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ORIGINAL ARTICLE

The Green *Tetrahymena utriculariae* n. sp. (Ciliophora, Oligohymenophorea) with Its Endosymbiotic Algae (*Micractinium* sp.), Living in Traps of a Carnivorous Aquatic Plant

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ABSTRACT

The genus *Tetrahymena* (Ciliophora, Oligohymenophorea) probably represents the best studied ciliate genus. At present, more than forty species have been described. All are colorless, i.e. they do not harbor symbiotic algae, and as aerobes they need at least microaerobic habitats. Here, we present the morphological and molecular description of the first green representative, *Tetrahymena utriculariae* n. sp., living in symbiosis with endosymbiotic algae identified as *Micractinium* sp. (Chlorophyta). The full life cycle of the ciliate species is documented, including trophonts and theronts, conjugating cells, resting cysts and dividers. This species has been discovered in an exotic habitat, namely in traps of the carnivorous aquatic plant *Utricularia reflexa* (originating from Okavango Delta, Botswana). Green ciliates live as commensals of the plant in this anoxic habitat. Ciliates are bacterivorous, however, symbiosis with algae is needed to satisfy cell metabolism but also to gain oxygen from symbionts. When ciliates are cultivated outside their natural habitat under aerobic conditions and fed with saturating bacterial food, they gradually become aposymbiotic. Based on phylogenetic analyses of 18S rRNA and mitochondrial *cox1* genes *T. utriculariae* forms a sister group to *Tetrahymena thermophila*.

THE genus *Tetrahymena* (Ciliophora, Oligohymenophorea) includes probably the best and most intensively studied ciliate species at present (Collins 2012; Kher et al. 2011; Plattner et al. 2009). A quick literature search (August 2016) in the Web Of Science™ Core Collection remarkably reflects the extensive research activities. Using the term "*Tetrahymena*" in the online search field "Topic" resulted in ~10,330 hits. This value even exceeds search results obtained for the ciliate genus *Paramecium* (~5,940 hits). In contrast with nearly all other ciliates, some *Tetrahymena* species can be grown as pure osmotrophs in axenic cultures (Cassidy-Hanley 2012), i.e. without accompanying bacteria. This aspect also contributes to the wide use of *Tetrahymena* strains as model organisms (Collins 2012).

In nature these primarily bacterivorous ciliates can be found in all freshwater habitats, with a clear preference for the benthic zone (Doerder and Brunk 2012). Nevertheless, a few *Tetrahymena* species were described to be facultative or obligate parasites (e.g. of insects; Jerome et al. 1996), whereas others are even histophagous (Strüder-Kypke et al. 2001). At present, more than 40 species within the genus were described (Lynn and Doerder 2012; Quintela-Alonso et al. 2013), i.e. ciliates got a species name although the precision and extent of species descriptions varied. On the one hand, a few species have been characterized in detail based on morphological features in combination with molecular marker genes (e.g. Quintela-Alonso et al. 2013). On the other hand, several species were named solely on the basis of molecular or

biochemical methods but adequate descriptions of their morphological characteristics and life cycles are still missing (e.g. *Tetrahymena farahensis* which was described based on sequences by Zahid et al. 2014). However, this is an ambivalent aspect, as a "pure morphology based discrimination" of several known *Tetrahymena* species seems to be impossible (Lynn and Doerder 2012). Thus, nowadays combinations of classical approaches with molecular phylogenetic analyses are needed, based not only on 18S rRNA but mainly on the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) genes (Chantangsi et al. 2007; Kher et al. 2011).

Nevertheless, all currently described *Tetrahymena* species from natural habitats have two characteristics in common: (i) No natural species was reported to harbor symbiotic algae, as it is known for several representatives of various freshwater genera (*Coleps, Euplotes*, *Paramecium*, *Stokesia* – to name just a few examples). (ii) All *Tetrahymena* species are aerobic ciliates and thus live in oxygenated habitats, although ciliates seem to prefer and tolerate microaerobic zones (Doerder and Brunk 2012).

Here, we present the "first" green *Tetrahymena* species, i.e. the ciliate harbors symbiotic green algae. Ciliates were discovered in a real exotic habitat—they live as symbionts in traps of the aquatic carnivorous plant *Utricularia reflexa* (Lentibulariaceae, Lamiales). The green *Tetrahymena* form a central element of the microbial food web in the anoxic trap fluid. For a detailed description of the autecology of this *Tetrahymena* species we refer to our accompanying manuscript by Šimek et al. (2016). Here, we give a detailed morphological and molecular characterization of the ciliate including its life cycle, resulting in the description of the new species *Tetrahymena utricullariae* n. sp. Ciliates were isolated and maintained as green but also as aposymbiotic cultures outside their natural habitat. Finally we investigated if algal symbionts were "typical" endosymbionts (in terms of taxonomic affiliation) described for ciliate hosts.

MATERIALS AND METHODS

Trap fluids and cultivation of ciliates and algae

The aquatic plant *U. reflexa* Oliv. originates from the Okavango Delta, Botswana and is cultivated at the Institute of Botany CAS (Třeboň, Czech Republic) since the year 2005. Whole plant shoots were transferred in 1-liter aquaria to the University of Zurich (Switzerland) in the years 2014 and 2015. For a detailed description of the plant itself and its cultivation conditions we refer to Adamec (2012, 2015) and Šimek et al. (2016).

To gain living ciliates, single traps were cut from the shoot and transferred into a small drop (300 μl) of 0.2 μm pre-filtered mineral water (Volvic, France). The trap wall was opened with two dissecting needles to release ciliates in the surrounding water. Most stages of the complex life cycle could be already observed after the opening of traps. In vivo observations were done with a Zeiss Axio Imager M1 (Zeiss, Jena, Germany) at magnifications of 100–1,600X with bright-field, phase and interference contrast. Photomicrographs were taken with a Canon EOS 1000D controlled by the software AxioVision (Zeiss). Single green ciliates were collected with a sterilized glass pipette. For the cultivation of ciliates we tried both, to create clonal isolates and cultures originating from several individuals. First, ciliates were transferred in 96 microwell plates (each well containing 200 μl of pre-filtered Volvic). Plates were kept at aerobic conditions at 18 °C, and at a 12 h day (10 μmol/m^2/s)/12 h night cycle. Half of the wells were amended with each 50 μl of a mixed bacterial suspension growing on wheat grains. In case of obvious growth of ciliates in wells, cells were transferred into Tissue Culture Flasks 25 cm^2 (TPP®; Techno Plastic Products AG, Switzerland), filled with 50 ml of pre-filtered Volvic (either enriched with bacteria or not). Cultures of green ciliates could be maintained in a freshwater medium WC (Guillard and Lorenzen 1972) and Volvic (1:1) mixture for several months. Nevertheless, several ciliate isolates kept under aerobic conditions with over-saturating bacterial food gradually lost their algal symbionts (during several weeks) – even when cultures were grown at a 12 h day/12 h night cycle. Here, ciliates switched toward complete heterotrophy, feeding exclusively on bacteria.

For the isolation of algal symbionts, single green ciliates were gently squeezed in a small drop (20 μl) of 0.2 μm pre-filtered Volvic with a dissecting needle until cell rupture happened and algal symbionts were released. Several algal cells were collected with a sterilized ultrafine glass capillary and transferred into 96 microwell plates. Each well contained 200 μl of freshwater medium WC. When successful growth was observed, algae were transferred to Tissue Culture Flasks 25 cm^2 (TPP®), filled with 30 ml of freshwater medium WC.

Silver impregnations

For the quantification of algal symbionts per ciliate we applied the quantitative protargol staining (QPS) method (Skibbe 1994 with modifications after Pfister et al. 1999). This method allowed for the collection of a high number of ciliates for silver impregnation. Samples were fixed with Bouin's solution (picric acid, formaldehyde, and acetic acid) adjusting a final concentration of 5% (vol/vol, Pfister et al. 1999). Subsamples (100–300 μl) were filtered on 0.8 μm pore-size cellulose nitrate filters (with counting grid; Sartorius) and silver impregnation with protargol was conducted according to the protocols mentioned above.

For analyses of ciliary patterns and measurements of morphometric parameters we used the protargol impregnation "Procedure A" after Foissner (2014). Silverline patterns were characterized after the dry silver nitrate staining procedure (Foissner 2014). Fixed specimens were observed with the microscopic equipment described above. Measurements of morphometric parameters (Table 1) derived from a green clonal culture and were done with the image analysis software Lucia (Laboratory Imaging, Prague).

Table 1. Morphometric parameters of living trophonts and theronts, and protargol impregnated trophonts of the green ciliate species *Tetrahymena utricullariae* n. sp.isolated from *Utricularia reflexa*

Symbiont bearing green ciliates	Average	M	SD	SE	CV	Min	Max	*n*
Body, length – living trophonts (μm)	36.7	36.7	4.0	0.7	11.0	30.0	45.3	31
Body, width – living trophonts (μm)	27.4	27.7	2.1	0.4	7.8	21.8	30.1	31
Body, volume – living trophonts (μm^3)	14,543	14,766	2,985	536	21.0	7,988	20,214	31
Body, length – living theronts (μm)	49.8	49.1	5.8	1.3	11.5	41.0	59.0	20
Body, width – living theronts (μm)	20.1	19.8	2.3	0.5	11.2	17.2	25.4	20
Number of algal symbionts per ciliate	52.0	49.5	15.7	2.5	30.1	26.0	89.0	40
Size of living algal symbionts (μm)	5.9	5.8	0.6	0.1	11.1	4.8	7.3	31
Body, length – protargol impregnated trophonts (μm)	32.6	33.7	4.4	0.9	13.6	22.9	38.2	25
Body, width – protargol impregnated trophonts (μm)	19.7	19.6	2.0	0.4	10.3	14.6	23.3	25
Anterior body end to buccal cavity, distance (μm)	3.7	3.6	0.9	0.2	24.5	2.2	5.7	25
Anterior body end to macronucleus, distance (μm)	11.1	10.9	2.3	0.5	20.3	5.7	16.8	25
Anterior body end to excretory pore, distance (μm)	26.5	26.6	3.7	1.4	13.8	20.1	32.0	7
Macronucleus, length (μm)	9.6	9.5	1.7	0.3	17.9	6.5	13.6	25
Macronucleus, width (μm)	8.4	8.2	1.5	0.3	18.4	5.2	12.8	25
Micronucleus, length (μm)	4.3	4.4	0.5	0.1	11.7	3.2	5.4	25
Micronucleus, width (μm)	3.1	3.2	0.4	0.1	13.8	2.2	3.9	25
Buccal cavity, length (μm)	6.9	6.9	1.0	0.2	14.1	5.1	8.3	25
Buccal cavity, width (μm)	3.9	3.8	0.8	0.2	19.8	2.4	5.4	25
Adoral membranelle number 1, length (μm)	3.4	3.4	0.4	0.1	13.2	2.5	4.2	25
Adoral membranelle number 2, length (μm)	3.4	3.4	0.5	0.1	13.5	2.7	4.4	25
Adoral membranelle number 3, length (μm)	1.8	1.8	0.3	0.1	15.5	1.2	2.3	25
Excretory pores, number	1.3	1.0	0.5	0.2	38.0	1.0	2.0	7
Somatic kineties, number	23.0	23.0	0.9	0.2	3.9	21.0	25.0	25
Postoral kineties, number	2.0	2.0	0.0	0.0	0.0	2.0	2.0	25
Kinetids in a dorsal kinety, number	36.5	36.0	4.7	0.9	13.0	30.0	46.0	25
Aposymbiotic ciliates								
Body, length – living trophonts (μm)	40.5	41.0	5.1	0.9	12.6	26.2	48.1	31
Body, width – living trophonts (μm)	24.2	24.1	1.9	0.3	7.9	19.5	27.8	31
Body, volume – living trophonts (μm^3)	12,649	13,157	3,131	562	25	5,186	17,858	31
Body, length – living theronts (μm)	51.1	51.8	3.2	0.6	6.3	43.9	56.0	26
Body, width – living theronts (μm)	16.5	16.3	2.0	0.4	12.2	12.7	21.0	26

CV, coefficient of variation in %; M, median; Max, maximum; Min, minimum; *n*, number of measured specimens; SD, one standard deviation; SE; standard error of average.

Algal symbionts were identified as *Micractinium* sp. Data based, if not mentioned otherwise, on mounted, protargol impregnated trophonts from a green clonal culture. In addition morphometric parameters of living trophonts and theronts of an aposymbiotic culture are listed.

DNA extraction and sequencing

DNA from a clonal aposymbiotic *T. utricullariae* culture was extracted with the DNeasy Blood and Tissue kit (Qiagen, Vento, the Netherlands). The aposymbiotic culture originated from a symbiont bearing clonal culture (basis for morphometric measurements) which was kept under aerobic conditions with over-saturating bacterial food. Subsequently, the partial *cox1* gene was amplified using Platinum® PCR Super Mix High Fidelity (Invitrogen), the forward primer COI-FW 5′-ATGTGAGTTGATTTTATAGAGCAGA-3′ (Chantangsi et al. 2007) and the reverse primer FolB 5′-TA AACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al. 1994). The PCR reaction was performed under the following conditions: initial denaturation at 94 °C for 180 s, five cycles of 94 °C for 30 s, 45 °C for 60 s, 68 °C for 75 s and 35 cycles of 94 °C for 30 s, 55 °C for 60 s, 68 °C for 75 s, and final extension at 68 °C for 600 s (modified from Strüder-Kypke and Lynn 2010). GenElute™ PCR Clean-Up Kit (Sigma, St. Louis, MO) served as tool for the purification of the PCR products, which were subsequently sequenced with the same primers and ABI Big-Dye chemistry on an ABI 3130x Genetic Analyzer (Applied Biosystems, Waltham, MA).

The amplification of the 18S rRNA gene of *T. utricullariae* was done with GoTaq® Green Master Mix (Promega, Madison, WI) and the primer pair EK82f 5′-GAAACTGC GAATGGCTC-3′ (Auinger et al. 2008)/EUK239r 5′-TGATCC TTCYGCAGGTTCAC-3′ (Moon-van der Staay et al. 2001). The conditions for the PCR were as follows: denaturation at 94 °C for 300 s, 30 cycles of 94 °C for 15 s, 50 °C for 60 s, 72 °C for 180 s and a final extension of 300 s at 72 °C. PCR products were purified and sequenced as described above.

In order to sequence the ribosomal genes (18S rRNA gene, ITS1, 5.8S rRNA gene and ITS2) of the algal symbiont, DNA was extracted with the DNeasy Blood and Tissue kit (Qiagen) from a pure culture. PCR reactions were

run using GoTaq® Green Master Mix (Promega) and the following primer pairs: EUK238f 5′-ACCTGGTTGATCCTGC CAG-3′/EUK239r (Moon-van der Staay et al. 2001) and INT-4F 5′-TGGTGAAGTGTTCGGATTGG-3′/HLR3R 5′-TCCC AAACAACCCGACTCT-3′ (Hoshina et al. 2005) under the previously described conditions. The generated amplicons were purified and sequenced as described above. All sequences were deposited in GenBank with the accession numbers (LT605001 – LT605003).

Phylogenetic analysis

Two phylogenetic trees for *T. utriculariae* n. sp. (18S rRNA and *cox1* genes) and one for the algal symbiont (18S rRNA gene combined with ITS2) were calculated with different tools. The online aligner SINA (Pruesse et al. 2012) and the software package ARB (Ludwig et al. 2004) were used for the alignment and manual curation of the 18S rRNA genes (1,613 positions). Only sequences of *Tetrahymena* spp. (52 isolates) which had additionally available *cox1* sequences were included in the analysis (see Table S1). Sequences from the genus *Ichthyophthirius* (KJ690571 and U17354) were used as outgroup. The RAxML algorithm (Stamatakis et al. 2008) served as tool for the calculation of a bootstrapped (1,000 iterations) maximum likelihood (ML) tree using the GTR (General Time Reversible) model with Γ distribution for rate heterogeneity among sites. Posterior probabilities (four chains; 100,000 generations) from Bayesian interference (BI) obtained with the ExaBayes software package (©The Exelixis Lab) were added to the ML tree where topologies of the trees generated by the two different methods were congruent. Finally, branches with low bootstrap support (< 50%) were collapsed.

The phylogenetic analysis of the *cox1* sequences was built based on the alignment by Quintela-Alonso et al. (2013). The alignment was supplemented and calculated again with all newly available sequences of *Tetrahymena cox1* genes from GenBank using the freely available software Unipro UGENE 1.23. Short sequences (≤ 634 nt) together with duplicates (identical sequences with two accession numbers) were deleted (Table S1) and the alignment was manually corrected. In total, it compromised 188 sequences (987 positions) of *Tetrahymena* isolates along with seven sequences of *Ichthyophthirius multifiliis* as outgroup. For the phylogenetic analysis we applied the neighbor-joining algorithm with an interior-branch-test (Dopazo 1994; Li 1989; Nei et al. 1985; Rzhetsky and Nei 1992), implemented in the program MEGA v7.014 (Tamura et al. 2011), using the Jukes Cantor distance model with 1,000 replicates and pairwise deletion (Quintela-Alonso et al. 2013). As previously described, the interior branch test is highly appropriate in cases where the tree topology is predefined (Sitnikova 1996). Finally, the phylogenetic distance was calculated by using the pairwise distance, also supplied by MEGA.

The phylogenetic analysis of the algal symbiont (Chlorophyta, Trebouxiophyceae, Chlorellaceae) was done based on the concatenated 18S rRNA gene sequence (without introns) and the ITS2 sequence (2,219 positions). Primary sequence information combined with their individual secondary structures increases the accuracy and robustness of the resulting phylogenetic tree (reviewed in Wolf et al. (2014)). Thus, the state of the art methodology recently described by Heeg and Wolf (2015) was applied for the phylogenetic reconstruction of Chlorellaceae including the algal symbiont of *T. utriculariae*. From this publication we also extracted the 60 sequences (concatenated 18S rRNA gene and ITS2) with individual secondary structures (see file S1 of the supporting information in Heeg and Wolf (2015)). First, the secondary structures had to be added to the gene sequences of the algal symbiont. The RNA structure was taken from *Actinastrum hantzschii* FM205841 (99.76% sequence similarity) and the secondary structure of its ITS2 was predicted by homology modeling using a relevant template (Selig et al. 2008). Subsequently, a "xfasta file" (sequence-structure information of the concatenated 18S rRNA gene + ITS2) of the algal symbiont was generated and added to the 60 extracted Chlorellaceae sequences (with *Chloroidium ellipsoideum* FM946015 and *Chlorella saccharophila* FM946000 as outgroup). The software 4SALE v1.7 (Seibel et al. 2008) was subsequently used for a global multiple sequence alignment with simultaneous consideration of the primary sequence and the secondary structure. Using this alignment a ML tree was calculated with "phagorn" (Schliep 2011) which is implemented in R (R Core Team 2014). We used the R script available from the 4SALE homepage at http://4sale.bioapps.biozentrum.uni-wuerzburg.de. Finally, a BI tree was calculated as described above using the alignment generated with 4SALE. In case of congruent topologies of both trees, posterior probabilities were added to the ML tree.

RESULTS

Description of *Tetrahymena utriculariae* n. sp

Sizes of living symbiont bearing trophonts (Table 1) are 36.7 × 27.4 μm ($n = 31$), resulting in an average cell volume of 14,543 μm^3. Measurements on protargol-impregnated ciliates show a shrinkage of fixed and stained specimens (Table 1). Ovate ciliate cells (trophonts) are neither very flexible nor contractile. The globular macronucleus of protargol-impregnated specimens (9.6 × 8.4 μm) is in the central or slightly posterior position (Fig. 1B, 2B). The micronucleus (4.3 × 3.1 μm) is positioned at the macronucleus (Fig. 1B, 2C), sometimes difficult to see in vivo but clearly recognizable after staining with DNA specific dyes (e.g. DAPI) or after protargol impregnation. The oral apparatus is typical for the genus *Tetrahymena*. It consists of one undulating membrane on the right side of the buccal cavity and three oral membranelles on the left (Fig. 1A and Table 1). The buccal cavity has a size of 6.9 × 3.9 μm on average in portargol-impregnated specimens (Table 1). The contractile vacuole is slightly subterminal and has in most cases one and occasionally two excretory pores in dorsal position (Fig. 1B). Trophonts have on average 23 somatic kineties including two

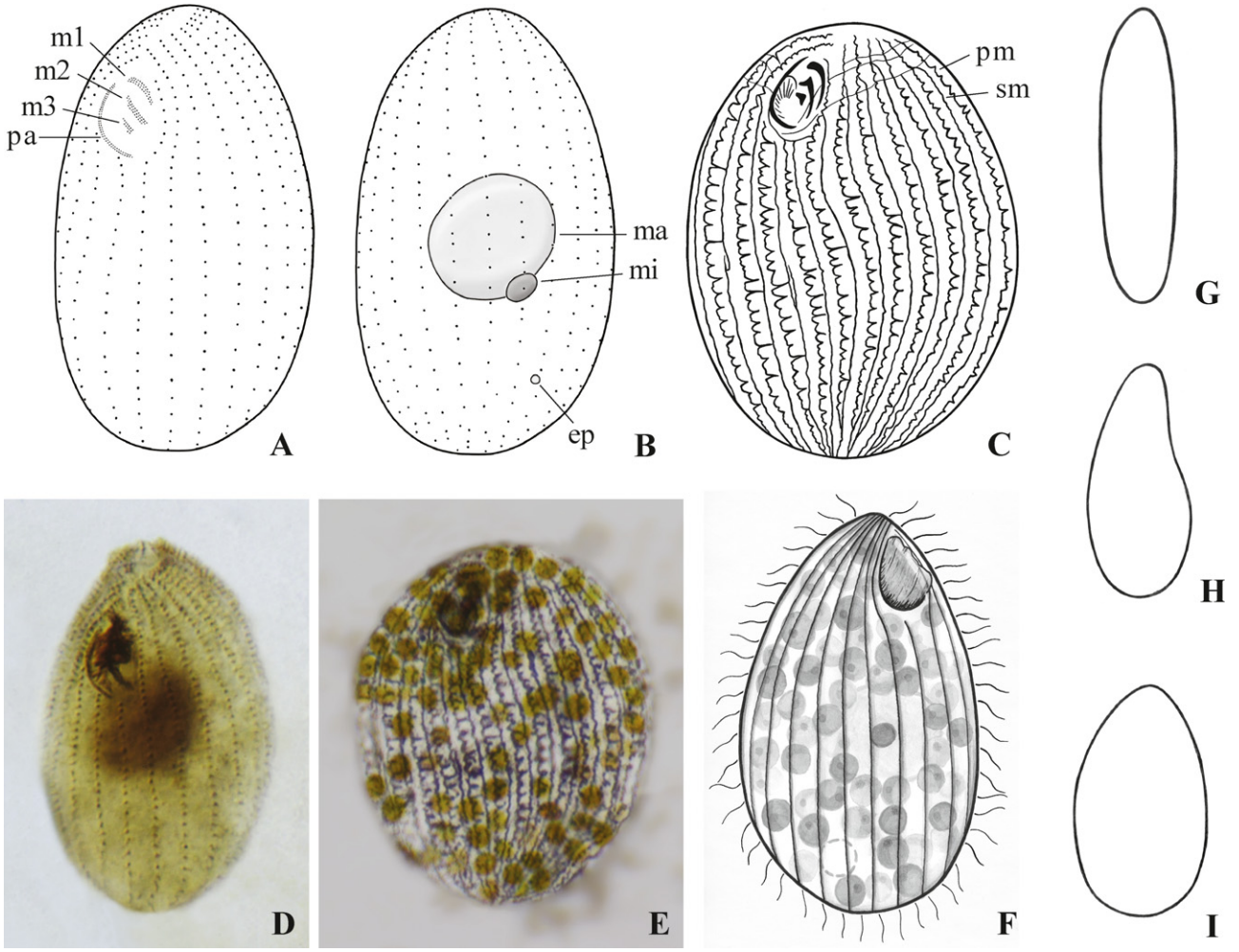

Figure 1 *Tetrahymena utriculariae* n. sp. after protargol impregnation (**A, B, D**), after "dry" silver nitrate impregnation (**C, E**), and from life (**F–I**). (A, B) Ciliary pattern of ventral and dorsal side of a trophont. (C) Silverline pattern of ventral side of a trophont. (D) Photomicrograph of a protargol stained green trophont, ventral view. (E) Photomicrograph of a "dry" silver nitrate impregnated green trophont, ventral view. (F) Right lateral view of a living green trophont. (G–I) Shape of a typical theront (G), lateral (H) and ventral (I) view of a typical trophont. ep, excretory pore; m1–m3, adoral membranelles; ma, macronucleus; mi, micronucleus; pa, paroral membrane; pm, primary silverline meridian; sm, secondary silverline meridian.

postoral kineties (Fig. 1A, B and Table 1). The silverline pattern (Fig. 1C,E) is of type 2, i.e. it shows primary meridians connecting the kinetids and secondary meridians (for further explanation of silverline patterns we refer to Quintela-Alonso et al. 2013). Trophonts harbor on average 52 algal symbionts (average diameter and standard deviation of living algae = 5.9 ± 0.6 μm) uniformly distributed within the ciliate cell (Fig. 2 and Table 1).

Tetrahymena utriculariae shows a "*Tetrahymena pyriformis* – like" life cycle (Fig. 2, 3). The real dominant life stage in trap fluids are green trophonts. Besides trophonts, conjugating individuals, dividers, and occasionally resting cysts could be observed. In stressed clonal cultures we could induce the formation of theronts. These elongated, spindle-shaped cells (49.8 × 20.1 μm; Table 1) show a higher swimming speed than gliding trophonts. Theronts have one clearly recognizable (in vivo) elongated caudal cilium (Fig. 2H) which is not observed for trophonts.

The cultivation of green ciliates under aerobic conditions and sufficient bacterial food particles resulted occasionally in a loss of algal symbionts. We could even generate algal free, aposymbiotic ciliate cultures. Sizes of living aposymbiotic trophonts are 40.5 × 24.2 μm (n = 31), resulting in an average cell volume of 12,649 μm^3 (Table 1). These aposymbiotic isolates can be maintained for several months, however, only by regular feeding with new bacterial food sources. We observed all life stages documented for the green counterparts also for the aposymbiotic cells (Fig. 2, 3), except conjugating cells. However, aposymbiotic ciliates can be re-infected by the addition of the isolated algal symbiont within several weeks (Šimek et al. 2016). Notably, colorless ciliates were never observed in trap fluids.

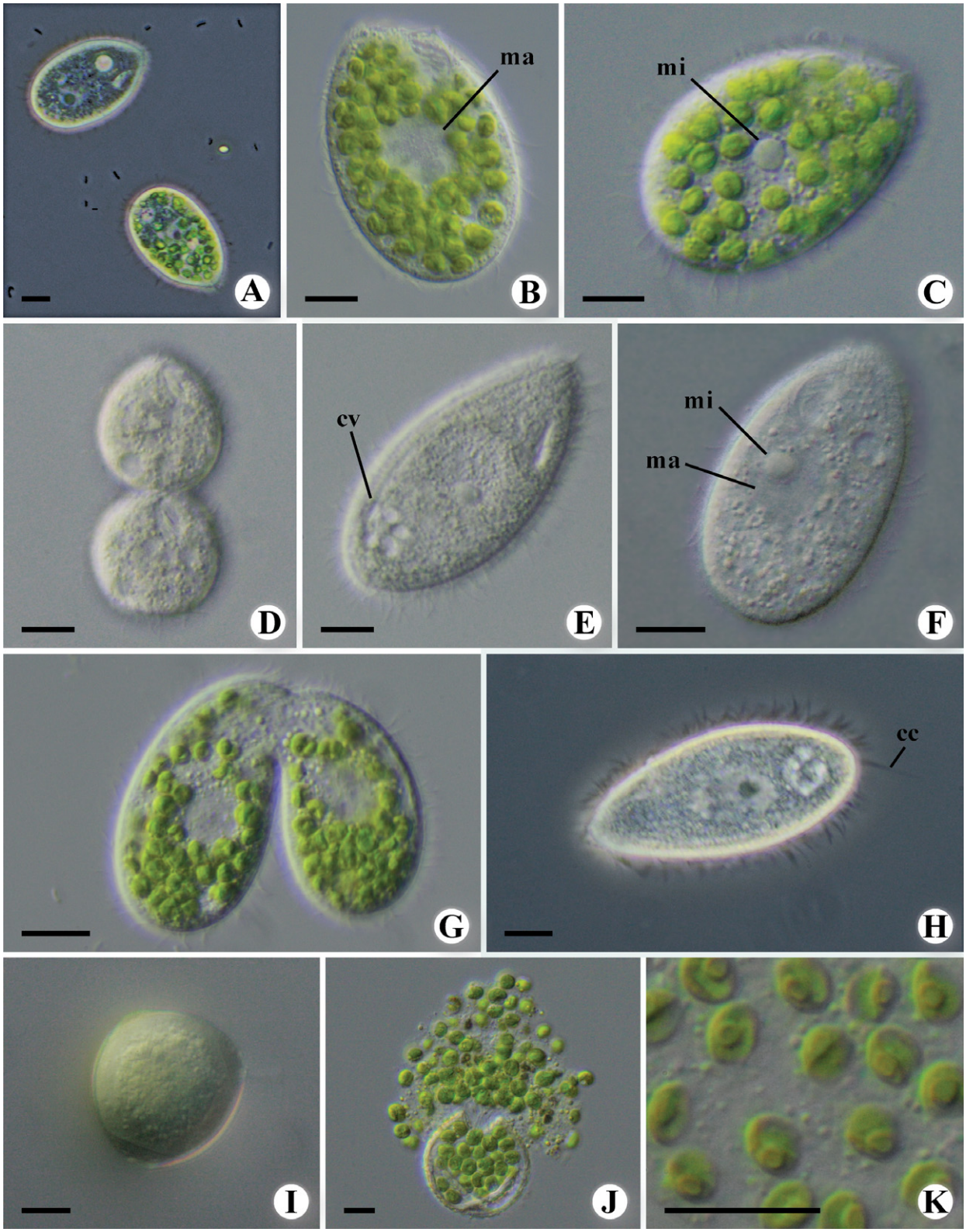

Figure 2 Photomicrographs of living green and aposymbiotic *Tetrahymena utriculariae* n. sp. (**A**) An aposymbiotic and a green trophont. (**B, C**) Green trophonts. (**D**) Late, aposymbiotic divider. (**E, F**) Aposymbiotic trophonts. (**G**) Conjugation pair of green ciliates. (**H**) Aposymbiotic theront. (**I**) Resting cyst. (**J**) A green cyst was dissected under microscopic control. (**K**) The isolated algal symbiont *Micractinium* sp. cc, caudal cilium; cv, contractile vacuole; ma, macronucleus; mi, micronucleus. Scale bars = 10 μm.

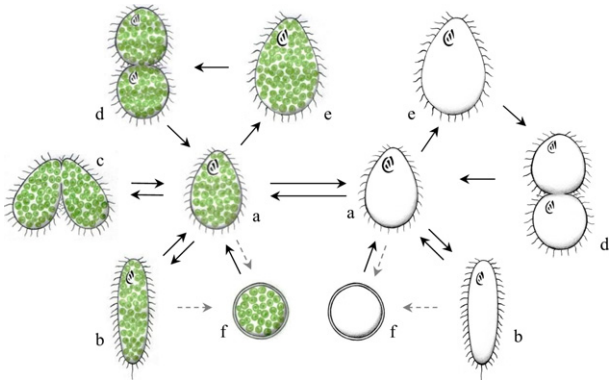

Figure 3 Observed stages within life cycles of green (left) and aposymbiotic (right) *Tetrahymena utricullariae* n. sp. Observed transformations are indicated by black arrows. Possible, but not observed transformations are shown by dashed gray arrows. a, throphont; b, theront; c, conjugation pair; d, cell dividers; e, enlarged trophont before cell division; f, resting cyst. Modified from Lynn and Doerder (2012).

Molecular identity and phylogenetic analysis of *Tetrahymena utricullariae*

The partial 18S rRNA gene sequence of *T. utricullariae* (1,643 unambiguous nucleotides, GC content 43.21%) was deposited under the accession number LT605001 at GenBank. The next hit at public databases for the 18S rRNA gene was *Tetrahymena malaccensis* (M26360) with a similarity of 99.76%. The sequence of the partial *cox1* gene (985 unambiguous nucleotides) was deposited under the accession number LT605002 at GenBank. The highest similarity (90.26%) was found with a *cox1* sequence of *T. thermophila* (GU439297).

As the phylogenetic analysis of the 18S rRNA gene alone (Fig. S1) was not sufficient, the *cox1* gene was used to determine the phylogenetic position of *T. utricullariae* (Fig. 4). In total 188 sequences were implemented in the analysis (Table S1), which showed a highly similar topology as previously published analyses (Chantangsi et al. 2007; Kher et al. 2011; Quintela-Alonso et al. 2013). The new species nested with medium support on a separate branch between the *T. thermophila* cluster (Fig. 4), containing 28 highly similar sequences, one sequence of *T. malaccensis*, one sequence of *T. farahensis* and a cluster of 10 sequences from a new *Tetrahymena* species, yet unnamed (Kher et al. 2011). The difference of the new species compared to its probably next relative, *T. thermophila*, was 9.74%, while the distance to *T. malaccensis* (EF070291) was 11.37%.

Habitat and ecology of *Tetrahymena utricullariae*

Tetrahymena utricullariae numerically dominates the eukaryotic community of commensals/mutual partners in traps of *U. reflexa*, reaching abundances of up to 50,000 cells per milliliter of trap fluid (Šimek et al. 2016). Green *T. utricullariae* inside traps are bacterivorous, with individual ingestion rates ranging from 260 to 340 bacteria/ciliate/h. Due to the high numbers of ciliates inside trap

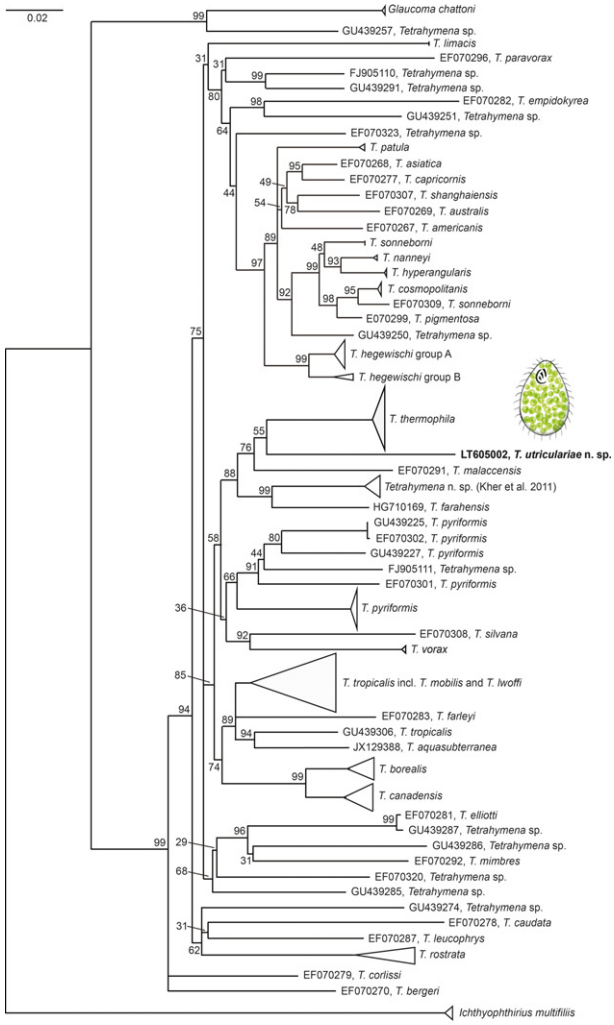

Figure 4 Phylogenetic analysis of 189 *Tetrahymena* isolates including *T. utricullariae* n. sp. (bold). A neighbor-joining tree was generated with an interior-branch test based on *cox1* gene sequences. *Ichthyophthirius multifiliis* was used as outgroup. Accession numbers for sequences inside collapsed cluster are listed in Table S1. Numbers at the branches represent bootstrap values. Scale bar: number of substitutions per site.

fluids, their total grazing rates lead to a fast bacterial turnover (Šimek et al. 2016).

The biogeography of *U. reflexa* is not confined to Botswana but the plant is endemic to Africa. We checked numerous feeding traps of seven other *Utricularia* species (*U. australis*, *U. bremii*, *U. aurea*, *U. inflata*, *U. purpurea*, *U. stygia*, *and U. vulgaris)* for the occurrence of *T. utricullariae*, however, at present we have to state that this ciliate species could be only found in *U. reflexa* (for further details see Šimek et al. 2016).

Molecular identity and phylogenetic analysis of the algal symbiont

The sequence of the algal symbiont, spanning almost the entire 18S rRNA gene, the ITS1, the 5.8S rRNA gene, the

ITS2 and the partial 28S rRNA gene (2,681 nt), was deposited under the accession number LT605003 at GenBank. The next hit at public databases was *Micractinium* sp. ehime (JX889639) with a sequence similarity of 98.5%. In the recent publication from Heeg and Wolf (2015) a detailed phylogenetic analysis of taxa belonging to the Chlorellaceae (sensu stricto + species name available) was conducted. Sequence-structure analysis of the 18S rRNA genes and the ITS2 genes alone was compared with the phylogenetic reconstruction using both of these genes in combination. The authors assumed to have more accuracy using the concatenated data set. For reasonable comparison, we thus followed their approach and included the same taxa and merely added the sequence of the algal symbiont (Fig. 5). According to our phylogenetic analysis, the algal symbiont clustered with high bootstrap support inside the *Micractinium* spp. cluster, basal to *M. pusillum* (FM205875), *M. belenophorum* (FM205879 and FM205880), and *A. hantzschii* (FM205841, FM205884). This was also true for the BI tree but the algal symbiont directly clustered with *M. pusillum* making up the sister group to the two *M. belenophorum* and the two *A. hantzschii* sequences (no posterior probabilities for the respective branches). The relative great distance and the separated positioning prevented us from affiliating the algal symbiont to a described species. It rather represents a novel species in the genus *Micractinium*.

DISCUSSION

Morphological comparison with congeners

A morphological comparison of *T. utriculariae* with closely related congeners (*T. farahensis*, *T. malaccensis*, *T. thermophila*) is difficult as the first two species were established without clear morphological descriptions (Table 2). We had to exclude the phylotype *Tetrahymena* n. sp. presented by Kher et al. (2011) from the comparison as it was only characterized by *cox1* gene sequences without defining a type strain (see Table S1 for further details).

The most striking difference within the congeners concerns the presence/absence of symbiotic algae in natural ciliate populations. Only *T. utriculariae* harbors endosymbionts, although this character may be lost under defined culture conditions (aerobic cultivation with dense bacterial food organisms). *Tetrahymena utriculariae* can form cysts, which was observed neither for *T. thermophila* nor for *T. malaccensis* (Table 2). All three species have at least in natural populations a micronucleus. The morphological comparison of *T. utriculariae* with the closest relative, *T. thermophila* (Collins 2012), shows some slight differences: *T. utriculariae* has on average 23 kineties (range 21–25), whereas *T. thermophila* has on average 17–21 kineties (range 15–25). The average number of kinetids in a kinety are 36.5 and 30 for *T. utriculariae* and *T. thermophila*, respectively. *Tetrahymena utriculariae* has usually one excretory pore (range one to two), whereas *T. thermophila* has on average two (range 1–3). A clear difference is obvious from the sizes of living trophonts. Even the aposymbiotic trophonts of *T. utriculariae* (on average 40.5 × 24.2 μm) are by 10 μm smaller than typical specimens of *T. thermophila* (on average 50 × 30 μm; Collins 2012).

Morphological comparison with similar species

Tetrahymena is a young genus-group name (Furgason 1940) and its type species, *T. pyriformis* (*T. geleii* is considered as a synonym; Aescht 2001), was classified in various other genera, e.g. *Glaucoma*, *Leucophrys*, and *Sathrophilus* (Foissner et al. 1994). Thus, we did a literature search for species showing similarities to *T. utriculariae* in the genus *Tetrahymena* and the mentioned genera.

Dujardin (1838) described a green species, *Glaucoma viridis*, which might have some similarities to *T. utriculariae*. In June 1837, the author discovered a mass development of *G. viridis* in an old wine barrel, containing tartar (dried rest of the wine) and rainwater. Obviously, quality of the one month old water was not the best anymore as the author spoke about '*l'eau s'était putréfiée*', meaning moldy water (Dujardin 1841). *Glaucoma viridis* had a size of 30–50 μm and a buccal cavity which seemed to be typical for the genus *Glaucoma* (see figure G4 on Plate 15 in Dujardin 1838 and figure 9 on Plate 8 in Dujardin 1841). Nevertheless, the buccal cavity spanned over at least one-third of the total cell length. This morphological feature allows the differentiation between *G. viridis* and *T. utriculariae*. According to Dujardin (1841), *G. viridis* contained several green "large" vacuoles which had "twelve to thirteen nodules". However, the author did not describe if these green vacuoles were ingested or symbiotic algal cells (likewise it is not obvious from the original drawings mentioned above). Unfortunately, there seems to be no other record of this species after the description by
Dujardin (1838, 1841).

Within the genus *Sathrophilus* we found one species similar to *T. utriculariae*. *Sathrophilus chlorophagus* (Kahl 1931) Corliss, 1960 has a similar size and shape (see figure 60, panel 33 in Kahl 1931). Free living specimens harbored often small green algae but were occasionally colorless. Cells had one elongated caudal cilium, conspicuous long cilia and a large buccal cavity which spanned over one-third of the total cell length. The latter two morphological features allow the differentiation between *S. chlorophagus* and *T. utriculariae*. *Sathrophilus chlorophagus* was abundant in a pond during winter but the exact type locality was not defined. Again, we could not find new records of this species after the description by Kahl (1931).

Another green ciliate with a similar shape as *T. utriculariae* was described by Penard (1922) as *Ophryoglena viridis* (see figure 146 in Penard 1922). Nevertheless, the ciliature of the buccal cavity, the high number of postoral kineties and the larger size of *O. viridis* (80 × 54 μm) allow a clear differentiation between the two species.

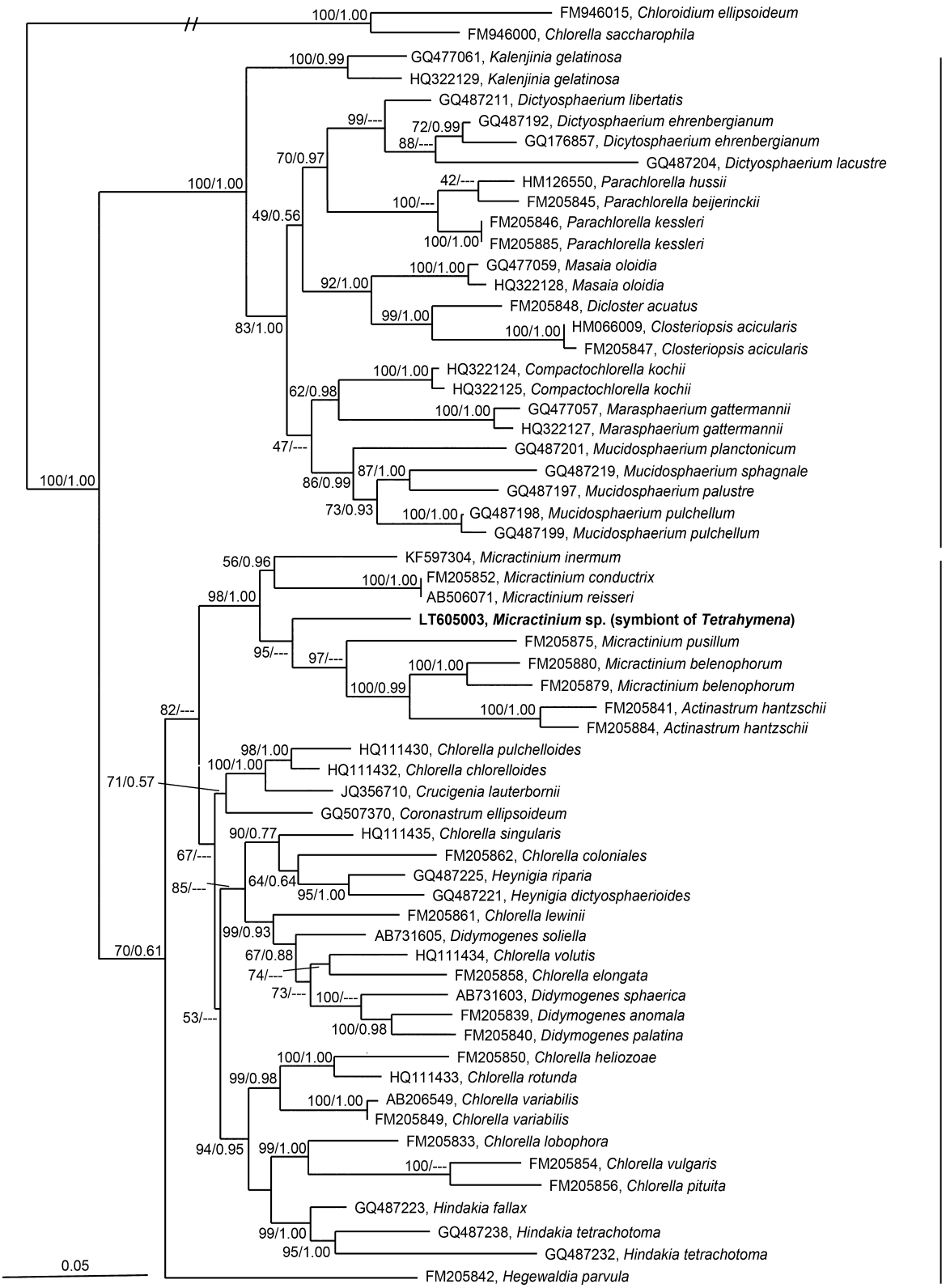

Figure 5 Maximum likelihood (ML) tree with posterior probabilities from Bayesian interference (BI) based on the concatenated ITS2 + 18S rRNA gene sequence-structure of 61 chlorellacean isolates. *Chloroidium saccharophilum* and *Chloroidium ellipsoideum* were used as outgroup. The algal symbiont (bold) affiliated with the *Micractinium* cluster. Numbers at the branches represent ML bootstrap value/BI posterior probabilities. Scale bar: number of substitutions per site.

Table 2. Brief characterization of *Tetrahymena* species related to *Tetrahymena utriculariae* n. sp. according to the *cox1* gene phylogeny

	Habitat	Algal endo-symbionts	Cysts	Mating	Micronucleus present (+)/ absent (−)	*Cox1* gene GenBank accession number	18S rDNA GenBank accession number	References
Tetrahymena utriculariae n. sp.	Traps of the aquatic plant *Utricularia reflexa*[a]	Yes[b]	Yes	Conjugation	+	LT605002	LT605001	This study
Tetrahymena farahensis	Wastewater treatment plant, Pakistan	No	Nd	Nd	Nd	HG710169	HE820726	Zahid et al. (2014)
Tetrahymena malaccensis	Swamp, K. Rantau Abang, Malaysia	No	No	Conjugation	+	EF070291	M26360	Simon et al. (1985)
Tetrahymena thermophila	Woods Hole, USA[c]	No	No	Conjugation	+/−	EF070310	M10932	Nanney and McCoy (1976)

Nd, not determined.

A detailed morphological comparison with the closest congener, *T. thermophila*, is given in the text. Table modified from Lynn and Doerder (2012).

[a]A detailed discussion of the type locality is given in the text.

[b]Symbiont bearing cultivated specimens may lose their symbionts when kept under aerobic conditions and dense bacterial food organisms. Nevertheless, we never found aposymbiotic cells in the natural habitat of *T. utriculariae*, i.e. in the traps of *Utricularia reflexa*.

[c]Strain B derived from a cross between wild strains WH-6 and WH-14.

Phylogenetic analysis and relation to other *Tetrahymena* species

The *cox1* gene is considered the most reliable DNA barcode to discriminate among different *Tetrahymena* species, as it is improving the resolution of most clades within the phylogenetic analysis, compared to the 18S and 28S rRNA gene (Chantangsi et al. 2007; Lynn and Strüder-Kypke 2006; Simon et al. 2008). When comparing phylogenetic analyses of 18S rRNA (Fig. S1) and *cox1* (Fig. 4) genes, a similar topology in general, with few exceptions, is recovered, indicating a monophyletic character of the genus *Tetrahymena*.

In our analysis of the *Tetrahymena cox1* dataset we recovered a topology supporting previous studies (Kher et al. 2011; Lynn and Doerder 2012; Quintela-Alonso et al. 2013). The application of the interior-branch-test, which is suitable for a given topology, increased the reliability and hence the bootstrap support within the tree (Quintela-Alonso et al. 2013; Sitnikova 1996). Analyzing the genetic distance of the *cox1* gene compared to other species, revealed a high distance of 9.74%. The intraspecific difference found regarding the *cox1* gene was described previously to range from 0% to 3.5%, with *T. thermophila* having the largest intraspecific range (Doerder 2014). Based on the high genetic distance, the ecological/physiological characteristics, and the habitat specificity (Šimek et al. 2016) we describe the strain from Třeboň (Czech Republic) as a new species, *T. utriculariae* n. sp.

Phylogenetic analysis of the algal symbiont (Chlorophyta, Trebouxiophyceae, Chlorellaceae)

Surprisingly, the addition of only one taxon, the algal symbiont of *T. utriculariae*, to the selected sequences of Heeg and Wolf (2015) changed the topology of the phylogenetic tree remarkably (compare our Fig. 5 with fig. 7 in Heeg and Wolf (2015)). Whereas the structure inside the *Parachlorella*-clade could be reproduced, there were structural changes inside the *Chlorella*-clade. Most conspicuous was the difference between the topologies concerning the *Micractinium* spp. isolates to which the algal symbiont clustered. In contrast with the polyphyly of this genus in the 18S rRNA + ITS2 gene tree from Heeg and Wolf (2015), in our tree all *Micractinium* spp. made up a monophyletic cluster including only two additional sequences from *A. hantzschii*. In contrast with this discrepancy regarding the two trees based on the concatenated data set, the phylogenetic ITS2 tree (Heeg and Wolf 2015) also revealed the monophyly of *Micractinium* spp. It would be tempting to claim the algal symbiont to be the missing link connecting the two parts of the *Micractinium* spp. cluster in the 18S rRNA + ITS2 gene tree. But since the importance of taxon sampling was reported earlier and also pointed out by Heeg and Wolf (2015), it should probably rather be treated as an extreme example illustrating the impact of taxon sampling on phylogenetic analysis.

Symbiosis with the alga *Micractinium* sp

Mutualistic symbioses between freshwater ciliates and green algae (often affiliated with Chlorophyta) can be found in various genera (e.g. *Askenasia*, *Coleps*, *Euplotes*, *Halteria*, *Paramecium*, *Stokesia*, *Vorticella*, etc. – for an overview see Foissner et al. (1999)). In nearly all cases, we find within the same genus both, "obligate" green and colorless species. The best studied symbiosis with algae concerns the species *Paramecium bursaria*. It is a real mutual relationship, whereby both partners profit from each other (see Fujishima and Kodama (2014) and

references therein). However, it was also shown, that ciliates can lose their symbionts due to experimental manipulations, e.g. cultivation in complete darkness with sufficient bacterial food. Nearly all algal symbionts can live outside the hosts as free cells, thus they can be successfully cultivated in monocultures (Pröschold et al. 2011). A reinfection of aposymbiotic *P. bursaria* (Summerer et al. 2007) but also of other ciliate species with isolated algal strains is possible within a few days to weeks (see also fig. 4 in Šimek et al. 2016).

All these aspects are also valid for *T. utriculariae* and its symbiont *Micractinium* sp., thus it seems to be a well-established ciliate algae symbiosis. However, one might speculate about the origin of this symbiosis. In principle, there are two ways how algae are taken up by ciliate hosts: (i) by incidental ingestion or (ii) by repetitive phagocytosis of algae as positively selected food source (Fujishima and Kodama 2014). As most *Tetrahymena* species are rather bacterivorous, an incidental incorporation of algal cells seems more likely. In case of *T. utriculariae* we suppose that the lack of oxygen in traps (Adamec 2007) was the major selective force driving the evolution of this ciliate alga symbiosis. Notably, caught metazoan prey first dies in traps of *U. reflexa* due to anoxic conditions and not primarily due to enzymatic lysis (Adamec 2007). All *Tetrahymena* species are aerobes, as they don't have adequate organelles, i.e. hydrogenosomes which are found in anaerobic ciliates. Thus, the symbiosis with oxygen producing algae is the prerequisite for *T. utriculariae* to survive in this habitat. That algal symbionts can indeed supply their ciliate hosts with oxygen was already demonstrated for *Paramecium bursaria* (Reisser 1980) and natural ciliate assemblages (Finlay et al. 1996).

Bacterial uptake rates of *T. utriculariae* (Šimek et al. 2016) may point to a second role of algal symbionts. Individual bacterial ingestion rates were low in comparison to other similar sized bacterivorous ciliate species and thus not allowing for the assumed rapid ciliate growth in trap fluid. Probably algal symbionts do also support *T. utriculariae* with diverse metabolites, however, we have no proof yet for this assumption.

Interestingly, Nakajima et al. (2009, 2013) could induce the uptake of a green alga (*Micractinium* sp. ehime) as symbiont by *T. thermophila* in a five year long-term co-cultivation microcosm, with *Escherichia coli* as bacterial food source. It seemed that even a more cooperative algal phenotype evolved after five years of co-cultivation which allowed the ciliate ancestor a longer lifespan in experimental tests (Germond et al. 2013). However, ciliates were permanently confronted with an extraordinary high number of algae ($5*10^6$ algal cells/ml) during co-cultivation (Nakajima et al. 2009), which does not reflect natural circumstances. In contrast, numbers of *Micractinium* sp. in trap fluids of *U. reflexa* were always below the limit of detection and also abundances reported for freshwater systems are much lower. However, it is remarkable that the *Micractinium* sp. strain used in their experiment was found to be next hit in public databases to the algal symbiont of *T. utriculariae*. In combination with the close relatedness of the hosts, this opens up speculations about an ancient origin of the symbiosis between *T. thermophila* and *Micractinium* sp. Most probably prerequisites for the seemingly "de novo" established symbiosis reported by Nakajima et al. (2009, 2013)), have been acquired already during a former coexistence. In other words ancestors of the symbiotic partners might have lived together and established the symbiotic interaction already in former times.

Habitat specificity of *Tetrahymena utriculariae*

Its natural environment, fluids inside traps of *U. reflexa*, is a harsh habitat, characterized by (i) very low, often anoxic conditions (Adamec 2007), (ii) low pH values (average 5.1), but (iii) extremely high concentrations of dissolved nutrients (Sirová et al. 2009). We checked numerous feeding traps of seven other *Utricularia* species, but detected *T. utriculariae* only in two *U. reflexa* populations form Botswana and Zambia (see also Šimek et al. 2016). It is important to note that *U. reflexa* is endemic to Africa. The here studied aquatic plants have been collected in the Okavango Delta (Botswana) and are cultivated at the Institute of Botany CAS (Třeboň, Czech Republic) since 2005. Thus, we have to debate whether *T. utriculariae* invaded plants during cultivation in the culture collection, or ciliates were transported together with *U. reflexa* inside the feeding traps at that time. The aquaria with *U. reflexa* are not covered, thus, it cannot be excluded that *T. utriculariae* has been introduced from the Czech nature, e.g. also with zooplankton used for plant feeding. However, several arguments supply the second thesis that this ciliate species was transferred together with *U. reflexa* from Africa to Europe: (i) *T. utriculariae* has been found neither in other native Czech *Utricularia* species nor in species of the same genus which have been co-cultivated for several months together with *U. reflexa* (for further details see Šimek et al. 2016). (ii) Green ciliates were discovered in cultivated *U. reflexa* already in August 2009 (see fig. 2A in Płachno et al. 2012) but ciliates were miss-identified as *Paramecium bursaria*. The anterior position of the cytostome and cell shapes, documented in fig. 2A of Płachno et al. (2012), definitely speak for the first photographic evidence of *T. utriculariae*. We have observed this ciliate species for the first time in the year 2014 in the Czech Republic, when we inspected fresh trap fluids of *U. reflexa*. (iii) All autecological data about *T. utriculariae* indicate that ciliates are not only commensals but probably have a mutualistic relationship with *U. reflexa*, forming a complex symbiosis (Šimek et al. 2016). Ciliates are protected inside the traps from predators and supported with bacterial food and probably saturating dissolved nutrients. On the other hand, ciliates are efficient and, moreover, the only regulators of the bacterial standing stock inside traps. It is very unlikely, that this relationship developed within a few years during the period when *U. reflexa* was cultivated in Třeboň (Czech Republic). (iv) *T. utriculariae* seems to be not a cosmopolitan species. Regarding the extensive research activities on *Tetrahymena* and related

genera, the peculiar green species would have been noticed by morphologists or recently, its sequence would have appeared in sequencing data. Both evidences were not provided until now (but see also our comparison with similar species records above).

In summary, arguments prevail which speak for an endemic, very specialized ciliate species. Concerning the type locality we mention that *U. reflexa* plants were collected from the Okavango Swamp north from Maun in Botswana. However, our type material of *T. utricullariae* was isolated from *U. reflexa* specimens cultivated in the aquatic plant collection of the Institute of Botany CAS, Section of Plant Ecology in Třeboň (Czech Republic). To get a final proof that *T. utricullariae* is indeed a common symbiont of African *U. reflexa* plants it will be worth to conduct an expedition to the Okavango Delta in the near future.

TAXONOMIC SUMMARY

Class Oligohymenophorea de Puytorac et al., 1974
Order Tetrahymenida Fauré-Fremiet in Corliss, 1956
Family Tetrahymenidae Corliss, 1952
Genus *Tetrahymena* Furgason, 1940
Tetrahymena utricullariae n. sp.

Diagnosis. Green ciliates with symbiotic algae of the genus *Micractinium* (Chlorophyta, Chlorellaceae). Size of living ovate trophonts 36.7 × 27.4 μm on average. Theronts (49.8 × 20.1 μm) ellipsoidal. On average 52 algae per ciliate cell. One globular macronucleus. One globular micronucleus. Contractile vacuole subterminal. On average 23 somatic kineties including two postoral ones. Cytostome typical for the genus. Life cycle includes trophonts, theronts, conjugating cells, dividing cells and occasionally resting cysts. Theronts with one elongated caudal cilium. Aposymbiotic specimens (without algal symbionts) with same life cycle, except conjugating cells (not observed up to now).

Type material. The slide with the protargol-impregnated symbiont bearing holotype and several paratypes as well as a paratype slide with "dry" silver-nitrate impregnated symbiont bearing and aposymbiotic ciliates have been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, under the accession numbers 2016/116 and 2016/117, respectively. Relevant specimens have been marked by black circles on the coverslip. In addition, one paratype QPS (quantitative protargol staining) slide with several symbiont bearing specimens has been also deposited (accession number 2016/118).

Type habitat. Inside carnivorous traps of the submerged aquatic plants *Utricularia reflexa*.

Type locality. Okavango Swamp north from Maun in Botswana (for some uncertainties see discussion). *Utricularia reflexa* specimens, from which the type material of *T. utricullariae* was isolated, are cultivated in the aquatic plant collection of the Institute of Botany CAS, Section of Plant Ecology, 379 82 Třeboň, Czech Republic.

Etymology. The species-group name *utricullariae* refers to the aquatic plant *Utricularia reflexa*. *Utricullariae* is the genitive of *Utricularia* and means that the described ciliates are closely associated with these plants. Up to now, the ciliate species was only detected in the carnivorous traps of
U. reflexa.

Gene sequence. The GenBank accession numbers for the partial 18S rRNA and *cox1* gene sequences of the ciliate are LT605001 and LT605002 respectively. The GenBank accession number for the partial 18S rRNA gene, the ITS1, the 5.8S rRNA gene, the ITS2 and the partial 28S rRNA gene of the symbiotic algae (*Micractinium* sp.) is LT605003.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Maximum likelihood (ML) tree with posterior probabilities from Bayesian interference (BI) based on the 18S rRNA gene sequences of 52 *Tetrahymena* and two *Ichthyophthirius* isolates as outgroup.

Table S1. Species of *Tetrahymena* and *Ichthyophthirius* used for *cox1* and 18S rRNA gene analyses.