The new photobiont *Ctenocladus verrucariae sp. nov.* (*Ulvales, Ulvophyceae*) found in various lichens of the family *Verrucariaceae* (*Eurotiomycetes*)

Tatyana Darienko, *Experimental Phycology and Culture Collection of Algae, University of Göttingen, Nikolausberger Weg 18, D-37073 Göttingen, Germany*

Thomas Pröschold, *Research Department for Limnology at Mondsee, University of Innsbruck, Mondseestr. 9, A-5310 Mondsee, Austria* (correspondence: Thomas.Proeschold@uibk.ac.at)

The genus *Ctenocladus* was described by Borzì (1883: 27) from a marine habitat. The characteristic morphological feature of the type species, *C. circinnatus* Borzì, is the unilateral branching of the filaments. However, Darienko & Pröschold (2017) demonstrated a high phenotypic plasticity among the different strains available in culture. For example, the deposited strain CCMP 2158 does not show the typical morphology of this genus in culture. The other strains, which were almost identical in sequence with CCMP 2158, were isolated from sandstone and salt marshes at high carbonate concentrations (solonetz-solonchak soils). This genus can form crustose thalli with irregularly branched filaments according to Darienko & Pröschold (2017).

Ctenocladus has been considered a rare genus. It has been reported from saline inland waters and soils in Italy, Spain, Ukraine, Russia, and North America (Darienko & Pröschold 2017; Samylina & al. 2014; Ariño & al. 1996; Herbst & Castenholz 1994; Blinn & Stein 1970; Borzì 1883). Habitats with high carbonate concentrations are typical for the genus *Lochmiopsis*, described by Woronichin & Popova (1929: 17) from soda lakes in Siberia (Russia); it was also recorded from such soils in different Asian deserts (Novichkova-Ivanova 1984). *Lochmiopsis* has a morphology similar to *Ctenocladus*, but its type species, *L. siberica* Woronichin & Popova, showed a high phenotypic and ecological plasticity (Ruinen 1933). Ruinen studied material collected from the type locality and from California and found similar morphology and ecology in both populations. Considering her findings, Smith (1950) treated the genus *Lochmiopsis* as a later synonym of *Ctenocladus*, a taxonomic opinion that is currently accepted by most phycologists (e.g., Blinn & Stein 1970). Blinn & Stein compared all specimens of *Ctenocladus circinnatus* and *Lochmiopsis siberica* deposited in 13 major herbaria and critically evaluated the material. They concluded that both species are conspecific and widely distributed along the 110–120° West longitude. The authors also found that the cell dimensions of collected field samples and cultured material is not a reliable criterion for separation of species within the genus *Ctenocladus*. The branching pattern as described by Borzì (1883) appeared to be at right angles only in magnesium-dominated salt solutions. Unfortunately, no authentic ("type") material of species of both genera is available in culture collections and sequencing data are needed to clarify their taxonomic status. However, Darienko & Pröschold (2017) found *C. circinnatus* in similar habitats to those from which *Lochmiopsis siberica* was described confirming their conspecificity.

Liu & al. (2016) and Darienko & Pröschold (2017) demonstrated that the genus *Ctenocladus*, as represented by the generitype *C. circinnatus,* belonged to the *Ulvales* (*Ulvophyceae*). Interestingly, the type strain of *Pseudopleurococcus printzii* Vischer (1933: 34) is closely related to the investigated strains of *Ctenocladus* and represents the second species of this genus (Darienko & Pröschold, 2017).

Here we report on an investigation of two photobionts of different lichens belonging to the genera *Verrucaria* and *Hydropunctaria* (*Verrucariaceae*) using an integrative approach (morphology and phenotypic plasticity, multiple gene phylogenies of SSU and ITS). Thüs & al. (2011) found several photobionts such as *Chloroidium* Nadson, *Dilabifilum* Tschermak-Woess (now *Pseudendoclonium*

Wille), *Elliptochloris* Tschermak-Woess, and others in various *Verrucariaceae*. The dominant green algal genus was *Diplosphaera* Bialosuknia found in the *Verrucariaceae*. Except for *Pseudendoclonium* (Ulvophyceae), all other green photobionts belonged to the *Trebouxiophyceae*. In addition, photobionts isolated from different *Verrucaria* species often assigned to *Dilabifilum* are referable to other genera of the *Ulvophyceae* (Darienko & Pröschold 2017).

The photobionts of the lichens *Hydropunctaria scabra* (Vězda) Keller, Gueidan & Thüs (previously known as *Verrucaria scabra* Vězda; for the taxonomic revision of these lichens, see Gueidan & al. 2009) and *Verrucaria margacea* (Wahlenberg) Wahlenberg were isolated by H. Thüs. Thüs (2002) identified the photobionts of both lichens as *Dilabifilum incrustans* (Vischer) Tschermak-Woess based solely on morphology. In contrast, phylogenetic analyses (Fig. 1) have revealed that both investigated strains represent a new species of *Ctenocladus*, *C. verrucariae*, which also belongs to the *Ulvophyceae*.

The strains SAG 2039 and SAG 2052 were isolated from the hosts as described by Thüs (2002) and Thüs & al. (2011). They were cultivated on liquid and on agarised basal medium (ES; medium 1 in Schlösser 1994) and modified Bold's Basal Medium (3N-BBM; medium 26a in Schlösser 1997) as well as in Seawater and Brackish Water Media (SWES and 1/2SWES; media 5 and 6 in Schlösser 1994). The strains were cultivated at 18°C, with 30 µmol photons m^{-2} s^{-1} provided by daylight fluorescent tubes (Osram L36W/954 Lumilux De Luxe Daylight, Munich, Germany), and a light: dark cycle of 14:10 h for 1–5 weeks for morphological investigations. Light microscopic investigations were conducted using an Olympus BX-60 microscope and the photomicrographs were taken with a ProgRes C14plus camera using the ProgRes CapturePro imaging system (version 2.9.0.1, both from Jenoptik, Jena, Germany).

The genomic DNA of the strains was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The SSU and ITS rDNA was amplified in two PCR reactions using the Taq PCR MasterMix Kit (Qiagen, Hilden, Germany) with the primer combinations EAF3/N1400R and N920F/ITS055R (Darienko & al. 2019). The SSU and ITS rDNA sequences are available in the EMBL, GenBank, and DDBJ sequence databases under the accession numbers OM985641 (SAG 2039) and OM985642 (SAG 2052), respectively. The SSU rDNA sequences were aligned and included into a data set of a total of 35 sequences (1772 bp) of representatives of the *Ulvales* (*Ulvophyceae*) using the sister order *Sykidiales* as outgroup (see Darienko & al. 2021). The data set was aligned according to the secondary structures. The secondary structures were folded using the software Mfold (Zuker 2003), which uses the thermodynamic model (minimal energy) for RNA folding. The structures were visualized using the program VARNA (Darty & al. 2009). GenBank accession numbers of all sequences used are given in Fig. 1. The phylogenetic analyses were conducted using the program PAUP, version 4.0b169 (Swofford 2002) with the automated model selection tool. The robustness of the tree was calculated using the methods described in Darienko & Pröschold (2017, 2021).

The results of these phylogenetic analyses clearly demonstrated that the two strains SAG 2039 and SAG 2052 represent a new species of *Ctenocladus* (Fig. 2) as follows.

Ctenocladus verrucariae Darienko & Pröschold, *sp. nov.* (Fig. 2)
Description: Algae developing as pseudoparenchymatic thalli, forming at the margins bilateral branched filaments. Cells of branched filaments in young culture 4.2-5.8 µm wide and 14.0-37.0 µm long, cylindrical, curved, sometimes uneven in thickness. Chloroplast parietal, occupying 25–75% of cell with a single pyrenoid. Pyrenoid surrounded by 2 or 4 large starch grains. Cells uninucleate. In actively growing culture end cells often contain two nuclei and pyrenoids. In

older cultures (> 5 weeks) the thalli are usually fragmented in sarcinoid cell packages or short filaments. The cell size of older filaments is slightly shorter (up to 25.0 µm) and 4.7-5.9 µm wide. Chloroplast usually covers all the perimeter of the cell wall. The cells of sarcinoid packages or single cells (probably representing preakinetes) are irregularly oval up to pear-shaped 11.2–16.3 µm, surrounded by a thick cell wall. Such cells give rise to new colonies consisting of branched filaments. The reproduction by vegetative cell division or by production of zoospores. Formation of zoospores is very rare event. Zoosporogenesis was observed in the cells of packages and zoosporangia contained 4 zoospores. The zoospores possess an eyespot and after settling were 5.3 µm in diameter.

Diagnosis: Differs from other species of *Ctenocladus* genetically by SSU and ITS sequences (OM985642).

Holotype: Strain SAG 2052 cryopreserved in a metabolically inactive stage at EPSAG.

Type locality: Switzerland: Davos Valley, bank of brook Drustschabächel (1570 m), photobiont of *Verrucaria margacea* (Wahlenberg) Wahlenberg.

The second strain, SAG 2039, was isolated from the lichen *Hydropunctaria scabra*, which was collected in the Black Forest near St. Wilhelmer Talbach, Germany, ca. 700 m, at low water level. The morphology of this strain (Fig. 3) is similar to SAG 2052 if grown under the same conditions. Interestingly, both strains grew well in freshwater, brackish and marine media. They differed in morphology if grown on brackish and seawater medium by having more branched filaments with more rounded cells compared to the growth on freshwater medium.

The two photobionts (SAG 2039 and SAG 2052) were completely identical in SSU rDNA sequences and differed only in one base in ITS-1. The SSU phylogeny revealed that both strains clearly belong to the genus *Ctenocladus*, but they formed a distinctive lineage within this genus (Fig. 1). Three additional lineages were apparent in our analyses, *C. circinnatus*, *C printzii*, and two specimens assigned to *C. circinnatus* by Liu & al. (2016). Interestingly, the latter lineage is separated from the other strains of this species. The tree topology was confirmed by all bootstrap and Bayesian analyses including those of complex evolutionary models implemented in MrBayes and PHASE.

To decide if the lineages represent separate species, we analyzed the ITS rDNA sequences and used the ITS-2/CBC approach, which was introduced for species delimitation. The phylogeny of the complete ITS (Fig. 4), which ITS-1, 5.8S and ITS-2 confirmed the separation into three lineages (unfortunately, no ITS sequences are available for the two specimens studied by Liu & al., 2016). The ITS-2/CBC approach clearly demonstrated that the three lineages represent species, which are supported by CBCs and HCBCs in the conserved region of ITS-2. The secondary structure of the photobionts (Fig. 6) differed from those presented for *Ctenocladus circinnatus* in Darienko & Pröschold (2017). The three species, *C. circinnatus*, *C. printzii* and the newly described *C. verrucariae* have a unique number barcode, namely CTE1, CTE2 and CTE3 in Fig. 4, respectively.

Since its description by Borzì (1883), the genus *Ctenocladus* was considered to have uncertain affiliations with other genera and classes. Schmidle (1901) treated *Ctenocladus* as a Section of *Gongrosira* Kützing, and Printz (1964) followed this opinion. By contrast, Smith (1950) recognized *Ctenocladus* as a distinct genus and transferred species of the genus *Lochmiopsis*, originally described by Woronichin & Popova (1929), to *Ctenocladus*. Most authorities (e.g., Fritsch 1945; Prescott 1968) at that time followed Smith. They all considered that *Ctenocladus* to be closely related to the *Trentepohliales*, which was confirmed by the presence of pit-like structures as intercellular cytoplasmic connections within the cell walls (Blinn & Morrison 1974). However, the autecology of *Ctenocladus* is clearly different from those of other *Trentepohliales* (e.g., Blinn &

Stein 1970; Blinn & Morrison 1974; Blinn 1970; 1971). Unfortunately, no cultured material of samples studied by the authors mentioned above is available for comparative morphological and molecular investigations. Darienko & Pröschold (2017) demonstrated that *C. circinnatus* has a wider distribution than previously known. They also showed that *Ctenocladus* forms a distinct genus within *Ulvales* (*Ulvophyceae*), not close to the *Trentepohliales*. Interestingly, closely related to *C. circinnatus* is a species that Vischer (1933) described as *Pseudopleurococcus printzii* Vischer, although he assigned this species to this genus with reservation. The genus *Pseudopleurococcus* was described by Snow (1899); both of his included species, *P. vulgaris* Snow and *P. botryoides* Snow, were found as epiphytes on the bark of trees. According to Chodat (1902), the descriptions of both species were incomplete, and separation is almost impossible. Vischer (1933) followed Chodat's opinion, but he left the assignment of his species to *Pseudopleurococcus* for further investigations. Despite the close relationship of *Ctenocladus circinnatus* and *Pseudopleurococcus printzii*, which was therefore transferred to *Ctenocladus* by Darienko & Pröschold (2017), Škaloud & al. (2018) considered *Ctenocladus* and *Pseudopleurococcus* as distinct clades in the family *Ctenocladiaceae* Borzì, without any explanation or evidence. The present study clearly shows with the finding of a new species that *Ctenocladus* is now represented by three species including *C. printzii*. *Pseudopleurococcus* as mentioned by several authors (Vischer 1933; Chodat 1902; Bourrelly 1990) should be considered a dubious taxon. Tschermak-Woess (1970) also questioned the taxonomic validity of *Pseudopleurococcus sensu* Snow, and it was regarded as a "*genus imperfectum*" by Vischer (1933) and Binz & Vischer (1956); Tschermak-Woess (1970) proposed a new generic name *Dilabifilum* for Vischer's species of *Pseudopleurococcus*. The genus *Dilabifilum* with its type species *D. arthropyreniae* (Vischer & Klement) Tschermak-Woess and *D. incrustans* was transferred to *Pseudendoclonium* based on phylogenetic analyses (Darienko & Pröschold 2017). Even if *Pseudopleurococcus* were to be resurrected in further studies, the generic name *Ctenocladus* would have nomenclatural priority.

Pleurococcoid green algae growing on barks of trees have been intensively studied by numerous authors (e.g., Brand 1925; Vischer 1960; Gärtner 1994; Ettl & Gärtner 2014). Phylogenetic analyses of those algae have demonstrated that most of them belong to the *Trebouxiophyceae* (e.g., Zhu & al. 2018). Of the *Ulvophyceae*, mostly *Trentepohliales* are found on bark of trees. The only isolate from bark, which probably belongs to *Ctenocladus*, is NIES-159 named as *Pseudopleurococcus printzii* var. *longissimus* Watanabe (1983: 259).

With the newly described species *Ctenocladus verrucariae*, the genus *Ctenocladus* showed a wider ecological spread, as might be expected: *Ctenocladus circinnatus*, which occurs in inland saline environments, and *C. printzii* found in freshwater habitats, *Ctenocladus* also can be discovered as photobionts of lichens belonging to the *Verrucariaceae*. *Ctenocladus printzii* was also found on bark of elms (*Ulmus*; Watanabe 1983) and as a photobiont of *Verrucaria aquatilis* Mudd (Tschermak-Woess 1970). However, Thüs & al. (2011) found *Pseudendoclonium* (as *Dilabifilum*) as a photobiont of this *Verrucaria* species demonstrating the high flexibility of photobionts among the *Verrucariaceae*. Ulvophycean photobionts have been discovered in several marine species of *Verrucaria* [e.g., *V. maura* Wahlenberg, *V. mucosa* Wahlenberg, *V. rheitrophila* Zschacke, and *Wahlenbergiella striatula* (Wahlenberg) Gueidan & Thüs], the photobionts of which belong to the recently established genera of the *Ulvophyceae*: *Paulbroadya*, *Lithotrichon*, and *Halofilum* (Darienko & Pröschold, 2017).

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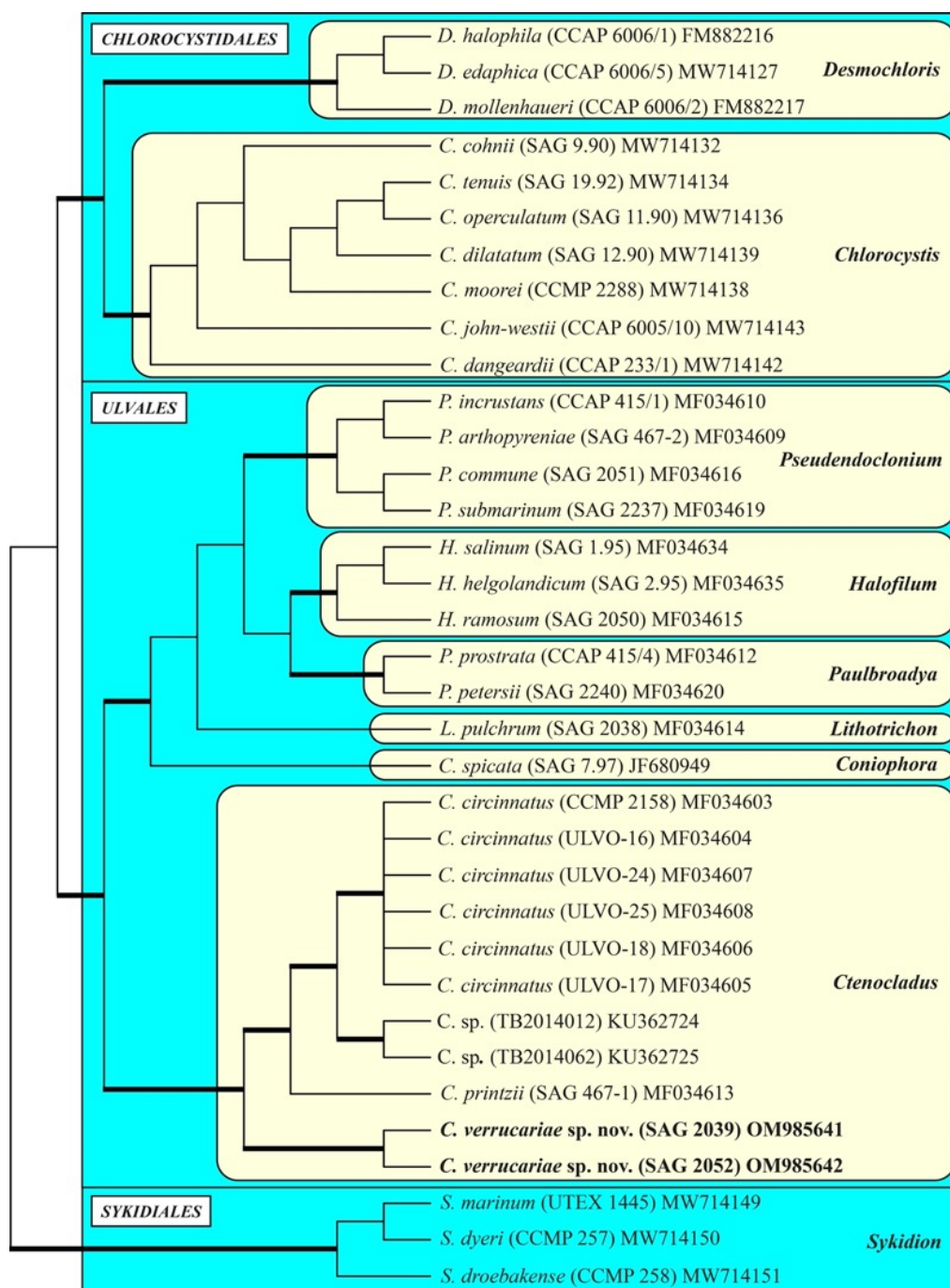
Fig. 1. Molecular phylogeny of the Ulvales based on SSU rDNA sequence comparisons. The phylogenetic tree shown was inferred using the maximum likelihood method based on a data set of 1772 aligned positions of 35 taxa using PAUP 4.0a build169. For the analysis, the TrN+I+G (base frequencies: A 0.2515, C 0.2130, G 0.2752, U 0.2603; rate matrix A-C 1.0000, A-G 1.8191, A-U 1.0000, C-G 1.0000, C-U 4.2180, G-U 1.0000) with the proportion of invariable sites (I = 0.6692) and gamma shape parameter (G = 0.6592) was chosen, which was calculated as the best model by the automated model selection tool implemented in PAUP. The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values > 70% calculated with PAUP using maximum likelihood, neighbour-joining, maximum parsimony, and RAxML using maximum likelihood). The sister group of the *Sykidiales* was chosen as the outgroup. The newly sequenced strains were highlighted in bold.
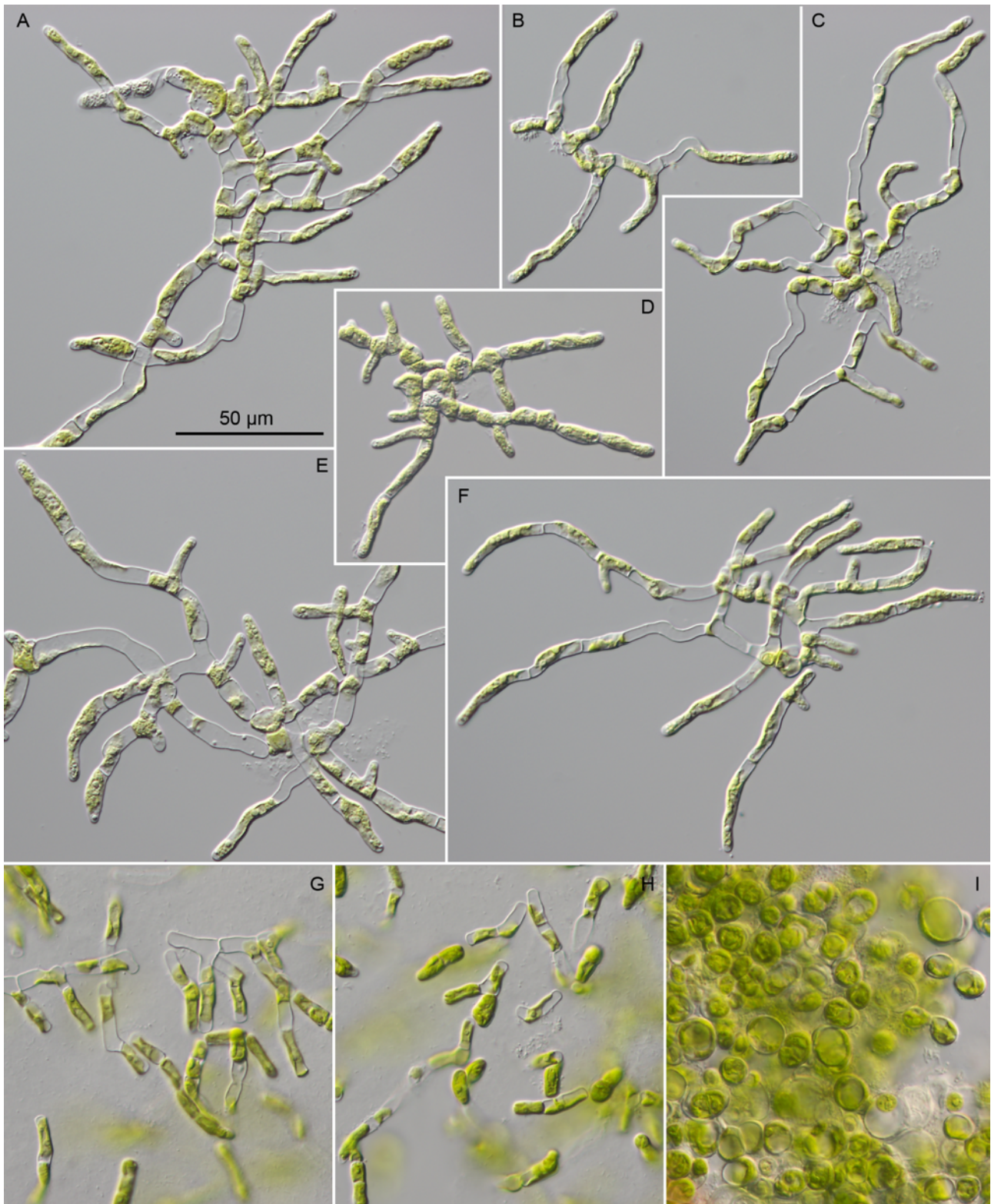
Fig. 2. Morphology and phenotypic plasticity of SAG 2052 *Ctenocladus verrucariae sp. nov.* (Authentic strain of the holotype) in 3N-BBM medium, scale bar = 50 μm for all pictures (A–I). A-F. young thalli of one-week-old culture, G-H. disintegrated filaments in five-week-old culture, I. cell packages and preakinetes in five-week-old culture.
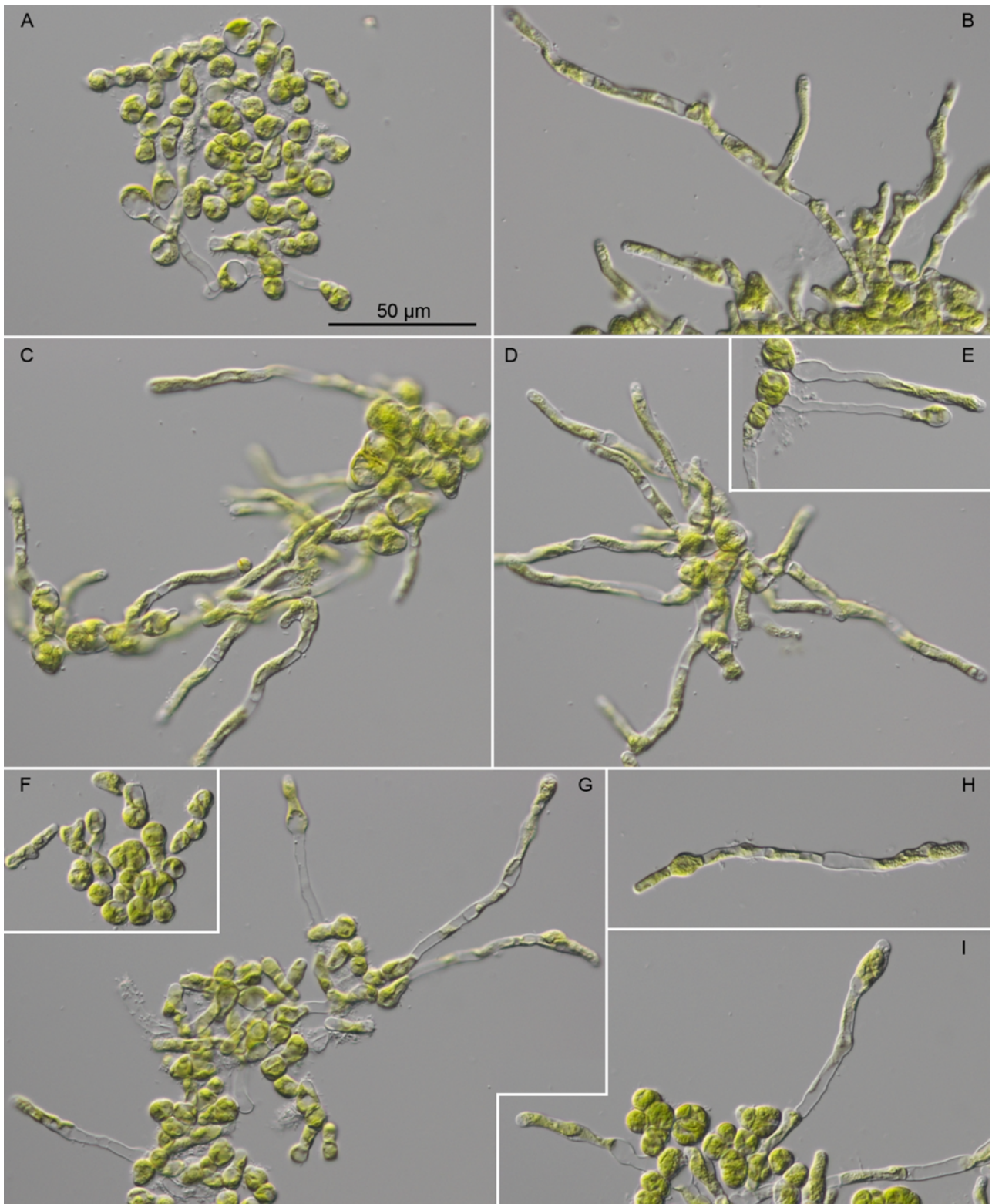
Fig. 3. Morphology and phenotypic plasticity of SAG 2039 *Ctenocladus verrucariae sp. nov.* in 3N-BBM medium, scale bar = 50 μm for all pictures (A–I).
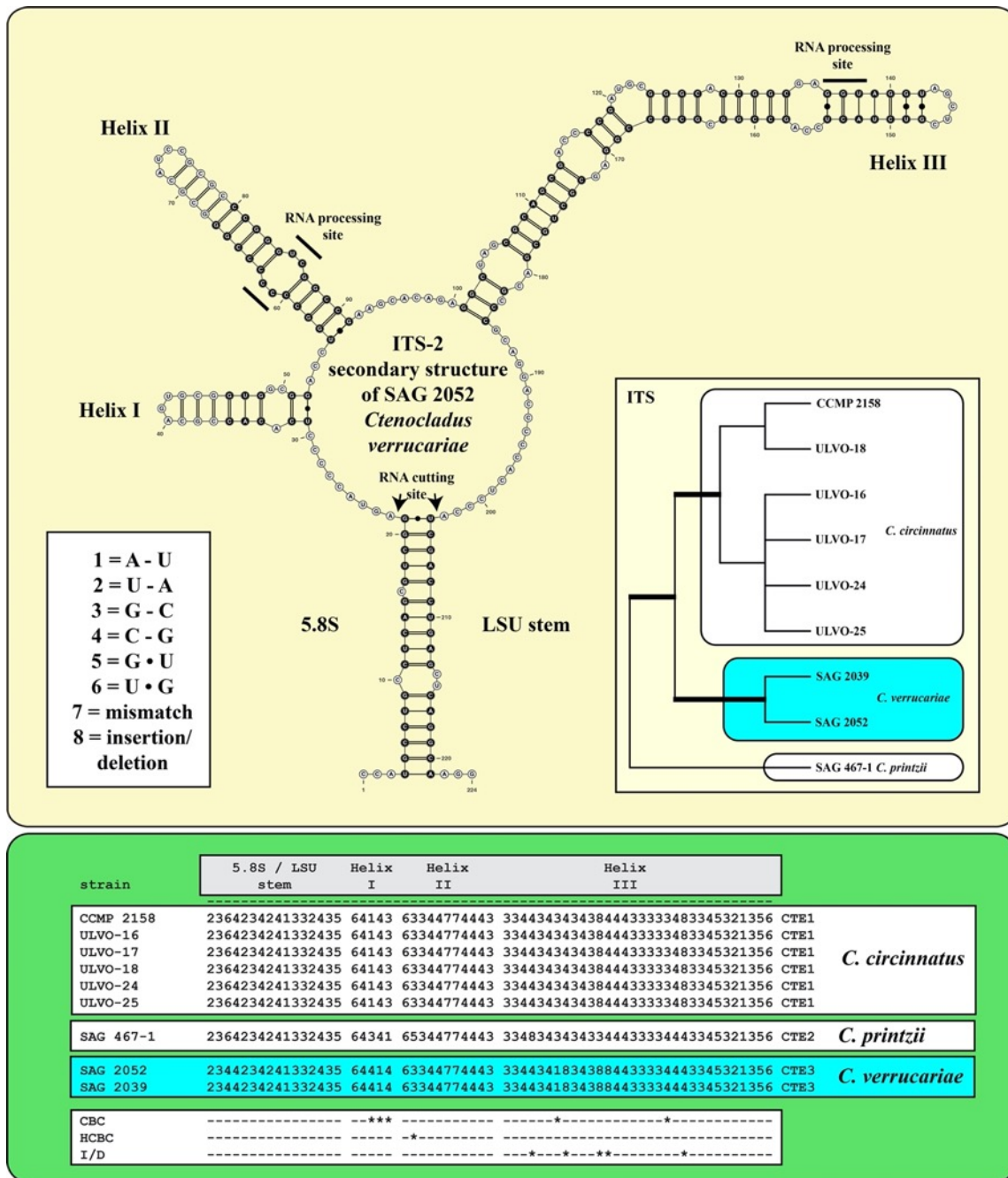
Fig. 4. ITS-2 rRNA secondary structure of *Ctenocladus verrucariae sp. nov.* and ITS rDNA phylogeny of *Ctenocladus* (inserted tree) as well as the ITS-2 barcodes of *C. circinnatus*, *C. printzii*, and *C. verrucariae* determined by the ITS-2/CBC approach. The number codes correspond to the bases highlighted in black in the ITS-2 structure. The phylogenetic tree of ITS shown was inferred using the maximum likelihood method based on a data set of 750 aligned positions of 9 taxa using PAUP 4.0a build169. For the analysis, the TIM+I (base frequencies: A 0.1594, C 0.4016, G 0.3129, U 0.1261; rate matrix A-C 1.0000, A-G 1.9259, A-U 2.4879, C-G 2.4879, C-U 3.3309, G-U 1.0000) with the proportion of invariable sites (I = 0.3101) and gamma shape parameter (G = equal) was chosen, which was calculated as the best model by the automated model selection tool implemented in PAUP. The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values > 70% calculated with PAUP using maximum likelihood, neighbour-joining, maximum parsimony, and RAxML using maximum likelihood).