Laetitia sardoa gen. & sp. nov., a new member of the Chlorellales (Trebuoxiophyceae, Chlorophyta) isolated from Sardinia Island

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ABSTRACT

A Sardinian small trebouxioiphycean alga, Laetitia sardoa, is described as a novel species and genus. This green alga was collected in an oligotrophic freshwater habitat, and its morphology was investigated. The strain established belongs to the order Chlorellales, of the class Trebouxiophyceae, as inferred from phylogenetic analyses of nuclear-encoded 185 rDNA and chloroplast-encoded ribulose-bisphosphate carboxylase-oxygenase gene (rbcL). The analyses supported Laetitia as a distinct lineage, sister to a Marvania clade. The taxonomy of this order has long been problematic due to the lack of distinguishable morphological characteristics. With the characterization of this microalgal strain, we confirmed the necessity of using molecular analyses to determine these cryptic algae. Moreover, our study contributes to the knowledge of the taxonomy and ecology of Chlorellales (Chlorophyta). In addition, the discovery of this new genus in the Ogliastra’s Tacchi suggests the potential diversity of this area, which up to now has remained largely unknown, and increases the general knowledge of the microalgal flora of this region.

INTRODUCTION

The Mediterranean basin is usually considered to be quite rich in terms of biological diversity and, in particular, Sardinia (Italy) represents a biodiversity hotspot (Moris 1837–1859; Grill et al. 2007; Cappadone et al. 2019). In Sardinia, areas with high levels of endemism generally coincide with mountains (Grill et al. 2007). The historic region of Ogliastra, being situated in the Central East area, is particularly relevant as it includes ecosystems with high biodiversity. One of the most important landscapes is the Jurassic dolomitic-limestone plateau called ‘Tacchi’.

Molecular diversity and phylogeny of the microalgal flora from freshwaters and the terrestrial habitats in this area are almost unexplored. In fact, most of the existing studies in Ogliastra focused on the vascular flora and endemic species (e.g. Bocchieri & Mossa 1986; Bocchieri & Mulas 1990; Bocchieri 1998; Loi & Lai 2001; Bocchieri & Iriti 2003; Loi et al. 2004; Sanna et al. 2006; Arrigoni 2007; Bocchieri et al. 2009; Fenu et al. 2010; Malavasi 2010).

The freshwater and terrestrial algal flora of Ogliastra Tacchi have previously been investigated by microscopic observations (Malavasi 2012), but no studies specifically focusing on coccoid green algae were performed in this area. Currently, only two eukaryotic microalgae were described from a molecular characterization point of view (Škaloud et al. 2014; Sausen et al. 2018). During a microalgal floristic investigation in the territory of Seui (Ogliastra), we established a new isolate of a coccoid green alga.

By light microscopy, this strain morphologically resembled the Nannochloris-like species. Most described genera of Chlorellales present morphological similarities to one another. Examples of similar-looking genera include Marvania Hindák, Nannochloris Naumann and Picochlorum Henley, Hironaka, Guillou, M.A. Buchheim, J.A. Buchheim, M.W. Fawley & K.P. Fawley. It is important to note that the name Picochlorum is illegitimate according to ICN Art. 52.1 (Turland et al. 2018) since, as originally circumscribed by Henley et al. (2004), it included the type species of Nannochloropsis, N. oculata (Droop) D.J. Hibberd. Over time, several authors have pointed out that molecular and phylogenetic support has become indispensable for the determination of Chlorella- and Nannochloris-like species (e.g. Huss & Sogin 1990; Henley et al. 2004; Bock et al. 2011; Somogyi et al. 2011, 2013; Krienitz et al. 2015; Hodač et al. 2016; Metz et al. 2019; Temraleeva et al. 2022). Indeed, the limited number of morphological characteristics and the small dimensions of vegetative cells make the morphological determination of these cryptic strains difficult, if not impossible.

The primary aim of this study was to characterize this isolate by the combination of morphological, ultrastructural and molecular investigations. In this paper, based on the description of the new SCCA 034 strain, we confirmed the necessity of molecular analyses as a first tool to evaluate the diversity and identity of unicellular green coccoids. However, morphological and ultrastructural investigations remain important to characterize and describe new taxa.

MATERIAL AND METHODS

Isolation and cultivation

The strain SCCA 034 was isolated on 7 June 2010 in the locality of Mount Arquerì (Central-eastern Sardinia, Italy,
39°51.60’N, 9°22.31’E) near an artificial spring (Fig. 1), from an oligotrophic freshwater habitat.

This geographic area is characterized by state-owned forest that stands out on the slopes of Monte Arbu and Tonnerri. The coolest slopes are dominated by Quercus ilex Linnaeus, and further down there is a strip of Mediterranean scrub.

The European Community has identified in this area the ‘Monti del Gennargentu’ as a Site of Community Importance (SIC). Specifically, the Natura (2000), which is a network of core breeding and resting sites for rare and threatened species, has recognized this SIC as an important area for reproduction for many species of community interest present in Sardinia (Natura 2000). The following priority habitats are present in this SIC: 6220, Pseudo-steppe with grasses and annuals of the Thero-Brachypodietea; 9580, Mediterranean Taxus baccata Linnaeus woods.

In this site, it is possible to find a large number of endemic and rare vascular plants, such as Lamyropsis microcephala (Moris) Dittrich & Greuter, Euphrasia genartgentea (Feoli) Diana, Tanacetum audiberti De Candolle and Paeonia corsica Sieber ex Tausch.

A part of the source’s wall was covered by moss and green mucilage (Fig. 1). The collection was made by picking up the ‘green material’ from the wall of the artificial spring with a pocket-knife, together with spring water. See Malavasi (2012) for more details, description of the site and its algal flora.

The alga was isolated by micropipetting with glass capillaries (Surek & Melkonian 2004), with an Olympus CKX41 inverted light microscope (Olympus, Tokyo, Japan). After isolation, stock cultures were established under controlled laboratory conditions (25°C, 12:12 h light:dark cycle, 80–100 μmol photons m⁻² s⁻¹). The alga was maintained in sterile liquid and on agarized Bold’s Basal Medium (BBM; Bischoff & Bold 1963).

**Documentation and observation**

Light microscope observations were made using two- to three-week old cultures (after subculturing) with an Olympus CKX41 light microscope, and morphometric measurements were made using LMicro camera imaging software. Photographs were taken with a Visitron Systems Spot Pursuit (Visitron Systems, Puchheim, Germany) or with a Canon EOS 40D system (Canon, Tokyo, Japan).

For observations in transmission electron microscopy (TEM), samples were fixed at 5°C in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0, overnight, post-fixed for 2 h at 5°C in 1% osmium tetroxide in 0.05 M phosphate buffer (Glauert 1984) and then immersed in 1% uranyl acetate in methanol for 1 h at room temperature. After dehydration through an ethanol series (70%, 96% and 100%), cells were embedded in Spurr’s resin (Spurr 1969) via isobutanol. Ultrathin sections, cut with a diamond knife on an Ultracut E (Reichert-Jung, Wien, Austria), were post-stained with lead citrate and examined using a JEOL 1011 TEM (JEOL Ltd., Tokyo, Japan). Photomicrographs were obtained using a Veleta CCD camera equipped with image analysis software (Olympus Soft Imaging Solution).

**DNA extraction, PCR amplification and sequencing**

Fresh culture was centrifuged and used for DNA extraction following the CTAB protocol (Cubero et al. 1999).

The nuclear-encoded SSU rDNA was amplified using the primers 18S-F (5’-AAC CGT GAT CCT GCC AGT-3’) and 18S-R (5’-TGA TCC TTC TGC AGG TTC ACC TAC G-3’) (Katana et al. 2001). The PCR amplification was carried out under the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min; final extension at 72°C for 10 min.

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**Fig. 1.** Sampling site of *Loetitia sardoa* SCCA 034, near an artificial spring, Mount Arqueri, Seui (Central-eastern Sardinia, Italy).
The chloroplast-encoded \(\text{rbcL}\) was amplified using the primers PRASR1–3 (5’-TTG TCA ATA GTA TCA AAT TC-3’; Sherwood et al. 2000) and \(\text{rbcL}-203-5\)-MPN-5’ (5’-GAA TCW TCW ACW GGW ACT TGG ACW AC-3’; Nelsen et al. 2011).

The PCR amplification was carried out under the following conditions: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and elongation at 72°C for 1.3 min; final extension at 72°C for 10 min. The PCR reactions were performed in a total volume of 20 μl, containing 0.1 μl of Polymerase My Taq HS Red DNA, 6.7 μl of sterile Milli-Q water and 0.3 μl of each primer.

Furthermore, we obtained the ITS DNA sequence using the primers zeleny_F2 (5’-TTG TTA GTT GGT GGG TTG CC-3’; Moya et al. 2018) and the universal primer ITS4 (White et al. 1990). The sequence was deposited in GenBank with the accession number MZ955368. No phylogenetic analyses were performed based on this gene due to a limited number of available sequences (only 13 sequences) of related strains and by their high dissimilarity (number of saturated positions) that would have make a construction of unambiguous alignment very limited.

After checking the quality of PCR products on agarose gel, the PCR products were purified using Agencourt AMPure XP Magnetic Beads (Beckman Coulter) according to the manufacturer’s protocols. The purified PCR products were sequenced with the amplification primers at Macrogen in Amsterdam (Macrogen, Seoul, Korea).

The newly obtained sequences, excluding the primer regions, were deposited in GenBank, and their accession numbers are listed in bold in Table S1.

**Phylogenetic analyses**

The SSU \(\text{rDNA}\) and \(\text{rbcL}\) sequences downloaded from the GenBank were aligned with the newly obtained sequences by MAFFT v7 (Katoh & Standley 2013) using the Q-INS-I method. The 18S \(\text{rDNA}\) sequence of SCCA 034 comprised 1690 bp, including one incompletely sequenced intron excluded from the alignment. The \(\text{rbcL}\) sequence comprised 1297 bp. Final datasets consisted of 73 SSU \(\text{rDNA}\) and 20 \(\text{rbcL}\) Chlorellales sequences, using the Oocystaceae as the outgroup (Table S1). Substitution models, were estimated with the Bayesian Information Criterion using jModelTest v2.1.4 (Darriba et al. 2012) as follows: GTR + I + G for SSU \(\text{rDNA}\) (p-inv 0.47, gamma shape 0.39), GTR + I + G for the first codon position of \(\text{rbcL}\) (p-inv 0.70, gamma shape 1.39), JC + I for the second codon position of \(\text{rbcL}\) (p-inv 0.91) and GTR + I + G for the third codon position of \(\text{rbcL}\) (p-inv 0.16, gamma shape 1.20).

The phylogenetic trees were inferred by Bayesian Inference in MrBayes v3.2.6 (Ronquist et al. 2012) for each locus separately, as well as on a concatenated dataset using the four partitions. Two parallel MCMC runs, with one cold and three heated chains, were run. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The first 25% of the trees were discarded as burn-in in each run. Fifty percent majority-rule consensus trees were obtained using the sumt option. The bootstrap analyses were performed by Maximum Likelihood analyses (ML) in RAxML 8.0.0 (Stamatakis 2014) using two independent runs and 1,000 pseudoreplicates. The analyses were run on the CIPRES Science Gateway v3.3 web portal (Miller et al. 2010).

**RESULTS**

**Phylogenetic analyses**

The SSU \(\text{rDNA}\) phylogenetic analysis revealed that the strain SCCA 034 represents a novel lineage within the Chlorellales (Fig. 2). The strain was inferred as a sister lineage to the genus *Marvania*, although with a moderate statistical support (0.96 PP). The concatenated SSU \(\text{rDNA}\) + \(\text{rbcL}\) phylogenetic analysis (Fig. S1) did improve the resolution of node support at the backbone of the tree and inferred the strain SCCA 034 as a distinct lineage, sister to a clade comprising a number of genera, including *Nannochloris*, *Picochlorum*, *Marvania* and *Pumilosphaera* Darienko & Pröschold. However, when comparing separate SSU \(\text{rDNA}\) (Fig. 2) and \(\text{rbcL}\) (Fig. S2) phylogenies, two strains exhibited a clear topological incongruence. First, *Radiofilum conjunctivum* Schmidle GR–2 was inferred as a distinct clade within Chlorellaceae, but \(\text{rbcL}\) analysis placed it as a member of Oocystaceae. Second, *Picochlorum eukaryotum* (invalidly published name) UTEX 2491 was inferred far from the *Picochlorum* clade by \(\text{rbcL}\) analysis. Therefore, the concatenated phylogeny should be considered cautiously since it might be negatively affected by conflicting phylogenetic signals.

**Morphology and ultrastructure**

The strain formed mostly spherical cells. In younger cells, the dimensions were 1.9–3.9 μm in diameter, and the cell wall was delicate and adherent (Figs 3, 4, 6, 8, 13, 16). During the cell cycle, older cells increased in size and the cell wall became well developed (Figs 5, 7, 11, 12, 15, 17). When mature, the cells were 4.0–4.5 μm in diameter (Figs 9, 11, 12, 15).

Chloroplasts are parietal and cup-shaped (Figs 3, 8, 14), sometimes occupying most of the cell (Figs 6, 7, 11, 12). Irregular colonies of small cells were occasionally observed in old cultures (over 1 month) on the solid medium. Autosporangia were also spherical, 5.0–6.5 μm in diameter (Figs 10, 20). The number of autosporangia per autosporangium varied widely, from two to eight (Figs 19, 20). Small autosporangia with slightly unequal autosporangia were rarely observed (Fig. 18). The mother cell wall of a two-celled autospore is visible in Fig. 21.

Transmission electron microscopy confirmed the condensed internal organization of the cell typical for small coccoid chlorophytes. Young autospores possessed a relatively thin cell wall, and they stayed sometimes connected by the remnants of the mother cell wall. The large nucleus was usually pressed to the cell periphery. Autosporangia contained one to several small vesicles with oil-like homogenous content (Fig. 22). These vesicles merged and filled almost the whole content in old stationary cells (not shown). The cell wall became thicker during the growth of the cell and the nucleus was surrounded by the chloroplast and pushed to a central position. Elongated mitochondria (Fig. 23, arrowheads), circular in cross-section, contained tubular cristae. Within the chloroplast thylakoids were
Fig. 2. Phylogenetic position of *Laetitia sardoa* sp. nov. based on SSU rDNA, obtained by Bayesian Inference analysis (BI). For each sequence, GenBank accession numbers, taxonomic designations, and, if known, strain information is provided. Support values shown on respective branches in following order: BI posterior probability/maximum likelihood (ML) bootstrap values. Values lower than 50% bootstrap support (from 1,000 pseudoreplications) or 0.8 posterior probability not shown. The newly obtained sequence is given in bold. Scale bar shows the estimated number of substitutions per site.
organized in lamellae. Starch grains were located in the chloroplast stroma between lamellae (Figs 23, 24). No pyrenoid was observed. The ultrastructure confirmed the fully developed cell wall of individual autospores within the autosporangium and the degradation of the mother cell wall before release of the autospores (Fig. 25). The cell wall comprised two layers: a probably cellulosic inner fibrillar wall, 70–100 nm thick (Fig. 26, fl), and an outer layer of trilaminar structure, a narrow dark-light-dark domain 15–20 nm thick (Fig. 26, tl). The trilaminar layer seemed to be relatively resistant, and this portion of the walls accumulated in the medium after degradation of the fibrillar layers (Figs 26, 27).

**TAXONOMY**

*Laetitia* Malavasi, Škvorová, Němcová & Škaloud gen. nov.

DESCRIPTION: Solitary cells that form regular aggregates; uninucleate; spherical, rarely irregular in shape. The chloroplast is single and parietal, lacking a pyrenoid. Cell walls are thin and smooth. Asexual reproduction by autospores, leading to two or more daughter cells, released by rupture of the parental wall. Sexual reproduction was not observed. The genus differs from other genera by the 18S rDNA and rbcL sequences.

ETYMOLGY: From Latin noun Laetitia, fecundity, fertility, luxuriance. The genus is named in memory of the mother of the first author, Letizia Atzeni.


Laetitia sardoa Malavasi, Škvorová, Němcová & Škaloud sp. nov.

DESCRIPTION: An alga with the general morphological characteristics of the genus. Vegetative cells solitary, uninucleate. Young cells mostly spherical, 1.9–3.9 μm; mature cells 4.0–4.5 μm. Cells have a parietal, cup-shaped chloroplast without pyrenoid. Asexual reproduction via two to eight autospores, 5.0–6.5 μm. Sexual reproduction was not observed.

HOLOTYPE: Strain SCCA 034, permanently cryopreserved in a metabolically inactive state (cryopreservation in liquid nitrogen) in the Culture Collection of Algae of the Charles University in Prague (http://botany.natur.cuni.cz/ algo/caup.html) with the code CAUP H 9001. Drawings of the holotype are provided in Fig. 28.

AUTHENTIC STRAIN: Living cultures of the alga are maintained at the Sardinian Culture Collection of Algae (SCCA) with the strain code SCCA 034.
Recently, Hodač et al. (2016) distinguished several genus-level lineages within the Chlorellaceae based on analyses of SSU rDNA sequences. The newly proposed genus *Laetitia* clearly represents a new phylogenetic lineage related to the genus *Marvania* and the lineage of sequences recognized by Hodač et al. (2016) as ‘OTU4 Marvania relatives’ and recently described by Temraleeva et al. (2022) as the new genus *Edaphochloris* Temraleeva, Krivina & Boldina.

In our tree, we included two *Edaphochloris* strains: *E. andreyevii* Temraleeva, Krivina & Boldina JL 4–6 and *Edaphochloris* sp. LH08AG1034. Interestingly, our SSU phylogeny did not support the close relationship between the genera *Marvania* and *Edaphochloris* as revealed by both Hodač et al. (2016) and Temraleeva et al. (2022), apparently due to the addition of the *Laetitia* sequence. Such a change points to the difficulty of inferring relationships between the genera of Chlorellales based on SSU rDNA sequences only.

As discussed by Eliáš & Neustupa (2009), *Marvania geminata* Hindak is very closely related to *Marvania cocoides* Henley, Hironaka, Guillou, M.A. Buchheim, J.A. Buchheim, M.W. Fawley & K.P. Fawley (CCAP 251/1b) originally described as *Nannochloris cocoides* Naumann. Indeed, Yamamoto et al. (2003) showed that the strain CCAP 251/1b is morphologically very similar to *M. geminata*. The species was formally transferred to the genus *Marvania* by Henley et al. (2004). Similarly, the newly proposed genus *Laetitia* morphologically resembles the *Nannochloris*-like species by possessing spherical cells with a simple parietal chloroplast without a pyrenoid. These available morphological data drew attention to the morphological similarities of these three genera and the difficulty to delimitate them using only light microscopy observations. Morphological and ultrastructural comparisons between the novel genus *Laetitia* and closely related genera are given in Table S2.

An important criterion to distinguish these genera is their special mode of cell division and autospore formation. The autosporation is the ancestral mode of cell division in the Trebouxiophyceae (Yamamoto et al. 2007). In *Laetitia*, the type of asexual reproduction is clearly autosporation (production of two, 4 or more autospores within the mother cell).

The genus *Nannochloris* propagates by binary division (Naumann 1921; Yamamoto et al. 2007), while species of *Marvania* propagate by budding (Hindák 1976; Tschermak-Woess 1999). In *Marvania*, the two daughter cells are of approximately equal size when their separation takes place (Sluiman & Reymond 1987; Eliáš & Neustupa 2009).

*Picochlorum* differs in size from *Laetitia; Picochlorum* is smaller, with a diameter of 1.5–3.0 μm. As reported by Hodač et al. (2016, fig. 3), all members of *Nannochloris*-like clades including the genus *Edaphochloris* produce the same cell shape (represented by globular cells with a parietal chloroplast).

The ultrastructure of *Picochlorum* is indistinguishable from that of *Laetitia*. During autosporation, autospores produce their own trilaminate cell wall (CW), which is, however, still covered by a very resistant mother cell wall (MCW). It is possible to note the difference in the number and size of starch grains within a chloroplast. However, this feature very probably depends on growth conditions. Moreover, the
ultrastructure of *Laetitia* differs from that of true *Marvania* (*M. geminata* SAG 12.88). In fact, as reported by Yamamoto *et al.* (2007), during the cell division of *Marvania*, extra layers of MCW cover one of the daughter cells, while the other cell remains uncovered.

The order Chlorellales is well known for its cosmopolitan distribution and great ecological versatility. To help visualizing the ecological preferences of all taxa included in the phylogenetic tree, their habitats are mentioned in Table S1. As was previously pointed out by Henley *et al.* (2004), the *Picochlorum* clade is a marine/saline lineage. Inside of the *Nannochloris* clade, there are strains from freshwater and soil habitats, whereas in the *Marvania* clade, only freshwater members have been revealed until now. Furthermore, it is important to note that most OTU4 members are soil algae.

According to Škaloud *et al.* (2018), in many algal groups, the boundary between marine and freshwater environments is crossed infrequently. This would imply that marine and freshwater taxa are not closely related since these transitions would represent rare evolutionary events. Here, we have described a new genus that probably originated just before such a transition. Indeed, together with the genus *Marvania*, *Laetitia sardoa* probably represents the closest freshwater relative of marine algal genus *Picochlorum*. Even though *Laetitia* was isolated from freshwater, its possible growth in the saline environment cannot be excluded on the basis of the evidence presently available. More work needs to be done to determine the ecological niche and geographic distribution of this genus.

In conclusion, our study has brought to light a new unicellular green alga, here recognized as a new species in a new genus. This discovery adds to our knowledge of microalgal flora diversity of the Sardinian region. In addition, *Laetitia* may represent a direct ancestor of algae that penetrated marine habitats to give rise to the genus *Picochlorum*.

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