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The green puzzle *Stichococcus* (Trebouxiophyceae, Chlorophyta): New generic and species concept among this widely distributed genus

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Abstract

Phylogenetic analyses have revealed that the traditional order Prasiolales, which contains filamentous and pseudoparenchymatous genera *Prasiola* and *Rosenvingiella* with complex life cycle, also contains taxa of more simple morphology such as coccoids like *Pseudochlorella* and *Edaphochlorella* or rod-like organisms like *Stichococcus* and *Pseudostichococcus* (called *Prasiola* clade of the Trebouxiophyceae). Recent studies have shown a high biodiversity among these organisms and questioned the traditional generic and species concept. We studied 34 strains assigned as *Stichococcus*, *Pseudostichococcus*, *Diplosphaera* and *Desmococcoccus.* Phylogenetic analyses using a multigene approach revealed that these strains belong to eight independent lineages within the *Prasiola* clade of the Trebouxiophyceae. For testing if these lineages represent genera, we studied the secondary structures of SSU and ITS rDNA sequences to find genetic synapomorphies. The secondary structure of the V9 region of SSU is diagnostic to support the proposal for separation of eight genera. The complex taxonomic history was summarized and revised. The ITS-2/CBC approach was used for species delimitation. Considering all these results, we revised the genera *Stichococcus*, *Pseudostichococcus*, *Diplosphaera* and *Desmococcus* and proposed four new genera and four new species for the science community. The usage of the V9 region and the ITS-2 barcodes discovered potential new species among the *Stichococcus*-like organisms in culture-independent studies.

Keywords: *Stichococcus; Diplosphaera*; *Pseudostichococcus*; *Desmococcus*; molecular phylogeny; integrative approach; systematics; terrestrial algae; lichen photobionts; generic and species concept

Introduction

The *Prasiola* clade of the Trebouxiophyceae comprises green algae with different morphological organization levels. Phylogenetic studies have revealed that representatives of the pseudoparenchymatous genera *Prasiola* and *Rosenviengiella*, branched filamentous *Prasiolopsis* and sarcinoid *Prasiococcus* are closely related to the filamentous taxa of *Stichococcus* and *Pseudostichococcus*, the package-like *Desmococcus* and *Diplosphaera*, the coccoid *Pseudochlorella*, *Edaphochlorella*, *Pseudomarvania* and the needle-like *Koliella*/*Raphidonema* (Figs 1–2). All these genera form a well-supported monophyletic lineage known as *Prasiola*-clade in the literature (Pröschold & Leliaert, 2007; Darienko *et al.*, 2010; Thüs *et al.*, 2011). Recently several new genera *Prasionema*, *Rosenviengiellopsis*, *Prasionella* were described based on phylogeny of *rbc*L and *tuf*A genes (Heesch *et al.*, 2016; Nelson *et al.*, 2018). The relationships within *Pseudochlorella* and *Edaphochlorella* were recently clarified (Darienko *et al.*, 2016). However, the generic concept among *Stichococcus*, *Pseudostichococcus*, *Desmococcus* and *Diplosphaera* is unresolved. Hodac *et al.* (2016) have shown that *Desmococcus* is closely related to the *Stichococcus* and was placed among algae with *Stichococcus*-like morphology. *Stichococcus*-like algae are very common and could be found in almost all types of habitats from freshwater, brackish, marine to hot acidic springs and snow (*i.e.*, Butcher, 1952; Kol, 1968; Ettl & Gärtner, 2014). Especially in samples collected from terrestrial habitats "*Stichococcus*" could be found in almost every soil sample. Originally the genus *Stichococcus* with its type species *S. bacillaris* was described by Nägeli (1849) from Switzerland. An authentic strain of this species does not exist. Since its first description, more than 100 species

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names could be found in the literature, but most of them are poorly described and very little is known about their morphology and ecology (Guiry & Guiry, 2019; INA: http://ucjeps.berkeley.edu/CPD/). *Stichococcus*-like algae are characterized by a simple morphology. They are mostly represented by cylindrical or short-cylindrical cells containing plate-like chloroplast without (or sometime with) pyrenoid. Sometimes the cells form short (from 2–3 cells) to middle long (20–30 cells) filaments. They reproduce by vegetative division. Only for *Pseudostichococcus monallantoides* zoospore formation was reported (Moewus, 1951). Because of their morphological simplicity many scientists put the special attention to the width of filaments and length:width ratio and pointed out that the length is variable feature (Raths, 1938; Printz, 1964). Some scientists have taken the life style (photobiont and species or genus of lichen) into account for the establishing of new species (Chodat, 1913; Letellier, 1918; Beck *et al.*, 2019). Grinzesco & Peterfi (1932) pointed out that *Stichococcus* is very closely related to the genus *Diplosphaera* and included *Diplosphaera* into *Stichococcus*. However, this conclusion was not supported by many scientists such as Vischer (1953, 1960), and Ettl & Gärtner (2014). They highlighted the difficulties of many described species and synonymized many *Stichococcus* species to eight existing species. In this group of closely related organisms, only one group is clearly morphologically separated from *Stichococcus*/*Diplosphaera*: the genus *Desmococcus*. This genus is characterized by a formation of package-like structures, which grow in branched filaments, producing akinetes with ornamented cell walls. In addition, *Desmococcus* can reproduce by zoospores. The genera *Stichococcus* and *Diplosphaera* usually reproducing only by vegetative cell division. The difference between both genera is that *Diplosphaera* can form packages (division in two dimensions), whereas *Stichococcus* makes division only in one dimension. The situation for taxonomy become more difficult because of lack of authentic strains. Only few authentic strains are known among *Stichococcus*-like taxa: *Diplosphaera mucosa* (SAG 48.86), *D. epiphytica* (SAG 11.88), *Desmococcus endolithicus* (SAG 25.92), *D. olivaceus* (SAG 1.92), *D. antarctica* (SAG 63.90), *Stichococcus jenerensis* (SAG 2138), *S. deasonii* (SAG 2139), and *Pseudostichococcus monallantoides* (SAG 380-1). Neustupa *et al.* (2007) and Hodac *et al.* (2016) have shown that *Stichococcus*-like organisms are polyphyletic, but the generic and species concept among taxa remain unresolved.

The aim of our study was the comprehensive analysis of *Stichococcus*/*Diplosphaera*/*Desmococcus*-like algae using the polyphasic approach and the development of a new generic and species concept for this group. To achieve the aim we sequenced nuclear-coding SSU and ITS rDNA as well as the chloroplast *rbc*L gene of selected strains.

Material and Methods

Strain origin, culture conditions and light microscopy

The investigated strains originated from the Culture Collection of Algae (SAG) at the University of Göttingen (Germany), the Culture Collection of University of Texas at Austin (UTEX, USA), and personal collections of one of the authors (TD). Details about origin and habitat are summarized in Table S1 (Supplemental Material). All strains were cultivated at 18°C, with 50 µmol photons/m^2s^1 provided by daylight fluorescent tubes, Osram L36W/954 Lumilux de lux daylight, Munich, Germany), and light:dark cycle of 16:8 hrs. *Stichococcus*-like green algae can occur in freshwater, terrestrial and marine habitats in temperate and cold regions. Therefore, we used modified Bold's Basal Medium (3N-BBM+V; medium 26a in Schlösser, 1997) as culture medium. To test the phenotypic plasticity, selected strains were cultivated under the same conditions in a marine medium (SWES; medium 5 in Schlösser, 1994) or at 8°C under freshwater conditions. The morphological observations were done on three-week-old cultures.

Three-week-old cultures were used for morphological identification with the keys of Ettl & Gärtner (2014), Printz (1964), and Heering (1914). In addition, the morphology of the strains was compared with the original species descriptions. For the light microscopic investigations, an Olympus BX-60 microscope was used (Olympus, Tokyo, Japan) and the micrographs were taken with a ProgRes C14plus camera using the ProgRes CapturePro imaging system (version 2.9.0.1), both from Jenoptik, Jena, Germany). This software was also used for measurements of length and width to compare the length:width ratio of the investigated strains. For this comparison over 50 cells of each strain were measured.

DNA extraction, PCR, and sequencing

The genomic DNA of the strains was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and following the instructions provided by the manufacturer. The SSU and ITS rDNA were amplified in PCR reactions using the Taq PCR MasterMix Kit (Qiagen, Hilden, Germany) either with the primers EAF3 and ITS055R (Marin *et al.,* 2003) or with two primer combinations EAF3/G800R and G500F/ITS055R (Darienko *et al.*, 2019). All PCR

products were purified and sequenced as described by Darienko *et al.* (2019). The *rbcL* gene of selected strains were amplified and sequenced as described in Darienko *et al.* (2018). The sequences are available in the EMBL, GenBank and DDBJ sequence databases under the accession numbers given in Table S1 and Figs 1–2.

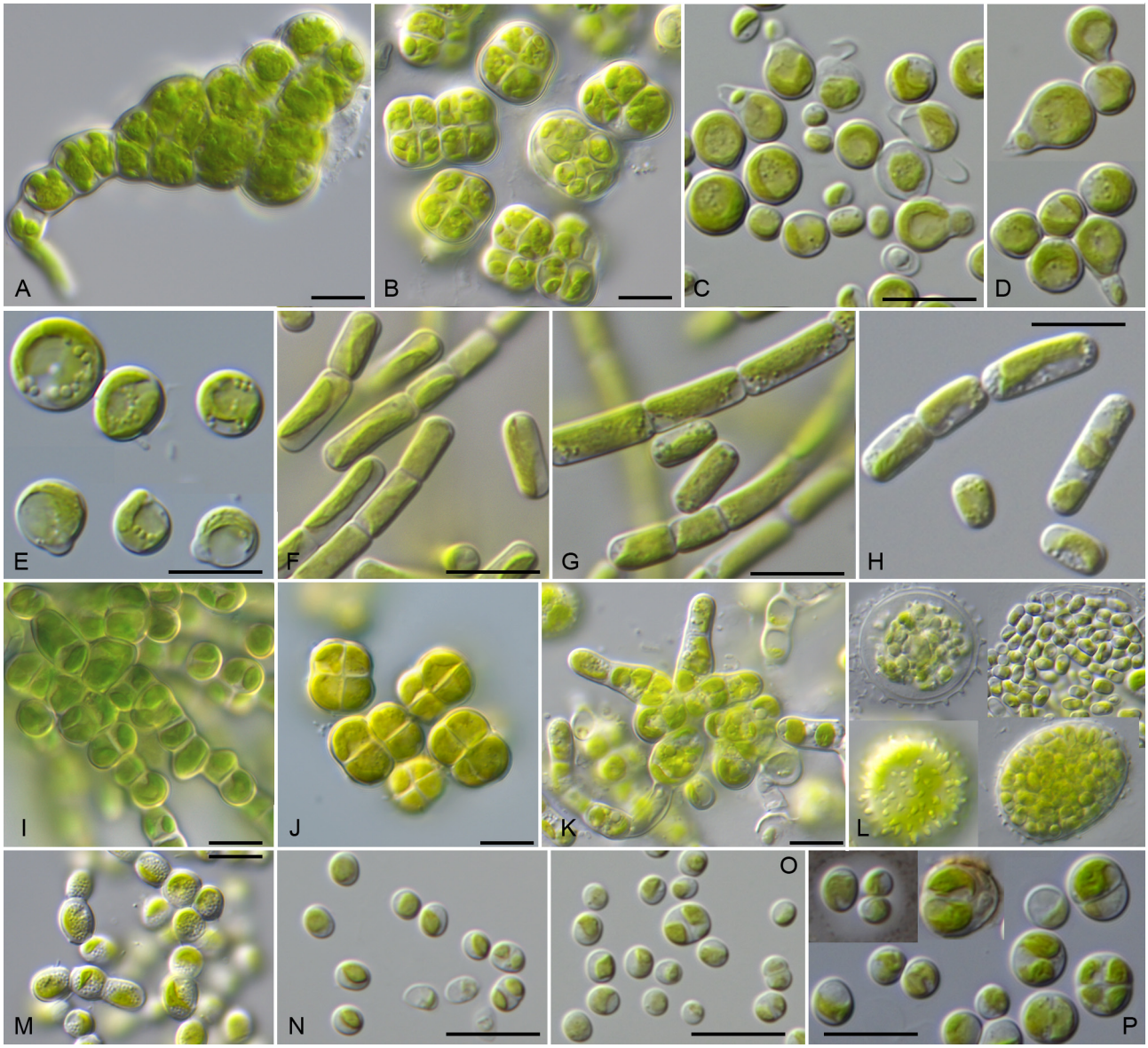

FIGURE 1. Morphology of the members of the *Prasiola* clade of the Trebouxiophyceae. **A.** SAG 26.83 *Prasiolopsis ramosa*, **B.** SAG 10.95 *Prasiococcus calcarius*, **C.–D.** SAG 2047 *Pseudomarvania ampulliformis*, **E.** SAG 2148 *Pseudomarvania aerophytica*, **F.** SAG 335-8 *Gloeotila scopulina*, **G.** SAG 56.91 *Gloeotila* cf. *protogenita*, **H.** SAG 379-2 *Stichococcus bacillaris*, **I.** SAG 25.92 *Desmococcus endolithicus*, **J.** SAG 1.92 *Desmococcus olivaceus*, **K.–L.** SAG 1.94 *Desmococcus olivaceus*, **M.** SAG 63.90 *Desmococcus antarcticus*, **N.** SAG 2049 *Stichococcus* sp., **O.** SAG 11.88 *Diplosphaera epiphytica*, **P.** SAG 48.89 *Diplosphaera mucosa*, scale bar = 10 µm.

Phylogenetic analyses

The SSU rDNA sequences of all strains were aligned according to their secondary structures. The ITS-1 and ITS-2 sequences of all strains were folded according to the protocol described in detail in Darienko *et al.* (2016). The alignments were separated into the following two datasets: (i) a dataset of 60 SSU rDNA sequences of *Prasiola* clade (1785 bp), and (ii) a concatenated dataset of 36 SSU and ITS rDNA (2629 bp). For comparison, additional datasets of 27 strains (the accession numbers of the used strains for these comparisons highlighted in yellow in Table S1) were created comprising (i) SSU and ITS rDNA (2629 bp), (ii) SSU, ITS rDNA and *rbc*L (3808 bp), (iii) ITS rDNA (853 bp), (iv) ITS-2 (395), (v) *rbc*L (all three codon bases; 1170 bp) and (vi) *rbc*L (only the first two codon bases; 780 bp). For all data sets the best evolutionary models were calculated with the program Modeltest 3.7 (Posada, 2008) using the Akaike Information Criterion (Akaike, 1974). The settings of the best models are given in the figure legends. The following methods were used for the phylogenetic analyses: distance, maximum parsimony, maximum likelihood,

and Bayesian inference. Programs used included PAUP version 4.0a166 (Swofford, 2002), RAxML version 8.2.12 (Stamatakis, 2014), MrBayes version 3.2.7a using the doublet approach (Ronquist *et al.*, 2012), and PHASE package 2.0 (Jow *et al.*, 2002, Higgs *et al.*, 2003, Hudelot *et al.*, 2003, Gibson *et al.*, 2005, Telford *et al.*, 2005). To find genetic synapomorphies in the SSU, the program DeSigNate (https://designate.dbresearch.uni-salzburg.at) was used.

ITS-2 secondary structures and ITS-2/CBC approach

The secondary structures of ITS-2 sequences were folded using the computer programs mfold (Zuker, 2003) and analyzed with the ITS-2/CBC approach, which is described in detail in Darienko *et al.* (2015) for *Coccomyxa.* The resulting barcodes of the *Stichococcus*-like organisms are given in the Fig. 7 and the diagnoses below.

Distribution pattern of *Stichococcus*-like organisms

To get an overview about the distribution, we used the V9 region of the SSU rDNA and the ITS-2 sequences for searching of entries in GenBank. The V9 and the ITS-2 haplotypes were used for the BLAST N searches (V9: 100% coverage, 100% identity, and ITS-2: 100% coverage, >97% identity; Altschul *et al.*, 1990). The metadata of the haplotypes (geographical origin and habitat) are summarized in the Supplemental Material (Tables S3–4) and in the Fig. 4.

Results

Molecular phylogeny of the *Prasiola* clade

All investigated strains belonged to the well-supported *Prasiola* clade of the Trebouxiophyceae (Chlorophyta). Phylogenetic analyses of the SSU rDNA revealed that the *Stichococcus*-like isolates formed seven independent lineages (Fig. 3). One out of them (*Stichococcus*) was sister of the genus *Desmococcus*. Both were closely related to the group of *Prasiola*-like organisms (*Prasiolopsis*, *Prasiococcus* and *Pseudomarvania*). All lineages were highly supported in all bootstrap and Bayesian analyses. The *Stichococcus*-clade (OTU6 *sensu* Hodac *et al.*, 2016) and the other six clades (OTU 1-5, 7-9) containing exclusively *Stichococcus*-like strains questioned the traditional generic concept of the genus *Stichococcus*. The subdivision into OTU groups of Hodac was mainly supported in our analyses with one exception. The groups OTU3/4/5 formed one clade (called *Tritostichococcus*) in our phylogenetic tree. Unfortunately, only the strain SAG 2406 (OTU4) of the groups designated by Hodac *et al.* (2016) was available for further investigations. The well-supported phylogeny represented in Fig. 3 required an accurate and complete SSU rDNA sequences. This was not an easy task, because many of the investigated strains had one or more introns in their SSU rDNA sequences (see intron positions in Table S1). Only 189 bases were variable in the data set of 1785 base positions when the *Prasiola*-like organisms and the outgroup were included. Among the SSU rDNA sequences of *Stichococcus*-like strains only 81 variable positions could be recognized.

To find synapomorphies of the eight clades in SSU, we analyzed all variable base positions in the SSU secondary structure with two methods, manually and with the program DeSigNate. 26 out of the 81 variable positions were non-homoplasious synapomorphies (NHS) of the clades; however, each lineage was only characterized by one or few NHS (see Table S2). The two clades *Deuterostichococcus* and *Tritostichococcus* had no NHS in their SSU rDNA sequences. Therefore, the high bootstrap and Bayesian support resulted only in analyses using complex evolutionary models, which included the SSU secondary structures.

Generic concept among the *Stichococcus*-like taxa

As demonstrated in Table S2, most of the variable base positions occurred in the V9 region of the SSU. Therefore, we compared the secondary structure of this region for each of the eight lineages (Fig. 4). Each of these clades called A-H were characterized by a unique loop region of the V9 (marked in white boxes in Fig. 4), which was diagnostic for each clade. Only the clades D and E showed few variations in V9 (D1-3 and E1-3). This region is often used as marker for environmental studies. Therefore, we tested if the *Stichococcus*-like organisms could be identified at generic level using the V9 region. The results of the BLAST N search (100% coverage, 100% identity) using all V9 variants were summarized in Fig. 4 and Table S3. 176 sequences (including our investigated strains) were found in GenBank. The eight clades were differently distributed around the world and occurred in different habitats. For example, most entries of *Tetratostichococcus* were from marine habitats in Asia. Photobionts of several lichens could be found within the genera *Deuterostichococcus*, *Diplosphaera*, *Tritostichococcus*, and *Pseudostichococcus*. Other details about origin and habitats of the GenBank entries is given in Fig. 4.

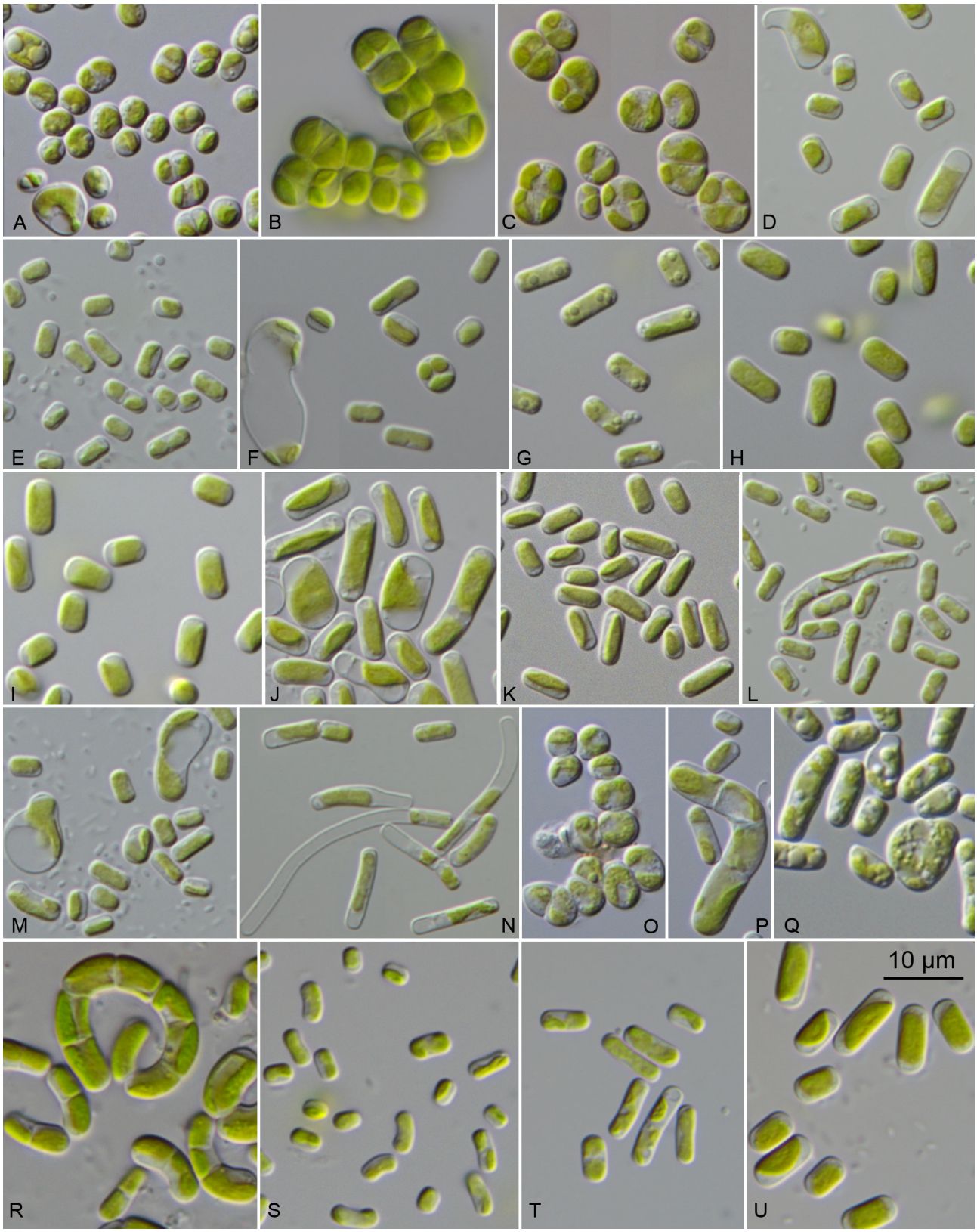

FIGURE 2. Morphology of the members of the *Prasiola* clade of the Trebouxiophyceae. **A.** SAG 49.86 *Diplosphaera* sp., **B.** SAG 2.82 *Chlorosarcina* sp., **C.** SAG 9.82 *Nannochloris normandinae*, **D.** SAG 2138 *Stichococus jenerensis*, **E.** SAG 2481 *Stichococcus* sp., **F.** SAG 107.80 *Stichococcus* sp., **G.** SAG 108.80 *Stichococcus* sp., **H.** SAG 2139 *Stichococcus deasonii*, **I.** SAG 2060 *Stichococcus* sp., **J.** SAG 2119 *Stichococcus* sp., **K.** SAG 10.97 *Gloeotila curta*, **L.** SAG 2482 *Stichococcus* sp., **M.** SAG 2406 *Stichococcus* sp., **N.** SAG 379-4 *Stichococcus fragilis*, **O.** SAG 2067 *Desmococcus spinocystis*, **P.** UTEX 2249 *Pleurastrum photoheterotrophicum*, **Q.** SAG 380-1 *Pseudostichococcus monollantoides*, **R.** ST-9 *Stichococcus coniocybes*, **S.** ST-7 *Stichococcus* sp., **T.** ST-6 *Stichococcus* sp., **U.** ST-2 *Stichococcus* sp., scale bar = 10 µm.

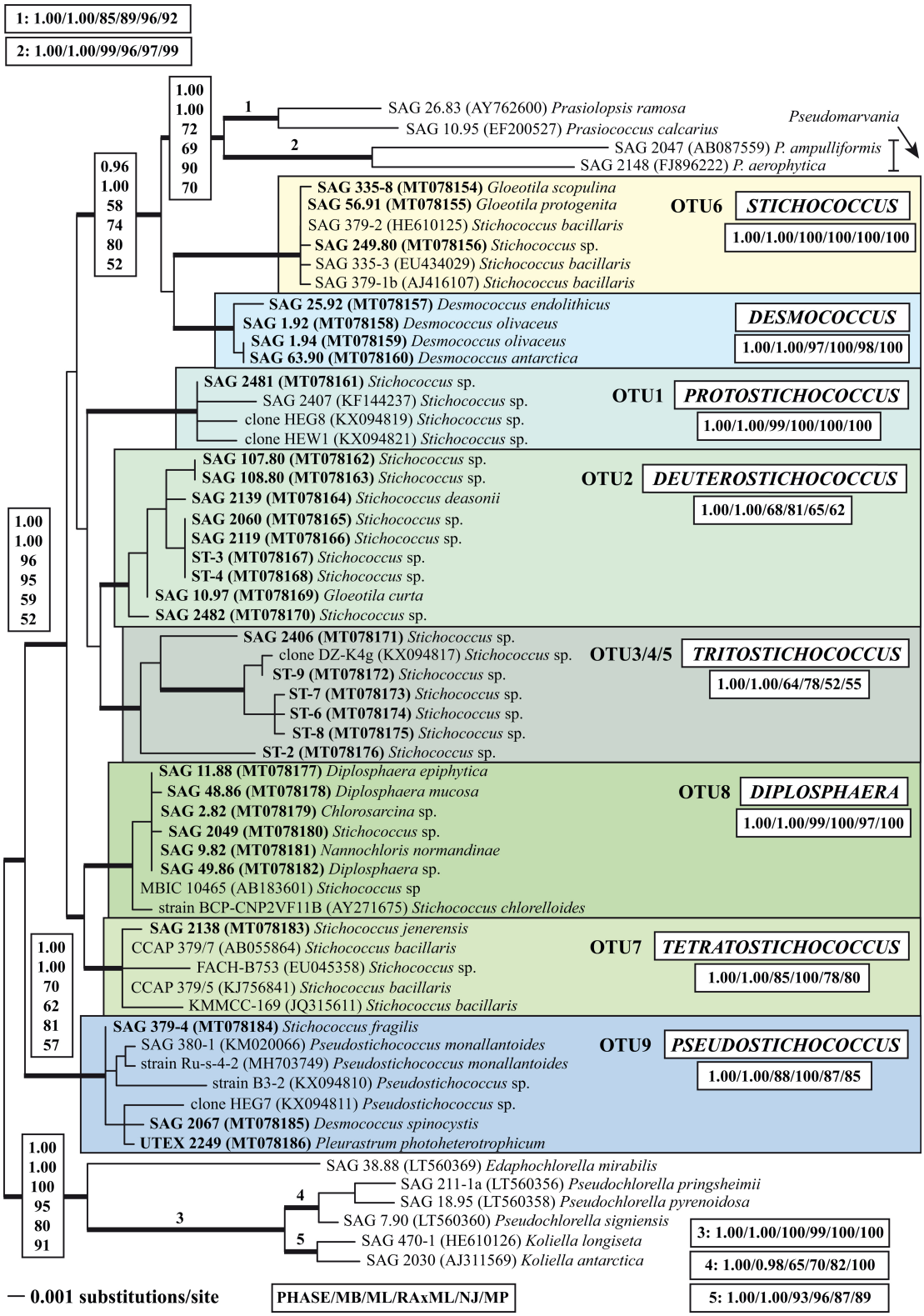

FIGURE 3. Molecular phylogeny of *Prasiola* clade of the Trebouxiophyceae based on SSU rDNA sequence comparisons. The phylogenetic tree shown was inferred using the maximum likelihood method based on the data sets (1785 aligned positions of 60 taxa) using PAUP 4.0a166. For the analyses the best model was calculated by Modeltest 3.7. The setting of the best model was given as follows: TrN+I+G (base frequencies: A 0.2536, C 0.2132, G 0.2755, T 0.2577; rate matrix A–C 1.0000, A-G 2.6026, A-U 1.0000, C–G 1.0000, C-U 8.6961, G–U 1.0000) with the proportion of invariable sites (I = 0.7623) and gamma shape parameter (G = 0.6744). The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values >50% calculated with PAUP using maximum likelihood, neighbor-joining, maximum parsimony and RAxML using maximum likelihood). Each taxa was named after the original designation in the public culture collection or in GenBank. The OTU designations followed the naming in Hodac *et al.* (2016). The clades are named after the genera (color-coded) proposed in this study.

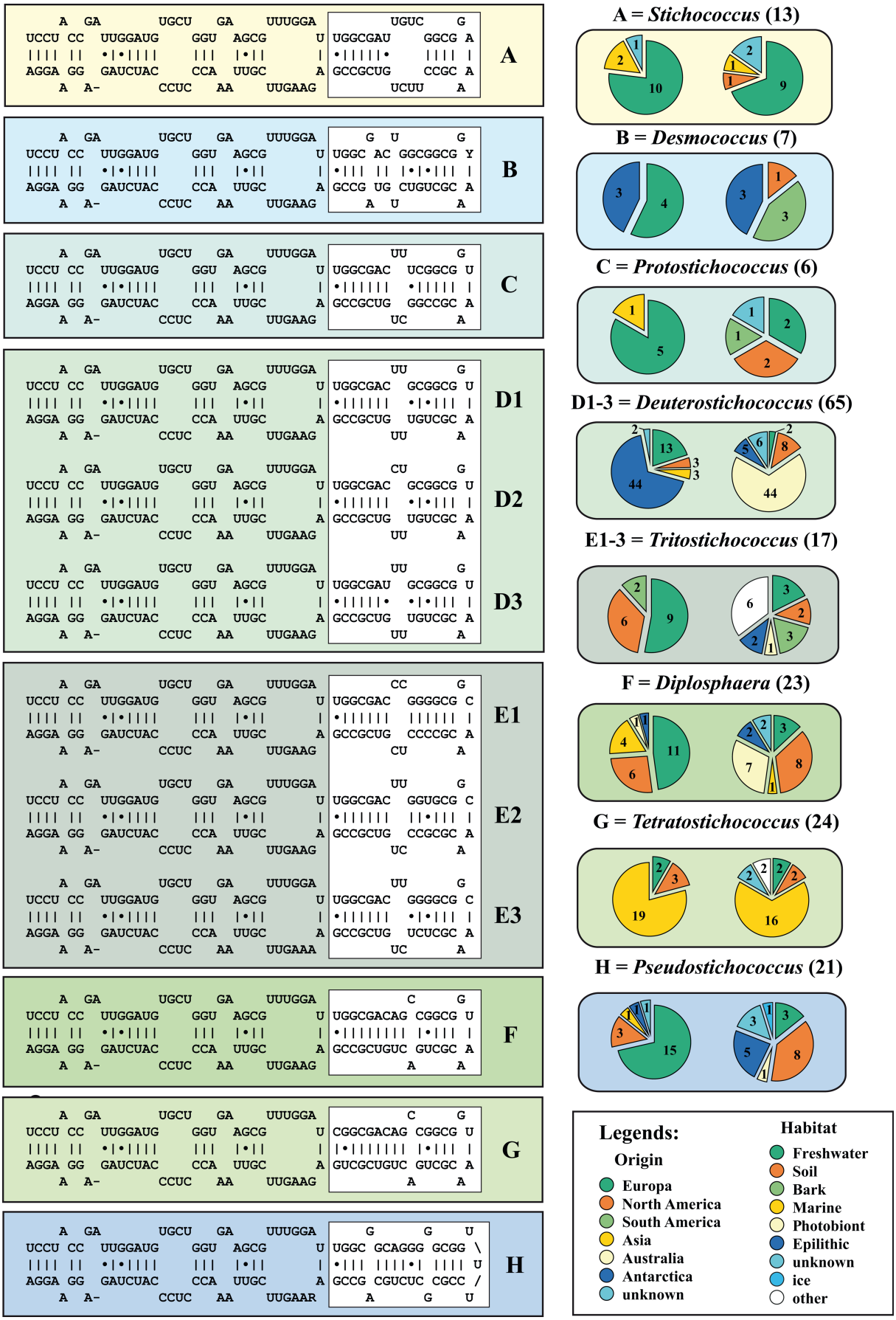

FIGURE 4. Secondary structure of the V9 region (Helix 49) of the SSU rDNA and distribution pattern of each genus. The variable region within the V9 are highlighted in white boxes. The distribution pattern of each genus are given after the geographical origin and after the habitat. The numbers of GenBank entries are given after generic names in brackets.

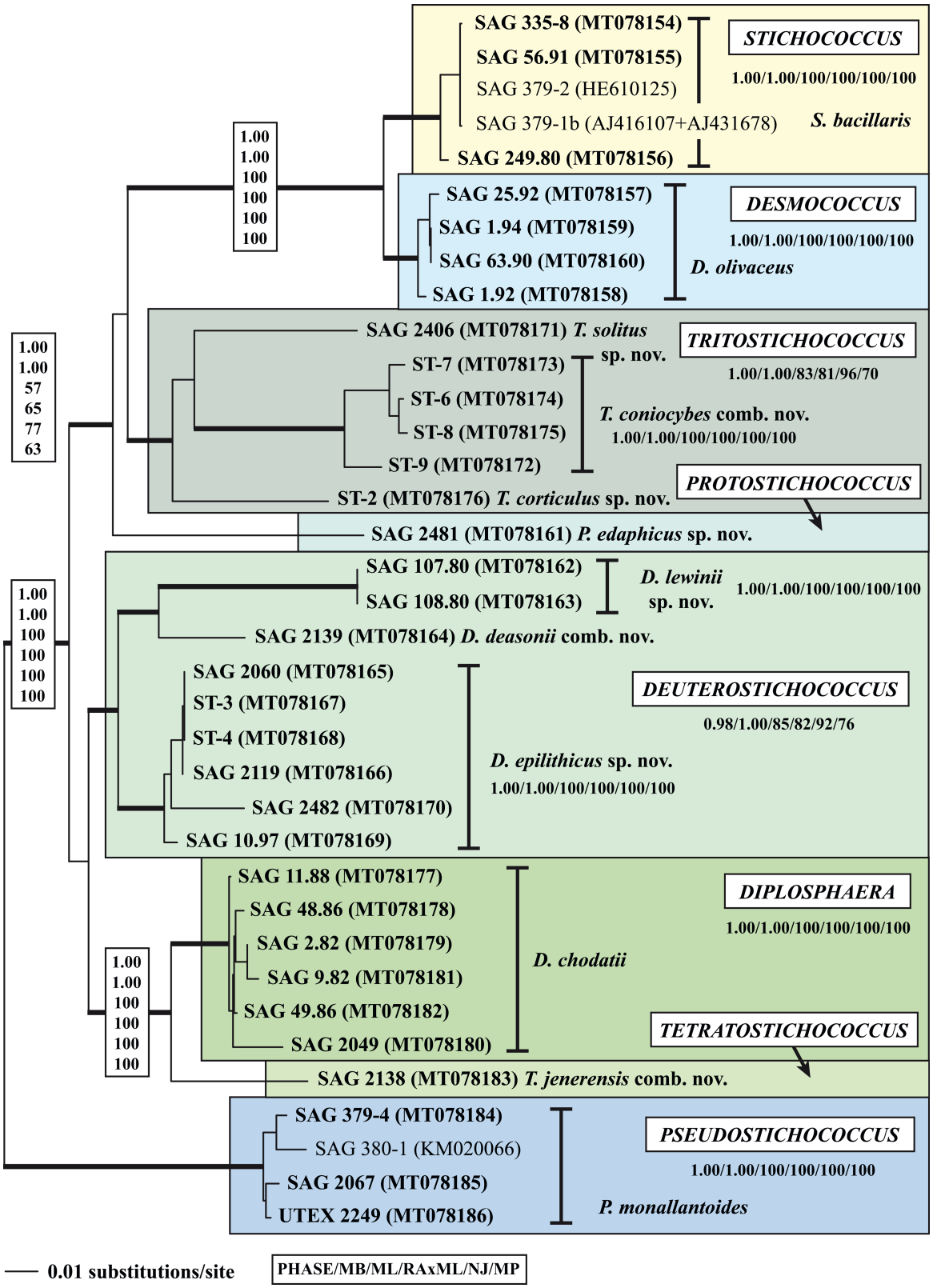

FIGURE 5. Molecular phylogeny of the *Stichococcus*-like organisms belonging to the *Prasiola* clade based on SSU and ITS rDNA sequence comparisons. The phylogenetic tree shown was inferred using the maximum likelihood method based on the data sets (2638 aligned positions of 36 taxa) using PAUP 4.0a166. For the analyses the best model was calculated by Modeltest 3.7. The setting of the best model was given as follows: GTR+I+G (base frequencies: A 0.2422, C 0.2564, G 0.2711, T 0.2303; rate matrix A–C 1.2334, A-G 1.9380, A–U 1.2950, C-G 0.7130, C-U 4.7292, G-U 1.0000) with the proportion of invariable sites (I = 0.6846) and gamma shape parameter (G = 0.4111). The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values >50% calculated with PAUP using maximum likelihood, neighbor-joining, maximum parsimony and RAxML using maximum likelihood). The clades are named after the genera (color-coded) proposed in this study.

To test further if the eight lineages represented genera, additional analyses were undertaken: (i) In addition to the SSU, the ITS rDNA were sequenced and analyzed. As demonstrated in Fig. 5, the phylogenetic analyses of the combined dataset (SSU+ITS) showed a higher support for the clades representing genera. The ITS-1, 5.8S, and ITS-2 sequences were aligned according to their secondary structures and the dataset was analyzed using the complex evolutionary models as highlighted above for the SSU rDNA sequences. (ii) For comparison, the plastid-coding *rbc*L gene was sequenced from selected strains. The resulting trees (using all three or only the first codon bases) were compared with the trees of SSU+ITS, ITS, and ITS-2 as well as all genes combined. As shown in Fig. 6, the concatenated dataset of SSU and ITS rDNA revealed the highest resolution among the *Stichococcus*-like organisms. The analyses of ITS, ITS-2 and *rbc*L (3 or 2 codon bases) alone reduced or show no support using bootstrap and Bayesian methods (branches marked with an asterisk in Fig. 6).

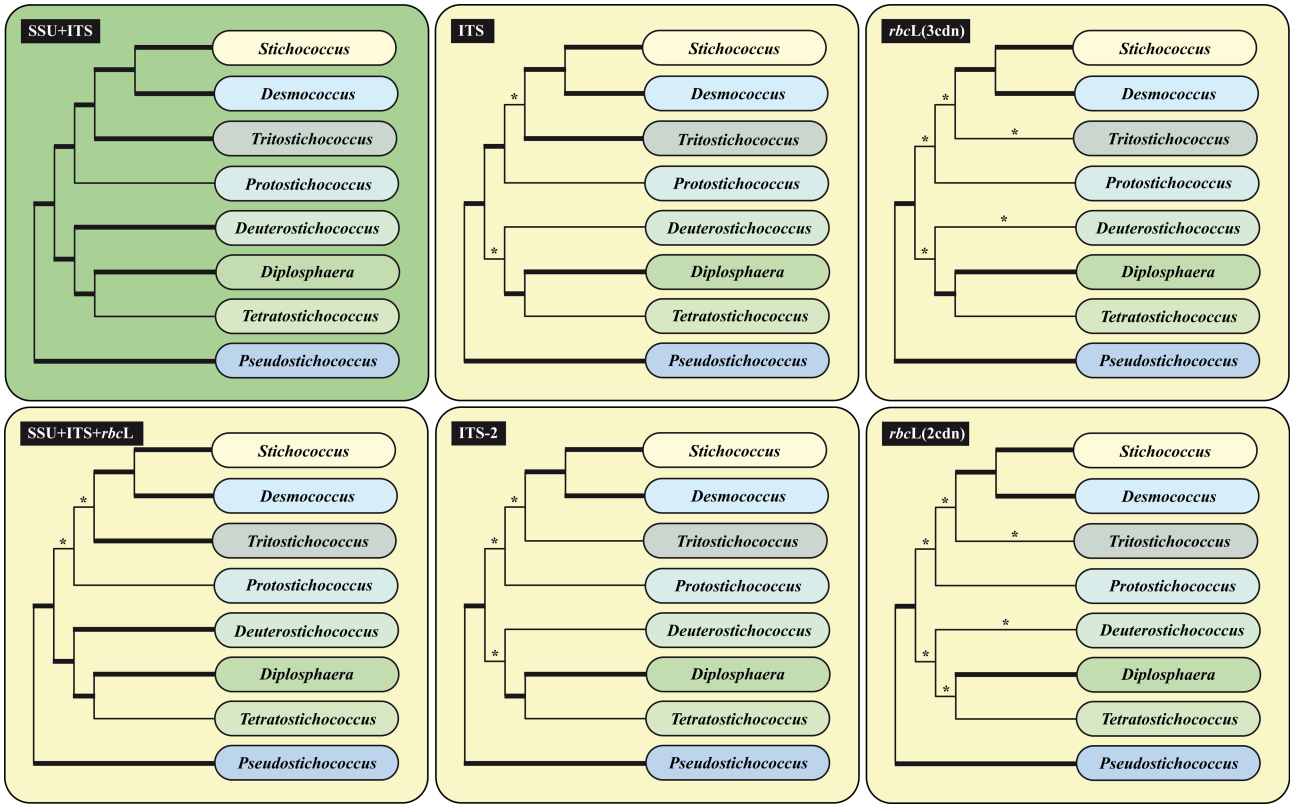

FIGURE 6. Molecular phylogeny of the *Stichococcus*-like organisms belonging to the *Prasiola* clade based on different sequence comparisons. The phylogenetic trees shown were inferred using the maximum likelihood method based on the data sets (27 taxa: 2638 aligned positions for SSU+ITS, 3808 for SSU+ITS+*rbc*L, 853 for ITS, 395 for ITS-2, 1170 for *rbc*L-3cdn, and 780 for *rbc*L-2cdn) using PAUP 4.0a166. For the analyses the best models were calculated by Modeltest 3.7. The setting of the best model was given as follows: (**SSU+ITS**) GTR+I+G (base frequencies: A 0.2417, C 0.2567, G 0.2718, T 0.2298; rate matrix A-C 1.2464, A-G 1.9043, A-U 1.2482, C-G 0.6997, C-U 4.9919, G-U 1.0000) with the proportion of invariable sites (I = 0.6909) and gamma shape parameter (G = 0.3997); (**SSU+ITS+*rbc*L**) GTR+I+G (base frequencies: A 0.2585, C 0.2249, G 0.2500, T 0.2666; rate matrix A-C 1.2577, A-G 2.4231, A-U 2.0869, C-G 1.1260, C-U 5.7459, G-U 1.0000) with the proportion of invariable sites (I = 0.6602) and gamma shape parameter (G = 0.6279); (**ITS**) GTR+I+G (base frequencies: A 0.2246, C 0.3155, G 0.2695, T 0.1904; rate matrix A-C 0.9335, A-G 1.8227, A-U 1.3977, C-G 0.4634, C-U 4.2638, G-U 1.0000) with the proportion of invariable sites (I = 0.3970) and gamma shape parameter (G = 0.7804); (**ITS-2**) GTR+I+G (base frequencies: A 0.2059, C 0.3097, G 0.2812, T 0.2032; rate matrix A-C 1.4399, A-G 2.9995, A-U 1.9939, C-G 0.6805, C-U 4.8834, G-U 1.0000) with the proportion of invariable sites (I = 0.3679) and gamma shape parameter (G = 1.3342); (***rbc*L-3cdn**) GTR+G (base frequencies: A 0.2826, C 0.1564, G 0.2160, T 0.3450; rate matrix A-C 1.3708, A-G 3.5871, A-U 3.2823, C-G 1.5354, C-U 10.5619, G-U 1.0000) with the gamma shape parameter (G = 0.1712); (***rbc*L-2cdn**) TrN+I+G (base frequencies: A 0.2637, C 0.2151, G 0.2839, T 0.2373; rate matrix A-C 1.0000, A-G 1.0632, A-U 1.0000, C-G 1.0000, C-U 4.0491, G-U 1.0000) with the proportion of invariable sites (I = 0.7852) and gamma shape parameter (G = 0.8121).

The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values > 50% calculated with PAUP using maximum likelihood, neighbor-joining, maximum parsimony and RAxML using maximum likelihood). The branches marked with an asterisk are not supported in our analyses.

Species concept among *Stichococcus* and related genera using the ITS-2/CBC approach

Among the highly supported lineages representing genera in Fig. 5, the genetic variability was very low. Only within *Deuterostichococcus* and *Tritostichococcus* a higher rate of base differences was detected. We applied the ITS-2/CBC approach (Pröschold *et al.*, 2018, Darienko & Pröschold, 2019, and references therein) to distinguish how many species can be found in each genus. This approach clearly demonstrated among the investigated strains that *Stichococcus*, *Desmococcus*, *Protostichococcus*, *Diplosphaera*, *Tetratostichococcus*, and *Pseudostichococcus* were monotypic genera (Figs 3,5,7 and Table S2). In contrast, three species could be discovered in the genera *Deuterostichococcus* and *Tritostichococcus*, respectively. All species are separated by unique CBCs and HCBCs in the barcode regions as highlighted in blue in Fig. 7. Hodac *et al.* (2016) have shown that the diversity among the *Stichococcus*-like organisms is much higher than expected. Therefore, we used the BLAST N search approach (100% coverage, >97% identity) to find the ITS-2 sequences of all *Stichococcus*-like entries in GenBank. 119 entries (including our investigated strains) were analyzed using the ITS-2/CBC approach. Few potentially new species were found among the genera *Deuterostichococcus*, *Tritostichococcus*, and *Pseudostichococcus* (accession numbers and origin of these entries were summarized in Table S4). Unfortunately, no additional data were available for these entries. Therefore, they were only marked with an asterisk in Fig. 7. Only three entries could be named (marked with # in Fig. 7). The ITS-2 sequences of those were described as *Stichococcus allas* and *S. antarcticus* by Beck *et al.* (2019). Our approach revealed that both authentic strains represented only one species. Unfortunately, these strains were not available in this study and no complete SSU rDNA were sequenced. To confirm that these strains belonged to *Deuterostichococcus*, we combined the available V9, ITS and *rbc*L into a dataset of selected taxa. The phylogenetic analyses (Fig. S1) clearly demonstrated that both strains represented one species of *Deuterostichococcus*, *D. allas* (see below in Taxonomic consequences).

BARCODE	5.8S/ LSU stem	Helix I	Helix II	Helix III	SPECIES
Barcode-6A	2342324241332415	64344	65344374643	33466342344336442846413814333343413483361365342 2	*Stichococcus bacillaris*
Barcode-6B	.....	.....	.....	.....2.....	*Stichococcus bacillaris*
Barcode-10A	2342324241332415	64344	65344374643	33466342344336488842444134433343413483361365342 2	*Desmococcus olivaceus*
Barcode-10B	.....	.....	.....	.....5.....	*Desmococcus olivaceus*
Barcode-3A	2342324241332435	64344	65344374643	33466348383338888842488158833543413483361365342 8	*Tritostichococcus corticulus*
Barcode-3B	.....	.....	3	.....8.8.338888842488118.335.....4.....2.	*Tritostichococcus corticulus*
Barcode-3C	.....	.....	3	.....8.8.331488842488118.335.....4.....2.	*Tritostichococcus corticulus*
Barcode-3D	.....	.....	3	.....8.8.331488842488118.335.....2.....2.	*Tritostichococcus* new species 1 *
Barcode-4A	.....	.....	3	.....3.4.514442418888113.335.....4.....8.	*Tritostichococcus solitus*
Barcode-4B	.....	.....	3	.....3.4.314482418888113.335.....4.....8.	*Tritostichococcus solitus*
Barcode-5A	.....	.....	1	.....3.4.342888842888238.553.....4.....2.	*Tritostichococcus coniocybes*
Barcode-1A	2342324241332435	64344	65344374643	33466343343314448888888881483554341348336136534 8	*Protostichococcus edaphicus*
Barcode-1B	.....	.....	.....	...6.....6..55.....	*Protostichococcus edaphicus*
Barcode-1C	.....	.....	.....	...4.....4..33.....	*Protostichococcus edaphicus*
Barcode-1D	.....	.....	.....	...6.....4..33.....	*Protostichococcus edaphicus*
Barcode-2A	2342324241332435	64344	65344374643	33463433454314424244414311853543413483321363348 8	*Deuterostichococcus lewinii*
Barcode-2B	.....	...4.	3	..4.3.....43..44242444431183 3.....21..5....	*Deuterostichococcus* new species 1 *
Barcode-2C	.....	...4.	3	..4.3.....83..824266888318888.....61..5....	*Deuterostichococcus epilithicus*
Barcode-2D	.....	...4.	3	..4.3.....83..824266888358888.....61..5....	*Deuterostichococcus epilithicus*
Barcode-2E	.....	...4.	3	..4.3.....83..824266888318888.....21..5....	*Deuterostichococcus epilithicus*
Barcode-2F	.....	...4.	3	..4.3.....83..864242418318888.....61..5....	*Deuterostichococcus epilithicus*
Barcode-2G	.....	...6.	3	..4.3.....83..864242418318888.....61..5....	*Deuterostichococcus epilithicus*
Barcode-2H	.....	...4.	2	..4.3.....43..424463264154833.....61..5....	*Deuterostichococcus deasonii*
Barcode-2I	.....	...4.	2	..4.3.....45..442483224154833.....61..5....	*Deuterostichococcus* new species 2 *
Barcode-2J	.....	...4.	2	..4.3.....43..424463224154833.....61..5....	*Deuterostichococcus* new species 3 *
Barcode-2K	.....	...4.	2	..4.3.....43..424641224512833.....61..5....	*Deuterostichococcus* new species 4 *
Barcode-2L	.....	...4.	3	..6.3.....43..424432264813833.....61..5....	*Deuterostichococcus allas #*
Barcode-2M	.....	...4.	3	..4.3.....43..424432264813833.....61..5....	*Deuterostichococcus allas #*
Barcode-2N	.....	...4.	3	..4.3.....43..424432664813833.....65..5....	*Deuterostichococcus allas #*
Barcode-2O	.....	...4.	3	..4.5.....83..824266888318888.....61..5....	*Deuterostichococcus epilithicus*
Barcode-8A	2342324241332435	64344	65344374643	33443433434314488843343131853343413483361365348 8	*Diplosphaera chodatii*
Barcode-8B	.....	.....	.....6.	.....	*Diplosphaera chodatii*
Barcode-7A	2342324241332435	64344	65344374643	33443433434314888843341138833343413483361365348 8	*Tetratostichococcus jenerensis*
Barcode-7B	.....	.....	.....	.....8.....3....	*Tetratostichococcus jenerensis*
Barcode-9A	2342324241332445	64344	65344374643	33434334333444888888888312353543413465321323348 8	*Pseudostichococcus monallantoides*
Barcode-9B	.....	...4.	.....	...3.....253.....34..	*Pseudostichococcus monallantoides*
Barcode-9C	.....	...4.	.....	...5.....635.....34..	*Pseudostichococcus monallantoides*
Barcode-9D	.....	...4.	.....	...3.....253.....18..	*Pseudostichococcus* new species 1 *
Barcode-9E	.....	...2.	.....	...5.....635.....34..	*Pseudostichococcus* new species 2 *

FIGURE 7. Comparison of the conserved region of ITS-2 among the species of the *Stichococcus*-like genera. Extraction of this region and translation into a number code for its usage as barcode. 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. Potential new species discovered by the GenBank search are highlighted with an asterisk. Compensatory base changes are marked in blue numbers.

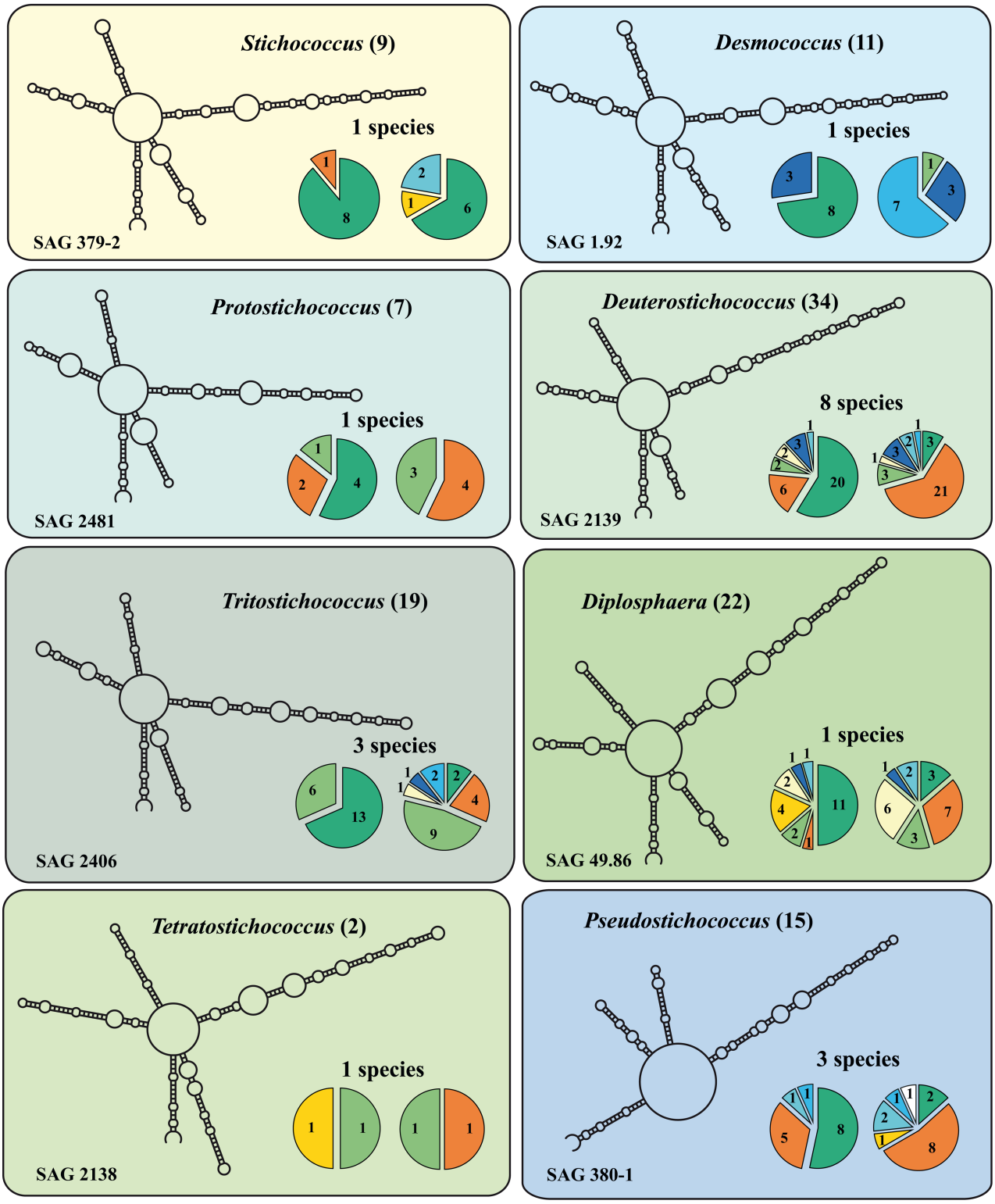

FIGURE 8. Distribution pattern of *Stichococcus*-like genera received by GenBank search of the ITS-2 rDNA sequences. The distribution pattern of each genus are given after the geographical origin and after the habitat. The numbers of GenBank entries are given after generic names in brackets. The secondary structure of the type species of each genus is given.

Morphology and phenotypic plasticity of *Stichococcus*-like taxa

As demonstrated above, the investigated strains belonged to eight different genera of the *Prasiola* clade (Trebouxiophyceae). To discover if the strains also differ in their morphology from each other, we studied the phenotypic plasticity under different conditions (standard conditions: 18°C, 16:8 h light in 3N-BBM+V, salinity stress: same conditions in SWES, and low temperature: 8°C, same conditions as standard). All morphological observations were done at three weeks old cultures.

The genus *Stichococcus* (Fig. 9A-I). All strains SAG 335-8, SAG 56.91, SAG 249.80 and SAG 379-2 were similar in morphology. The two SAG 335-8 and SAG 56.91 were originally designated as *Gloeotila scopulina* and *Gloeotila protogenita*, respectively. However, both strains formed filaments without mucilage, which partially disintegrated. The filaments of all strains consisted from cylindrical cells, possessed parietal chloroplast with pyrenoid, which was only visible after staining with Lugol solution. Median cell size was 8.3 µm x 3.6 µm with length:width ratio 2.2. The strain SAG 249.80 had similar cell size 9.6 µm x 3.8 µm and ratio 2.5. SAG 379-2 is characterized by slightly bigger cells 11.1 µm x 4.3 µm with ratio 2.7 and formed short easy disintegrated filaments consisting out of 3-5 cells. Under saline conditions all strains grew very well. The cells became shorter and thinner (median 5.1-5.7 µm x 2.4-3.1 µm with ratio 2.0-1.8) and formed long curved filaments. The strain SAG 379-2 formed 4-6 celled filaments, which slightly spiral. Cultivated at 8°C, cells often became irregular, sometimes from one side pointed. Cells became strongly vacuolized. The median cell size was 10.2 µm x 4.0 µm and is slightly bigger than under standard conditions (9.6 µm x 3.8 µm). The strain SAG 335-8 showed a similar reaction. The long filaments disappeared and formed filaments of 3-8 cells. Some cells became shorter and thicker, other cells become from one side swollen. On each pole of cell large vacuoles were present. Median cells size was 10.9 µm x 4.2 µm. SAG 379-2 showed the strongest influence under temperature stress. Different reactions were observed. Some cells formed 3-8 celled filaments, some cells became irregularly twisted. Occasionally cells started to form 3-4 chloroplast per cell, what could be interpreted as "autospore" formation. Median cell size was 9.5 µm x 4.3 µm, which was smaller than under standard condition. The strain SAG 249.80 formed short filaments consisting of 2-5 cells.

The genus *Desmococcus* (Fig. 1K-N). The four investigated strains showed the same morphology, which was described for *D. olivaceus* by Ettl & Gärtner (2014). The typical akinete formation and the production of *Stichococcus*-like autospores were observed in all strains. At low temperature the cells showed no morphological changes. Under marine conditions, all strains died after three weeks.

The genus *Protostichococcus* (Fig. 9J-L). The strain SAG 2481 was characterized by the smallest cells (median cell size 4.7 x 2.5 µm). The cell morphology was similar to *Stichococcus* described above with one exception, the absence of a pyrenoid in SAG 2481. Under marine conditions, the alga formed short filaments consisting of 2-3 cells. The cells became almost twice as long as under standard conditions. The median cell size was 7.0 x 2.4 µm with ratio 2.7. The cells were often curved, sometimes elongated up to 12.5 µm. Under low temperature, the cells became longer and were mostly vacuolized.

The genus *Deuterostichococcus* (Fig. 10A-L, 11A-D). The nine strains were similar in morphology with one exception. Only the strain SAG 2139 differed from the other strains by presence of a single pyrenoid. All strains had cylindrical cells with variable cell size. The median cell size of different strains varied from 5.4 x 2.5 until 9.6 x 3.7 µm (ratio 2.0-2.9). Under salinity stress, the strains showed different reactions. The cells of SAG 107.80 became longer in comparison with freshwater medium and reached a median cell size of 7.4 x 2.7 µm with a ratio of 2.6. Sometimes long cells (~11.0 µm) were observed. This alga was always unicellular. The strain SAG 2060 formed 2-3 celled filaments, sometimes with cell length up to 15 µm containing 3-4 chloroplasts (delayed division). The median cell size was 7.7 x 3.0 µm (ratio 2.6). The cells became only slightly longer and thinner than on 3N-BBM+V. The strain SAG 2139 changed the cell shape and size on SWES medium. The median cell size reached 7.0 x 3.1 (ratio 2.1). Cells became irregularly, kidney-shaped, ovoid and curved, connected to 2-3 celled loose filaments. Cells contained several large oil drops. SAG 2482 formed very curved short filaments. Cells were irregular, sigmoid (median cell size: 8.0 x 2.3 µm, with ratio 3.3). At low temperature, most of the strains became longer without changes of the width. The median cell sizes increased almost twice as long as under standard conditions. Sometimes the cells became pointed from one side. The formation of 2-5 celled filaments were observed.

The genus *Tritostichococcus* (Fig. 12A-L, 13A-C). The cells of all strains were cylindrical with parietal chloroplast without pyrenoid. The median cell size was 5.8 x 2.6 µm with a ratio of 2.1. Sometimes swollen cells with large vacuoles were present in culture, which were twice bigger than normal cells. Under marine medium, the cell size increased up to 7.7 x 2.8 (ratio 2.6). Sometimes long cells with several chloroplasts and the delay of the cell division were observed. Cells gathered together in loose filamentous-like spiral structures. At 8°C, the strains showed the same morphological changes as described for *Protostichococcus* and *Deuterostichococcus.*

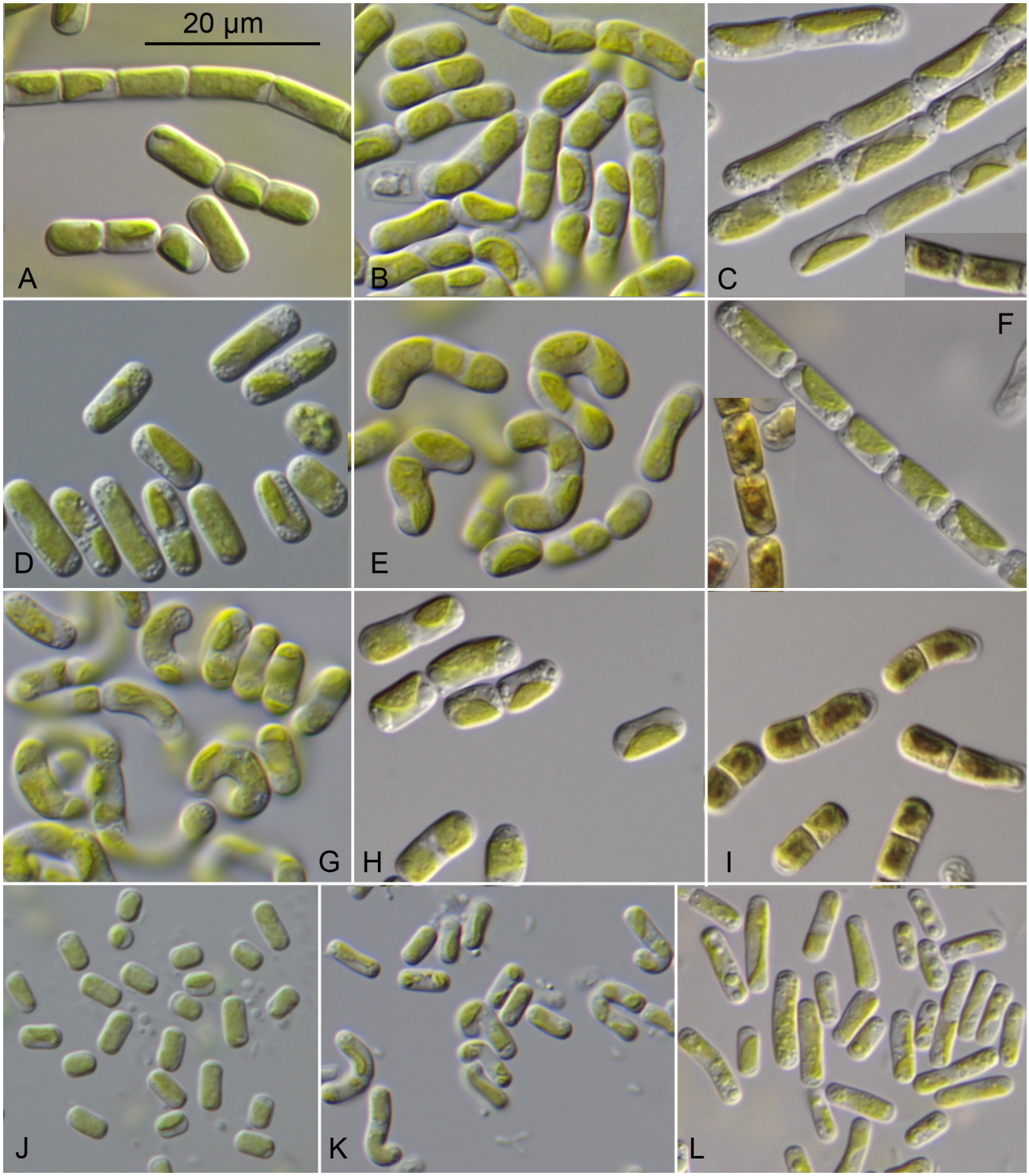

FIGURE 9. Morphology and phenotypic plasticity of the investigated strains belonging to *Stichococcus* (**A.–I.**) and *Protostichococcus* (**J.–L.**) after growth of three weeks under different conditions. **A.** SAG 335-8 (3N-BBM+V), **B.** SAG 335-8 (SWES), **C.** SAG 335-8 (3N-BBM+V, 8°C), **D.** SAG 379-2 (3N-BBM+V), **E.** SAG 379-2 (SWES), **F.** SAG 379-2 (3N-BBM+V, 8°C), **G.** SAG 249.80 (1/2 SWES), **H.** SAG 249.80 (SWES), **I.** SAG 249.80 (3N-BBM+V, 8°C), **J.** SAG 2481 (3N-BBM+V), **K.** SAG 2481 (SWES), **L.** SAG 2481 (3N-BBM+V, 8°C).

The genus *Diplosphaera* (Fig. 11E–M). Three out of six strains, SAG 11.88, SAG 2049 and SAG 49.86 were identical in their morphology. They were unicellular or 2- rarely 4-celled packages, without mucilage. The cells were almost spherical, 3.4 x 2.8 µm in size with a ratio of 1.1. Chloroplasts were parietal without pyrenoid. The strain SAG 48.86 exhibited the same cell size, but the cells were surrounded by mucilage. Sometimes the cells with warts and brown cell wall could be observed (probably akinetes?). The strain SAG 2.82 formed packages containing 8–16 cells,

which usually did not disintegrate. SAG 9.82 characterized by slightly longer cells up to 5.0 x 3.8 µm with a ratio of 1.3. All strains except SAG 48.86 were without mucilage. Under marine conditions, all strains reacted with an increase of the cell size (4.8 x 3.7–6.2 x 5.2 µm). At low temperature of 8°C, the cells of all strains became elongated reaching the length:width ratio of almost 2.1.

The genus *Tetratostichococcus*. The cells of SAG 2138 formed single cylindrical cells with a size of 6.3 x 3.8 µm (ratio 2.9). The parietal chloroplast consisted an inconspicuous pyrenoid. In old culture swollen cells were rarely present. Detailed morphological description was provided by Neustupa *et al.* (2007). The strain was not observed under marine conditions and at low temperature.

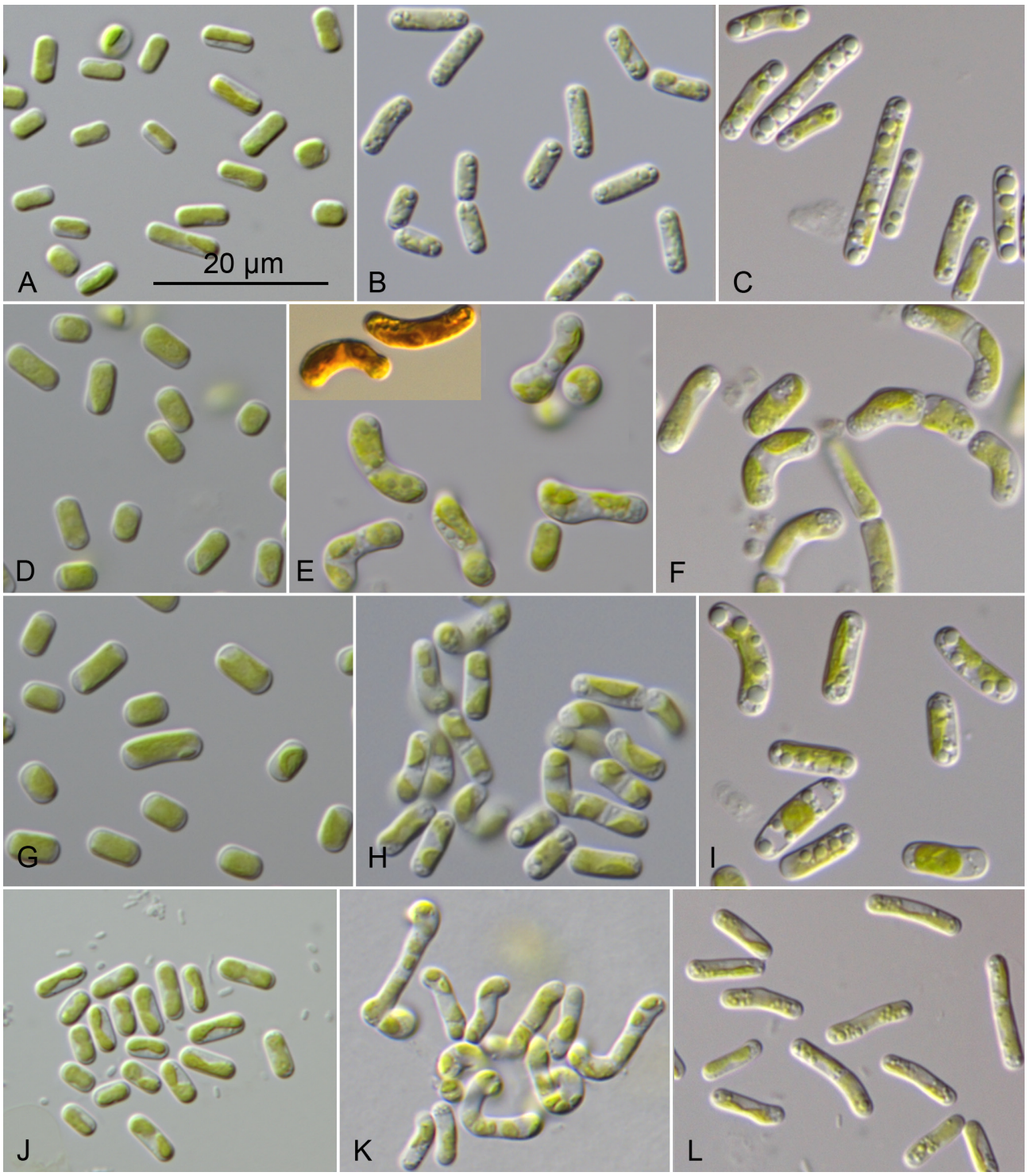

FIGURE 10. Morphology and phenotypic plasticity of the investigated strains belonging to *Deuterostichococcus* after growth of three weeks under different conditions. **A.** SAG 107.80 (3N-BBM+V), **B.** SAG 107.80 (SWES), **C.** SAG 107.80 (3N-BBM+V, 8°C), **D.** SAG 2139 (3N-BBM+V), **E.** SAG 2139 (SWES), **F.** SAG 2139 (3N-BBM+V, 8°C), **G.** SAG 2060 (3N-BBM+V), **H.** SAG 2060 (SWES), **I.** SAG 2060 (3N-BBM+V, 8°C), **J.** SAG 2482 (3N-BBM+V), **K.** SAG 2482 (SWES), **L.** SAG 2482 (3N-BBM+V, 8°C).

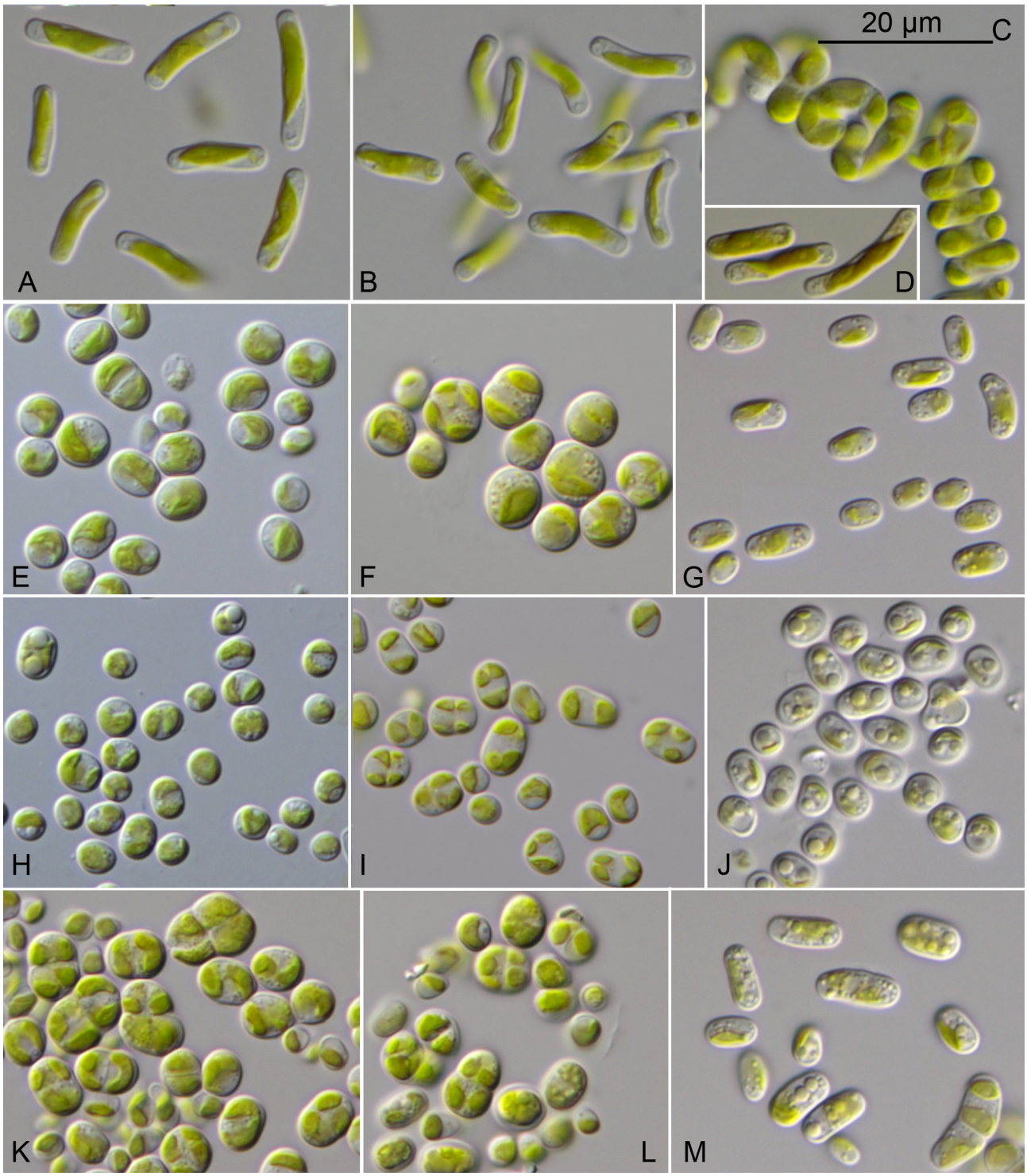

FIGURE 11. Morphology and phenotypic plasticity of the investigated strains belonging to *Deuterostichococcus* (**A.–D.**) and *Diplosphaera* (**E.–M.**) after growth of three weeks under different conditions. **A.** strain ST-10 (3N-BBM+V), **B.** strain ST-10 (1/2 SWES), **C.** strain ST-10 (SWES), **D.** strain ST-10 (Lugol stained, 3N-BBM+V), **E.** SAG 48.86 (3N-BBM+V), **F.** SAG 48.86 (SWES), **G.** SAG 48.86 (3N-BBM+V, 8°C), **H.** SAG 49.86 (3N-BBM+V), **I.** SAG 49.86 (SWES), **J.** SAG 49.86 (3N-BBM+V, 8°C), **K.** SAG 9.82 (3N-BBM+V), **L.** SAG 9.82 (SWES), **M.** SAG 9.82 (3N-BBM+V, 8°C).

The genus *Pseudostichococcus* (Fig. 13D–L). The four strains SAG 379-4, SAG 380-1, SAG 2067, and UTEX 2249 were morphologically most diverse. The strain SAG 379-4 originally designated as *Stichococcus fragilis* showed with median cell size of 8.2 x 2.2 µm, sometimes even up to 25–30 x 2.7–2.9 µm. The authentic strain of *Pseudostichococcus monallantoides* (SAG 380-1) had cylindrical cells with the median cell size of 5.7 x 2.2 µm (ratio 2.6). Cells appeared sometimes irregular and rarely formed 2–4-celled packages. The strain UTEX 2249 originally

designated as *Pleurastrum photoheterotrophicum* was mostly unicellular, but formed sometimes 3–4 celled filaments. Some cells were curved. The strain SAG 2067 had oval cells with round cell ends (median cell size 5.1 x 3.9 µm and ratio 2.1) and formed 2–4 celled packages. Some packages produced brownish cell wall with warts. Under marine conditions, the cells of strain SAG 380-1 became bigger in size (median cell size 8.9 x 3.6 µm, ratio 2.4). The strain 2067 was unicellular, ellipsoidal, with a median cell size of 5.5 x 4.6 µm (ratio 1.2) and was strongly vacuolized. The cells of UTEX 2249 became often irregular, sigmoid, sometimes swollen on one side and curved (median cell size 7.0 x 3.1 µm, ratio 2.2). Rarely 2–3 celled filaments could be observed. Occasionally elongated cells up to 11.2 x 3.0 µm (ratio 3.8) were formed in culture. At 8°C the strains SAG 380-1 and SAG 2067 became bigger and vacuolized, but without any morphological changes. Only the cells of strain UTEX 2249 became longer (up to10.8 µm), pyriform, needle-like, from one side pointed and cell wall thickening.

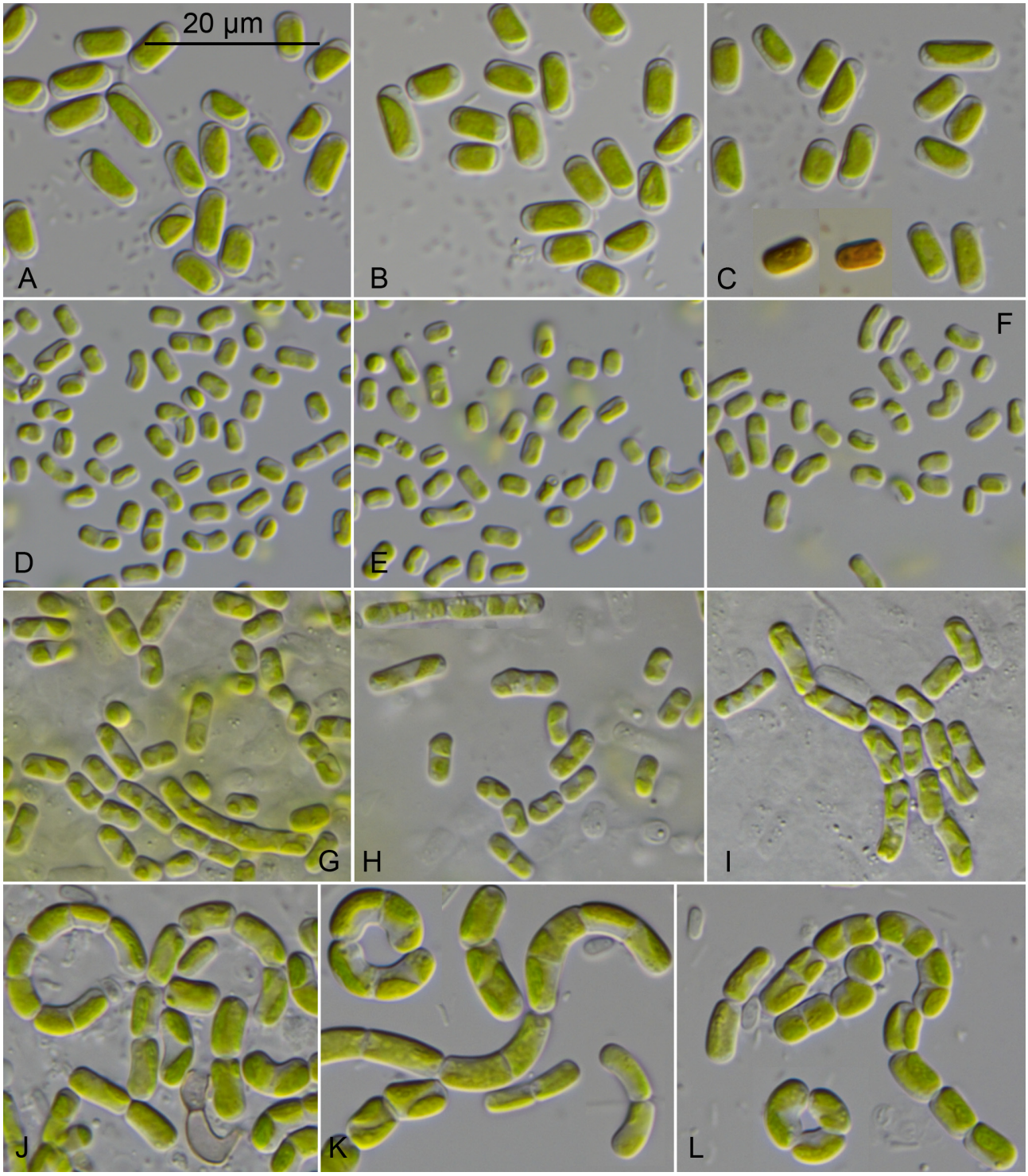

FIGURE 12. Morphology and phenotypic plasticity of the investigated strains belonging to *Tritostichococcus* after growth of three weeks under standard conditions (3N-BBM+V). **A.-C.** strain ST-2, **D.–F.** strain ST-7, **G.–I.** strain ST-8, **J.–L.** strain ST-9.

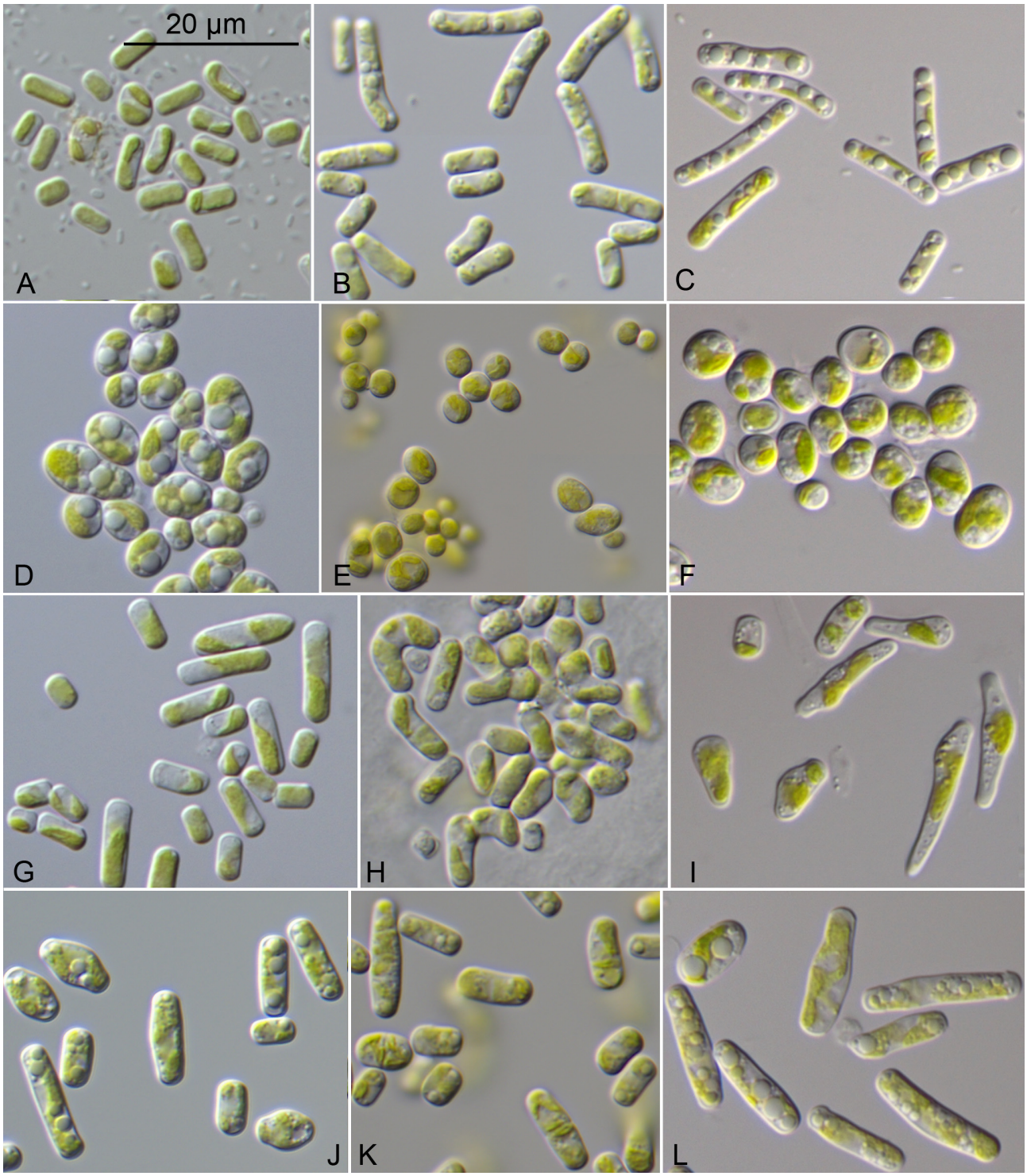

FIGURE 13. Morphology and phenotypic plasticity of the investigated strains belonging to *Tritostichococcus* (**A.–C.**) and *Pseudostichococcus* (**D.–L.**) after growth of three weeks under different conditions. **A.** SAG 2406 (3N-BBM+V), **B.** 2406 (SWES), **C.** 2406 (3N-BBM+V, 8°C), **D.** SAG 2067 (3N-BBM+V), **E.** SAG 2067 (SWES), **F.** SAG 2067 (3N-BBM+V, 8°C), **G.** UTEX 2249 (3N-BBM+V), **H.** UTEX 2249 (SWES), **I.** UTEX 2249 (3N-BBM+V, 8°C), **J.** SAG 380-1 (3N-BBM+V), **K.** SAG 380-1 (SWES), **L.** SAG 380-1 (3N-BBM+V, 8°C).

For comparison, the length:width ratios of all *Stichococcus*-like strains were given in boxplots (Fig. 13). As demonstrated, only the genus *Diplosphaera* can be clearly separated from the others. The median ratio was 1.1–1.3, whereas the ratios of the other genera were higher than 2 with one exception. The strains SAG 2067 belonging to *Pseudostichococcus* had also a ratio of 1.2. The median length of all strains is more variable (3–12 µm) as the median width (2–4 µm). Details about length and width is given in Fig. S2.

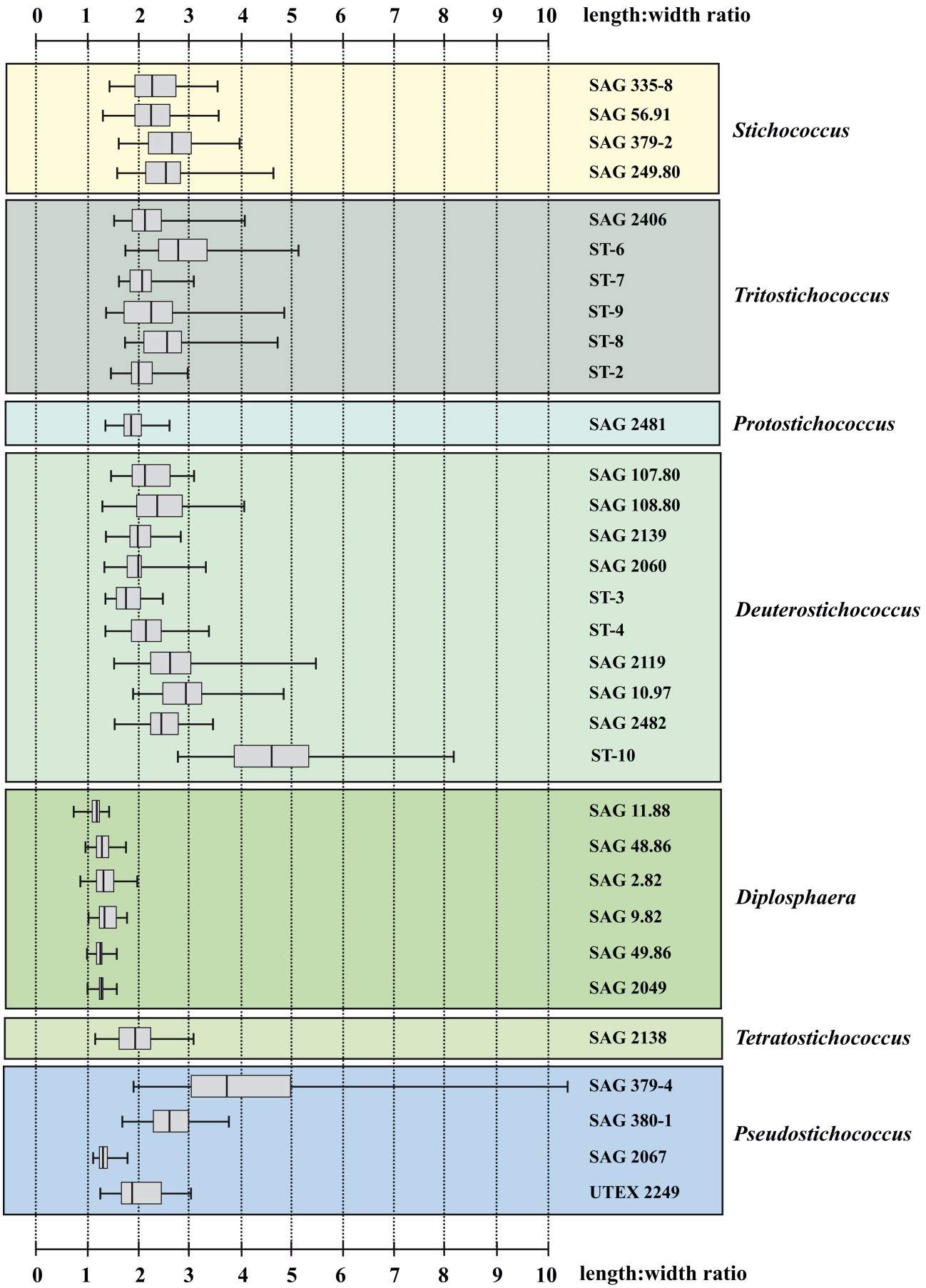

FIGURE 14. Length:width ratios of the investigated strains belonging to the different genera presented in box-and-whisker diagrams.

Discussion

Polyphyly and distribution of *Stichococcus*-like algae

All investigated *Stichococcus*-like strains belonged to the *Prasiola* clade and formed seven independent lineages within this clade. The rod-like morphotype is distributed in all of the seven clades, which makes it difficult or even impossible to assign the strains at generic and species level as shown in Fig. 1. Hodac *et al.* (2016) demonstrated that *Stichococcus*-like organisms represented nine lineages (called OTU 1-9), which already questioned the generic concept of the traditional genus *Stichococcus*. Our analyses revealed only eight lineages because additional sequences united the OTUs 3-5 to one lineage, *Tritostichococcus*. The difficulties to assign the strains is already reflected in the naming of them in public culture collections. Most of them were designated as *Stichococcus* sp., and even the generic assignment is difficult, then some strains were named as different species of *Gloeotila* or as *Nannochloris*, *Chlorosarcina* and *Pleurastrum*. In addition, the achievement of a robust phylogeny of *Stichococcus*-like organisms is complicated for two reasons. (i) For phylogenetic analyses using complex evolutionary models (including secondary structure models), accurate and complete SSU and ITS rDNA sequences were necessary. However, many of the GenBank entries were incomplete and contained several sequencing errors, which can only be discovered by analyzing of the secondary structures. Such incomplete sequences or sequences with errors can result in incorrect tree topologies and lower support. However, sequencing errors could be discovered analyzing pairing regions in the secondary structures because base changes in those regions may require another changes on the compensatory site. If that is not the case, it resulted in mismatches, which indicated possible occurring errors. (ii) Most of the *Stichococcus*-like organisms contained one or more introns within the SSU rDNA, which makes sequencing difficult and required a lot of additional primer reactions to achieve the accurate and complete sequences. For both reasons, we included only few GenBank entries in our analyses. For example, we excluded two authentic strains, *Stichococcus allas* and *S. antarcticus*, from our analyses because the strains were not publicly available and no complete SSU rDNA are available in GenBank. Even the ITS rDNA sequences were incomplete and the available *rbc*L sequences were only 760 bases long (Beck *et al.*, 2019). To discover the phylogenetic position of both these species, we combined the V9, ITS and *rbc*L to a small dataset. The analyses (Fig. S1) revealed that both strains belonged to the genus *Deuterostichococcus* and represented only one species, *D. allas comb. nov.* (see below).

For understanding the distribution pattern of *Stichococcus*-like organisms, it is necessary to discriminate these taxa at least at generic level. Therefore, we searched based on the SSU secondary structures for synapomorphies for each genus. As shown in Table S2 and Fig. 4, the V9 region was ideal for this purpose. Each genus can be identified by the V9 region and the folding of its secondary structure revealed possible sequencing errors among GenBank entries. We used the BLAST N search using the V9 region (100% coverage and 100% identity) to get the GenBank entries for the distribution pattern of each genus. The patterns after geographical origin and habitats showed no clear distribution of the genera (Fig. 4). Only slight tendencies can be revealed. For example, most entries of *Tetratostichococcus* originated from Asian marine habitats. Most photobionts of lichens belonged to the genera *Deuterostichococcus* and *Diplosphaera*. However, no clear conclusions can be made from this analysis, because only few studies addressing this question are available, then no or only few records of *Stichococcus*-like organisms from Africa, Australia and South America are published. Therefore, the conclusion made by Hodac *et al.* (2016) that strains from temperate zones are closely related to those from the tropics, is premature and need comprehensive studies. Our experiments to cultivate the strains under freshwater and marine conditions as well as at low temperature clearly demonstrated that *Stichococcus*-like organisms are able to adapt to different environmental conditions and explained the worldwide distribution in almost all habitats.

New generic and species criteria using an integrative approach

As highlighted above, our analyses revealed that a new generic and species concept is required. The phylogenetic tree of SSU rDNA sequences already showed that the genus *Stichococcus* in traditional sense is split into seven lineages. For better phylogenetic resolution, we sequenced the ITS rDNA sequences, which is commonly used for discrimination at species level. In addition, we also sequenced the plastid-coding *rbc*L gene of selected taxa because this gene is often used for species delimitation especially of lichen photobionts. Our analyses supported the separation of *Stichococcus*-like organisms into seven clades (Figs 6–7). However, the bootstrap and Bayesian support differed depending on genes used for analyses. The designated genera in Fig. 3 got the highest support using the concatenated dataset of SSU and ITS (Figs 5–6). The *rbc*L phylogeny showed only a weak support for some genera when all three codon base positions were used, and even decreased if only the first two bases were analyzed. As consequence, *Stichococcus*-like

organisms should be analyzed by the usage of an integrative approach, which has been introduced for different lineages of green algae especially of trebouxiophycean genera and species (Darienko *et al.*, 2016). This approach always used the phylogeny of SSU and ITS as backbone, which has also been applied here in this study. The ITS-2/CBC approach resulted in unique barcodes, which can be analyzed for discrimination of taxa at species level. The Fig. 7 demonstrated that most genera were only represented by a single species. Only in the genera *Deuterostichococcus* and *Tritostichococcus* three species could be discovered, respectively. In addition, the ITS-2 BLAST N search algorithm (100% coverage and >97% identity) was applied to figure out more about the distribution of the species. The entries found in GenBank were also analyzed with the ITS-2/CBC approach, which resulted in the discovery of potential new species in the genera *Deuterostichococcus, Tritostichococcus* and *Pseudostichococcus* (Fig. 7). Unfortunately, no morphological and other data of these entries are available in publications. Therefore, these taxa cannot be described here. Similar to the V9 results described above, the distribution pattern about the genera and species could be discovered using the ITS-2 search results (see Fig. 8). Generally, *Stichococcus*-like organisms are widely distributed in almost all habitats (Ettl & Gärtner, 2014), but they are not recorded in environmental studies based on HTS approaches. The reason for this is probably the difficulties to amplify *Stichococcus* using PCR. Most HTS approaches uses the V4 or V9 regions of the SSU. As demonstrated here, most SSU sequences of *Stichococcus* contained introns nearby these variable regions and were probably discriminated during the amplification.

The genera *Stichococcus*, *Pseudostichococcus*, *Diplosphaera*, *Chodatia*, *Gloeotila* and *Chlorospira*—historical overview

For the taxonomic revision of *Stichococcus*-like organisms, it is necessary to get an overview about the described genera and species, then as highlighted above the strains showed a high phenotypic plasticity cultivated under different conditions. The polyphyly of the genus *Stichococcus* requested a decision, which of the phylogenetic lineage remains as *Stichococcus* and which needs to be transferred to new or to existing genera. According to the International Code for Nomenclature of Algae, Fungi and Plants (ICN), older generic and species names have priority against newer described ones. Therefore, we checked all old names of green algae with rod-like morphology. The genus *Stichococcus* was described by Nägeli (1849) who described three species (*S. bacillaris*, S. *minor* and *S. major*). The latter species were synonymized to *S. bacillaris* by Rabenhorst (1868) leaving *S. bacillaris* as type species. Since the first description, several species of *Stichococcus* were described. Heering (1914) revised the described taxa and excluded all pyrenoid-bearing algae from this genus. He also provided a more precise diagnosis of *Stichococcus*, which is still commonly used. He synonymized five species to *S. bacillaris* leaving seven species in this genus. Grinzesco & Peterfi (1932) provided the new morphological revision of this genus resulting in the description of three new species. They also did not accept some of Heering's taxonomical changes and recognized *S. minor* as separate species. Since then several species of *Stichococcus* were described or transferred to this genus. An overview about the currently 21 accepted species (including nine varieties) is given in Table S5.

The major problem for taxonomic revision is the lack of authentic material and the high phenotypic plasticity as highlighted above. In case of the genus *Pseudostichococcus* described by Moewus (1951), the authentic strain was available. This genus differed from *Stichococcus* by its marine occurrence and the formation of zoospores. In our morphological investigations no zoospores could be observed in the authentic strain as well as in the other strains of this clade. Our study also showed that marine isolates can occur in different clades and is therefore not a diagnostic feature for discrimination. The difficulties to identify species of *Stichococcus* is documented in the status of the genus *Diplosphaera*, which was described by Bialosuknia (1909) from the lichen *Lecanora tartarea*. From the description, it is not clear if *D. chodatii* is the photobiont of this lichen or only an epiphyte of it. Since its description, *Diplosphaera* had a debatable history. It was several times transferred to *Stichococcus* or recognized as separate genus by several authors (Chodat, 1913; Heering, 1914; Grinzesco & Peterfi, 1932; Vischer, 1953, 1960). For example, Chodat (1913) transferred it to *Stichococcus diplosphaera,* which was illegitimate according to the ICN. Heering (1914) corrected this combination to *Stichococcus chodatii* (Bialosuknia) Heering. Grintzesco & Peterfi (1932), Printz (1964), Ramanathan (1964) followed Heering and accepted synonymy of *Diplosphaera*. In contrast, Vischer (1960) emended the diagnosis of *Diplosphaera* and re-established the genus. Ettl & Gärtner (2014) followed Vischer and accepted *Diplosphaera* as separate genus. Broady (1982) described the second species of the genus, *Diplosphaera mucosa*, which differed from the type by the presence of mucilage. We investigated six strains designated as *Diplosphaera*. The authentic strain *D. mucosa* (SAG 48.86) showed until now the typical morphology and mucilaginous layer was always present, independently of presence or absence of any stress. The strains SAG 2049 and SAG 11.88 are characterized by identical morphology. The strain SAG 9.82, the authentic strain of *Nannochloris normandinae* (Tschermak-Woess, 1981), showed the typical *Diplosphaera* morphology, except of cells were slightly longer as other three strains. SAG

2.82, a derivate of SAG 9.82, was the only strain, which formed packages of four or more cells, which was illustrated by Vischer (1960). The investigation of morphology under low temperature showed that strains changed the cell length and became longer and similar to small *Stichococcus* (Fig. 11F,I,L), which could be an explanation why many scientists doubt the separation of both genera *Stichococcus* and *Diplosphaera*. Unfortunately the small morphological differences were not supported in the molecular phylogeny. Only the strain SAG 2049 showed several differences in ITS-1 sequences and one HCBC in the conserved region of ITS-2 (ITS-2 Barcode 8B), but exhibit the typical morphology. *Diplosphaera* is also known as photobiont of different lichens (Zeitler, 1954; Thüs *et al.*, 2011; Fontaine *et al.*, 2012, 2013). Our analyses showed that the lichenized *Diplosphaera* represented the same species and were almost identical with free-living.

Another genus with *Stichococcus*-like morphology was described by Kol (1934). She found a species in Pennine Alps (Switzerland) and named it *Chodatia tetrallantoides.* The cells are curved with rounded poles and after division are loosely connected forming 2–4 celled filaments. The cell size varies from 1.5 x 6.0–7.5 µm (ratio 3–4). She separated this genus from *Stichococcus* by its cell division through slant division. However, this feature could be also observed in *Stichococcus allas*, which was described by Reisigl (1964). He also found this species in the Austrian Alps (Weißkugel, Ötztal Alps). This alga also formed short pseudofilaments, which easy disintegrated. Cells are curved with round cells. Cell sizes are 1.5–3.0 x 3.5–12.0 µm with length:width ratio of 3–4. Both species are also similar in their ecology. Unfortunately the generic name *Chodatia* was pre-occupied for the tetrasporacean alga described by Hansgirg (1903). The difference in slant or strait division should be ignored, because this feature is variable according to Hoham (1973). Fukushima (1963) synonymized *Chodatia tetrallantoidea* to *Stichococcus bacillaris*, Hindak (1996) to the *Stichococcus tetrallantoideus* (Chodat) Hindak, which is invalid because the generic name is illegitimate.

As shown in Fig. 3, some of investigated strains were designated as different species of *Gloeotila*, a genus that was described by Kützing (1843). The type description with its type species, *G. oscillarina*, is very poor and caused many different interpretations and misinterpretations later on. Hazen (1902) examined the existing herbarium specimens from that time and established an lectotype of *Gloeotila*. The modern description of *Gloeotila* was provided by Heering (1914). His emended generic description comprises taxa with long or short filaments often surrounded by thick mucilagenous layer. Vegetative cells consist parietal chloroplasts without pyrenoids reproducing by zoospores. Usually only one zoospore was produced per cell. The akinetes were also known. Currently this genus includes 16 species and three doubtful species. Asexual reproduction was observed only for some species. No authentic strains or epitypes are known. At the present time no strain corresponded with the diagnosis of this genus and no isolates were available in the public culture collections. All of our investigated strains differed in morphology (no formation of mucilage layers and zoospores) compared to the description of *Gloeotila* and cannot therefore be assigned to this genus.

Korshikov (1939) described the genus *Chlorospira* for a (pseudo)filamentous alga without mucilage, which is spirally twisted. Cells are cylindrical, possessing chloroplasts without pyrenoids. The genus comprises three species, one out of them was synonymized to *Planktolyngbya*, a cyanobacterium, and the others were synonymized to *Stichococcus* (*S. crassus* (Korshikov) Hindak, *S. irregularis* (Korshikov) Hindak) by Hindak (1996). None of our strains formed twisted spirally filaments under standard conditions. For the four newly described lineages none of the published names can be applied. If someone finds the type species of *Chlorospira*, *C. crassa*, which can be assigned to one of our clades, this genus should then be transferred to *Chlorospira*.

The genus *Desmococcus*

The phylogenetic position of *Desmococcus* as sister of *Stichococcus* (in revised form) required several taxonomic revisions proposed below. In contrast to *Stichococcus*-like organisms, *Desmococcus* species can be easily differentiated by morphology from those taxa. However, *Desmococcus* has also a difficult taxonomical history, but in contrast to *Diplosphaera* was always recognized by phycologists, because distinct morphological features such formation of branched filaments, production of zoospores, formation of spiny or bold akinetes. The nomenclatural situation of *Desmococcus* was clarified by Laundon (1985). At present this genus includes five species *Desmococcus olivaceus* (Pers. Ex Ach.) Laundon, *D. spinocystis* Gärtner & Ignolic, *D. endolithicus* Broady & Ingerfeld, *D. antarcticus* Rybalka, Wolf, Andersen & Friedl, *D. apatococcus* Stockmayer. Unfortunately, only the authentic strains of *D. endolithicus* (SAG 25.92) and *D. antarcticus* (SAG 63.90) are available in public culture collection. The authentic strain of *D. spinocystis* (SAG 35.83) is extinct, but the SSU sequence is available under GenBank AJ431572 and clearly shows position within the genus *Desmococcus*. The strain SAG 2067 assigned as *D. spinocystis* belonged to the genus *Pseudostichococcus* and showed different morphology compared to *Desmococcus*. All strains had only minor differences in ITS 1-2 sequences and were therefore synonymized to one species, *D. olivaceus* (see below).

Taxonomical changes and consequences

For the taxonomy of *Stichococcus*-like organisms, the ratio of length to width was introduced to classify these algae. Heering (1914), Grintzesco & Peterfi (1932) and Raths (1938) subdivided them into three groups: 1. cells as long as wide or slightly longer as wide, 2. cells about six times longer than wide, and 3. cells 10 to 20-times longer than wide. As demonstrated in Fig 13 and Fig. S2, this grouping cannot be applied to our strains and can therefore not be used for classification.

As demonstrated above, several taxonomic changes and descriptions of new genera and species were necessary, which are given here as follows:

Stichococcus Nägeli emend. Pröschold & Darienko (Fig. 9A–I)

Emended description. Filaments usually few-celled (2–4 cells) or rarely long, easy fragmenting into single cells, uniseriate and unbranched, straight on freshwater medium or curved under marine condition. Cell walls thin, without mucilaginous layer. Cells cylindrical with length/wide ratio around 3. Cells possess rounded ends. Chloroplast single, parietal, not lobed with smooth margin, with or without pyrenoid. Pyrenoid (if present) is barely visible, oval, located in the middle of chloroplast. Cells with single central nucleus and often with polar vacuoles. Reproduction by fragmentation of filaments and by vegetative cell division.

Diagnosis: Differs from other genera by V9 region of its SSU sequence (V9 type A in Fig. 4).

Type species: *Stichococcus bacillaris* (Nägeli) Nägeli 1849.

Stichococcus bacillaris (Nägeli) Nägeli 1849, *Gattungen einzelliger Algen*: 76, Tab. IV, G fig.1 (L).

Basionym: *Protococcus bacillaris* Nägeli ex Kützing 1849, *Sp. Alg.*: 198.

Synonyms: *Stichococcus pallescens* Chodat 1909, Étude critique et expérimentale sur le polymorphisme des algues: 118, 120, pl. 13: fig. 19A, E; *Stichococcus dubius* Chodat 1913, *Monogr. Alg. Cult. Pure*, 160, fig. 136; *Stichococcus nivalis* Chodat 1922, *Bull. Soc. Bot. Geneve, sér.* 2, 13: 79; *Stichococcus bacillaris* var. *viridis* Nakano 1917, *J. Coll. Sc. Imp. Univ. Tokyo*, 40: 98, pl. I, III; *Stichococcus bacillaris* var. *nivalis* (Chodat) Printz 1964, *Hydrobiologia*, 24: 41; *Stichococcus bacillaris* var. *fungicola* Lagerheim 1884, *Öfvers. K. Svensk. Vet.-Akad. Förh.* 41: 106; *Stichococcus minor* Nägeli 1849, *Gattungen einzelliger Algen*: 77; *Stichococcus major* Nägeli 1849, *Gattungen einzelliger Algen*, 77, Tab. IV, G fig.2; *Stichococcus bacillaris* var. *major* (Nägeli) Rabenhorst 1868, *Flora Europea Algarum*, III: 48; *Stichococcus bacillaris* var. *minor* (Nägeli) Rabenhorst 1868, *Flora Europea Algarum*, III: 48; *Stichococcus membranaefaciens* Chodat 1913, *Monogr. Alg. Cult. Pure*, 161, fig. 138; *Stichococcus cylindricus* Butcher, 1952, *J. Mar. Biol. Ass. UK*, 31: 182, pl. I, fig. 26.

Emended diagnosis: SSU and ITS sequences (GenBank: HE610125), ITS-2 Barcode: 6a in Fig. 7.

Epitype (designated here): The strain SAG 379-2 permanently cryopreserved at SAG in metabolically inactive stage.

Comment: The strains SAG 379-1a, SAG 379-1b, SAG 379-1d, and SAG 379-2 were identical in M13-DNA-fingerprinting (Oppermann *et al.* 1997). The first three strains were isolated from Switzerland, the country of origin. The last strain originated from Germany. All these strains were used in different studies as references for *Stichococcus bacillaris*. Take this into account, we propose to designate the strain SAG 379-2 as epitype.

Protostichococcus Pröschold & Darienko, *gen. nov.* (Fig. 9J–L)

Description. Algae are usually unicellular. Two-celled filaments were observed only under marine condition. On freshwater medium cells short cylindrical till cylindrical, 3.8–5.8 x 2.0–2.8 µm and length/wide ratio among 1.5–2.5, with rounded ends. Cell wall thin, without mucilaginous layer. Chloroplast single, parietal, not lobed with smooth margin, with or without pyrenoid. Cells with single central nucleus. Reproduction by vegetative cell division.

Diagnosis: Differs from other genera by V9 region of its SSU sequence (V9 type C in Fig. 4).

Type species (designated here): ***Protostichococcus edaphicus*** Pröschold & Darienko *sp. nov.*

Protostichococcus edaphicus Pröschold & Darienko *sp. nov.*

Description: with the features of the genus. SSU-ITS sequences (GenBank: MT078161) and ITS-2 Barcode: 1a in Fig. 7.

Diagnosis: Differs from other *Stichococcus*-like algae by SSU-ITS sequences.

Holotype (designated here): The strain SAG 2481 permanently cryopreserved at SAG in metabolically inactive stage.

Etymology: The name *Protostichococcus* are created from oldgreek "πρῶτος" [protos]—first and *Stichococcus*.

The name reflected the first appearance after *Stichococcus* in our phylogenetic tree (Fig. 3). The species epithet *edaphicus* is of Greek origin and means "living in soil".

Deuterostichococcus Pröschold & Darienko, *gen. nov.* (Fig. 10A–L, 11A–D)
Synonym: *Chodatia* Kol 1934 (illeg.) *non Chodatia* Hansgirg 1903.

Description. Algae are usually unicellular rarely forms uniseriate and unbranched filaments out of 2–4 cells, which are easy fragmenting. Filaments are straight and short on freshwater medium or curved and longer under marine condition. Cell walls thin, without mucilaginous layer. Cells short cylindrical till cylindrical, with length/wide ratio among 1.9–2.9, with rounded ends. Chloroplast single, parietal, not lobed with smooth margin, with or without pyrenoid. Cells with single central nucleus. Reproduction by fragmentation of filaments and by vegetative cell division.

Diagnosis: Differs from other genera by V9 region of its SSU sequence (V9 type D1-3 in Fig. 4 corresponding to the studied species).

Etymology: The name *Deuterostichococcus* are created from oldgreek "δεύτερος" [deúteros] - second and *Stichococcus*. The name reflected the second appearance after *Stichococcus* in our phylogenetic tree (Fig. 3).

Type species (designated here): ***Deuterostichococcus deasonii*** (Neustupa, Elias & Sejnohova) Pröschold & Darienko *comb. nov.*

Deuterostichococcus deasonii (Neustupa, Elias & Sejnohova) Pröschold & Darienko *comb. nov.*
Basionym*: Stichococcus deasonii* Neustupa, Elias & Sejnohova 2007, *Nova Hedwigia*, 84: 60, fig. 1.

Synonyms: *Hormidium marinum* Deason 1969, *Trans. Am. Microscop. Soc.*, 88, 240–246; *Klebsormidium marinum* (Deason) P.C. Silva, K.M. Mattox & W.H. Blackwell 1972, *Taxon* 21: 643.

Emended description: SSU-ITS sequences (GenBank: MT078164) and ITS-2 Barcode: 2H in Fig. 7.

Epitype (designated here): The strain SAG 2139 permanently cryopreserved at SAG in metabolically inactive stage.

Deuterostichococcus allas (Reisigl) Pröschold & Darienko *comb. nov.*
Basionym: *Stichococcus allas* Reisigl 1964, *Österr. Bot. Z.*, 111 : 489, fig. 41.

Synonym: *Chodatia tetrallantoidea* Kol 1934 (illeg.), *Stichococcus tetrallantoideus* (Kol) Hindak (illeg.), *Stichococcus antarcticus* A. Beck & Bechteler 2019, *Symbiosis*, 79: 14, fig. 2e.

Emended description: ITS-2 Barcode: 2L-N.

Deuterostichococcus epilithicus Pröschold & Darienko *sp. nov.*
Description: Algae are usually unicellular. Under salinity stress can form unbranched filaments out of 2–4 cells, which are easy fragmenting. On freshwater medium cells are cylindrical with rounded ends, 5.1–7.6 x 2.9–3.8 µm with the length:width ratio of 1.7–2.7. Cell walls thin, without mucilaginous layer. Chloroplast parietal, not lobed with smooth margin, without pyrenoid. Cells with single central nucleus. Reproduction by fragmentation of filaments and by vegetative cell division. SSU and ITS sequences (GenBank: MT078165) and ITS-2 Barcode: 2C in Fig. 7.

Diagnosis: Differs from others closely related species differs in SSU - ITS and *rbc*L sequences.

Holotype (designated here): The authentic strain SAG 2060 permanently cryopreserved at SAG in metabolically inactive stage.

Etymology: The species epithet *epilithicus* is of Greek origin and means "epilithic".

Comment: The strains SAG 2060, SAG 2119, SAG 2482, SAG 10.97, ST-3, ST-4, and ST-10 differ slightly in their ITS and *rbc*L sequences, but they showed no CBC in the conserved region of ITS-2 (see ITS-2 Barcodes 2C-G and 2O in Fig.7).

Deuterostichococcus lewinii Pröschold & Darienko *sp. nov.*
Description: Algae are usually unicellular. Cells are cylindrical with rounded ends, 4.1–7.9 x 2.1–3.4 µm with the ratio 2.5. Cell walls thin, without mucilaginous layer. Chloroplast single, parietal, not lobed with smooth margin, without pyrenoid. Cells with single central nucleus. Reproduction by fragmentation of filaments and by vegetative cell division. SSU and ITS sequences (GenBank: MT078162) and ITS-2 Barcode: 2A in Fig. 7.

Diagnosis: Differs from others closely related species differs in SSU - ITS and *rbc*L sequences.

Holotype (designated here): The authentic strain SAG 107.80 permanently cryopreserved at SAG in metabolically inactive stage.

Etymology: The species epithet honors Prof. Dr. Ralph Lewin who isolated strain SAG 107.80, in recognition of the many contributions he made to our knowledge about the green algae.

Tritostichococcus Pröschold & Darienko *gen. nov.* (Fig. 12A–L, 13A–C)

Description. On freshwater medium algae are unicellular or two celled. Slightly curved and longer (rarely until 20 cells) filaments could be observed under marine condition. Cell walls thin, with rounded ends. Cells cylindrical, with length:width ratio around 2.1. Chloroplast single, parietal, without pyrenoid. Cells with single central nucleus. In culture often present big oval cells, which possess one big vacuole. Reproduction by fragmentation of filaments and by vegetative cell division.

Diagnosis: Differs from other genera by V9 region of its SSU sequence (V9 type E1-3 in Fig. 4 corresponding to the studied species).

Etymology: The name *Tritostichococcus* are created from oldgreek "τρίτος" [trítos]—third and *Stichococcus*. The name reflected the third appearance after *Stichococcus* in our phylogenetic tree (Fig. 3).

Type species (designated here): *Tritostichococcus solitus* Pröschold & Darienko *sp. nov.*

Tritostichococcus solitus Pröschold & Darienko *sp. nov.*

Description: with features of the genus, without pyrenoid. SSU and ITS sequences (GenBank: MT078171) and ITS-2 Barcode: 4A in Fig. 7.

Diagnosis: Differs from others closely related genera and species differs in SSU - ITS and *rbc*L sequences.

Holotype (designated here): The authentic strain SAG 2406 permanently cryopreserved at SAG in metabolically inactive stage.

Etymology: The species epithet is of Latin origin and means "common".

Tritostichococcus coniocybes (Letellier) Pröschold & Darienko *comb. nov.*

Basionym: *Stichococcus coniocybes* Letellier 1918. *Bull. Soc. Bot. Genève, ser.* 2, 9 : 401, fig. F-II, text figs. 4, 5.

Synonyms: *Stichococcus pallescens* var. *lucida* Raths 1938, *Ber. Schweiz. Bot. Ges.*, 48: 396, fig. 38; *Stichococcus chloranthus* Raths 1938, *Ber. Schweiz. Bot. Ges.*, 48: 380, fig. 17; *Stichococcus bacillaris* var. *mucigena* Raths 1938, *Ber. Schweiz. Bot. Ges.*, 48: 383, fig. 20; *Stichococcus bacillaris* var. *subaurifera* Raths 1938, *Ber. Schweiz. Bot. Ges.*, 48: 386, fig. 23; *Stichococcus bacillaris* var. *elegans* Raths 1938, *Ber. Schweiz. Bot. Ges.*, 48: 390, fig. 29; *Stichococcus bacillaris* var. *crassa* Raths 1938, *Ber. Schweiz. Bot. Ges.*, 48: 391, fig. 32; *Stichococcus bacillaris* var. *maxima* Raths 1938, *Ber. Schweiz. Bot. Ges.*, 48: 393, fig. 35; *Stichococcus bacillaris* var. *tenuis* Raths 1938, *Ber. Schweiz. Bot. Ges.*, 48: 398, fig. 44.

Emended diagnosis: On freshwater medium filaments are 4–15 celled, twisted. Cell walls thin, without mucilaginous layer. Cells cylindrical, usually slightly curved, 5.8–13.0 x 3.0–4.6 µm with length:width ratio between 1.5–3.9. Cells possess rounded ends. Chloroplast single, parietal, without pyrenoid. Cells with single central nucleus. Air filaments were not observed. Reproduction by fragmentation of filaments and by vegetative cell division. SSU and ITS sequences (GenBank: MT078172) and ITS-2 Barcode: 5A in Fig. 7.

Diagnosis: Differs from others closely related genera and species differs in SSU - ITS and *rbc*L sequences. Differs from *Stichococcus undulatus* by absence of air filaments and smaller cell size.

Comment: At present the phylogenetic positions of lichen symbionts among genera *Coniocybe* s.l. and *Chaenotheca* was not investigated. According to the Tibell (2001), the genus *Chaenotheca* is associated with four different algal genera: *Stichococcus*, *Trentepohlia*, *Dictyochloropsis* and *Trebouxia*. Only *Stichococcus antarcticus,* known as symbiont of two lichens *Placopsis antarctica* and *P. contortuplicata,* was investigated (Beck *et al.* 2019) and belonged to the genus *Deuterostichococcus* (see above). Our strain ST-9 isolated from *Chaenothecopsis sp.* (collected Uholka-Shyrokyy Luh Wilderness, part of the Carpathian Biosphere Reserve, Ukraine*)* correspond in the cell size and L/W ratio with type description provided by Letellier. It only differs from the type by filament formation. The formation of filaments is dependent on culture conditions and therefore we think that our isolate represented the same alga observed by Letellier (1918). *Tritostichococcus coniocybes* clearly differs from *Stichococcus antarcticus* and represents a separate species. According to our opinion, the lichen genera *Chaenotheca*, *Chaenothecopsis* and *Coniocybe* contain the same photobiont (*T. coniocybes*). The strains ST-6, ST-7 and ST-8 are morphologically similar to the lichen photobiont ST-9, but were free-living and differ only in few bases among the SSU and ITS rDNA sequences.

Tritostichococcus corticulus Pröschold & Darienko *sp. nov.*

Description. Algae are usually unicellular. Cells are cylindrical with rounded ends, cell size 6.3–10.1 x 3.1–4.1 µm with L/W ratio between 1.7–2.9. Cell walls thin, without mucilaginous layer. Chloroplast single, parietal, not lobed with smooth margin, with pyrenoid. Pyrenoid without starch grains and only visible after staining with Lugol. Cells

with single central nucleus. Reproduction by fragmentation of filaments and by vegetative cell division. SSU and ITS sequences (GenBank: MT078176) and ITS-2 Barcode: 3B in Fig. 7.

Diagnosis: Differs from others closely related species differs in SSU - ITS and *rbc*L sequences.

Holotype (designated here): The authentic strain ST-2 permanently cryopreserved at CCAP in metabolically inactive stage.

Etymology: The species epithet is of Latin origin and means "living on the bark tree".

Tetratostichococcus Pröschold & Darienko, *gen. nov.*

Description: Algae are usually unicellular rarely forms uniseriate and unbranched filaments out of 2–4 cells. Filaments are short and straight on freshwater medium or curved and longer under marine condition. Cell walls thin, without mucilaginous layer. Cells cylindrical, with length/wide ratio around 2.1. Cells possess rounded ends. Chloroplast single, parietal, not lobed with smooth margin, with pyrenoid. Cells with single central nucleus. Reproduction by fragmentation of filaments and by vegetative cell division.

Diagnosis: Differs from other genera by V9 region of its SSU sequence (V9 type G in Fig. 4).

Etymology: The name *Tetratostichococcus* are created from oldgreek "τέταρτος" [tétartos]—fourth and *Stichococcus*. The name reflected the fourth appearance after *Stichococcus* in our phylogenetic tree (Fig. 3).

Type species: *Tetratostichococcus jenerensis* (Neustupa, Elias & Sejnohova) Pröschold & Darienko *comb. nov.*

Tetratostichococcus jenerensis (Neustupa, Elias & Sejnohova) Pröschold & Darienko *comb. nov.*

Basionym: *Stichococcus jenerensis* Neustupa, Elias & Sejnohova 2007, *Nova Hedwigia*, 84: 61, fig.2.

Emended diagnosis: SSU and ITS sequences (GenBank: MT078183) and ITS-2 Barcode: 7A in Fig. 7.

Pseudostichococcus L. Moewus emend. Pröschold & Darienko (Fig. 13D–L)

Emended description: Algae are solitary or formed only two-celled rarely four-celled filaments or sometimes packages of four cells. Cells are cylindrical with rounded cell ends. Chloroplast plate-like, without pyrenoid, covering half of cell. Usually several large oil drops are present. Reproduction by fragmentation of filaments and by vegetative cell division.

Diagnosis: Differs from other genera by V9 region of its SSU sequence (V9 type H in Fig. 4).

Type species: *Pseudostichococcus monallantoides* L. Moewus.

Pseudostichococcus monallantoides L. Moewus 1951: 305, 316, figs. 7 a–m, 8, 9.

Emended diagnosis: SSU and ITS sequences (GenBank: KM020066) and ITS-2 Barcode: 9C in Fig. 7.

Epitype (designated here): The authentic strain SAG 380-1 permanently cryopreserved at SAG in metabolically inactive stage.

Comment: In original description, the formation of zoospores was reported. In our investigations we never observed zoospore formation.

Pseudostichococcus monallantoides* var. *exiguus (Gerneck) Pröschold & Darienko *comb. nov.*

Basionym: *Stichococcus exiguus* Gerneck 1907, Beih. Bot. Centralbl., 21: 262, pl. XII: figs. 66–76.

Synonym: *Stichococcus fragilis* Gerneck 1907 *non Stichococcus fragilis* (A. Braun) Gay 1891 (illeg.).

Emended diagnosis: SSU and ITS sequences (GenBank: MT078184) and ITS-2 Barcode: 9A in Fig. 7.

Epitype (designated here): The strain SAG 379-4 permanently cryopreserved at SAG in metabolically inactive stage.

Comment: *Stichococcus mirabilis* Lagerheim probably belongs to this variety. However, the only sequenced strain CCAP 379/3, which is not longer available, represents the same species, *Pseudostichococcus monallantoides* var. *exiguus.* The duplicate strain SAG 379-3a does not contain any *Stichococcus*, but *Geminella*.

Diplosphaera Bialosuknia emend. Vischer (Fig. 11E–M)

Diagnosis: Differs from other genera by V9 region of its SSU sequence (V9 type F in Fig. 4).

Type species: *Diplosphaera chodatii* Bialosuknia emend. Vischer

Diplosphaera chodatii Bialosuknia 1909, *Bull. Soc. Bot. Genève, II., Ser.* 1: 103, emend. Vischer 1960, *Schweiz. Z. Hydrologie*, 22: 338–339, fig. 6.

Synonyms: *Stichococcus diplosphaera* Chodat 1913, *Monogr. Alg. Cult. Pure*: 163; *Stichococcus chodatii* (Bialosuknia) Heering 1914, *Süsswasserfl., Heft* 6, *Chlorophyceae* III: 52; *Protococcus chodatii* (Bialosuknia) R. Chodat & F. Chodat 1924, *C.R. Soc. Phys. Hist. Genève*, 41: 106; *Diplosphaera epiphytica* Darienko & Pröschold,

2018, *Notulae Algarum*, 54: 2, figs 2; *Nannochloris normandinae* Tschermak-Woess 1981, *Nova Hedwigia* 35: 72, figs 4,5.

Emended diagnosis: SSU and ITS sequences (GenBank: MT078182) and ITS-2 Barcode: 8A in Fig. 7.

Epitype: The strain SAG 49.86 permanently cryopreserved at SAG in metabolically inactive stage.

Diplosphaera chodatii* var. *mucosa (Broady) Pröschold & Darienko, *comb. nov.*

Basionym: *Diplosphaera mucosa* Broady 1982, *Nova Hedwigia*, 36: 473, figs. 99–104, 129–131.

Emended diagnosis: SSU and ITS sequences (GenBank: MT078178) and ITS-2 Barcode: 8A in Fig. 7.

Epitype: The strain SAG 48.86 permanently cryopreserved at SAG in metabolically inactive stage.

Comment: This variety differs from the other by production of mucilage, which is stable in culture. However, this strain showed only few differences in the SSU and ITS rDNA sequences.

Desmococcus Brand 1925 emend Vischer 1960

Diagnosis: Differs from other genera by V9 region of its SSU sequence (V9 type B in Fig. 4).

Type species: *Desmococcus olivaceus* (Persoon ex Acharius) Laundon

Desmococcus olivaceus (Persoon ex Acharius) Laundon 1985, *Taxon*, 34 : 672.

Additional synonyms: *Desmococcus endolithicus* Broady & Ingerfeld 1993, *Eur. J. Phycol.*, 28: 28, figs 3I–N; *Desmococcus antarcticus* Rybalka, Wolf, Andersen & Friedl 2013, *BMC Evol. Biol.*, 13: 10.

Emended diagnosis: SSU and ITS sequences (GenBank: MT078158) and ITS-2 Barcode: 10A in Fig. 7.

Epitype (designated here): The strain SAG 1.92 permanently cryopreserved at SAG in metabolically inactive stage.

The following species of *Stichococcus* are doubtful and need to be excluded from this genus:
S. lacustris Chodat, *S. atomus* Skuja, *S. crassus* (Korshikov) Hindak, *S. minutissimus* Skuja, *S. castellanus* González Guerrero, *S. variabilis* West & West, *S. sequoieti* Arce, *S. spiroides* (G.S.West) Hindák, *S. filiformis* Hindák, *S. marinus* (Wille) Hazen, *S. pelagicus* (Nygaard) Hindák, and *S. bacillaris* var. *maximus* Hansgirg.

Conclusions

Stichococcus-like organisms are widely distributed in almost all habitats. As shown they are distributed in seven phylogenetic lineages representing genera. All of our investigated strains exhibited a maximal length of 12 µm. However, in the literature *Stichococcus* species with cell length longer than 15 µm were described. For example, Vinatzer (1975) described *S. undulatus* with a length of 30 (42) µm. Unfortunately, no strain with cells longer than 15 µm is available in culture. Therefore, studies of those specimens are necessary and maybe present new phylogenetic lineages within the *Prasiola* clade if the approach as described in this study, is used.

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Supplemental material

Figure S1. Molecular phylogeny of selected *Stichococcus*-like organisms based on V9, ITS rDNA and *rbc*L sequence comparisons. The phylogenetic tree shown was inferred using the maximum likelihood method based on the data sets (1774 aligned positions of 30 taxa) using PAUP 4.0a166. For the analyses the best model was calculated by Modeltest 3.7. The setting of the best model was given as follows: SYM+I+G (base frequencies: equal; rate matrix A-C 1.2133, A-G 2.3840, A-U 2.0017, C-G 1.1190, C-U 5.1631, G-U 1.0000) with the proportion of invariable sites (I = 0.4621) and gamma shape parameter (G = 0.6603). The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values >50% calculated with PAUP using maximum likelihood, neighbor-joining, maximum parsimony and RAxML using maximum likelihood). The clades are named after the genera (color-coded) proposed in this study.

Figure S2. Median values of the cell size of the investigated strains belonging to the different genera used for the box-and-whisker diagrams presented in Fig. 14.

Table S1. Strains used in this study

Table S2. Variable positions among the SSU rDNA sequences of the investigated strains analyzed manually and with the usage of the program DeSigNate. The base positions of the V9 are highlighted in black in the header.

Table S3. GenBank entries (accession numbers) found by BLAST N search (100% coverage; 100% identity) using the V9 region (Helix 49) of the SSU rDNA of each genus. To each entry the geographical origin and after the habitat are given. The investigated strains are marked in green.

Table S4. GenBank entries (accession numbers) found by BLAST N search (100% coverage; 100% identity) using the ITS-2 rDNA of each genus. To each entry the geographical origin and after the habitat are given. The investigated strains are marked in green.

Table S5. Overview about currently accepted species and varieties as well as doubtful names of *Stichococcus*.