Revista Mexicana de Biodiversidad

Revista Mexicana de Biodiversidad 97 (2026): e975732

Taxonomy and systematics

COI based phylogeny and morphological characterization of a Brazilian population of *Lambornella trichoglossa* (Ciliophora: Tetrahymenidae)

Filogenia basada en COI y caracterización morfológica de una población brasileña de Lambornella trichoglossa *(Ciliophora: Tetrahymenidae)*

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Received: 28 July 2025; accepted: 22 October 2025

Abstract

In this work, the morphological characterization and molecular phylogeny (SSU rDNA and COI) of *Lambornella trichoglossa* Foissner, 2003, found in phytotelmata environments in southeastern Brazil is presented. The morphological study performed with silver impregnation techniques demonstrated parameters similar to those recorded in the type population, and additional new information was also obtained, including the conjugation process and the occurrence of specimens with an increased number of caudal cilia. The phylogenetic reconstructions based on both markers suggest the inclusion of *L. trichoglossa* in the genus *Tetrahymena* Furgason, 1940. The COI sequence obtained in this work is the first mitochondrial sequence of the genus *Lambornella* deposited in GenBank and published in a scientific article.

Keywords: *Tetrahymena*; Phytotelmata; Molecular phylogeny

Resumen

En este trabajo se realizó la caracterización morfométrica y la filogenia molecular (SSU rDNA y COI) de *Lambornella trichoglossa* Foissner, 2003, encontrada en ambientes de fitotelmata en el sureste de Brasil. El estudio morfológico realizado con técnicas de impregnación de plata demostró parámetros similares a los registrados en la población tipo y también se obtuvo nueva información adicional, incluyendo el proceso de conjugación y la presencia de especímenes con un mayor número de cilios caudales. Las reconstrucciones filogenéticas basadas en

ISSN versión electrónica: 2007-8706; Universidad Nacional Autónoma de México, Instituto de Biología. https://doi.org/10.22201/ib.20078706e.2026.97.5732

ambos marcadores sugieren la inclusión de *L. trichoglossa* en el género *Tetrahymena* Furgason, 1940. La secuencia COI obtenida en este trabajo es la primera de tipo mitocondrial del género *Lambornella* depositada en GenBank y publicada en un artículo científico.

Palabras clave: *Tetrahymena*; Fitotelmas; Filogenia molecular

Introduction

The genus *Lambornella* has its systematic history marked by alterations. The type species, *Lambornella stegomyiae* Keilin, 1921, is a mosquito (Diptera, Culicidae) parasite. It was reclassified in the genus *Tetrahymena* Furgason, 1940 by Corliss (1960). However, the description of *Lambornella clarki* Corliss, 1976, another parasitic species, revalidated the genus, indicating the formation of cuticular cysts and the number of post-oral kineties as diagnostic features. Nevertheless, Strüder-Kypke et al. (2001) suggested the non-validity of the genus *Lambornella* once again, due to its position within the *Tetrahymena* clade, based on phylogenetic analyses inferred from small subunit ribosomal DNA (SSU). Results presented by Bourland and Stüder-Kypke (2010), and Dunthorn et al. (2012), also based on SSU sequences, demonstrated a similar grouping.

Lambornella trichoglossa Foissner, 2003 is the only free-living species of the genus. It occurs endemically in phytotelmata environments (Foissner, 2003). Despite the recurrent records in the Neotropical region (Buosi et al., 2014, 2015; Durán-Ramírez et al., 2015; Foissner, 2003; Foissner et al., 2003), no studies have investigated *Lambornella*'s phylogenetic position based on mitochondrial markers. According to Chantangsi and Lynn (2008), the mitochondrial gene cytochrome c oxidase-subunit I (COI) is effective in elucidating recent phylogenetic events in the genus *Tetrahymena*. In this context, the present work aimed to investigate the molecular phylogeny of *L. trichoglossa* based on the mitochondrial marker COI. Morphological analysis of the specimens was also performed in order to confirm specific identification and investigate possible morphological variation.

Materials and methods

One hundred milliliters of phytotelmata content were collected from 12 bromeliads belonging to *Portea petropolitana* at the Botanical Garden of the Federal University of Juiz de Fora (21°43'74" S, 43°22'06" W), in September, 2019. On the same day of collections, the samples were analyzed under a stereoscopic microscope with transmitted light. Active ciliates were picked with glass micropipettes and processed, according to Foissner (2014), to perform silver carbonate and dry silver nitrate impregnation techniques. From the collection day, over a period of 7 days, 20 specimens of *L. trichoglossa* were screened from the samples and measured *in vivo* to check for possible alterations in body length. Obtained data were analyzed using the Shapiro-Wilk normality test, and the means recorded on the first and seventh days were compared using the Student's t-test. All statistical analyses related to the morphological data were conducted using PAST software, version 4.03 (Hammer et al., 2001).

Thirty specimens of *L. trichoglossa* were picked from the samples and fixed in absolute ethanol for molecular analysis. Total DNA extraction was performed using the Blood and Tissue kit (Qiagen®), following the manufacturer's guidelines. Primers F388dT and R1184dT (Strüder-Kypke & Lynn, 2010) were used to amplify the COI gene in 25-microliter reactions. The SSU marker was also sequenced for comparison with the type population. Therefore, primers 18S F9Euk and 18S R1513 (Schrallhammer et al., 2013) were used.

PCR products were visualized in a 1% agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen®), following the manufacturer's guidelines. Subsequently, the material was sent for sequencing according to the Sanger method in 7 µl reactions, using the M13 forward and M13 reverse primers (Messing, 1983) for COI, and 18S R536, 18S F783, 18S F919, and 18S R1052 for SSU (Modeo et al., 2006). Sequencing reactions were performed on an ABI PRISM® 3100 sequencer (Applied Biosystems).

Obtained sequences were added to the respective datasets, jointly with COI and SSU sequences of approximately 20 species of the genus *Tetrahymena*, as well as outgroups obtained from GenBank accessed in May 2025. Sequences were aligned using MAFFT software, version 7 (Katoh et al., 2017). The resulting alignment was edited in the GBlocks platform, version 0.91b (Talavera & Catresana, 2007). The determination of the best nucleotide substitution model (GTR + G + I to COI, and TN93 + G to SSU) was performed with the aid of MegaX software (Kumar et al., 2018), using the

maximum likelihood method, considering all sites. The maximum likelihood phylogenetic analysis was inferred using RAxML software, version 8 (Stamatakis, 2014), at its default settings, with 500 bootstrap replicates for COI, and 1,200 for SSU. The evolutionary distances of COI and SSU sequences were computed in Mega X, using the Kimura 2-parameter method (Kimura, 1980). A third dataset with 74 COI sequences of *Tetrahymena* species was prepared, in addition to outgroups, with the aim of performing the pairwise distance calculations with a greater number of species (Supplementary material). For this analysis, the Maximum Composite Likelihood model (Tamura et al., 2004) was used, with the aid of MegaX (Kumar et al., 2018).

Results

Table 1 presents the morphological data obtained. Morphological characterization of the *L. trichoglossa* population analyzed in this study resembles the type population described by Foissner (2003), presenting slightly higher values for body size and the number of somatic kineties. However, postoral kineties appear in lower numbers. Another important morphological characteristic distinct from the type population detected in the present study was the occurrence of specimens with a greater number of caudal cilia (Fig. 1h). These organisms have a complex of caudal cilia, as shown in Figure 1h and Supplementary material. However, they were not included in the morphological statistical analysis due to the unsatisfactory results of the impregnation techniques in their oral and some somatic structures. Opportunely, conjugating forms of *L. trichoglossa* were recorded, which is unprecedented for the species (Fig. 1a). The temporal analyses on body length performed demonstrated a significant decrease in the average size of the organisms over 7 days (Fig. 2, Table 2).

The obtained SSU sequence was deposited in GenBank with accession number MN567691. It matches 99.97% of the type population's sequence (AJ810078) (Table 3). The phylogenetic reconstruction inferred by the maximum likelihood method revealed that the new sequence is grouped with *L. trichoglossa* (AJ810078), *T. corlissi* (U17356), and *T. berger* (AF364039) (Fig. 3).

Lambornella trichoglossa's COI sequence was deposited in GenBank with accession number MN477017. It has 774 nucleotides, with the following percentage of bases: A = 33%, C = 12%, G = 13.4%, and T = 41.6%.

Table 1
Morphological data on silver carbonate-impregnated specimens of *Lambornella trichoglossa*.

	$\bar{x}$	M	SD	SE	CV	Min	Max	n
Body, lenght	269.75	269.43	26.72	4.88	9.906	226.65	327.91	30
Body, width in ventral view	73.105	73.295	16.05	2.93	21.96	47.44	107.77	30
Body lenght: Lateral width, ratio	3.82	3.906	0.81	0.14	21.19	2.606	5.36	30
Anterior body end to membranelle 1, distance	47.91	48.73	7.407	1.35	15.46	34.14	63.87	30
Oral opening, width	32.45	31.42	5.11	0.93	15.77	21.28	44.12	30
Anterior body end to macronucleus, distance	94.55	92.88	13.86	2.53	14.66	74.16	126.35	30
Macronucleus, length	49.72	43.96	11.48	2.09	23.08	36.92	77.75	30
Macronucleus, width	51.27	48.71	9.42	1.72	18.38	33.74	66.86	30
Macronucleus, number	1	1	0	0	0	1	1	30
Micronucleus, width	5.67	5.5	1.15	0.21	20.408	4	9.56	27
Micronucleus, number	1.03	1	0.19	0.035	18.68	1	2	27
Somatic kineties, total number	52.33	52.5	3.79	0.69	7.24	45	62	30
Somatic kineties, postoral number	6.1	6	0.84	0.15	13.85	5	8	30
Caudal cilia, number	1	1	0	0	0	1	1	30

Measurements in µm. $\bar{x}$, arithmetic mean. M, median. SD, standard deviation. SE, standard error of arithmetic mean. CV, coefficient of variation (%). Min, minimum. Max, maximum. n, number of individuals investigated.

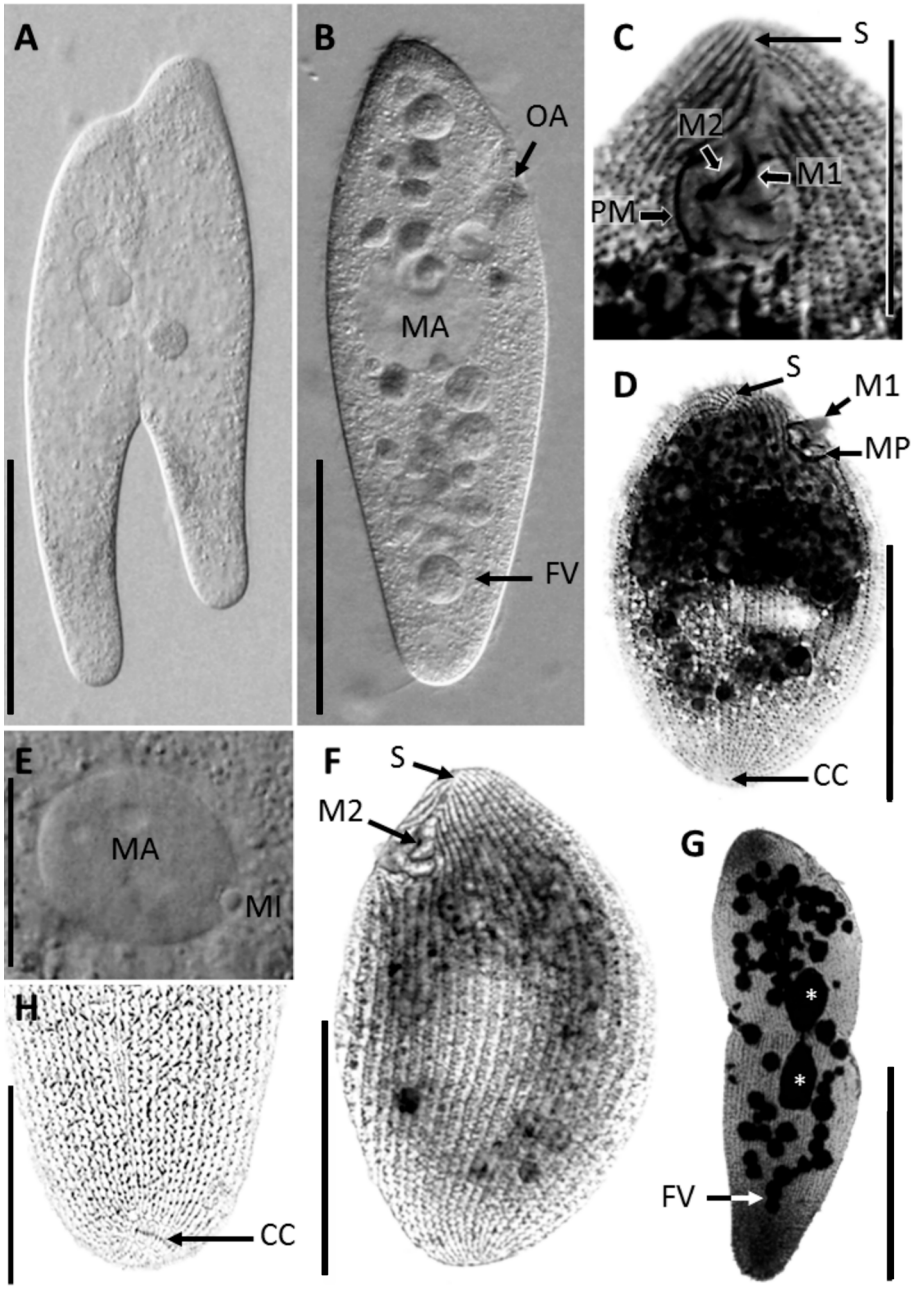

Figure 1. Morfological characterization of *Lambornella trichoglossa*. A, B, E. *In vivo*. C, D, F. Dry silver impregnation. G, H. Silver Carbonate impregnation. A, First recorded conjugating specimens; B, lateral view; C, oral apparatus; D, specimen with normal number of caudal cilium (dorsal view); E, nuclear apparatus; F, ventral view, showing oral apparatus and postoral kineties, below; G, specimen recorded during divisional morphogenesis, with multiple food vacuoles scattered throughout the body; H, detail of caudal cilia complex recorded in larger specimens. OA (oral apparatus), FV (food vacuoles), S (preoral suture), PM (paroral membrane), M1 and M2 (adoral membranelles), MA (macronucleus), MI (micronucleus), CC (caudal cilia). Asterisks indicate macronucleus during divisional morphogenesis process. Scale bars in A, B, D, F, G = 100 µm. In C, E H = 50µm.

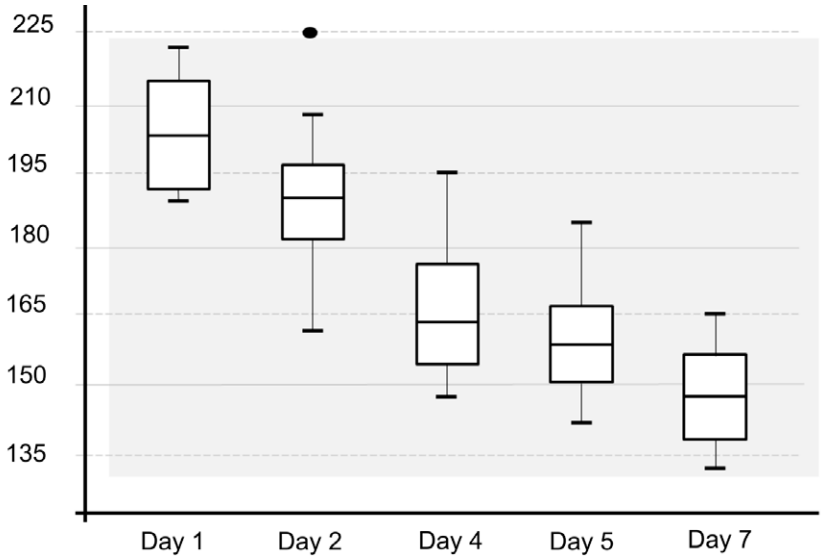

Figure 2. Body length reduction of *L. trichoglossa* over a 7-day period.

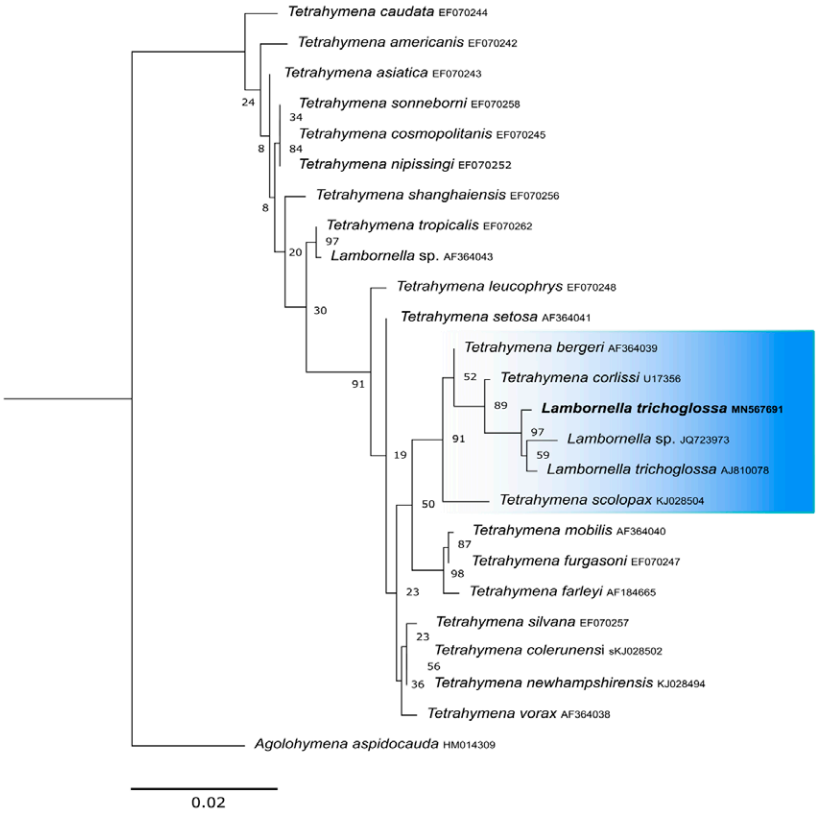

Figure 3. Maximum likelihood (ML) tree inferred from small subunit ribosomal DNA (SSU rDNA). Bold: sequence obtained in the present work. Numbers at nodes represent the bootstrap values of ML out of 1,200 replicates.

Table 2
Statistical data on the difference in body length of *L. trichoglossa* 7 days after collection.

Number of measured specimens	
1st day	20
7th day	20
Body length mean (μm)	
1st day	205.66
7th day	148.255
Maximum length (μm)	
1st day	222.79
7th day	164.84
Minimum length (μm)	
1st day	190.18
7th day	132.43
Standart deviation	
1st day	10.97027077
7th day	10.06698748
Variance	
1st day	120.3468408
7th day	101.3442368
Shapiro-Wilk test (α = 0.05)	
1st day	W: 0.9273
	p-value: 0.1371
7th day	W: 0.9556
	p-value: 0.4607
Student's t-test	
	t: 17.241
	p: $1.47.10^{-19}$
	Critical *t*-value (*p* = 0.05): 2.0244

Phylogenetic reconstruction using the maximum likelihood method demonstrated *L. trichoglossa* clustering in a clade with the same topology revealed by the SSU phylogeny; it also grouped with *T. corlissi* (EF070279) and *T. bergeri* (EF070270) with a support value of 86%, besides *T. scolopax* (KJ028669) (Fig. 4). The average paired difference of the 74 *Tetraymena* sequences used in the phylogenetic analyses was 12.99%. The *L. trichoglossa* sequence differs by 13.04% from the *T. corlissi* sequence (Table 4). Although the value is slightly above the average for the *Tetrahymena* species analyzed, this percentage is lower than the difference observed between *T. corlissi* and *T. caudata* (14.29%), *T. paravorax* (14%), and *T. glochidiophila* (16.39%), for example.

Discussion

The similarity of the morphological parameters between the specimens analyzed in the present study and the data presented in the description of *L. trichoglossa* ensures the correct taxonomic identification of the organisms. It should be noted that the characterization performed by Foissner (2003) occurred a few days after collection, whereas in the present work, the morphological study techniques were performed a few hours after collection. The temporal analyses on body length performed in the present work demonstrated a decrease in the average size of the organisms over time (Fig. 2), which would justify the slightly higher average values compared to the type population.

Regarding the occurrence of specimens with a greater number of caudal cilia, polymorphic life cycles are a common characteristic of the genus *Tetrahymena*. Lynn and Doerder (2012) mentioned the occurrence of changes in size, body shape, organization, morphology, and cilia of the oral apparatus of *Tetrahymena* species, due to environmental changes. The aforementioned authors highlighted the great genotypic and phenotypic plasticity found in the clade.

Phylogenetic reconstructions performed with both markers demonstrate the internal position of *L. trichoglossa* in the genus *Tetrahymena*, corroborating analyses based on the SSU marker presented by Strüder-Kypke et al. (2001), Bourland and Strüder-Kypke (2010), and Dunthorn et al. (2012). Several authors demonstrated the division of the genus *Tetrahymena* into 2 strongly molecularly supported clades, Australis and Borealis (Chantangsi, 2007; Chantangsi & Lynn, 2008; Lynn et al., 2018; Nanney, 1998; Strüder-Kypke et al., 2001). This division was observed in the inferred reconstructions by the SSU marker, performed in the present study, demonstrating the inclusion of *L. trichoglossa* into the Borealis clade. Doerder (2018) states that this clade has greater molecular diversity and more species than the Australis clade, thus being an important source of diversity for the genus. *Lambornella trichoglossa*, included in this clade, is the greatest example of phenotypic diversification, given its morphological and ontogenetic particularities demonstrated by Foissner (2003).

The phylogenetic analyses inferred by the COI gene demonstrated the inclusion of *L. trichoglossa* in a

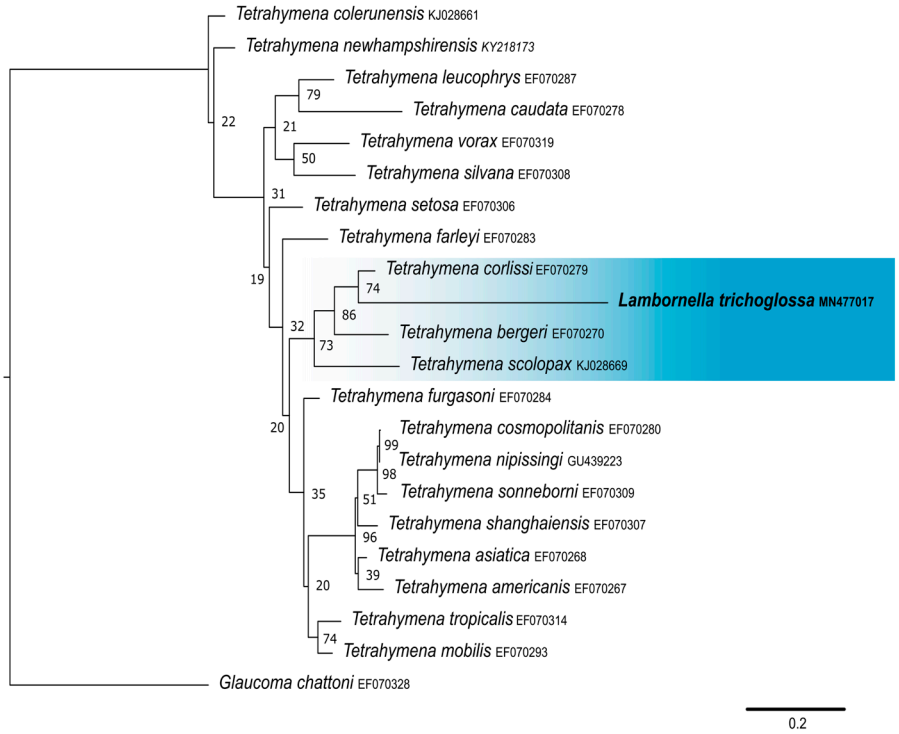

Figure 4. Maximum likelihood (ML) tree inferred from cytochrome oxidase subunit I (COI). Bold: sequence obtained in the present work. Numbers at nodes represent the bootstrap values of ML out of 500 replicates.

Table 3

Pairwise distance between SSU sequences of *Lambornella trichoglossa* and *Tetrahymena* spp.

MN567691	***L. trichoglossa***								
AJ810078	*L. trichoglossa**	0.0029							
JQ723973	*Lambornella* sp.**	0.0053	0.0047						
U17356	*T. corlissi*	0.0059	0.0063	0.0077					
AF364039	*T. bergeri*	0.0071	0.0092	0.0089	0.0046				
EF070247	*T. furgasoni*	0.0130	0.0149	0.0149	0.0099	0.0092			
AF364040	*T. mobilis*	0.0130	0.0151	0.0149	0.0104	0.0092	0.0006		
KJ028502	*T. colerunensis*	0.0132	0.0139	0.0139	0.0087	0.0080	0.0051	0.0058	
KJ028504	*T. scolopax*	0.0135	0.0156	0.0156	0.0094	0.0067	0.0135	0.0142	0.0125

Bold font: sequence obtained in the present study. * Sequence obtained from the type population (Dunthorn et al., 2012). ** Sequences obtained from samples collected in Jamaica (Dunthorn et al., 2012).

Table 4
Pairwise distance between COI sequences of *Lambornella trichoglossa* and *Tetrahymena* spp.

MN477017	***L. trichoglossa***									
EF070279	*T. corlissi*	0.1294								
EF070270	*T. bergeri*	0.1450	0.0921							
EF070291	*T. malaccensis*	0.1536	0.1187	0.1320						
EF070268	*T. asiatica*	0.1557	0.1026	0.1113	0.1062					
KY218158	*T. alphathermophila*	0.1713	0.1152	0.1403	0.1175	0.1281				
EF070296	*T. paravorax*	0.1961	0.1400	0.1491	0.1413	0.1185	0.1407			
KJ028669	*T. scolopax*	0.1982	0.1364	0.1376	0.1461	0.1184	0.1534	0.1491		
EF070278	*T. caudata*	0.1857	0.1429	0.1492	0.1622	0.1331	0.1553	0.1735	0.1615	
MF693881	*T. glochidiophila*	0.2187	0.1639	0.1867	0.1626	0.1631	0.1703	0.1500	0.1926	0.2051

Bold font: sequence obtained in the present study.

clade called coxset A4 by Chantangsi and Lynn (2008). The species *T. corlissi* and *T. bergeri*, members of this clade, share the parasitic lifestyle and have particular morphological characteristics (Hoffman et al., 1975; Imai et al., 2000; Strüder-Kypke et al., 2001). Foissner (2003) performed infection tests with *L. trichoglossa* and different mosquito species, noting that the ciliate does not develop a parasitic association. However, congeners *L. stegomyiae* and *L. clarki* are parasites of culicid dipterans, demonstrating that this way of life is predominant in coxiset A4, corroborating Chantangsi and Lynn (2008). Probably, parasitism also represents the ancestral condition of this clade, since Strüder-Kypke et al. (2001) demonstrated that histophagy is a recurrent phenomenon in the evolution of *Tetrahymena*.

Traditionally recognized *Tetrahymena* species, described based on morphological or biochemical characteristics, and molecularly supported, may have high evolutionary difference values when comparing COI sequences. For example, *T. caudata* differs by over 15% from *T. corlissi*, *T. termophila*, and *T. glochidiophila*. Some species described by Doerder (2018) based mainly on molecular diversity differ by values close to 20%. Therefore, the 12.94% distance observed between the COI sequence of *L. trichoglossa* and *T. corlissi* does not represent a discrepancy concerning the molecular variability observed in *Tetrahymena*.

According to Chantangsi and Lynn (2008), the mitochondrial COI gene is effective in elucidating *Tetrahymena*'s recent phylogeny, while the SSU marker is more suited to assessing the deep phylogeny of the genus. The maintenance of topology in the reconstructions performed with both markers demonstrates that *L. trichoglossa*, *T. corlissi*, and T. *bergeri* share a long evolutionary history, and the differentiation of the species belonging to the genus *Lambornella* could be recent within the phylogeny of *Tetrahymena*. The phylogenetic position of *L. trichoglossa* also demonstrated by the COI gene, reinforces the proposals for synonymization of the genera *Lambornella* and *Tetrahymena*. Although *Lambornella*'s description precedes that of *Tetrahymena*, the principle of stability is appropriate to maintain the name of the latter taxon. In any case, it is necessary to establish an extended diagnosis for *Tetrahymena*, so that the species involved in new combinations are covered.

Finally, the molecular analysis of *L. trichoglossa* based on the COI marker supports the proposal for synonymization of the genera *Lambornella* and *Tetrahymena*. This systematic readjustment also points to the great molecular, morphological, evolutionary and ecological complexity of the genus *Tetrahymena*, which represents one of the greatest examples of evolutionary adaptation among eukaryotic organisms.

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