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Micractinium tetrahymenae (Trebouxiophyceae, Chlorophyta), a New Endosymbiont Isolated from Ciliates

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Abstract: Endosymbiosis between coccoid green algae and ciliates are widely distributed and occur in various phylogenetic lineages among the Ciliophora. Most mixotrophic ciliates live in symbiosis with different species and genera of the so-called *Chlorella* clade (Trebouxiophyceae). The mixotrophic ciliates can be differentiated into two groups: (i) obligate, which always live in symbiosis with such green algae and are rarely algae-free and (ii) facultative, which formed under certain circumstances such as in anoxic environments an association with algae. A case of the facultative endosymbiosis is found in the recently described species of *Tetrahymena*, *T. utriculariae*, which lives in the bladder traps of the carnivorous aquatic plant *Utricularia reflexa*. The green endosymbiont of this ciliate belonged to the genus *Micractinium*. We characterized the isolated algal strain using an integrative approach and compared it to all described species of this genus. The phylogenetic analyses using complex evolutionary secondary structure-based models revealed that this endosymbiont represents a new species of *Micractinium*, *M. tetrahymenae* sp. nov., which was further confirmed by the ITS2/CBC approach.

Keywords: *Micractinium tetrahymenae*; Tetrahymena; *Utricularia*; facultative endosymbiosis; ciliate-algae symbiosis

1. Introduction

The genus *Micractinium* with its type species, *M. pusillum*, was described by Fresenius [1] for a coccoid green alga, which formed colonies of 2–4 cells and produced bristles. Since the first description, several species of *Micractinium* were established based on cell shape, number of bristles, and arrangement of cells into colonies [2]. All species occurred in all kinds of freshwater habitats, such as lakes and small ponds, and were typical planktonic species. Phylogenetic analyses of *Micractinium* surprisingly showed that *M. pusillum* is closely related to the genus *Chlorella*, a unicellular green alga without any cell appendices. Luo et al. [3,4] have demonstrated that the colony and bristle formation was a response on grazing through the rotifer *Brachionus calyciflorus*. The SSU and ITS rDNA sequences revealed that *M. pusillum* represented a cryptic species complex [4,5]. In addition, Pröschold et al. [6] transferred the genus *Diacanthos* with its type species *D. belenophorus* to the genus *Micractinium*. Apart from these free-living species of *Micractinium*, Pröschold et al. [7] indicated that a green algal endosymbiont of the ciliate *Paramecium bursaria* also belonged to *Micractinium*. Brandt [8] was the first who discovered that "chlorophyll-bearing bodies" in *Paramecium bursaria* and

Stentor polymorphus were independent organisms and not plastids. Since then, endosymbiotic algae in ciliates, heliozoa, amoeba, or other invertebrates have been of special interests in phycology as well as in zoology, microbiology, and virology. Within ciliates, green algal endosymbionts are widely distributed. Around 40 species of ciliates and other protists live in symbiosis with green algae [9]. For most of these endosymbionts, the origin and phylogenetic position are unknown. The majority of the investigated green algae belong to the *Chlorella* clade of the Trebouxiophyceae ([7] and references therein). Interestingly, the endosymbionts do not form a single lineage within the *Chlorella* clade, but are closely related to free-living species of *Chlorella*, *Micractinium* [7], and *Meyerella* [10], and sometimes formed an own genus like *Carolibrandtia* [11,12].

Symbiotic interactions between green algae and ciliates are known to be of different nature. Some mixotrophic ciliates always bear zoochlorellae in their cells and rarely occur algae-free. Such obligate endosymbiosis is found for example in *Paramecium bursaria*, one of best investigated ciliate species [13]. In contrast, several ciliates live only facultatively in symbiosis with green algae. One of these ciliates is the recently described *Tetrahymena utriculariae*, which lives in symbiosis with the alga *Micractinium* [14]. *T. utriculariae* lives inside bladder traps of *Utricularia reflexa*, a carnivorous aquatic plant. The ciliate survives the typically anoxic and nutrient-rich milieu inside traps, most likely because of its green algal endosymbionts. Cultivated outside the traps under oxygenic conditions, the ciliates lose their endosymbionts and switch to a heterotrophic way of life. This clearly indicated that the green algal endosymbiont *Micractinium* has a special function by providing oxygen to its hosts [15].

The aim of this study was to clarify the phylogenetic position and the taxonomic status within *Micractinium*. We isolated the strain from its host *Tetrahymena utriculariae* and deposited it under the number SAG 2587 in the Culture Collection of Algae at the University of Göttingen. We used an integrative approach (morphology and phenotypic plasticity, SSU, and ITS rDNA sequences including their secondary structures) for comparing this strain with existing described species of *Micractinium*.

2. Material and Methods

The strain SAG 2587 was isolated from the host as described in Pitsch et al. [14] and cultivated on agarized basal medium with peptone (ESP; medium 1b in [16]). For morphological investigations, we cultivated the strain 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 for two to three weeks. The light microscopic investigations were conducted using an Olympus BX-60 microscope (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).

The genomic DNA of the strain was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the instructions provided by the manufacturer. The SSU and ITS rDNA was amplified in PCR reactions using the Taq PCR MasterMix Kit (Qiagen, Hilden, Germany) with the primers EAF3 and ITS055R [17]. The PCR product was purified and sequenced as described by Darienko et al. [18]. The SSU and ITS rDNA sequence is available in the EMBL, GenBank, and DDBJ sequence databases under the accession number MT359915. This sequence was aligned and included into a data set of a total of 40 sequences (2602 bp) of representatives of the Chlorellaceae (Trebouxiophyceae). The data set was aligned according to the secondary structures. The secondary structures were folded using the software mfold [19], which uses the thermodynamic model (minimal energy) for RNA folding. GenBank accession numbers of all sequences used are given in the figure. For the phylogenetic analyses, the dataset with unambiguously aligned base positions was used. To test which evolutionary model fitted best for the data set, we calculated the log-likelihood values of 56 models using the automated model selection tool implemented in PAUP, version 4.0b167 [20], and the best model according to the Akaike criterion by PAUP was chosen for the analyses. The setting of the best model is given in the figure legend. The following methods were used for the phylogenetic

analyses: distance, maximum parsimony, maximum likelihood, and Bayesian inference. Programs used included PAUP version 4.0b167 [20], RAxML version 8.2.12 [21], MrBayes version 3.2.7a [22], and the PHASE package 2.0 [23–27]. For the Bayesian calculations, the secondary structure models of SSU and ITS (doublet in MrBayes and RNA7D in PHASE) were also taken into account.

3. Results

Micractinium tetrahymenae Pröschold, Pitsch, & Darienko sp. nov. (Figure 1A)

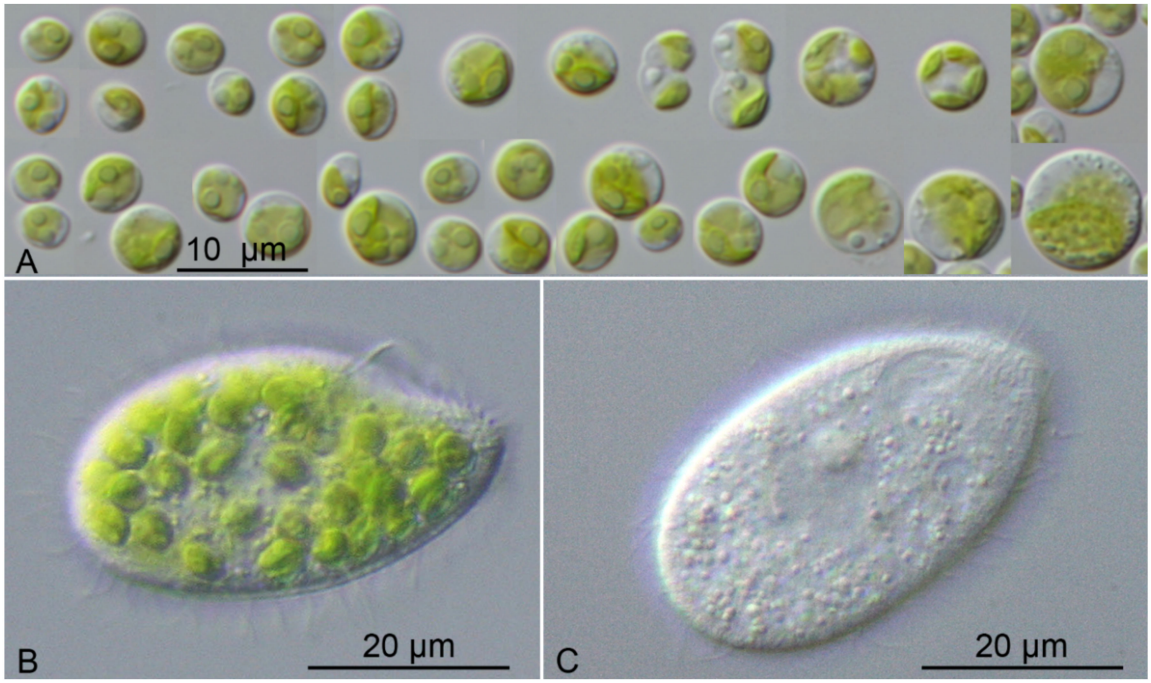

Figure 1. A. Morphology and phenotypic plasticity of *Micractinium tetrahymenae*, strain SAG 2587, **B.-C.** *Tetrahymena utriculariae* under anoxic (B) and oxygenic (C) conditions.

Description: Young cells are solitary, ellipsoidal up to broadly ellipsoidal; 3.1–4.2 µm in size. Mature vegetative cells are broadly ellipsoidal up to spherical, 4.8 × 4.9 µm up to 7.1 × 7.6 µm in size; rarely pyriform under suboptimal condition, 8.5 × 5.3 µm. Old cells are spherical up to 9.3 µm in diameter. Chloroplast is parietal cup-shaped possessing a single pyrenoid surrounded by starch grains. Cytoplasm is vacuolized. Asexual reproduction by autosporulation. The autospores are produced by 2–4 per cell. Autosporangia are 4.6 × 6.2 µm up to 6.3 × 7.4 µm. Release of autospores occurs after rupture of the mother cell wall. Bristle formation was not observed.

Diagnosis: Differs from morphologically similar *M. conductrix* and other free-living species of *Micractinium* through genetic signatures in SSU and ITS-2 rDNA sequences as well as in ITS-2 Barcode (see Section 4.2).

Holotype (designated here): The authentic strain SAG 2587 is cryopreserved in a metabolically inactive state at SAG under the number Z000694542.

Type locality: Facultative endosymbiont of *Tetrahymena utriculariae* (Oligohymenophorea, Ciliophora).

Etymology: The name reflected the appearance in the host organism.

Phylogenetic position and genetic signatures of the endosymbiont of *Tetrahymena utriculariae*: The SSU and ITS rDNA sequences of strain SAG 2587 (MT359915) were completely identical with those deposited in GenBank by Pitsch et al. [14] under the number LT605003. This endosymbiont clearly is the sister of *Micractinium pusillum*, based on the phylogenetic analyses of SSU and ITS rDNA sequences (Figure 2). The genus *Micractinium* is only highly supported in Bayesian analyses using the complex evolutionary models, which included the doublet and RNA7D functions (secondary structure

models implemented in MrBayes and PHASE, respectively; see details in Material and Methods). The maximum likelihood analyses using bootstrapping resulted in a high to moderate support for the genus *Micractinium*. In contrast, the common branch of the genus *Chlorella* was not supported in Bayesian analyses and only got moderate values in bootstrap calculations. All analyses showed that the separation of *Micractinium* and *Chlorella* is not supported using simple evolutionary models and distance or parsimony methods (data not shown). However, both genera together were highly supported in all analyses questioning the separation into two genera. The other genera belonging to the *Chlorella* clade were highly supported in all of our analyses.

Within *Micractinium*, *M. tetrahymenae* sp. nov. is closely related to *M. pusillum*. The genetic variability of SSU rDNA among the species of *Micractinium* was very low (only 28 variable positions of 1783 bp = 1.6%). Even variable regions such as V4 showed only little changes (5 bases). Only the V9 region was partly diagnostic (Figure 3), being unique for both, *M. conductrix* and *M. pusillum*. In contrast, The V9 of *M. tetrahymenae*/*M. belenophorum* and *M. inermum*/*M. simplicissimum*/*M. singulare*/*M.variabile* were identical, respectively. The variability among the species was higher in the ITS-1 and ITS-2. The general structures of *M. tetrahymenae* are presented in Figure 4 and were similar to those of the members of *Micractinium* and other genera of the *Chlorella* clade. The ITS-1 and ITS-2 showed the typical four helices called helices 1–4 of ITS-1 and helices I-IV for ITS-2 according to Coleman and Mai [28]. The differences among the species in ITS-1 and ITS-2 showed that all species could be distinguished by characteristic compensatory base changes (CBCs and HCBCs) and loops (highlighted in white boxes in Figures 5 and 6). The base pair differences of V9 (SSU) and the conserved region of ITS-2 among the *Micractinium* species are summarized in Figure 7. In total, ten CBCs, seven HCBCs, and six insertion/deletions could be discovered (highlighted with an asterisk in Figure 7). By replacing base pairs with a number code, representatives of *Micractinium* received a unique barcode based on which species could be clearly recognized.

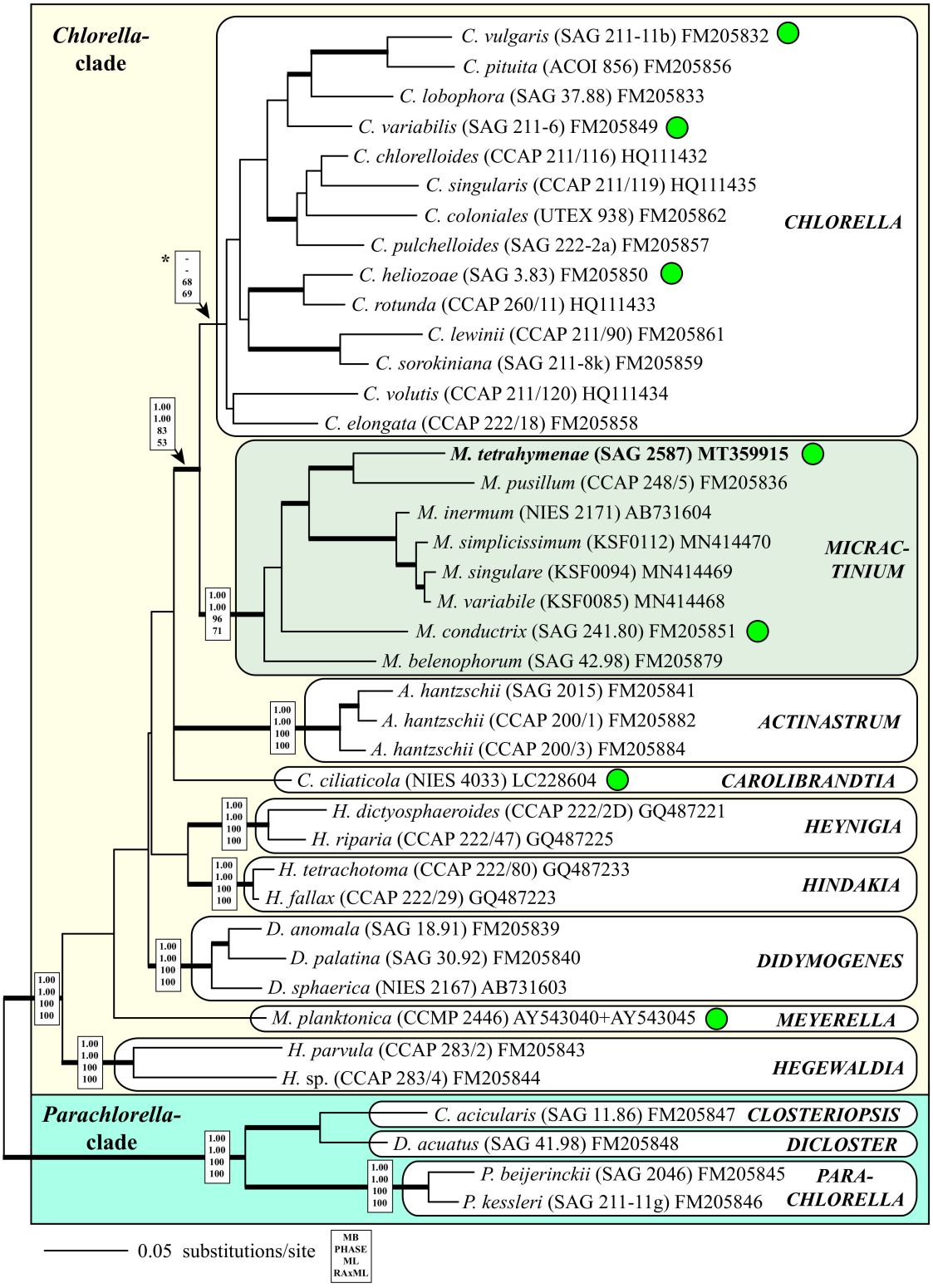

Figure 2. Comparison of the V9 of SSU and the conserved region of ITS-2 among the eight *Micractinium* species. Compensatory base changes (CBCs and HCBCs) and insertion/deletion are marked with an asterisk.

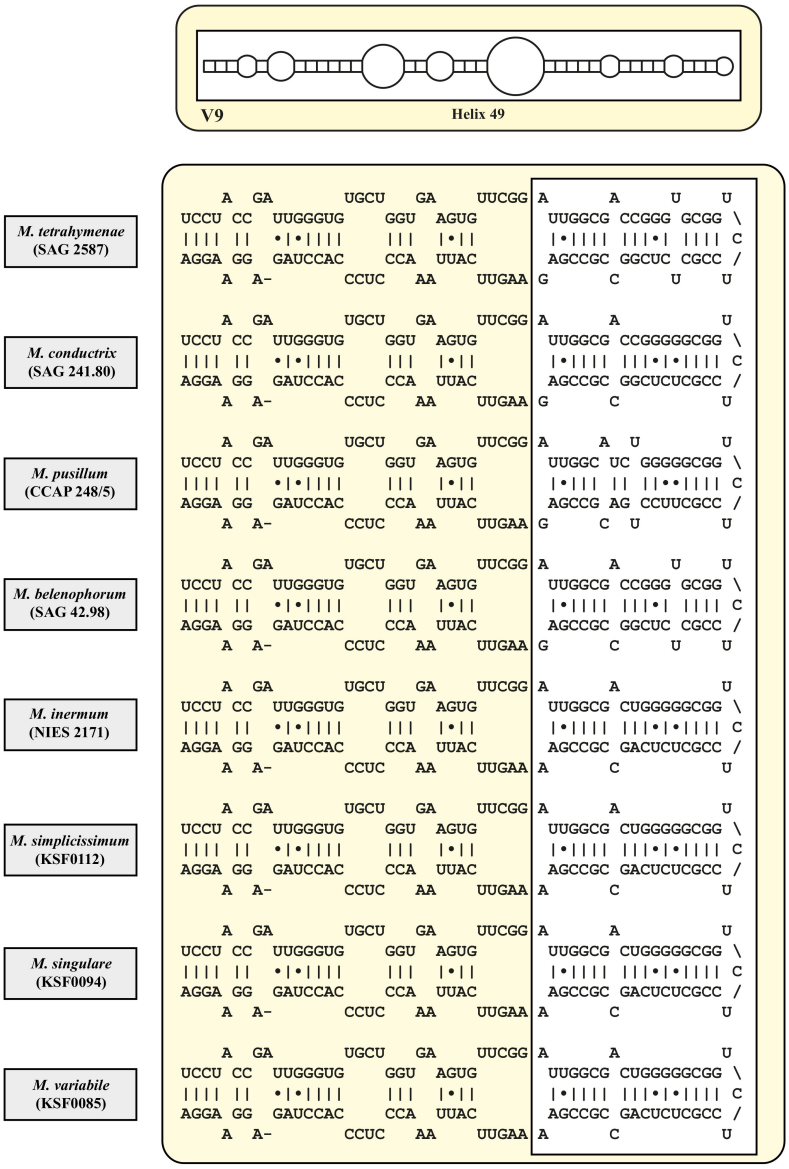

Figure 3. Molecular phylogeny of the Chlorellaceae based on SSU and ITS sequence comparisons. The phylogenetic trees shown were inferred using the maximum likelihood method based on the data sets (2602 aligned positions of 40 taxa), using PAUP 4.0a167. For the analyses, the best model was calculated by PAUP. The setting of the best model was given as follows: GTR + I + G (base frequencies: A 0.2112, C 0.2784, G 0.2743, T 0.2361; rate matrix A-C 0.7316, A-G 0.9716, A-U 0.9475, C-G 0.6216, C-U 3.2173, G-U 1.0000) with the proportion of invariable sites (I = 0.7266) and gamma shape parameter (G = 0.6963). The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with MrBayes and PHASE, 10 million generations; bootstrap values > 50%, calculated with PAUP, 1000 replicates using maximum likelihood, neighbor-joining, and maximum parsimony). The endosymbiotic species are marked with a green circle. The accession and strain numbers are given.

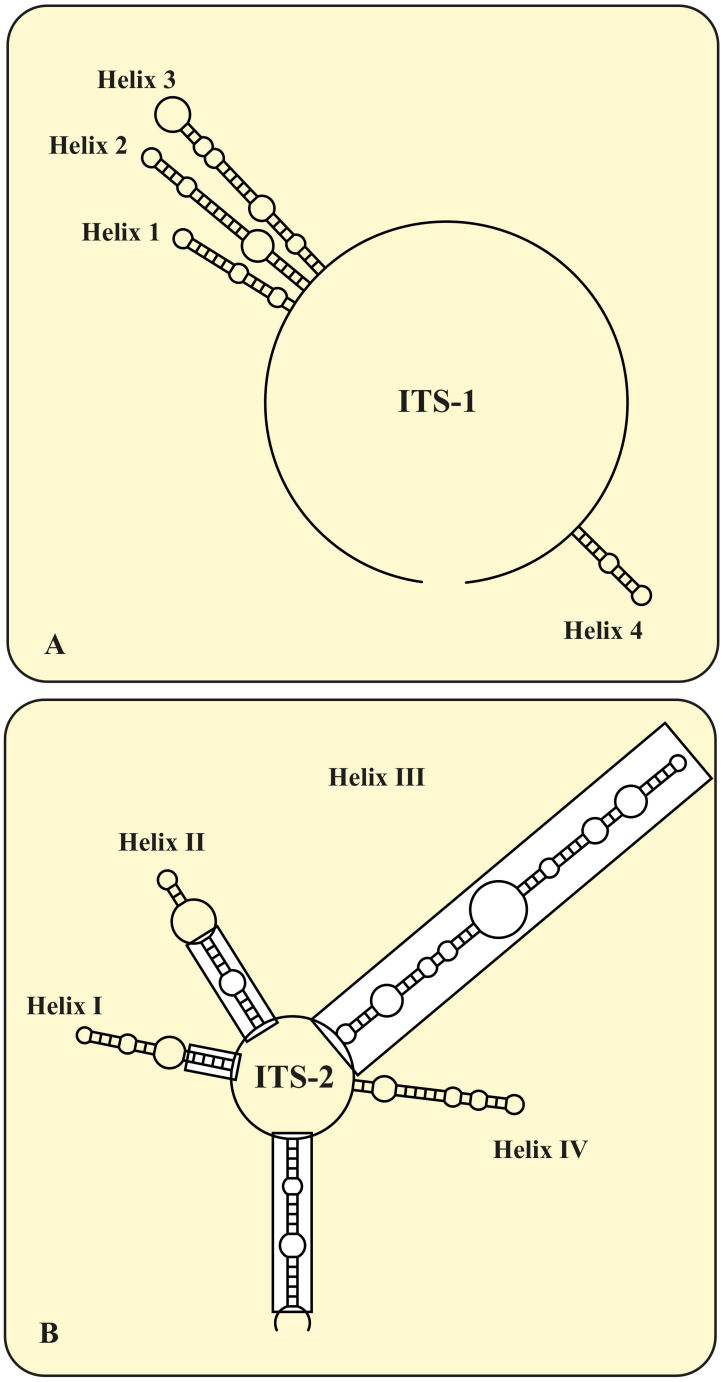

Figure 4. Secondary structure of the V9 region (Helix 49) of the SSU rDNA among the *Micractinium* species. The variable region within the V9 are highlighted in white boxes.

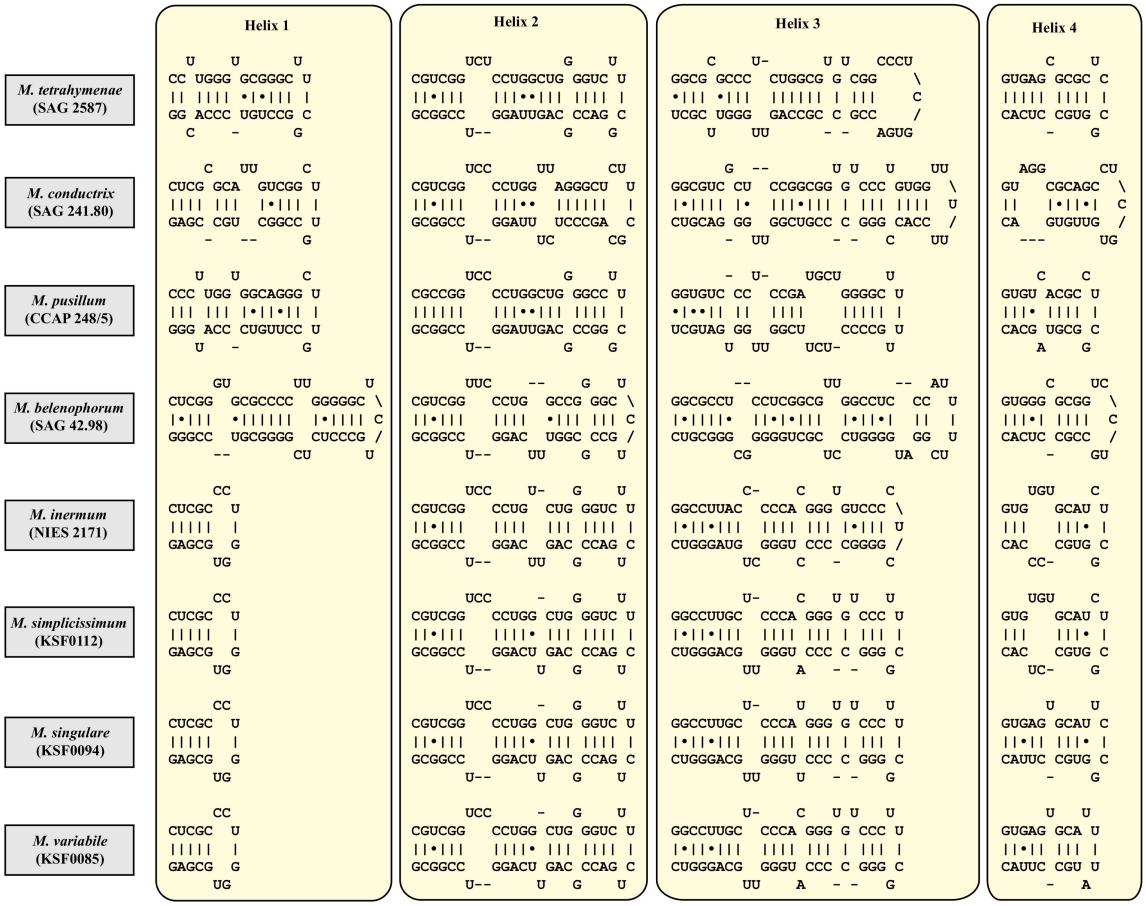

Figure 5. Secondary structure of the ITS-1 (**A**) and ITS-2 (**B**) rDNA of *Micractinium tetrahymenae*. The regions used for barcoding are highlighted in white boxes.

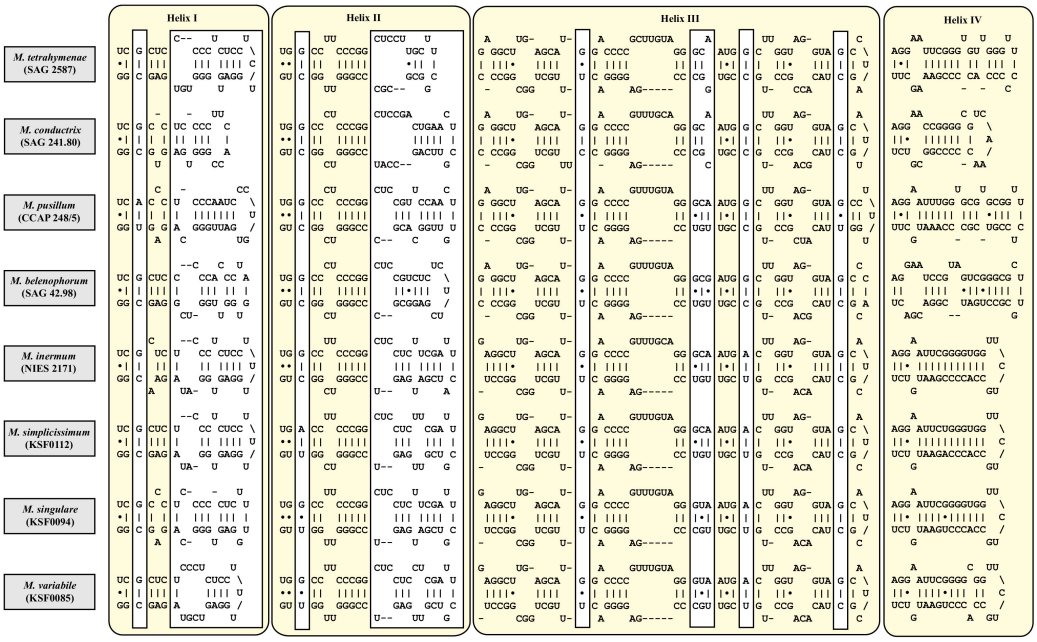

Figure 6. Variability of ITS-1 among the eight *Micractinium* species.

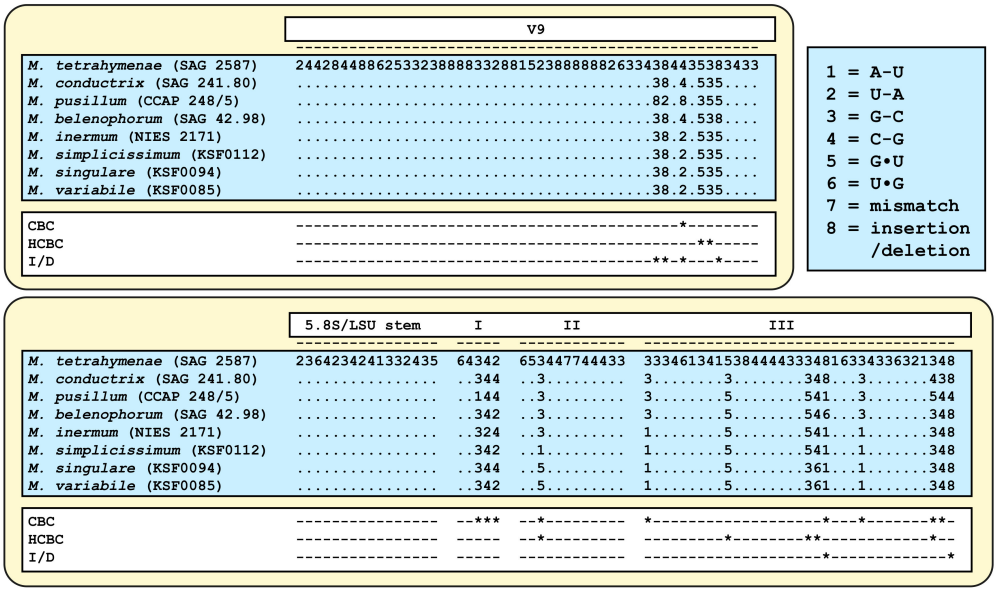

Figure 7. Variability of ITS-2 among the eight *Micractinium* species. The characteristic features within the conserved regions are highlighted in white boxes.

4. Discussion

4.1. Green Algae in Endosymbiosis Belonging to the Chlorellaceae

Zoochlorellae or *Chlorella*-like algae living as endosymbionts in ciliates and other protozoa are known for a long time ([7] and references therein). Interestingly, most of these green algae belonged to the *Chlorella* clade of the Trebouxiophyceae. Within this clade, six out of the seven species (highlighted with green circles in Figure 2) exclusively occurred in endosymbiotic associations. Only *Chlorella vulgaris* could be found free-living in various habitats (see details in reference [18]). *C. vulgaris*, *C. variabilis*, and *Micractinium conductrix* formed an obligate endosymbiont in *Paramecium bursaria* [7,29]. *Meyerella planctonica* is the endosymbiont of another green *Paramecium* (*P. chlorelligerum* [10]). The genus *Carolibrandtia* was discovered to be the endosymbionts of the ciliates *Pelagodileptus trachelioides*, *Cyclotrichium viride*, and *Stokesia vernalis* [11,12].

Micractinium tetrahymenae sp. nov. represented a second species within this genus that lives in symbiosis with a ciliate. In contrast to *M. conductrix*, a species which is the obligate endosymbiont of *Paramecium bursaria* [7,29] and *Coleps primhirtus* [30], *M. tetrahymenae* formed only under anoxic or microaerobic conditions a symbiotic association with *Tetrahymena utriculariae* [14]. This demonstrated that *M. tetrahymenae* is a facultative endosymbiont. However, whether this species can also occur free-living needs further investigations. No entry in GenBank could be found in the BLASTn search (100% coverage, 97% identity) using our SSU and ITS sequence (2452 bp). It is also unknown if *Tetrahymena utriculariae* would be able to live in symbiosis with other green algae belonging to the Chlorellaceae.

4.2. Taxonomy and Systematics of the Genus Micractinium

Morphologically, both endosymbiotic *Micractinium* species were difficult to distinguish from each other. *M. tetrahymenae* sp. nov. was slightly smaller than *M. conductrix* (3–8 vs. 4–10 µm). Both species showed no bristle formation under the chosen culture conditions. Three other species of *Micractinium*, all occurring free-living, were known to be bristle-less (*M. inermum*, *M. simplicissimum*, and *M. singulare* [31,32]). Colony formation among *Micractinium* species was not always observed. The morphological features of all currently accepted species are summarized in Table 1.The taxonomy and systematics of spiny coccoid green algae is very confusing and unclear for two major reasons:

(i) Most species were described based on field samples and no type material of these species is available for comparative studies; often only pictures were presented as holotypes [2]; (ii) cultured material such as strains of *Micractinium* were unicellular and without any bristles, which made it almost impossible to distinguish them from members of the genus *Chlorella*. Luo et al. [3,4] demonstrated that bristle and colony formation is an inducible defense mechanism against grazing of the rotifer *Brachionus calcyflorum*. Phylogenetic analyses such as those presented in Figure 2 revealed the close relationship between *Chlorella* and *Micractinium*. In contrast to *Micractinium*, the monophyly of *Chlorella* was not supported in our analyses. However, the molecular signature described by Pröschold et al. [7], the CBC at the end of helix III in ITS-2 (G-C in *Chlorella* vs. C-G in *Micractinium*), remained.

Traditionally both genera belonged to two different families. The family Chlorellaceae comprised algae reproducing exceptionally by autospores without sexual reproduction or zoosporogenesis. Other important criteria for separation of Chlorellaceae was composition of cell wall, which consisted of 2–3 layers containing obligatory cellulose and an outside layer of sporopollenin [2]. Unfortunately, this feature was based on the investigation of *Chlorella fusca* (now *Scenedesmus abundans*, Chlorophyceae) and the cell wall of "true" *Chlorella* species did not contain sporopollenin [33]. In contrast, the family Micractiniaceae contains algae, in which sexual reproduction, but no production of zoospores, is known. The cells are arranged in colonies consisting out of 2 up to 256, and were covered with bristles. The cell walls contain cellulose, without sporopollenin [34,35]. In summary, the differences between both families were the presence of sexual reproduction and bristles in Micractiniaceae. However, phylogenetic analyses have revealed that both families were polyphyletic (see [36] and references therein).

Hegewald and Schnepf [34,37] revised the representatives of the family Micractiniaceae based on morphological, ultrastructural investigations using SEM and TEM. They studied living cultures and some formaldehyde-fixed type material to explore the nature of spines and bristles used for the differentiation at generic level within this family. By definition, bristles contained, in contrast to spines, no cellulose and only proteins in their appendices. In addition, the formation of both is different. Whereas spines were formed before the cell walls were produced, bristles were exhibited after the cells are covered by the rigid cell wall. Considering these features, they revised the genus *Micractinium* by transferring several species to this genus, which were originally as species of other genera, such as *Golenkinia* and *Golenkiniopsis*. The genus *Micractinium* comprised four species, *M. pusillum*, *M. appendiculatum*, *M. elongatum*, and *M. parvulum*, according to Hegewald and Schnepf [34,37], and the complicated synonymy were provided therein. However, the validation of these taxonomical combinations needs to be proven.

Table 1. Diacritical morphological features among the described species of *Micractinium*.

Species	Cell Shape	Cell Size [μm]	Chloroplast Shape	Pyrenoid	Bristles	Length of Bristles [μm]	Colony Formation	Life Style	Habitat	Reference Strain
M.pusillum	spherical	3.0–7.0–12.0	cup-shaped	+	up to 8 per cell	40–65–(100)	single, or up to 8–32	free-living	plankton of ponds	CCAP 248/5
M.elongatum	spherical	6.0–7.0	cup-shaped	+	1–2 per cell	up to 50.0	4 - celled	free-living	plankton	-
M.appendiculatum	ellipsoidal, ovoid	8.0	parietal	+	2–4 per cell	28.0–70.0	up to 64	free-living	plankton of ponds	-
M.belenophorum	ellipsoidal, broadly ellipsoidal	8.0–10.0 × 4.5–5.5	parietal	+	2 per cell at the poles	up to 55.0	up to 4	free-living	plankton of ponds and rivers	SAG 42.98
M.conductrix	spherical	4.0 -10.0	cup-shaped	+	-	-	-	endosymbiotic	*Paramecium bursaria*	SAG 241.80
M.extremum	spherical	5.2–6.4	cup-shaped	+	1–2 per cell	up to 30.0	8 celled	free-living	plankton	-
M.inermum	ellipsoidal up to spherical	5.0–5.4–3.2–3.7	cup-shaped	+	-	-	-	free-living		NIES 2171
M.quadrisetum	ovoid, ellipsoidal	6.0- 10.0 × 4.0–7.0	cup-shaped	+	1–4	23.0–50.0	16 celled and more	free-living	Plankton of ponds and rivers	-
M.simplicissmum	ellipsoidal up to spherical	5.5–5.7 × 3.3–3.9	cup-shaped	+	-	-	-	free-living		KSF0112
M.singulare	ellipsoidal up to spherical	7.2–7.4 × 4.5–4.7	cup-shaped	+	-	-	-	free-living		KSF0094
M.tetrahymenae	spherical		cup-shaped	+	-	-	-	endosymbiotic	*Tetrahymena utricullariae*	SAG 2587
M.variabile	ellipsoidal up to sphaerical	8.2–8.6 × 5.0–5.4	cup-shaped	+	4–8 (?)	10–30	solitary,+	free-living	plankton	KSF0085

The latter species was transferred to another genus, *Hegewaldia*, based on phylogenetic analyses of SSU and ITS rDNA sequences [6]. In addition, they also transferred *Diacanthos belenophorus* to *Micractinium*, which was assigned to the Micractiniaceae by Hegewald and Schnepf [38].

Interestingly, it is the occasional occurrence of the sexual reproduction in the family Micractiniaceae. The oogamy was observed in *Micractinium pusillum* by Nygaard [39], Lund [40], Korschikov [41], and Hegewald [42], and in *Hegewaldia parvula* by Iyengar and Balakrishnan [43], Starr [44], and Ellis and Machlis [45], originally assigned as *Golenkinia minutissima*. The ultrastructure of the spermatozoid was investigated by Moestrup [46], who showed that the spermatozoid had an untypical structure of the flagella (9 + 1). The presence of sexual reproduction in *Micractinium* and *Hegewaldia* and its absence in *Chlorella* could be potential criteria for distinguishing the genera. However, Fucikova et al. [47] found in *Chlorella* meiotic genes and genes that were transcribed during sexual reproduction, in only asexually reproducing trebouxiophytes. This questioned the traditional concept of genera.

As already pointed out Hegewald and Schnepf [34], even the formation of bristles considered as a good morphological feature, is not a stable feature. The morphology and length of bristles is polymorphic and dependent on temperature and media. For example, they observed that *Micractinium strigonense* Hortobagyi sometimes have different bristles (thick and delicate) and occurred sometimes without bristles. Considering these observations, they proposed to synonymize several species, which is unfortunately illegitimate.

The high phenotypic plasticity and the lack of stable morphological and ultrastructural characters requested a new generic and species concept within the Chlorellaceae and Micractiniaceae. These traditional families should be rejected according the phylogenetic analyses of molecular marker genes. Considering the SSU and ITS sequences, new species were described from Japan [31] and Antarctica [32]. The integrative approach used in this study clearly demonstrated that *Micractinium* contained eight species (Figure 2). The morphological features of those species as well as the remaining species of Hegewald and Schnepf [34,37] were compared in Table 1. The comparison and judgement of traditional features and molecular data is quite difficult. For example, both endosymbiotic species showed only small morphological differences and were not considered as members of *Micractinium* without phylogenetic analyses. However, our study showed that both are separate species based on the CBC approach, as demonstrated in Figure 7. On the other hand, molecular data provided an inflation of new species descriptions, when the traditional literature was not considered and no strains are available in public culture collections. As an example, Chae et al. [32] described *Micractinium variabile* based on SSU and ITS rDNA sequences. Morphologically, this species is very similar to *M. quadrisetum*, which is unfortunately not available in culture. Therefore, it is possible that both species represent only one species. According to the ICN, *M. quadrisetum* would have priority against *M. variabile*. As described in the results, only little genetic differences among *Micractinium* species could be discovered. In particular, *M. inermum* and the three species described by Chae et al. [32] had identical V9 regions and little differences in ITS-1 and ITS-2, but they differed by two CBCs and three HCBs (Figures 5–7). Considering the ITS-2/CBC approach, we do not propose any taxonomic changes without further investigations.

4.3. Ecology and Distribution of Micractinium

The genera *Chlorella* and *Micractinium* have different ecological patterns and are distributed in various habitats. Whereas *Chlorella* has a worldwide distribution in almost all kinds of habitats, it seems that *Micractinium* is restricted to freshwater habitats. Species of *Chlorella* were found aquatic in freshwater and marine habitats [18,36], symbiotic in ciliates and heliozoa [7], and terrestrial [48,49]. *Micractinium* species were only observed in freshwater habitats [2,31,32,36], in wet soils [5], and symbiotic in ciliates ([7,14] and this study). The occurrence of *M. tetrahymenae* in the traps of *Utricularia* is exceptional. Whereas *Tetrahymena* species are widely distributed in the bladder traps of different *Utricularia* species, only one record is known of the green *Tetrahymena utricularie* [14]. No other record of the occurrence of a *Micractinium* species in such traps have been reported in microbiome studies [50]. Simek et al. [15] studied the ecology and dynamic of

trap communities and found that the endosymbiosis of *Micractinium* in *Tetrahymena* contributed significantly for the survival of the ciliate in such harsh environment. No other *Utricularia* species have had mixotrophic ciliates in their traps until now [50].

4.4. Interactions between Tetrahymena Utriculariae and Micractinium Tetrahymenae

The role of the *Micractinium* symbiont in *Tetrahymena utriculariae* has been studied in experiments by Simek et al. [15]. The ciliate has a flexible life strategy. It can live in different aquatic environments under oxygenic conditions, or if captured in bladder traps of *Utricularia* under anoxic conditions. For this flexibility, the endosymbiotic *Micractinium* is absolutely necessary. It has been demonstrated that aposymbiotic *Tetrahymena* had the highest growth rate, if exclusively bacterial food is present. However, if cultivated with both bacterial food and symbiotic *Micractinium*, *Tetrahymena* had a reduced growth rate, but after 44 days of cultivation, 80% of the Tetrahymena cells reestablished the symbiosis with the algae [15]. These experiments clearly demonstrated that both organisms formed a symbiotic association depending on the environment. The main profit for the host is that the algae produced the oxygen through photosynthesis. If the algae also provided nutrients to the host, this has not been investigated so far. *Micractinium tetrahymenae* benefited from CO_2 production of the host and stable conditions inside the host, whereas outside in ciliates the environment was very harsh (low pH 4.3 and anoxic) [51]. The endosymbiosis with this *Micractinium* species is probably essential for *Tetrahymena*, because the green algae were included in cyst formation [14].

5. Conclusions

The newly described species is the second species of *Micractinium*, which lives in endosymbiosis with ciliates. If this species is exclusively distributed in a symbiotic association like *M. conductrix*, it cannot be decided so far. No GenBank record has been reported nor could be found in BLAST searching. *Tetrahymena utriculariae* is also the only mixotrophic species of this genus. Both organisms were only found once so far, which is probably caused by the lack of investigations. Fortunately, different aspects of this ciliate-green algal association can be studied in detail, because species are available in culture. Nothing is known about the specificity of this symbiosis. The easiness of cultivating makes this ciliate and its endosymbiont the perfect model organisms to study associations between ciliates and green algae.

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