; Wang *et al.*, 2019), and the ITS2 spacer region, with its secondary structure analysis, provided additional insights (Ancona-Canché *et al.*, 2017; Hegewald *et al.*, 2010; Krivina *et al.*, 2024; Novakovskaya *et al.*, 2021).

Currently, the genus *Coelastrella* comprises 19 species (Guiry & Guiry, 2025), of these, eight taxa have been described since 2019 based on an integrative approach: *C. affinis* Krivina, Sinetova, Zadneprovskaya, Shibzukhova, Lobakova & Temraleeva; *C. chongqingensis* Q.Wang, H.song & G.Liu; *C. cogersae* Suarez-Montes, Borrell & J.M.Rico; *C. ferroedaphica* Novakovskaya, Patova & Shadrin; *C. polaris* Krivina, Sinetova, Anissimova & Temraleeva; *C. tenuitheca* Q. Wang, H.Song, X.Liu, G.Liu & Z.Hu; *C. thermophila; C. yingshanensis* Q.Wang, H.Song, X.Liu, G.Liu & Z.Hu. This significant expansion of the species number suggests that *Coelastrella* diversity remains incompletely explored.

In this study, we characterized three green algal strains from soil samples collected at the Russian Far East. Molecular and phenotypic data suggested that these strains represent a new species.

MATERIAL AND METHODS

Study site, culture conditions and microscopy

Soil samples were collected in broad leaved coniferous mixed forest (Haplic Cambisol soil type) in July 2021 in the temperate monsoon climate zone of the Jewish Autonomous Region and Primorsky Territory, Russian Far East (Fig. S1, Table 1).

Sampling was carried out according to a standard protocol (Kuzyakhmetov & Dubovik, 2001). The strains of green algae were isolated by the micro-pipette method (Andersen, 2005) and then cultured in liquid and agar Waris-H nutrient medium (McFadden & Melkonian, 1986) at 20–22°C with a photon fluence of 17.9–21.4 μmol photons•$m^{-2}s^{-1}$ in a 16:8 h light/dark cycle. The strains were maintained in the culture collection of the Laboratory of Botany in the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Russia (strain numbers VCA-272, VCA-273, and VCA-274). Morphology of vegetative and reproductive cells was examined using an Olympus BX 53 light microscope (Olympus Corporation, Tokyo, Japan) equipped with Nomarski DIC optics. The cultures were repeatedly examined throughout their life cycle stages, i.e. in cultures of different (3, 6, 12 months) ages.

Cell wall structures were visualized by scanning electron microscopy (SEM) following the protocol of Novakovskaya *et al.* (2021), with modifications. The samples used for SEM analysis were cultivated in liquid Waris-H medium for at least 3–4 weeks. Microscopic slides (d = 12 mm) were coated with poly-L-lysine solution to aid cell adhesion. The gradual dehydration of the cells was achieved by dipping them into an ethanol series of 10%, 30%, 50%, 70%, 90%, and 96% for 10 min each, followed by transferring them into an ethanol: isoamyl acetate solution (1:1), and subsequently into 100% isoamyl acetate. Thereafter, the samples were critical point dried. The prepared samples were sputtered with an alloy of gold–palladium (Au–Pd, 6:4) and studied with a Merlin SEM (Carl Zeiss, Jena, Germany) at the Instrumental Centre of Biotechnology and Gene Engineering of FSCEATB FEB RAS. The fluorescence of chloroplasts in living cells was examined with a CLSM 710 LIVE (Carl Zeiss, Oberkochen, Germany) at the same instrumental centre. Chloroplast autofluorescence was recorded in the additional emission channel after 600 nm, using a Plan-Apochromat 63×/1.40 Oil DIC M27 objective with digital zoom. For visualization of nuclei, live algal cells were stained with Hoechst 33342 (Thermo Fisher Scientific, Rockford, USA). To increase membrane permeability 1 μl of 0.01% Triton X-100 was added to the culture. Z-stack of cells was recorded and analysed with ZEN 2011 software.

DNA extraction, amplification, and sequencing

For DNA analysis, the cultures were harvested during the exponential growth phase and concentrated by centrifugation.

Table 1. Location of the sample sites, strain information, and habitat characterization.

№	Strain	GenBank accession numbers (SSU+ITS/tufA)	Sampling site	Coll. Date
1	VCA-272 (holotype)	PV081916/PV067674	State Nature Reserve 'Bastak' (Jewish Autonomous Region), 49°05′N 133°04′E	01 Jul. 2021
2	VCA-273	PV081918/PV067676	The vicinity of the villages of Roshchino and Nezametnoye (Primorsky Territory), 45°57′N 134°57′E	15 Jul. 2021
3	VCA-274	PV081917/PV067675	The vicinity of the villages of Roshchino and Nezametnoye (Primorsky Territory), 45°57′N 134°57′E	15 Jul. 2021

Total genomic DNA was extracted, as described previously by Abdullin *et al.* (2021). For the amplification of the SSU rDNA and the ITS region, the following primers were used: 82F (López-García *et al.*, 2003), ITS4R (White *et al.*, 1990). PCR parameters were described previously Mikhailyuk *et al.* (2018). The PCR products were purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, CA, USA) and sequenced in both directions using an ABI 3500 genetic analyser (Applied Biosystems, Waltham, MA, USA) with a BigDye terminator v.3.1 sequencing kit, with the amplification primers, plus SSU528F-800 (Hoef-Emden & Melkonian, 2003), 920F (Marin *et al.*, 1998), n1400R (Marin *et al.*, 2003), and Bd18SF1 (Goka *et al.*, 2009). The *tuf*A sequence was amplified using the primer pair *tuf*AF and *tuf*AR (Famà *et al.*, 2002). Sequences were edited and assembled with the Staden Package v.1.4 (Bonfield *et al.*, 1995). The sequences covering a partial SSU rDNA region, the complete ITS region, and the *tuf*A gene were deposited in GenBank (accession numbers in Table 1).

Alignment, secondary structure modelling, and datasets

To clarify the phylogenetic position of the new strains, four datasets were used (Fig. S2). The datasets were enriched with sequences that showed similarity as inferred from the BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 10 January 2025). Sequence alignment was conducted using the SeaView program (Galtier *et al.*, 1996) with manual correction based on the secondary structure information for the ITS rDNA dataset. Alignments were trimmed by hand to remove introns in SSU rDNA and ambiguously aligned regions in ITS rDNA.

The Mfold web server (Zuker, 2003) was used with the default settings to predict the ITS2 secondary structures, which were then visualized using the program 4SALE (Seibel *et al.*, 2008). The ITS2 model was constructed based on the consensus secondary structure model of *Coelastrella* proposed by Kaufnerová & Eliáš (2013). The ITS2 sequence alignment was translated into the barcode alignment by replacing each base-pair by a number (A–U = 1; U–A = 2, G–C = 3, C–G = 4, G•U = 5, U•G = 6, mismatch = 7, deletion or unpaired or single bases = 8) as described in Novakovskaya *et al.* (2021).

Phylogenetic analysis

Phylogenetic trees were constructed using maximum likelihood (ML) in IQTREE 2 (Minh *et al.*, 2020) and Bayesian inference (BI) in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Akaike information criterion (Akaike, 1974) implemented in jModelTest 2.1.1 (Darriba *et al.*, 2012) was used to determine the most appropriate DNA substitution model for the datasets (Table S1). In BI, four parallel MCMC runs were carried out for two (SSU rDNA, SSU and ITS rDNA), five (ITS rDNA), and one (*tuf*A) million generations. Sampling was carried out every 100 generations. Convergence of the chains was assessed, and stationarity was determined according to the 'sump' plot with the first 25% of samples discarded as burn-in; posterior probabilities were calculated from the remaining trees. The robustness of the ML trees was estimated using ultrafast bootstrap (1000 replicates (UFBP); Hoang *et al.*, 2018) and posterior probabilities (PP) in the BI. Pairwise distances (*p*-distances) were estimated using MEGA 11 (Tamura *et al.*, 2021).

RESULTS

Morphological observation and taxonomic implications

Vegetative cells of the studied strains were solitary and characterized by a spherical, or rarely, ellipsoidal shape, with several small vacuoles and without polar thickenings (Figs 1, 2). The chloroplast was parietal cup-shaped in young cells and with several lobes in mature cells (Figs 2, 3), with a single pyrenoid surrounded by a starch envelope (Figs 1, 2). A central nucleus is seen in vegetative cells (Fig. 3). Asexual reproduction occurred via formation of 2–8 autospores (Figs 4, 5). Neither coenobia formation nor sexual reproduction involving flagellated cells was observed. Light and electron microscopy confirmed a smooth outer wall and the absence of meridional ribs on the cell wall (Figs 7, 8). Old cells (Fig. 6) had small orange globules (possibly containing lipid droplets and carotenoids). Prolonged cultivation resulted in an overall orange colouration of the culture (Fig. 9).

The studied strains (VCA-272, VCA-273, and VCA-274), exhibited typical *Coelastrella*-like morphology (Figs 1–8, S3–14; Table S2). They differ from other *Coelastrella* species that lack meridional ribs on the cell wall (*C. tenuitheca* and *C. chongqingensis*) by cell dimensions, the maximum number of autospores in the autosporangium, and its distinct phylogenetic position based on SSU, ITS rDNA, and *tuf*A gene phylogenies (see below). Based on this we propose a new species for these strains.

Coelastrella laevis V.Yu.Nikulin, R.Z.Sushchenko, Sh.R. Abdullin, A.Yu.Nikulin & A.A.Gontcharov *sp. nov.*

Figs 1–9

DESCRIPTION: Cells solitary, spherical (4.9–7.1 μm), rarely ellipsoidal (5.6–9.1 × 5.9–9.7 μm), uninucleate, with a thin, smooth cell wall (lacking meridional ribs) without polar thickenings, and with several small vacuoles. The chloroplast cup-shaped in young cells and with several lobes in mature cells, parietal; a single pyrenoid surrounded by starch envelope. Asexual reproduction by 2–8 autospores. Sexual reproduction and flagellate cells unknown. GenBank accession numbers: SSU+ITS, PV081916; *tuf*A, PV067674.

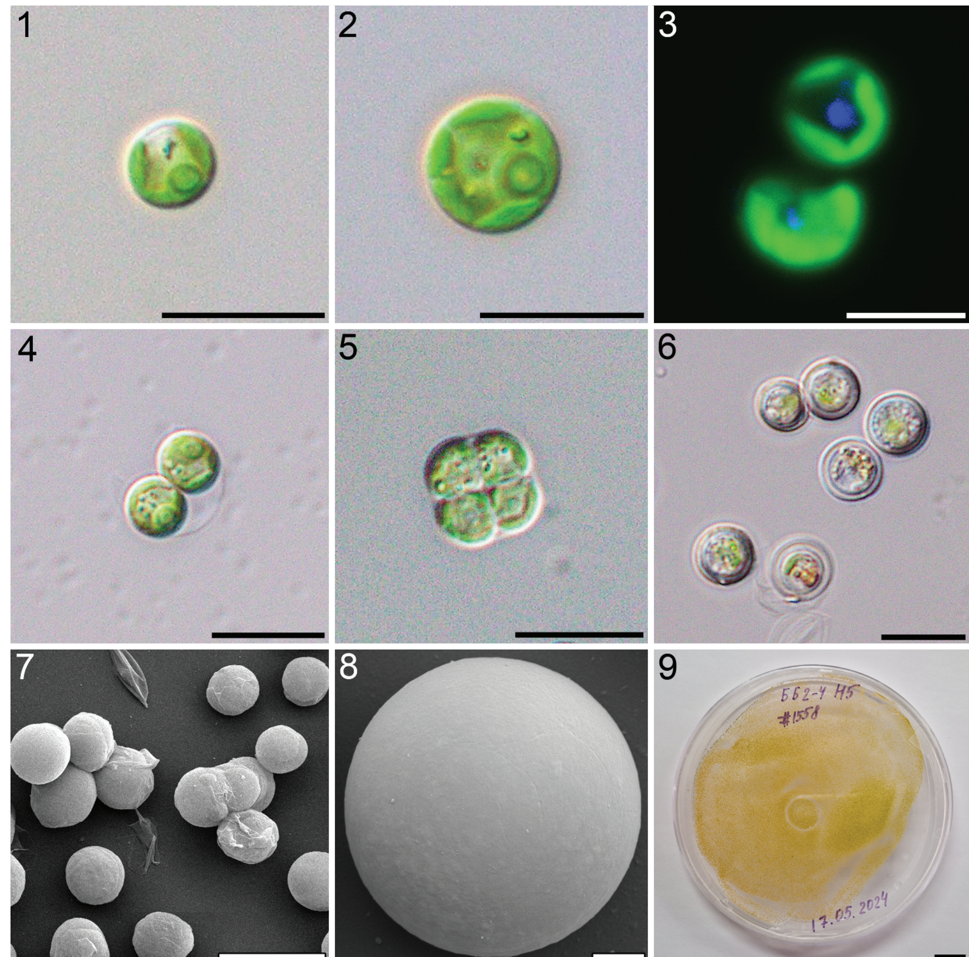
HOLOTYPE: strain VCA-272 (voucher number VLA-CA-1558), dried biomass of a unialgal population deposited in the Herbarium of the Federal Scientific Center of the East Asia Terrestrial Biodiversity (VLA), Vladivostok, Russia.

TYPE LOCALITY: Russia, Jewish Autonomous Region, State Nature Reserve 'Bastak' (49°05′N 133°04′E), on forest soil (Haplic Cambisol).

ETYMOLOGY: From Latin *laevis* (smooth), referring to its smooth surface.

Phylogenetic analyses

Phylogenetic analyses of SSU rDNA and ITS both indicated the monophyly of *Coelastrella* (Figs S15, S16) and indicated that our new strains were distinct. A combined analysis of the SSU and ITS rDNA placed *C. laevis sp. nov.* as a robust



Figs 1–9 Micrographs illustrating general morphology of *Coelastrella laevis sp. nov*. (strain VCA-272) and a photograph of culture on solid medium.
Fig. 1. Young cell. Scale bar = 10 µm.
Fig. 2. Mature cell. Scale bar = 10 µm.
Fig. 3. 3D confocal microscopy visualization of chloroplast (green) and nucleus (blue). Scale bar = 5 µm.
Fig. 4. Autosporangium with two autospores. Scale bar = 10 µm.
Fig. 5. Autosporangium with four autospores. Scale bar = 10 µm.
Fig. 6. Old cells showing orange inclusions. Scale bar = 10 µm.
Fig. 7. General SEM view. Scale bar = 10 µm.
Fig. 8. Cell with a smooth cell wall. Scale bar = 1 µm.
Fig. 9. Orange pigmentation of an ageing culture on solid medium. Scale bar = 1 cm.

lineage within the *Coelastrella* clade, although without support (Fig. 10). *Coelastrella* also included *Asterarcys quadricellularis* and *Enallax costatus* (Figs S15, S16). Resolution within the *Coelastrella* clade was weak in all analyses. *Coelastrella laevis* strains differed from each other by a substitution in the SSU rDNA exon region (VCA-272), seven substitutions in the intron region (VCA-274) and the presence of one (VCA-272 and VCA-273) or two (VCA-274) introns. The sequence divergence of SSU rDNA gene among *Coelastrella* species was from 0.17–7.66% (Table S3).

The ITS dataset included all available *Coelastrella* sequences from the GenBank database. Phylogenetic analyses identified most species clades with high to moderate support, but branching pattern of the basal clades remained unresolved (Fig. S16). *Coelastrella laevis* accessions formed a clade showing no affinity to any other lineage. A well-supported clade comprising 20 unidentified *Coelastrella* accessions was established. Thus, the ITS data suggest the presence of hidden diversity in *Coelastrella*. The ITS region showed a high sequence divergence, ranging from 2.72–16.72% (Table S4). ITS sequences of *C. laevis* differed from those in other *Coelastrella* species by more than 9.49%.

To identify sequence variations in *C. laevis* that distinguish it from other species, we constructed its ITS2 secondary structure model (Fig. S17). The predicted model revealed four helices and five single-stranded domains typical for *Coelastrella*. The studied strains displayed branched helix I, a common feature for members of the genus. Strains VCA-272 and VCA-273 shared identical sequences, whereas VCA-274 differed in three substitutions in single-stranded regions (apical and lateral loops of helices I and III).

Further insights into nucleotide signatures of *C. laevis* were obtained through comparative ITS2 secondary structure analysis, using the conservative region barcoding approach of Novakovskaya *et al.* (2021). The 5.8S-LSU stem region was conserved across all accessions (Fig. 11).

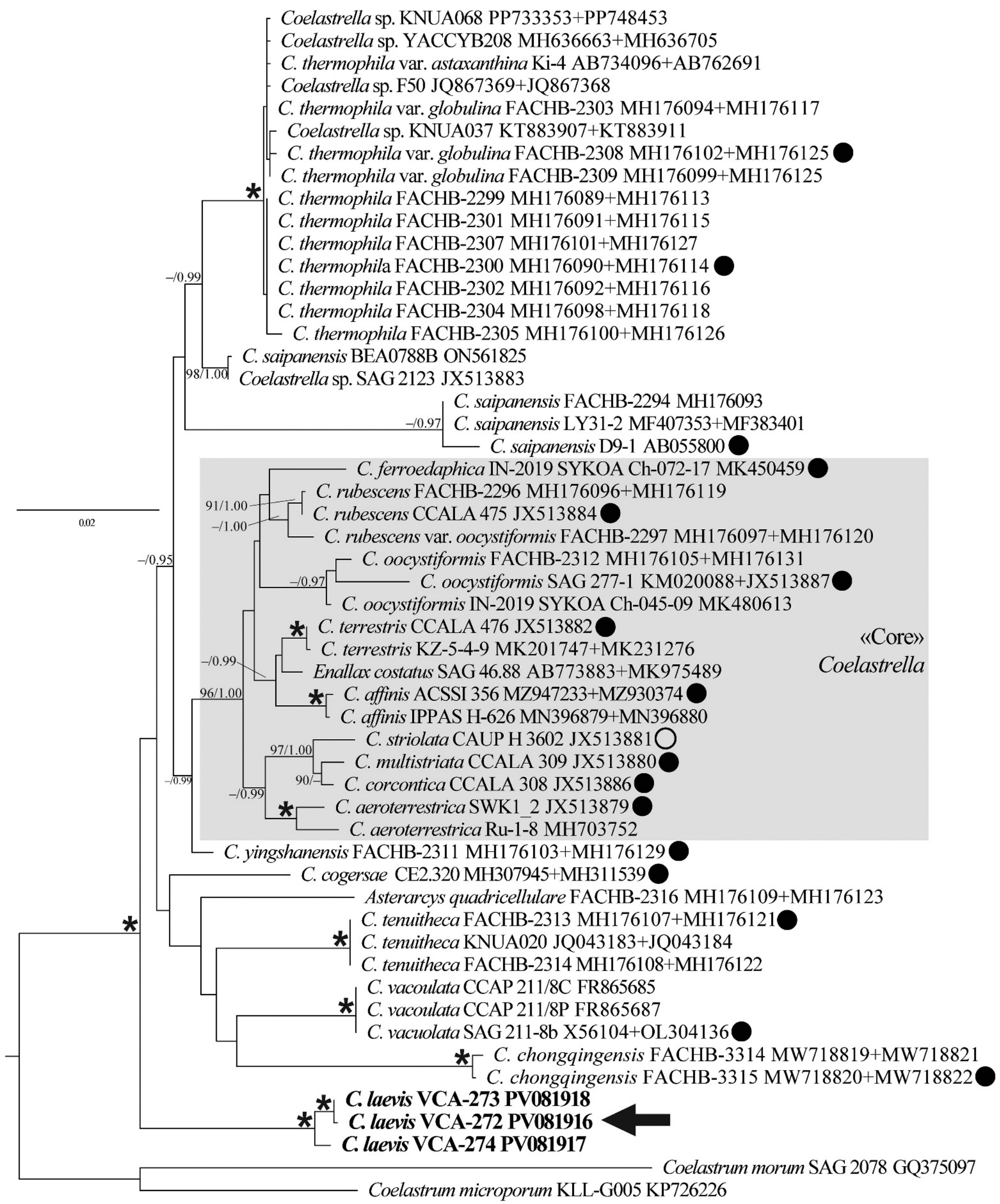


Fig. 10. ML phylogeny of the genus *Coelastrella* showing the position of *C. laevis sp. nov*. based on combined SSU and ITS rDNA sequence data (53 sequences, 2348 aligned positions). Black circles mark type strains. Unfilled circle = the type species of the genus. Support values [(UFBP) ≥ 90% and (PP) ≥ 0.95: ML/BI] are provided at branches. *Coelastrella laevis sp. nov*. strains are shown in boldface and indicated by an arrow. Nodes with 100% BP and 1.00 PP are marked with asterisk.

The barcoding analysis further indicates that helix I exhibited one CBC and one hCBC, attributed to the first base pair. Three variable positions were identified in helix II (one CBC, two hCBCs, and one mismatch). Helix III was the most variable with 13 variable positions, including three CBCs, nine hCBCs, and five positions with indels or mismatches. The strains studied were characterized by five unique substitutions/indels in helices II (one hCBC; Fig. 11) and III (one indel, two hCBCs and one CBC). The presence of specific CBC and hCBC patterns in helices II and III further supported recognition of *C. laevis* as a distinct species. The closest barcode match was *C. cogersae*, which shared three variable positions.

Phylogenetic analyses of chloroplast *tuf*A gene sequences (Fig. 12) confirmed the distinctiveness of *C. laevis* with high support (98/0.99) but was unable to resolve the branching order within the genus. The GenBank database currently contains *tuf*A sequences for eight *Coelastrella* species only, and for six of these sequences type strains are available. *Coelastrella laevis* samples had identical *tuf*A gene sequences, differing by 2.48–3.85% from other species (Table S5). Overall, intraspecific distance was low in the genus, ranging from 0–0.21%.

DISCUSSION

In the present study, a novel species, *Coelastrella laevis sp. nov*., was described using an integrative approach from soils in eastern Russia. Nuclear SSU and ITS rDNA sequence

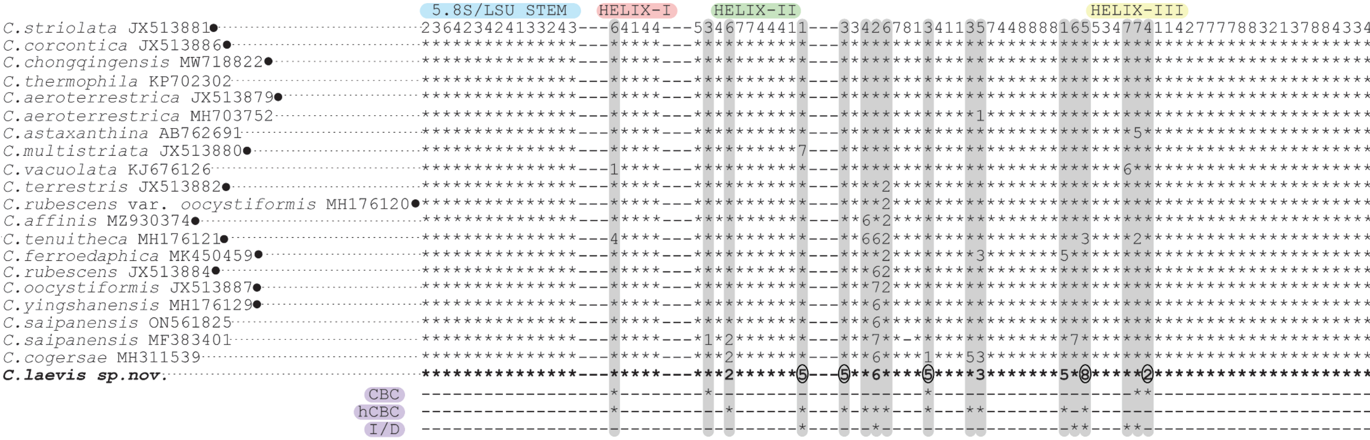


Fig. 11. Comparison of the conserved region of ITS2 among the species of *Coelastrella*. Number code for each base pair: 1 = A-U; 2 = U-A; 3 = G-C; 4 = C-G; 5 = G•U; 6 = U•G; 7 =mismatch; 8 = deletion, single or unpaired bases. The sequence of *C. striolata* is taken as a reference. Equal base-pairs are marked with asterisks. The barcode of new strains is shown in boldface. Grey shading indicates variable positions. Unique CBCs and hCBCs discussed in the text are circled. Black circles indicate type strains.

Fig. 12. ML phylogeny of the genus *Coelastrella* based on *tuf*A gene sequence data (900 aligned positions, 32 sequences) showing the position of *C. laevis sp. nov*. See Fig. 10 legend for details. Strains with similar morphotypes *sensu* Wang *et al*. (2019) are highlighted by different colours.

comparisons, as well as the chloroplast *tuf*A gene, consistently resolved the new species as a part of the *Coelastrella* clade, likely representing an early diverging lineage (*Coelastrella sensu lato* group). The species, represented by three strains, was found in two localities situated *ca*. 380 km apart. Only minor sequence variations in SSU and ITS rDNA (0.39% and 0.96%, respectively) were observed between strains, suggesting intraspecific variability rather than interspecific divergence. At the same time, ITS sequence divergence between *C. laevis* and the rest of the genus (>9.5%) was high, highlighting the distinctiveness of the new species. Furthermore, species-level differentiation was also evident in the ITS2 barcode regions, with five unique substitutions/indels in helices II and III serving as distinct molecular signatures of our alga.

Coelastrella sequence sampling ranged from 146 (ITS region) to 28 (*tuf*A) accessions in the datasets used. *Enallax costatus* and *Asterarcys quadricellularis* were also included because previous studies demonstrated their affinity to *Coelastrella* (Kaufnerová & Eliáš, 2013; Wang *et al*., 2019). Our datasets fully confirmed this close relationship and raised a question about the concepts of the genera *Enallax* and *Asterarcys* as they are nested in the *Coelastrella* clade. All markers (*tuf*A, ITS rDNA, and SSU+ITS rDNA) supported most *Coelastrella* species but mostly failed to resolve their relationships. Analyses of the ITS rDNA dataset revealed a well-supported divergent clade comprising 20 unidentified *Coelastrella* accessions, suggesting existence of an undescribed species within the genus. Notably, this lineage showed affinity to *Asterarcys quadricellularis*. Phenotypic characterization of these strains is needed to compare their features with those in *Asterarcys* and *Coelastrella*.

It should be noted that *C. laevis* possesses no unique morphological traits; rather, the new species is characterized by a combination of features found in other members of the genus (Table S2). Vegetative cells of *C. laevis* have smooth cell walls without meridional ribs and polar thickenings, which is typical for a number of *Coelastrella* species, representing morphotype 3 according to Wang *et al*. (2019). From these *C. laevis* differs by significantly smaller (4.9–7.1 μm) spherical or rarely ellipsoidal cells and a smaller number of autospores (8 at maximum; Table S2).

Coelastrella laevis resembles *Chlorella* and genera segregated from it recently, example *Micractinium* and some other genera in gross morphology, which poses a question: could the new species be related to morphospecies of green microalgae not tested yet with molecular tools? A combination of a smaller number of autospores (2–8 *vs*. 2–64), the presence of several vacuoles lacking in some *Chlorella* spp., as well as the chloroplast that fits tightly and occupies nearly the entire inner cell surface in mature cells distinguishes *C. laevis* from *Chlorella*-like algae. Representatives of *Ettlia, Chlorococcum*, and *Tetracystis*, also having similar appearance, but reproduce by zoospores that are not known for *C. laevis*. Thus, a set of phenotypic features allows the species differentiation even under LM.

Four distinct morphotypes based on phylogenetic data and morphological criteria were recognized in *Coelastrella* (Wang *et al*., 2019, 2024). Morphotype 1 exhibits typical characteristics of the original *Coelastrella* (globose to broadly ellipsoidal cells without or with very tiny polar thickenings and 16–40 meridional ribs; including the species *C. saipanensis, C. thermophila*), while morphotype 2 possesses traits of the original *Scotiellopsis* and *Enallax* (fusiform or citriform cells with 4–12 (20) meridional ribs and polar thickenings; *C. oocystiformis, C. rubescens* (Vinatzer) Kaufnerová & Eliáš, and *E. costatus*). Morphotype 3 is characterized by smooth cell walls lacking ribs (*C. chongqingensis, C. vacuolata*, and *C. tenuitheca*), whereas morphotype 4 is defined by four-cell coenobia within a mucilage envelope (*Asterarcys quadricellularis*). Wang *et al*. (2019) previously noted that species with morphotype 1 do not form a monophyletic group and a further study by Novakovskaya *et al*. (2021) indicated similar pattern for species classified as morphotype 2. Inclusion of *C. laevis* into a *tuf*A dataset now challenges earlier monophyly of morphotype 3. It should be noted that the presence of ribs in *C. vacuolata*, being a part of morphotype 3 remains uncertain. Kalina & Punčochářová (1987) described its cell wall as having a fine network of ribs visible only under electron microscopy (as *Graesiella vacuolata* (Shihira & Krauss) Kalina & Punčochářová). Some further studies reported ribbed cell walls in *C. vacuolata* (Kawasaki *et al*., 2020; Song & Lee, 2014), while others did not observe these structures (Senthil *et al*., 2019; Shetty *et al*., 2021). It is possible that the presence of the cell-wall decoration is age-dependent in *C. vacuolata*. Andreeva (1998) noticed that the type strain of this species forms robust meridional ribs easily detectable by light microscopy in aged liquid culture. In contrast to that, in frequently transferred actively growing cultures these ribs were not detectable. Shetty *et al*. (2021) used seven-day-old cultures for SEM, although 2–3 weeks-old cultures were typically used before. Keeping this in mind we repeatedly examined cell wall surface of *C. laevis* with SEM in 3–4 weeks and 5–-6 weeks-old cultures but did not detect any surface structures. Thus, we conclude that the absence of ribs in *C. laevis* is not age-dependent but a stable morphological trait.

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