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ORIGINAL ARTICLE

A New *Tetrahymena* (Ciliophora, Oligohymenophorea) from Groundwater of Cape Town, South Africa

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ABSTRACT

The identification of species within the genus *Tetrahymena* is known to be difficult due to their essentially identical morphology, the occurrence of cryptic and sibling species and the phenotypic plasticity associated with the polymorphic life cycle of some species. We have combined morphology and molecular biology to describe *Tetrahymena aquasubterranea* n. sp. from groundwater of Cape Town, Republic of South Africa. The phylogenetic analysis compares the *cox1* gene sequence of *T. aquasubterranea* with the *cox1* gene sequences of other *Tetrahymena* species and uses the interior-branch test to improve the resolution of the evolutionary relationships. This showed a considerable genetic divergence of *T. aquasubterranea* to its next relative, *T. farlyi*, of 9.2% (the average *cox1* divergence among *bona fide* species of *Tetrahymena* is ~ 10%). Moreover, the analysis also suggested a sister relationship between *T. aquasubterranea* and a big clade comprising *T. farleyi*, *T. tropicalis*, *T. furgasoni* and *T. mobilis*. The morphological data available for these species show that they share with *T. aquasubterranea* a *pyriformis*-like life style and at least two of them, *T. farleyi* and *T. mobilis*, a similar type II silverline pattern consisting of primary and secondary meridians. *Tetrahymena aquasubterranea* exhibits a biphasic life cycle with trophonts and theronts, is amicronucleate, and feeds on bacteria.

THE genus *Tetrahymena* belongs to the family Tetrahymenidae Corliss, 1952 in the hymenostome grouping of ciliates. Currently, it comprises 41 species (Lynn and Doerder 2012), but this number very likely underestimates the real species richness and genetic diversity within the genus. *Tetrahymena* is relatively easy to distinguish from other hymenostomes because of its clear-cut generic characteristics. Unfortunately, problems arise for the discrimination of species due to their essentially identical morphology, the occurrence of cryptic and sibling species, and the phenotypic plasticity associated with the polymorphic life cycle of some species. The value of considering groups or combinations of characteristics was already addressed by different researchers, particularly Loefer (1967) and Corliss (1970). The latter proposed the recognition of four main features, which can be used for a reliable identification. These are: (1) the infraciliature and other cortical features; (2) the life cycle; (3) the habit; and (4) physiological and biochemical properties. The use of these characteristics has shown that they are insufficient to unequivocally separate "biological" species of *Tetrahymena* (Chantangsi et al. 2007; Corliss 1973; Simon et al. 2008).

In this context, the advent of molecular methods in the 1990s provided a very different perspective. The DNA-based molecular approaches quickly emerged as a valuable tool to define the phylogenetic relationships and to identify cryptic species in the genus *Tetrahymena* (Brunk et al. 1990; Jerome and Lynn 1996). Furthermore, it showed that: (1) the genetic diversity within and around already known lineages and apparently tightly defined morphotypes was much higher than expected, suggesting cryptic diversification and a much larger number of sister taxa (Barth et al. 2006; Chantangsi et al. 2007; Katz et al. 2011; Lynn and Strüder-Kypke 2006; Sadler and Brunk 1992; Ye and Romero 2002), and (2) the occurrence of many homoplasies within protist evolution (Patterson 1999; Pawlowski and Burki 2009). These two facts add

"another layer of poorly resolved complexity to the already muddled story emerging from earlier morphological studies" (Boenigk et al. 2012).

With the subsequent development of gene-sequencing technologies, the DNA-based identification became a solid alternative taxonomic approach for identifying organisms (Dawkins 1998; Tautz et al. 2002, 2003). The DNA barcoding increased its popularity for species identification, mainly because it is thought that it can be used without needing taxonomic expertise. Among the known DNA barcodes, like the small subunit (SSU) or the large subunit (LSU) of the ribosomal DNA (rDNA), the mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene was proposed as an ideal universal marker for global biological identification of animal species (Hebert et al. 2003a,b). The *cox1* was recently proved to be a useful tool for assigning *Tetrahymena* isolates to species level, particularly amicronucleate tetrahymenas, which have never been observed to mate when brought into the laboratory (Chantangsi et al. 2007; Chantangsi and Lynn 2008; Kher et al. 2011), or to unveil a much greater number of physiological or molecular species hidden behind the traditional morphospecies (Patterson and Lee 2000).

We combined morphology and molecular biology to characterize *Tetrahymena aquasubterranea* as a new species, as this approach recently emerged as an effective tool due to its ability to conquer the challenges of classifying organisms as *Tetrahymena* prone to simple or convergent morphologies.

MATERIAL AND METHODS

Sampling site and cell culture

Tetrahymena aquasubterranea was isolated from groundwater samples collected from a 12-m deep closed drinking water well in the suburb of Tokai, Cape Town, Republic of South Africa, in July 2007. The groundwater was pumped out and transferred to sterile containers without contact to soil or surface water. Cultures of several strains were isolated at the University of Cape Town where *T. aquasubterranea* was found free living in the samples and further analysed at the Institute of Zoology in Cologne (Germany).

Clonal cultures were established in 1-ml multi-well plates and later transferred to 50-ml tissue culture bottles by mixing Eau de Volvic and Wright's Chu medium (Guillard and Lorenzen 1972) in a ratio 2:1 and adding a grain of wheat or a couple of grains of quinoa to support bacterial growth. Cultures used for silver staining were established in Petri dishes, in the above-mentioned media or in tap water adding an excess of crushed wheat grains to stimulate bacterial growth. The formation of theronts was induced by starvation.

Morphological methods

The morphological characterization of *T. aquasubterranea* was based on the methods as described in Foissner (1991), i.e. in vivo observation using a high-power oil immersion objective and interference contrast; different silver impregnation techniques (silver nitrate, silver carbonate, protargol), and scanning electron microscopy (SEM). Deciliated specimens were obtained with the method described by Foissner (2003). In vivo measurements were conducted at magnifications of 100–1,000×. Counts and measurements on prepared specimens were performed at a magnification of 1,000×. Illustrations of live specimens were based on free-hand sketches and micrographs, while those of prepared cells were made with a drawing device. For details on terminology, see Foissner and Xu (2007) and Lynn (2008).

DNA extraction, amplification and sequencing

DNA was extracted using CTAB protocol (Wylezich et al. 2010). The small subunit (SSU) rDNA was amplified using the universal primers 18SFor (5′-AACCTGGTTGATCCTGCCAGT-3′) and 18SRev (5′-TGATCCTTCCGCAGGTTCACCTAC-3′) (Medlin et al. 1988). PCR program started with an initial denaturation at 94 °C for 4 min followed by the cycles repeating 30 times denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min and elongation at 72 °C for 2 min and finalized by a final elongation at 72 °C for 7 min. The PCR products were purified using the E.Z.N.A. Cycle-Pure-Kit (Peqlab, Erlangen, Germany) and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Darmstadt, Germany) and FS Taq DNA polymerase. For cycle reactions, the primers 18SFor, 590F and 1280F for the forward strand and 600R, 1300R and 18SRev for the reverse strand were used (Quintela-Alonso et al. 2011; Wylezich et al. 2002). Cycle sequencing reactions were purified with AutoSeq G-50 columns (Amersham Biosciences, Braunschweig, Germany) and sequenced on an ABI Prism® 3100 Genetic Analyzer (Life Technologies). Sequence parts were aligned using BioEdit (Hall 1999).

For the large subunit (LSU) of the rRNA, the D1–D5 region was amplified by PCR using the primer pair fw1 (Sonnenberg et al. 2007) and D5-Rev2 (Wylezich et al. 2010). PCR conditions were as described elsewhere (Wylezich et al. 2010). Besides the primers used for amplification, the D1–D5 fragments were additionally cycle-sequenced with internal primers (D3-For or D3-For-n and D3D4-Rev); cycling reaction procedure and sequencing were the same as described above.

The partial cytochrome *c* oxidase subunit 1 (*cox1*) gene was amplified using the forward primer 288 5′-TCAGGTGCTGCACTAGC-3′ and the reverse primer FolB 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Lynn and Strüder-Kypke 2006). Reaction conditions were as follows: initial denaturation at 94 °C for 4 min followed by five cycles repeating denaturation at 94 °C for 1 min, primer annealing at 48 °C for 1 min and elongation at 72 °C for 2 min followed by 30 cycles with 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min and finalized by a final elongation at 72 °C for 7 min. PCR products were purified and sequenced in both directions (see SSU rDNA) with the forward and reverse PCR primers. Although fragments of

about 900 nucleotides long were obtained (the barcoding region of *cox1* in ciliates is ~ 980 nucleotides long), only the overlap region of the forward and reverse strand of the *cox1* amplicon was used for the phylogenetic analysis (~ 634 nucleotides long).

Phylogenetic analyses

The alignment of *Tetrahymena cox1* sequences used for the phylogenetic analysis in this study was kindly provided by Chandni Kher and was previously used in the study by Kher et al. (2011) to demonstrate that the *cox1* barcoding is an invaluable tool to identify and assign unknown *Tetrahymena* isolates to the species level, especially when used in conjunction with morphological studies. The *cox1* barcode dataset consists of 155 sequences from *Tetrahymena* isolates along with 5 sequences from *Glaucoma chattoni* and 7 sequences of *Ichthyophthirius multifilis* (Kher et al. 2011; we have integrated the *cox1* sequence of *T. aquasubterranea* within their original alignment). For the phylogenetic analysis, we applied the interior-branch test (Dopazo 1994; Li 1989; Nei et al. 1985; Rzhetsky and Nei 1992) supplied by MEGA v5.05 (Tamura et al. 2011) using the Kimura 2-parameter (K2P) distance model (Kimura 1980) with 1,000 replicates. We selected this test as we based our phylogenetic analysis on the *cox1* dataset of *Tetrahymena* species by Kher et al. (2011) and it has been described to be appropriate for evaluating the reliability of a predetermined tree topology (Sitnikova 1996). The genetic divergence was calculated using the K2P distance model.

RESULTS

Description of Tetrahymena aquasubterranea n. sp

Tetrahymena aquasubterranea exhibits a biphasic life cycle with trophonts (TR) and theronts (TH), whose size is strongly influenced by culture and preparation conditions. Using some in vivo measurements and the data from Table 1 (considering 5% shrinkage in the Chatton–Lwoff silver nitrate preparations), the following in vivo sizes can be calculated: TR 44–56 × 20–28 μm, usually about 50 × 25 μm; TH 48–67 × 15–18 μm, usually about 60 × 15 μm, i.e. about 16% larger than TR. TR from cultures older than 2 weeks about 22–45 × 10–22 μm, usually about 35 × 15 μm; TH generated from starved old cultured TR 35–55 × 10–15 μm, usually about 45 × 13 μm, i.e. about 26% larger than TR. Thus, the total length range of *T. aquasubterranea* TR and TH under different culture conditions is 22–56 μm and 35–67 μm respectively. Both TR and TH are very flexible, but not contractile.

The shape of both TR and TH is very variable. The ordinary shape of the TR is elliptical to ovate; occasionally, *Colpidium* shaped, i.e. the anterior quarter of the cell is slightly bent to the ventral side (Fig. 1, 7, 8). The TH are lenticular to elongate lenticular; occasionally, they are obpyriform or cylindroidal (Fig. 2, 9, 10).

The macronucleus is in or slightly posterior of mid-body; it is globular to ellipsoidal. The macronucleus of the TH is more ellipsoidal and longer than that of the TR, i.e. 9 × 7 μm vs. 8 × 8 μm on average respectively. A micronucleus is absent (Fig. 1, 2, 12, 14, 25–27; Table 1).

The contractile vacuole is distinctly subterminal and has two excretory pores near the posterior end of kineties 5 and 6. The pore in kinety 5 is slightly anterior to the pore in kinety 6. Occasionally, there are three excretory pores involving kineties 5–7 (Fig. 1, 2, 12, 14, 32–34; Table 1).

The cortex is very flexible and distinctly granulated by the mucocysts in the TH. The cytoplasm is colourless and contains lipid droplets 1–3 μm across, more densely packed in the TH. The food vacuoles of the TR are up to 10 μm across vs. only up to 5 μm in the TH (Fig. 1, 2). Unfortunately, we do not know the feeding behaviour of this species in nature; presumably, it is microphagous (bacteria-feeder). In the laboratory, it feeds on bacteria and some bacteria-sized starch grains are also ingested. The TR swim slower than the TH, mostly gliding. The TH swim continuously and rapidly, rotating about the main body axis or glide and wriggle among mud accumulations.

The cilia are about 5 μm long in vivo and narrowly spaced (i.e. on average 1.8 μm and 1.4 μm in preparations of TR and TH respectively). The arrangement and number of the ciliary rows are similar in TR and TH, while the number of kinetids is higher in the TH by about 53%. There are 17–19 meridional somatic kineties including two postoral kineties (Fig. 1, 2, 5, 6, 11–16, 18, 25–27, 31–34; Table 1). Although a polar basal body complex was not revealed by silver staining, there is some indication of a caudal cilium, i.e. some specimens (mostly TH) showed an oblique, rather rigid cilium only slightly longer than the ordinary somatic cilia and difficult to observe in vivo and in preparations (Fig. 27, arrowhead).

The oral apparatus is as typical for the genus *Tetrahymena*: the paroral or undulating membrane is on the right of the oral cavity and three oral polykinetids or membranelles are on the left. The oral cavity is longer and wider in the TR than in TH: 9 × 6 μm vs. 7 × 4 μm on average (Table 1). The paroral of the TR is composed of a higher number of kinetosomes than in the TH, i.e. ~ 28 vs. ~ 18, respectively, and with the longest cilia about twice the size of those in the TH, i.e. ~ 4.5 μm vs. ~ 2.5 μm respectively (Fig. 1, 2, 5, 6, 11, 13, 15–24).

The silverline pattern is of type 2, that is, composed of primary meridians connecting the kinetids and secondary meridians running parallel and regularly alternating with the primaries (vs. type 1 pattern composed only of primary meridians and of many short cross-silverlines). The primary meridians are slightly to distinctly sinuous and have short cross-silverlines forming branches, peaks, or loops extending left between the kinetids occasionally reaching the secondary meridian. These cross-silverlines are infrequent in the secondary meridians and, when present, are not as striking as those of the primaries. The secondary meridians curve right to meet the primary meridians usually in the anterior quarter of the cell, at about the level of the third intermeridional connective, although some of

Table 1. Morphometric data on the trophont (upper line) and the theront (lower line) of *Tetrahymena aquasubterranea* n. sp.

Characteristics[a]	Mean	*M*	SD	SE	CV	Min	Max	*n*	% Increase[b]	*P* [c]
Body, length in protargol preparations (μm)	42.2	43.0	2.6	0.5	6.1	37.0	46.0	27	−17.2	***
	51.0	51.5	3.2	0.6	6.2	46.0	58.0	27		
Body, width in protargol preparations (μm)	20.0	20.0	1.4	0.3	7.0	18.0	24.0	27	30.7	***
	15.3	15.0	1.3	0.3	8.7	12.0	17.0	27		
Body, length in Chatton–Lwoff silver nitrate preparations (μm)	46.9	47.0	3.5	0.8	7.6	42.0	53.0	21	−15.8	***
	55.7	57.0	4.2	0.9	7.6	46.0	64.0	21		
Body, width in Chatton–Lwoff silver nitrate preparations (μm)	22.7	23.0	2.0	0.4	8.8	19.0	27.0	21	51.3	***
	15.0	15.0	0.8	0.2	5.4	14.0	17.0	21		
Body length:width, ratio in protargol preparations	2.1	2.1	0.1	0.0	5.4	1.9	2.4	27	−38.2	***
	3.4	3.3	0.4	0.1	11.2	2.7	4.3	27		
Body length:width, ratio in Chatton–Lwoff silver nitrate preparations	2.1	2.1	0.1	0.0	6.0	1.9	2.3	21	−43.2	***
	3.7	3.8	0.3	0.1	8.5	3.1	4.3	21		
Anterior body end to buccal cavity, distance (μm)	3.4	3.2	0.6	0.1	16.1	2.0	4.0	27	−40.3	***
	5.7	6.0	0.8	0.2	14.1	4.8	8.0	27		
Anterior body end to macronucleus, distance (μm)	18.0	18.0	2.0	0.4	11.1	14.0	23.0	27	−14.3	***
	21.0	21.0	1.7	0.3	8.2	17.0	23.0	27		
Anterior body end to anterior most excretory pore of contractile vacuole, distance (μm)	35.3	35.0	2.0	0.4	5.7	30.0	39.0	27	−18.1	***
	43.1	43.0	2.7	0.5	6.3	38.5	50.0	27		
Macronucleus, length (μm)	8.5	8.0	0.9	0.2	11.2	6.8	11.0	27	−5.5	n.s.
	9.0	9.0	1.3	0.2	14.2	7.0	13.0	27		
Macronucleus, width (μm)	8.4	8.0	0.8	0.2	9.6	7.0	10.0	27	13.5	***
	7.4	7.2	0.7	0.1	9.5	6.0	9.0	27		
Buccal cavity, length (μm)	9.4	9.5	0.7	0.1	7.7	7.5	10.0	27	27.0	***
	7.4	7.0	0.5	0.1	6.8	6.5	8.0	27		
Buccal cavity, width (μm)	5.9	6.0	0.5	0.1	9.3	5.0	7.0	27	43.9	***
	4.1	4.0	0.4	0.1	9.6	3.0	5.0	27		
Adoral membranelle number 1, length (μm)	4.9	5.0	0.6	0.1	11.6	4.0	6.0	25	36.1	***
	3.6	4.0	0.5	0.2	13.9	3.0	4.0	11		
Adoral membranelle number 2, length (μm)	4.1	4.0	0.5	0.1	12.1	3.0	5.0	25	17.1	**
	3.5	3.0	0.5	0.2	15.1	3.0	4.0	11		
Adoral membranelle number 3, length (μm)	2.6	3.0	0.6	0.1	22.8	2.0	4.0	25	30.0	**
	2.0	2.0	0.0	0.0	0.0	2.0	2.0	11		
Excretory pores, number	2.1	2.0	0.3	0.1	12.9	2.0	3.0	27	5.0	n.s.
	2.0	2.0	0.0	0.0	0.0	2.0	2.0	27		
Somatic kineties, number	18.2	18.0	0.6	0.1	3.1	17.0	19.0	27	1.1	n.s.
	18.0	18.0	0.6	0.1	3.3	17.0	19.0	27		
Somatic kineties with a dikinetid at anterior end, number	11.6	12.0	0.9	0.2	8.0	9.0	13.0	21		
	-	-	-	-	-	-	-	-	-	-
Kinetids in a dorsal kinety, number	23.2	23.0	3.1	0.6	13.4	19.0	31.0	27	−34.8	***
	35.6	36.0	3.2	0.6	9.1	30.0	42.0	27		
Postoral kineties, number	2.0	2.0	0.0	0.0	0.0	2.0	2.0	27	0.0	n.s.
	2.0	2.0	0.2	0.0	9.8	1.0	2.0	27		

[a]Data based, if not mentioned otherwise, on mounted, protargol-impregnated and randomly selected specimens from pure cultures. CV, coefficient of variation in %; *M*, median; Max, maximum; Min, minimum; *n*, number of specimens investigated; SD, standard deviation; SE, standard error of mean.

[b]% increase in the mean for trophonts relative to theronts.

[c]*P* values from the Wilcoxon rank-sum test for differences between trophonts and theronts. Asterisks denote significant levels where $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n.s., not significant.

them can be locally interrupted or completely absent in some specimens. Both meridional lines usually contain irregularly distributed granules about the size of the kinetids, likely pores or granules related to the mucocysts. They are best revealed in Chatton–Lwoff silver nitrate preparations and are present in most of the specimens, both TR and TH, although their number and size varies among individuals (Fig. 3–6, 29–34). There are three intermeridional connectives encircling the oral portion. These delicate fibres usually impregnate faintly and are rarely all-visible at the same specimen (Fig. 5, 6, 30).

Occurrence and ecology

As yet, we found *T. aquasubterranea* only at the type locality, i.e. in the groundwater of a 12-m deep well in a

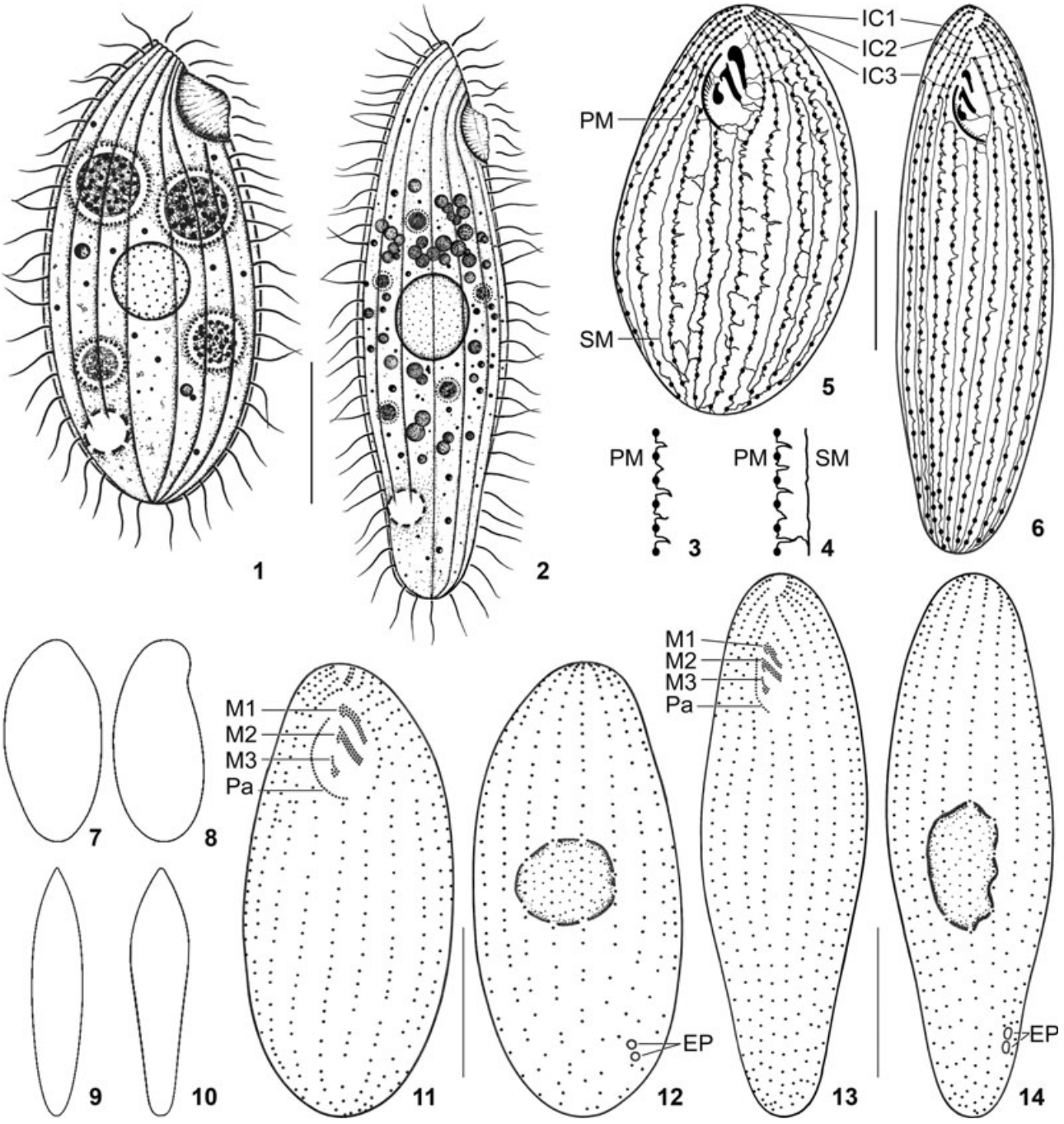

Fig. 1–14. *Tetrahymena aquasubterranea* n. sp., from life (1, 2, 7–10), after Chatton–Lwoff silver nitrate impregnation (3–6), and protargol impregnation (11–14). **1**, **2.** Right lateral view of a representative trophont (1) and theront (2). **3**, **4.** Type I (3) and Type II (4) silverline pattern in *Tetrahymena*. Type I consists only of primary silverline meridians with basal bodies of cilia. Type II consist of primary and distinct secondary silverline meridians. **5, 6.** Ciliary and silverline pattern of ventral side of a trophont (5) and a theront (6). **7–10.** Shape variability of trophonts (7, 8) and theronts (9, 10). **11–14.** Ciliary pattern of dorsal and ventral side and macronucleus of trophont (11, 12) and theront (13, 14) from the type series. EP, excretory pores; IC (1–3), intermeridional connectives; M(1–3), adoral membranelles; Pa, paroral membrane; PM, primary silverline meridian; SM, secondary silverline meridian. Scale bars = 15 μm.

sandstone area. *Tetrahymena aquasubterranea* was associated with several heterotrophic flagellates such as chrysomonads, bicosoecids, cercomonads, bodonids, choanoflagellates and apusomonads as well as with some amoebae (vannellids). Long-term cultures were easy to maintain in a Petri dish or culture flask with tap or mineral

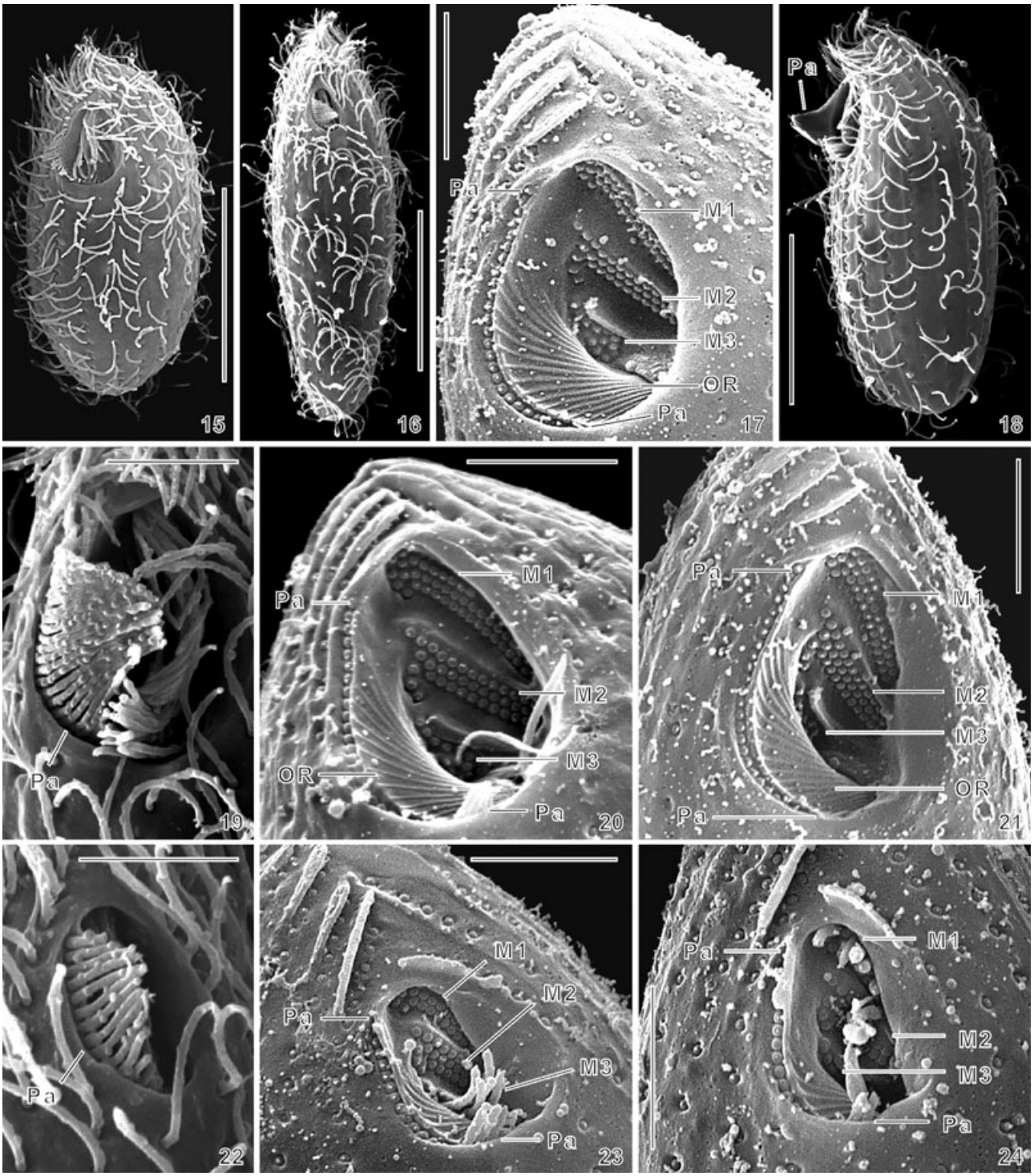

Fig. 15–24. *Tetrahymena aquasubterranea* n. sp. in the scanning electron microscope. **15**, **16.** Ventral view of a trophont (15) and of a theront (16). **17.** Ventral view of the ovate oral opening and the oral cavity of a deciliated trophont. There are three adoral membranelles and a paroral membrane from which the oral ribs extend towards the cytostome. **18.** Left side view of a trophont with extended paroral membrane. **19–24.** Ventral views of the oral cavity of three trophonts (19–21) and three theronts (22–24). Note the smaller and narrower oral opening and oral cavity, and the shorter paroral membrane of the theronts (19, 22). Micrographs (20, 21, 23, 24) show the oral apparatus of deciliated specimens with the basal bodies of the paroral on the right of the oral cavity and the three oral polykinetids on the left. M (1–3), adoral membranelles; OR, oral ribs; Pa, paroral membrane. Scale bars = 4 μm (Fig. 17, 19–24) and 20 μm (Fig. 15, 16, 18).

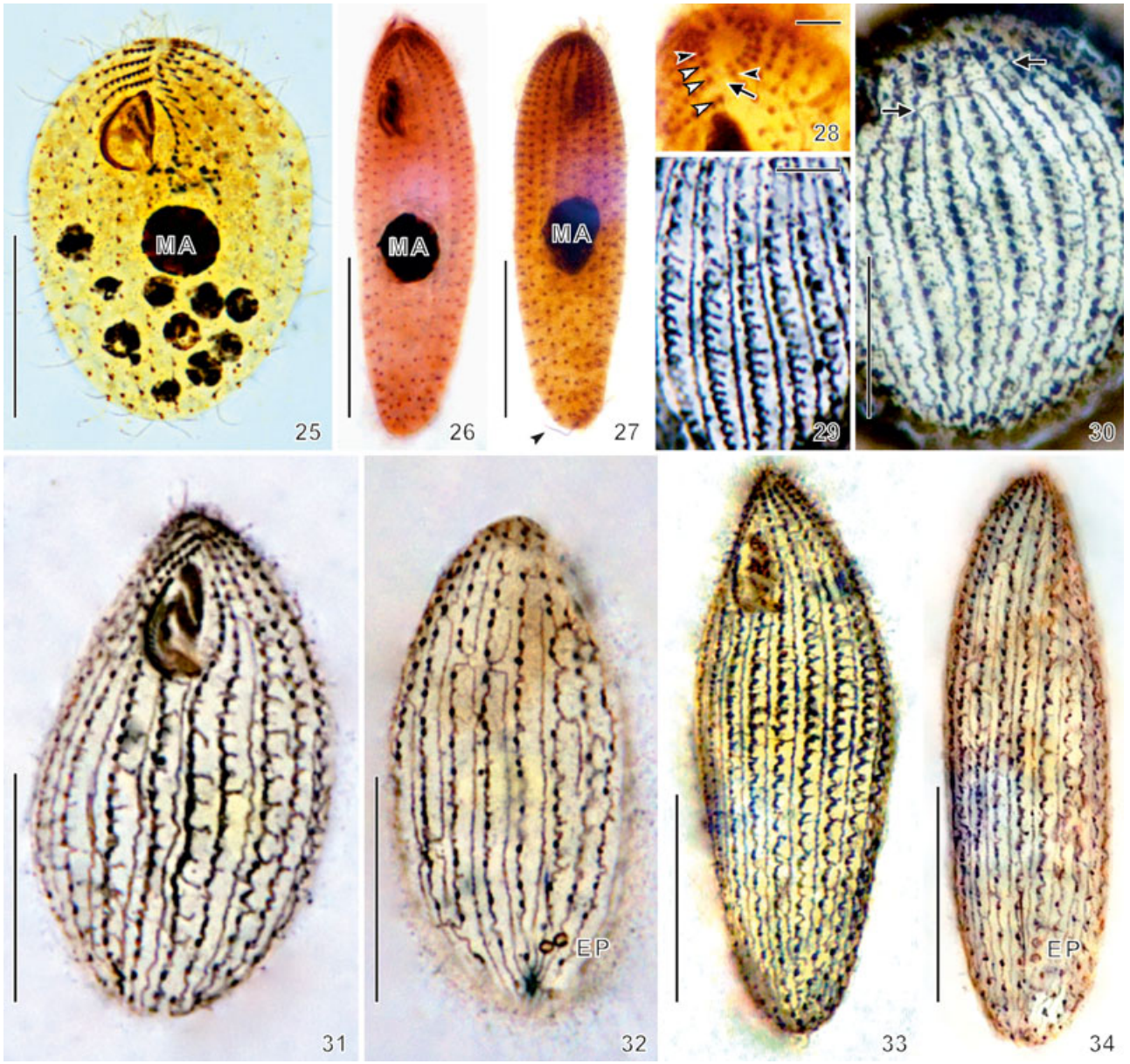

Fig. 25–34. *Tetrahymena aquasubterranea* n. sp., ciliary and silverline pattern of trophonts (25, 28–32) and theronts (26, 27, 33, 34) after silver carbonate impregnation (25), protargol impregnation (26–28), Klein–Foissner dry silver nitrate impregnation (29, 30) and Chatton–Lwoff silver nitrate (31–34) impregnation. **25.** Ventral view of a trophont, showing oral structures, macronucleus, and preoral suture. **26, 27.** Ventral and dorsal infraciliature of a theront, showing macronucleus and, possibly, a thicker caudal cilium (arrowhead). **28.** Ventral view of a trophont, showing the preoral suture (arrow) and the anterior end of the ciliary rows either having a dikinetid (black arrowheads) or a monokinetid (white arrowheads). **29.** Dorsal side silverline pattern of a trophont. **30.** Left-side silverline pattern of a trophont. Arrows mark intermeridional connectives. **31–34.** Ventral and right-side ciliary and silverline pattern of trophonts (31, 32) and theronts (33, 34). EP, excretory pores; M(1–3), adoral membranelles; MA, macronucleus. Scale bars = 2 μm (Fig. 28), 5 μm (Fig. 29), 15 μm (Fig. 30) and 20 μm (Fig. 25–27, 31–34).

water and some wheat grains to promote bacterial growth. Although *T. aquasubterranea* survived for a long time in culture, even at low concentrations of bacteria, we observed that the highest growth rates were achieved when the culture conditions turned slightly microaerobic by adding an excess of crushed wheat grains to stimulate the formation of a thick biofilm.

As *T. aquasubterranea* has a biphasic life cycle, TR cultures were starved for 5 weeks to induce the formation of TH. After 2 days, all the TR became TH, and after a week many TH became very small, broadly lenticular or ovate lying motionless on the bottom of the Petri dish. After five weeks, 99% of the cells had died. Resting cysts were not formed.

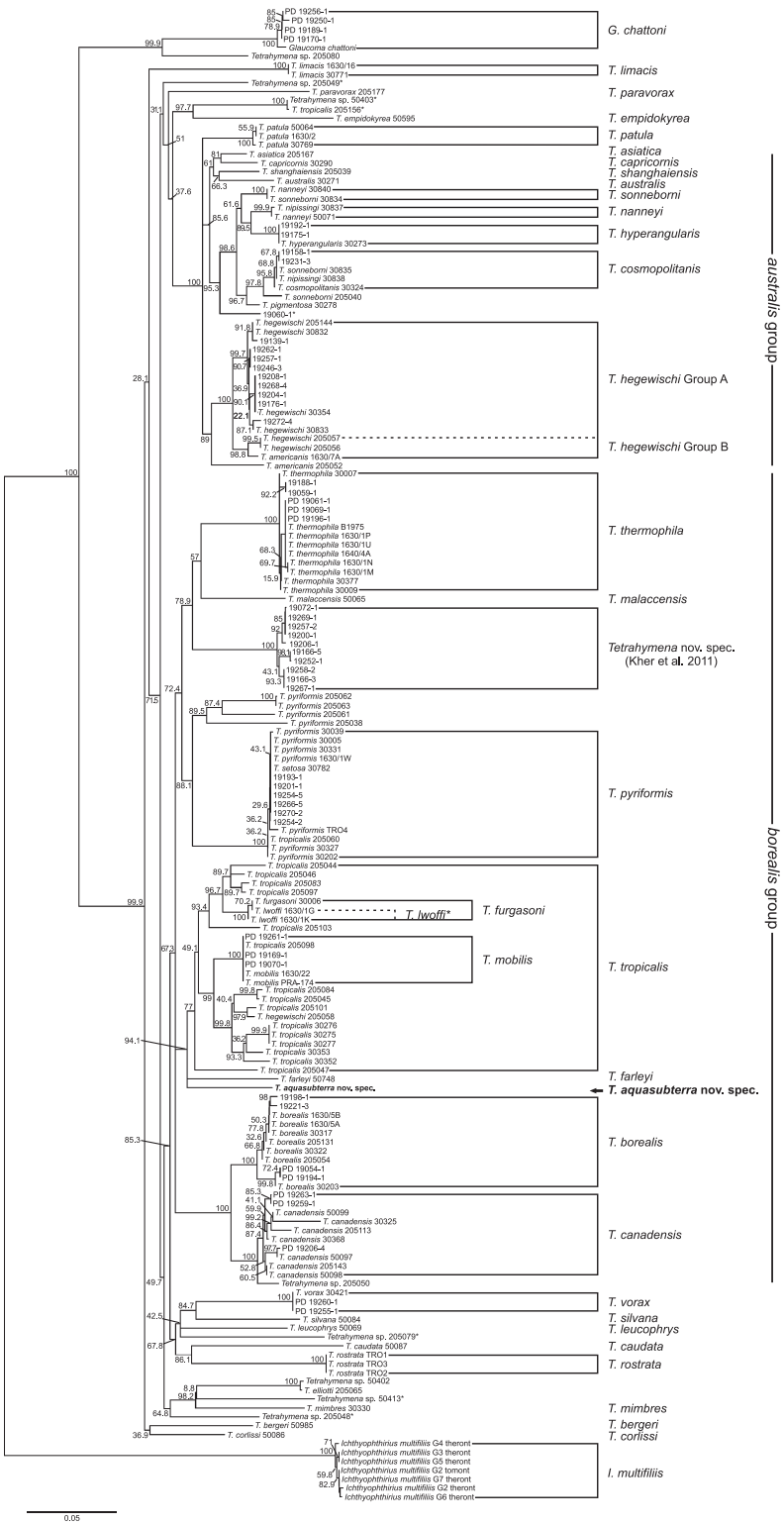

Fig. 35. Position of *Tetrahymena aquasubterranea* n. sp. in a phylogenetic tree containing 160 isolates from different *Tetrahymena* species. The tree was generated using the interior-branch test based on an 814-nucleotide stretch of cytochrome *c* oxidase subunit 1 (*cox1*) gene sequences. Genetic distances (scale bar = 0.05 nucleotide substitutions per site) were computed using the Kimura two-parameter model and the data were bootstrap resampled 1,000 times. Isolates marked with an asterisk should be assigned to new species. (Modified from the original *Tetrahymena cox1* barcode dataset and alignment of Kher et al. 2011 kindly provided by Chandni Kher).

Molecular characteristics

The amplified region of the mitochondrial *cox1* gene of the type strain HFCC 701 of *T. aquasubterranea* is 634 nucleotides long and has a GC-content of 26.0%. The SSU rDNA gene sequence is 1829 nucleotides long and shows a GC-content of 41.7%. The partial LSU rDNA gene sequence is 1144 nucleotides long with a GC-content of 46.6%. These sequences, *cox1*, SSU rDNA, and LSU rDNA, are available from GenBank under the accession numbers JX129388, JX129387 and JX271899 respectively.

The LSU rDNA and *cox1* gene sequences of another two strains isolated from the type locality (HFCC700 and HFCC702) are also available from the GenBank under the accession numbers JX271898 and JX442757 for HFCC 700, respectively, and JX271900 and JX442758 for HFCC 702 respectively.

Phylogenetic analysis

The preliminary analysis of the *cox1*, SSU and LSU rDNA gene sequences showed that there are no genetic differences between the three strains of *T. aquasubterranea* that we isolated from the type locality (HFCC 700, HFCC 701, and HFCC 702). Therefore, we included only the type strain HFCC 701 in the study. The sequences of SSU and LSU were not sufficient to recover the phylogenetic position of the studied *Tetrahymena* species (data not shown). Thus, we performed a phylogenetic analysis including the *cox1* gene sequence within the alignment of a set of 160 nucleotide sequences of *Tetrahymena* isolates previously used by Kher et al. (2011); Chandni Kher kindly provided us with their alignment, which they created combining 116 newly sequenced isolates with 60 sequences previously published by Chantangsi et al. (2007).

As expected, we obtained a *cox1* topology and relationships within the species of the genus *Tetrahymena* mostly congruent with those in previous publications (Fig. 35). *Tetrahymena aquasubterranea* nested within a well-supported clade including *T. farleyi*, *T. tropicalis*, *T. furgasoni,* and *T. mobilis* isolates, which suggests a sister relationship of the latter species with *T. aquasubterranea*. The last three species form a weakly supported clade dominated by *T. tropicalis* isolates and two separated and highly supported clades nested among them: (1) a clade containing *T. furgasoni* and *T. lwoffi*, the latter being considered a junior synonym of *T. furgasoni* (Meyer and Nanney 1987) and was thus excluded from the list of recognized species (Lynn and Doerder 2012); (2) the other clade includes *T. mobilis* isolates, among them the strain 160/33, selected as the type by Lynn and Doerder (2012) based on its morphology (Schiftner and Foissner 1998).

DISCUSSION

Comparison with related species

Most *Tetrahymena* species have a rather uniform somatic infraciliature, overlapping ranges in body size, similar body shape and number of ciliary rows, or have cosmopolitan distribution. Therefore, distinguishing *T. aquasubterranea* from other *Tetrahymena* species of the *pyriformis* group based on morphological criteria becomes difficult in practice. This is the case with *T. tropicalis*, *T. furgasoni*, *T. farleyi* and *T. mobilis*, all *pyriformis*-like species with sister relationship with *T. aquasubterranea*. Moreover, some of these species were established without morphological investigations, which contribute to the identification problems. For instance, Nanney and McCoy (1976) identified *T. tropicalis* and *T. furgasoni* according to breeding experiments and affinities of isozyme electrophoretic mobilities respectively. So, most of the morphological traits with diagnostic value, such as the presence/absence of secondary silverline meridians, the number of somatic kineties, the presence/absence of a caudal cilium, the formation and shape of reproductive and resting cysts (if present at all), and the life cycle were not described in most of the species related to *T. aquasubterranea*.

The biphasic life cycle exhibited by *T. aquasubterranea*, with trophonts and theronts, is also present in *T. mobilis* (Foissner, unpubl. data) and is apparently the most striking difference to *T. tropicalis*, *T. furgasoni* and *T. farleyi*, as no reference was made to any polymorphism in the description of these species. However, considering the lack of detailed morphological studies and the presence of a different kind of polymorphism in the genus *Tetrahymena*, related to the availability of food resources or to their potential to parasitize invertebrates or vertebrates (Corliss 1953), the occurrence of this feature might have been overlooked in these species (especially if trophont and theront are not as distinct as in *T. aquasubterranea*).

Tetrahymena aquasubterranea has a type II silverline pattern (with secondary meridians) that is also present in *T. farleyi* and *T. mobilis*, but remains undescribed for *T. tropicalis* and *T. furgasoni*. Whether or not the type II is the most common pattern among the species with a "*pyriformis*-like life style" (it appears also in *T. pyriformis*) could be only answered performing the missing morphological studies. The "*pyriformis*-like life style" was suggested by Lynn and Doerder (2012; together with *rostrata*-like and *patula*-like life styles) instead of the traditionally used infrageneric groupings, *pyriformis* group, *rostrata* group and *patula* group (Corliss 1970, 1973), to quickly identify the life cycle and general biology of a *Tetrahymena* species. A species with a "*pyriformis*-like life style" would be preferently bacterivorous with a potential to facultatively parasitize invertebrates and vertebrates, both living and dying (Corliss 1970, 1973).

Among the species related to *T. aquasubterranea*, only *T. farleyi* was reported as a parasite. It was discovered by Lynn et al. (2000) as the first *Tetrahymena* parasitizing a mammal (Dalmatian dog). Although it was first considered as a facultative parasite, Lynn and Doerder (2012) indicated recently that it might be an obligate parasite. However, we have identified *T. farleyi* free-living in groundwater samples from a 20-m depth well near the Zoological Research Station of the University of Cologne in Grietherbusch, Germany (unpubl. data; see Table 2).

Some of the characteristics of *T. aquasubterranea* and its related species are summarized in the Table 2.

Phylogenetic analyses of various *Tetrahymena* species

The *cox1* is currently considered the most useful DNA barcode to discriminate among species of *Tetrahymena* because it improves the resolution within some branches of the phylogenetic tree (Simon et al. 2008). The *cox1* sequences are able to distinguish among species with identical D2 LSU or SSU rDNA regions, as it happens with members of the "*australis*" group (Chantangsi et al. 2007; Lynn and Strüder-Kypke 2006). Phylogenetic trees based on mitochondrial *cox1* and nuclear SSU rDNA genes are in general agreement, although there are differences in detail that remain to be resolved (Chantangsi and Lynn 2008). According to the SSU rDNA and *cox1* topologies, the genus *Tetrahymena* appears to be monophyletic (Chantangsi and Lynn 2008; Kher et al. 2011; Strüder-Kypke et al. 2001). Moreover, the two major groups, "*borealis*" and "*australis*," originally suggested by various rDNA sequences including LSU rDNA sequences (Nanney et al. 1998), are also supported by the SSU rDNA and *cox1* sequences (Chantangsi and Lynn 2008).

We performed the phylogenetic analysis of *T. aquasubterranea* by adding its sequence within the *Tetrahymena cox1* barcode dataset of Kher et al. (2011), which has become the basis for recent works on *Tetrahymena* (Lynn and Doerder 2012). We obtained a *cox1* topology and relationships within the species of the genus *Tetrahymena* mostly similar with those of these authors. Nevertheless, to improve the resolution of the evolutionary relationships, we used the interior-branch test, as it has been described as appropriate for evaluating the reliability of a predetermined tree topology (Sitnikova 1996).

Our results adding the *cox1* sequence of *T. aquasubterranea* n. sp. to the *cox1* barcode dataset of Kher et al. (2011) and applying the interior-branch test, are not only in agreement but give more consistency to the results obtained by these authors. The phylogenetic analysis comparing the *cox1* gene sequence of *T. aquasubterranea* with the *cox1* gene sequences of other known *Tetrahymena* species shows a genetic divergence of *T. aquasubterranea* to its next relative, *T. farleyi*, of 9.2%. According to the results obtained by various authors (Chantangsi et al. 2007; Kher et al. 2011; Lynn and Doerder 2012), the average *cox1* divergence among bona fide species is ~ 10%, while the intraspecific genetic diversity among *Tetrahymena* species is less than 2%. Furthermore, the three strains of the new species isolated from the same groundwater well have identical *cox1* and, additionally, identical SSU and D1-D5 LSU rDNA gene sequences. Based on the considerable genetic distance of *T. aquasubterranea* n. sp. from other *Tetrahymena* species and using the DNA-based identification criteria, we have described the strain from South Africa as a new species.

The combined approach of morphology and the *cox1* barcode offered an effective way for the identification and description of *Tetrahymena aquasubterranea* n. sp. The interior-branch test seems to be a promising tool capable of adding more consistency to the already known phylogenetic relationships within the species of *Tetrahymena*,

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

Species name and authorship	Most frequently reported ecological habitus in the literature[a]	Silverline pattern[b]	Cysts	Micronucleus: present (+) absent (–)	Reference for original description
Tetrahymena aquasubterranea nov. spec.	Bacterivore	Type II	No	–	
Tetrahymena farleyi Lynn et al. 2000;	Facultative parasite (!)[c]	Type II	No	–?	*Acta Protozool.*, 39:289-294
Tetrahymena furgasoni Nanney and McCoy 1976;	Bacterivore	?	No	–	*Trans. Amer. Microsc. Soc.*, 95:664–682
Tetrahymena mobilis (Kahl, 1926) Lynn and Doerder 2012;	Bacterivore/ histophage	Type II	No	+	*J. Eukaryot. Microbiol.*, (Abstr. 84), 45 (Suppl.):15A
Tetrahymena tropicalis Nanney and McCoy 1976	Bacterivore	?	No	+/–	*Trans. Amer. Microsc. Soc.* 95:664–682

Tetrahymena lwoffi is considered a junior synonym of *Tetrahymena furgasoni* (Lynn and Doerder 2012; Meyer and Nanney 1987), and thus it is not in the list of recognized species of *Tetrahymena*; Lynn and Doerder (2012) established recently the combination *Tetrahymena mobilis* (Kahl, 1926) Lynn and Doerder (2012) for *Sathrophilus mobilis* (Kahl, 1926); references for original descriptions are provided only in this table due to space limitations.

[a]Species of *Tetrahymena* here labelled as bacterivores can be also facultative histophages and/or parasites (Lynn and Doerder 2012).

[b]The silverline patterns of *Tetrahymena* can be classified in Type I (only primary meridians connecting the kinetids) and Type II (primary meridians and secondary meridians running parallel and regularly alternating with the primaries).

[c]*Tetrahymena farleyi* was discovered by Lynn et al. (2000) and reported as the first *Tetrahymena* parasitizing a mammal (Dalmatian dog). Although it was first considered as a facultative parasite, Lynn and Doerder (2012) indicate that it might be an obligate parasite. However, we have found *T. farleyi* free-living in groundwater samples from a 20 m depth well located in an alluvial floodplain area of the Lower River Rhine near the Zoological Research Station of the University of Cologne in Grietherbusch (Germany; unpubl. data).

increasing the resolution within the phylogenetic trees when compared with previous studies.

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 aquasubterranea n. sp.

Diagnosis. *Tetrahymena* of the *pyriformis*-group. Size of trophonts (TR) about 50 × 25 μm, that of theronts (TH) about 60 × 15 μm in vivo. TR ellipsoidal to ovate. TH oblong with both ends tapered. Macronucleus globular. Amicronucleate. Single contractile vacuole subterminal, two excretory pores. On average 18 ciliary rows, two postoral. Oral apparatus typical of genus, paroral of TH distinctly shorter than that of TR. Type II silverline pattern.

Type material. A hapantotype consisting of two slides with protargol-impregnated specimens and four slides with silver nitrate-impregnated preparations (Klein–Foissner and Chatton–Lwoff methods) have been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz (LI), under the accession number [2012/114–119]. The hapantotype material constitutes the name-bearing type. Relevant specimens have been marked by black ink circles on the coverslip.

Type habitat. Groundwater from a 12-m deep closed drinking water well in a sandstone soil.

Type locality. Suburb of Tokai, south of Cape Town, Republic of South Africa (34°3′S 18°26′E).

Etymology. The species-group name *aquasubterranea* is a composite of the Latin noun *aqua*, *aquae* [genitive, f] (water) and the Latin adjective *subterrane·us*, *-a*, *-um* [m, f, n] (underground, subterranean), referring to the habitat where the species was discovered. This species group-name is a compound word (Article 11.9.1 of the ICZN 1999) whose separate words represent together a single entity (subterranean water) and are thus united without hyphen (Article 11.9.5 and 32.5.2.2 of the ICZN 1999). Furthermore, this species epithet is in apposition and thus does not need to agree in gender with the generic name (Article 34.2.1 of the ICZN 1999).

Gene sequence. The GenBank accession numbers for the SSU rDNA, LSU rDNA and *cox1* gene sequences from the type strain HFCC701 are JX129387, JX271899 and JX129388 respectively.

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