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Tetrahymena glochidiophila n. sp., a new species of *Tetrahymena* (Ciliophora) that causes mortality to glochidia larvae of freshwater mussels (Bivalvia)

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ABSTRACT: A ciliate protozoan was discovered whose presence coincided with a rapid decrease in the viability (i.e. ability to close valves) of glochidia of the freshwater mussel *Lampsilis siliquoidea*. Microscopic examination showed it to be a histophagous tetrahymenine ciliate. Small subunit (SSU) rRNA and cytochrome *c* oxidase subunit 1 (*cox*1) barcode sequences from cultured cells showed that it belongs to the same new species isolated from water samples as a free-living ciliate. Phylogenetic analyses place this new ciliate in the same clade with the macrostome species *Tetrahymena paravorax*, and we propose the name *T. glochidiophila* n. sp. for this new species. The phylogeny provides further support for the hypothesis that histophagy was a life history trait of the ancestor of *Tetrahymena*.

KEY WORDS: Glochidia · Ciliate · Histophagy · *Tetrahymena glochidiophila* · *Lampsilis siliquoidea* · Freshwater mussel parasite

INTRODUCTION

Species in the ciliate genus *Tetrahymena* have been recorded as facultative or obligate parasites of a variety of other organisms (Corliss 1973, Lynn & Doerder 2012), including insects, gastropods, and fish. A new species was also identified that had parasitized a dog (Lynn et al. 2000). Historically, these parasitic species have been primarily identified based on the host species that they were infecting (Corliss 1973). However, DNA barcoding using the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene has now provided an unambiguous method for identifying all species of *Tetrahymena* (Kher et al. 2011), and most of the parasitic species have now been barcoded (Lynn & Doerder 2012).

In addition to the parasitic species, which are also termed histophagous, because they ingest the cells and tissues of their hosts, some *Tetrahymena* species can transform from bacterivorous forms to macrostome forms (Corliss 1973, Lynn & Doerder 2012). These macrostome forms typically develop under starvation conditions or in the presence of a transformation factor secreted by prey (Corliss 1973), and once transformed are able to ingest conspecifics and other smaller ciliates with their enlarged mouths.

Phylogeny within the genus *Tetrahymena* was historically rationalized based on these life history traits: a bacterivorous group of species — the *pyriformis* complex; a macrostomatous group of species — the *patula* complex; and a parasitic group of species — the *rostrata* complex (Corliss 1973). However, genetic data have refuted the life history view and robustly indicate parallel evolution of these life history traits within the genus (see Fig. 3). Furthermore, these data suggested that histophagy or the potential for

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parasitism was a life history trait of the ancestor of *Tetrahymena* (Strüder-Kypke et al. 2001).

While performing toxicity tests with glochidia of cultured freshwater mussels *Lampsilis siliquoidea*, one of us (R.S.P.) observed a ciliated protozoan whose presence coincided with a rapid decrease in the viability of the glochidia. The ability to close their valves, an indicator of the viability of glochidia, is used as an endpoint in toxicity testing because as obligatory parasites glochidia need to close their valves to clamp down and encyst on their host's gill tissue (typically fish) to complete their life cycle (American Standard Testing Methods 2003). Isolation, cultivation, and gene sequencing of this ciliate provide evidence that it is a new histophagous species of *Tetrahymena*, namely *T. glochidiophila* n. sp., which is identical to an undescribed genetically identified isolate (F. P. Doerder unpubl.), and is related to the macrostome species *T. paravorax*. In this report, we characterize this species by its morphology and genetics, and provide further support for the hypothesis that histophagy was a life history trait of the ancestor of *Tetrahymena*.

MATERIALS AND METHODS

Bivalve collection

Gravid female freshwater mussels *Lampsilis siliquoidea* (Barnes, 1823), commonly called fatmuckets, were provided by Dr. Chris Barnhardt, Missouri State University (MSU). Mussels were shipped overnight from the MSU laboratory to Environment and Climate Change Canada, at the Canada Centre for Inland Waters, Burlington, Ontario, for the initiation of toxicity testing. The adult female mussels in which *Tetrahymena glochidiophila* n. sp. were found were approximately 2 yr old, and all gravid females were infected by *T. glochidiophila* n. sp. Mortality has also been observed in the glochidia of 2 other freshwater mussel species: pocketbook mussels *Lampsilis cardium* and wavy-rayed lampmussels *Lampsilis fasciola* (R. S. Prosser et al. unpubl. obs.).

Isolation and cultivation of ciliates

L. siliquoidea glochidia were examined for ciliates. Cells were removed by drawn-glass micropipettes from the mantle cavities of glochidia and transferred either to a bacterized spring water medium with rice and barley grains or to 1% (w/v) proteose peptone – 1% (w/v) yeast extract – 0.2% dextrose (PPYE) with antibiotics according to Doerder & Brunk (2012). One polyclonal culture (TGL1, Designation SD03326) in PPYE has been submitted to the *Tetrahymena* Stock Center at Cornell University (Ithaca, New York).

Water samples were also collected from ponds and streams in the USA and processed as described by Doerder & Brunk (2012). Following isolation as clonal populations, cells were cultured in Cerophyl™ inoculated with *Klebsiella pneumoniae* or in sterile PPY (1% (w/v) proteose peptone, 0.15% (w/v) yeast extract, 0.001 M $FeCl_3$). Table 1 provides a list of isolates, their collection sites, *Tetrahymena* Stock Center accession numbers, and associated GenBank accession numbers.

Staining of ciliates

Ciliates were removed directly from the mantle cavities of glochidia or from the bacterized medium by drawn-glass micropipettes and photographed with differential interference contrast (DIC) microscopy using a Zeiss Axiovert 135 or a Zeiss Axioplan 2 compound microscope. These histophagous and bacterivorous forms were also fixed in Champy's and Da Fano's Fluids in preparation for Chatton-Lwoff silver staining (Galigher & Kozloff 1971). These 2 forms were also stained with DAPI (Lessard et al. 1996) and photographed using fluorescence microscopy. Cells grown in Cerophyl™ were vitally stained with acridine orange and assessed by fluorescence microscopy for the presence/absence of the micronucleus.

DNA isolation and gene sequencing of ciliates

Ciliates (~30–50 cells) were hand-picked with drawn-glass micropipettes and rinsed in Castle Rock™ spring water prior to DNA extraction using the MasterPure™ Complete DNA & RNA Purification Kit. Cells of *Tetrahymena* nsp10 and 19518-2 grown by F.P.D. in Cerophyl™ (typically 15 ml) or PPY (8–12 ml) were harvested, and DNA was extracted and purified with a modified microwave procedure (Goodwin & Lee 1993) as previously described (Zufall et al. 2013). Standard PCR with primers as described by Doerder (2014), Strüder-Kypke et al. (2001), or Kher et al. (2011) was used to amplify DNA for the *cox*1 barcode region and nuclear small subunit (SSU) rRNA.

Table 1. Collecting data and GenBank (GB) accession numbers for wild isolates of *Tetrahymena* nsp10 and nsp52. TSC: *Tetrahymena* Stock Center, Cornell University, https://tetrahymena.vet.cornell.edu/. US states are: MA: Massachusetts; UT: Utah; PA: Pennsylvania; MI: Michigan; IA: Iowa; OH: Ohio; CO: Colorado; KY: Kentucky. Dates are given as mm/dd/yy

Isolate number	Species	Water name	Water type	US state	Latitude (°N)	Longitude (°W)	Date collected	Accession numbers			
								TSC cells	TSC DNA	*cox*1 (GB)	SSU rRNA (GB)
19187-3	nsp10	Fort Pond Marsh	Pond	MA	42.536	71.697	7/11/2006			KJ028731	
19374-2	nsp10	Otter Creek Reservoir	Lake	UT	38.167	112.018	5/21/2007			KJ028718	
19392-1	nsp10	Carp Russ Rd	Stream	PA	41.700	79.921	6/7/2007			KJ028717	
19420-4	nsp10	Houghton Lake	Lake	MI	44.300	84.725	6/13/2007			KY218149	
19449-2	nsp10	Don Williams Lake	Lake	IA	42.119	93.816	8/5/2007			KJ028715	
19599-4	nsp10	Lake Huron	Stream	MI	45.555	84.655	9/15/2007			KJ028744	
19640-1	nsp10	Hook Pond OPC	Pond	OH	39.728	81.707	10/28/2007			KY218150	
19673-4	nsp10	Caskie Rd private pond	Pond	OH	41.358	83.513	5/24/2008			KY218151	
19900-1	nsp10	Woodcock Creek Lake	Lake	PA	41.702	80.096	8/26/2008			KJ028670	
19903-2	nsp10	Woodcock Creek	Stream	PA	41.709	80.145	8/26/2008			KJ028668	
19922-1	nsp10	Tappan Lake	Lake	OH	40.356	81.209	9/2/2008			KY218152	
19925-1	nsp10	Leesville Lake	Lake	OH	40.465	81.187	9/2/2008			KY218153	
19995-1	nsp10	Lonetree Reservoir	Lake	CO	40.336	105.134	10/1/2008			KJ028663	
20082-3	nsp10	Walborn Reservoir	Lake	OH	40.965	81.200	3/24/2009		Yes	KJ028657	
20127-1	nsp10	SG69-6	Pond	PA	41.640	79.912	4/17/2009			KJ028650	
20161-1	nsp10	Ottawa Canal	Canal	OH	41.632	83.215	4/27/2009			KJ028644	
20195-4	nsp10	Grand River	River	MI	43.040	86.083	5/18/2009			KJ028641	
20214-1	nsp10	Bills Lake	Lake	MI	43.395	85.670	5/19/2009			KJ028639	
20227-1	nsp10	Clifford Lake	Lake	MI	43.301	85.182	5/19/2009			KJ028637	
20238-5	nsp10	Lincoln Lake	Lake	MI	43.242	85.365	5/19/2009			KJ028636	
20249-2	nsp10	Warner Lake	Lake	MI	42.146	85.049	5/21/2009			KJ028635	
20720-1	nsp10	Lake Manganese	Lake	MI	47.456	87.879	7/10/2010			KY218154	
20739-3	nsp10	Swan Lake	Lake	MI	46.163	88.399	7/11/2010		Yes	KJ028590	
20750-1	nsp10	Briar Hill Cr Pond	Pond	MI	46.100	88.385	7/12/2010			KJ028588	
20903-1	nsp10	Lampson Reservoir	Pond	OH	41.752	80.799	8/25/2010			KJ028578	
21345-1	nsp10	Greenbo Lake	Lake	KY	38.484	82.888	6/14/2011			KJ028544	KY399447
21485-1	nsp10	Grass Lake	Lake	MI	45.121	84.032	8/11/2011	SD03064	Yes	KJ028538	KY399448
21500-5	nsp10	Grand Lake	Lake	MI	45.299	83.526	8/11/2011			KJ028743	
21502-5	nsp10	Grand Lake	Lake	MI	45.299	83.526	8/11/2011			KJ028532	
19518-2	nsp52	Spring Cr Duhring	Stream	PA	41.516	78.994	8/15/2007		Yes	KJ028745	KJ028516
TGL-3	Tgloch	Missouri	Pond	MI	39.004	94.522	9/23/2016	SD03326	Yes	MF693880	MF693881

Genes were sequenced directly on both strands using BigDye Terminator v3.1 (Applied Biosystems) and general eukaryotic primers as previously described (Strüder-Kypke et al. 2001, Kher et al. 2011) by the Nucleic Acid/Protein Service (NAPS) Unit at the University of British Columbia (NAPS UBC, BC) or at the Core Facility of the Cleveland Clinic (Cleveland, OH). Sequences were imported into Geneious v6.1.8 or v7.1.3 (Biomatters) and assembled into contigs that were inspected by eye and modified to correct ambiguities and trim low-quality read ends. The newly generated sequences are available from GenBank (accession numbers MF693880 and MF693881).

Phylogenetic analyses

Trees for neighbor-joining (NJ) and maximum likelihood (ML) analyses were drawn and edited with Mega 7.0 (Tamura et al. 2013). Evolutionary distances were computed using the Kimura 2-parameter method, and the analysis used 1343 positions (excluding gaps) of SSU rRNA. GenBank accession numbers of sequences of type strains for named species are those listed in Lynn & Doerder (2012). Bootstrap values are based on 500 replicates.

When a pairwise difference was calculated, it was either as differences between 2 type strains or, in the

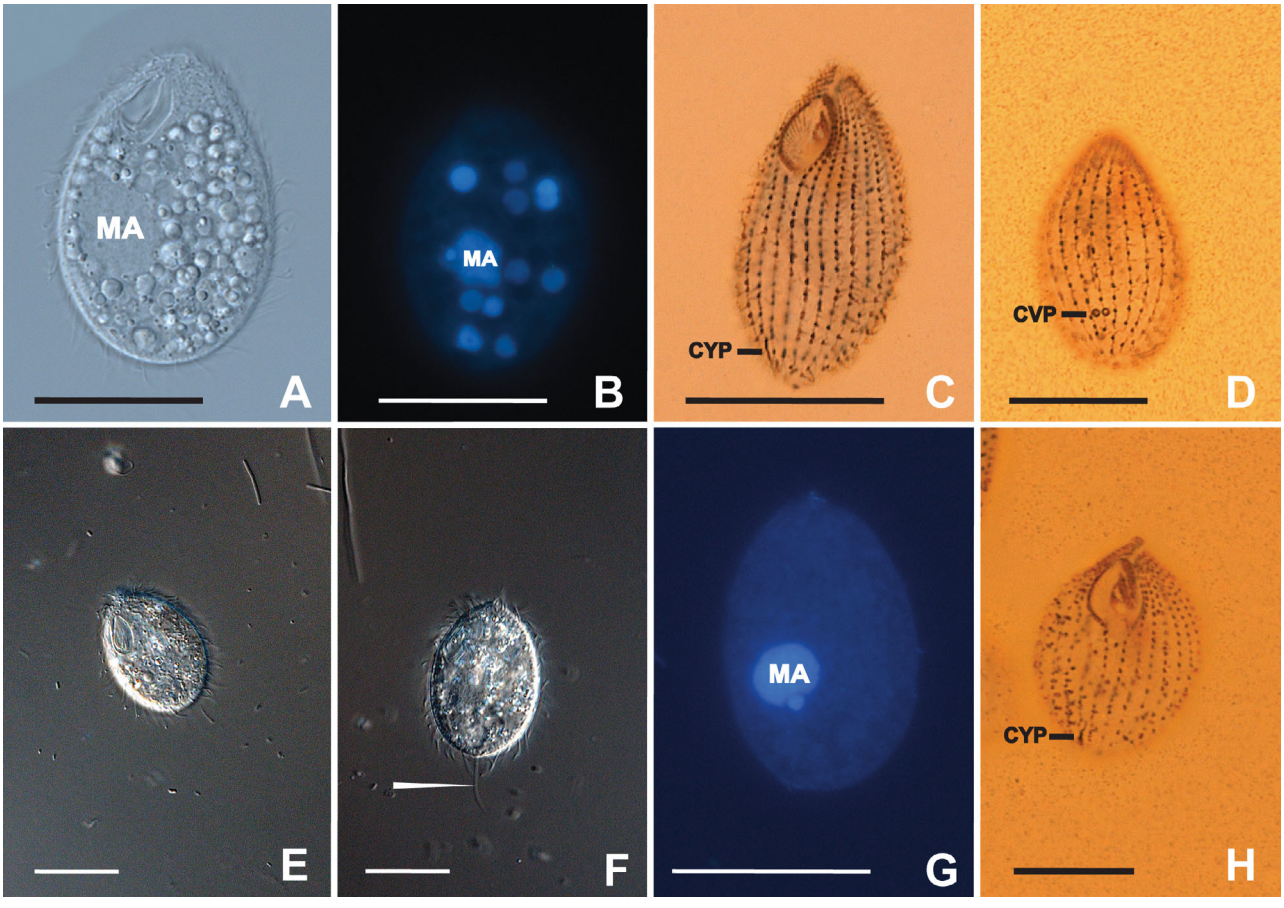

Fig. 1. *Tetrahymena glochidiophila* n. sp. (A–D) Histophagous forms isolated from the mantle cavity of glochidia larvae of the freshwater bivalve *Lampsilis siliquoidea*. (A) Differential interference contrast (DIC) image showing large anterior oral apparatus and macronucleus (MA). (B) DAPI-stained cell with macronucleus (MA) and the micronucleus to its left. All other DAPI-positive bodies are likely the ingested nuclei of cells from the glochidia larva (see Fig. S1 and Video in the Supplement at www.int-res.com/articles/suppl/d127p125_supp/). (C) Ventral view of a Chatton-Lwoff silver-stained holotype, a histophage showing the cytoproct (CYP). Note the oral apparatus with the paroral or undulating membrane along its right edge (see panel A) and the 3 oral polykinetids or membranelles along its left side. (D) Right-lateral view of a Chatton-Lwoff silver-stained histophage showing the 2 contractile vacuole pores (CVP). (E–H) Bacterivorous forms isolated from a bacterial culture. (E,F) DIC images of 2 bacterivores in which the caudal cilium (arrowhead in F) is visible in 1 of these. (G) DAPI-stained cell with macronucleus (MA) and the micronucleus just below it. (H) Ventral view of a Chatton-Lwoff silver-stained bacterivore showing the cytoproct (CYP). Note the relatively larger size of the oral area in the bacterivore and the more visible oral membranelles on its left side. Scale bars = 20 µm

case of clades, as the average of all pairwise differences of type strains of the clade and the compared species.

RESULTS

Distribution of *Tetrahymena* nsp10

Tetrahymena nsp10 was isolated as a free-living ciliate at 28 locations, from Colorado (CO) and Utah (UT) in the west, Massachusetts (MA) in the east, Michigan (MI) in the north, and Kentucky (KY) in the south; 13 of 29 isolates were from MI (Table 1). Most isolates were found in ponds or lakes; 4 were found in streams (or a canal). This distribution is roughly congruent with that of *Lampsilis siliquoidea*. Most isolates (27/29) possessed a micronucleus. Among 24 isolates examined, 23 *cox*1 haplotypes were found; their distribution gave no evidence of population structure. The nsp10 haplotype of isolate 20270-1 most closely resembling that of the *T. glochidiophila* n. sp. isolate, differing at 3/640 bases, was found in the upper peninsula of MI (Lake Superior watershed) (Table 1).

Behavior of *T. glochidiophila* n. sp.

T. glochidiophila n. sp., once introduced to glochidia, will rapidly penetrate the mantle cavity and begin ingesting cells and tissue (Fig. 1B, and see Fig. S1 and Video in the Supplement at www.int-res.com/articles/suppl/d127p125_supp/). Once depleted, the ciliates will leave the glochidia and disperse to infect other glochidia, rapidly killing all viable glochidia over a 24–48 h period (R. S. Prosser et al. unpubl. obs.).

Morphology of *T. glochidiophila* n. sp.

The histophagous form is ovoid, ranging in body length from 41–59 µm and body width from 19–31 µm with a length:width ratio of 0.50 (Table 2). The relatively prominent oral apparatus is about 12 µm in length and about 4.5 µm from the anterior end. The paroral or undulating membrane is prominent, as are the oral ribs extending from it to the cytostome (Figs. 1A,C & 2A). There are 23–26 somatic kineties, with 2 of these being postoral (Figs. 1C & 2A, Table 2). The 1 or 2 contractile vacuole pores are located between somatic kineties 7 and 8 and 8 and 9 (Fig. 1D). When feeding on glochidia cells and tissues, the cytoplasm is filled with food vacuoles (Fig. 1A). When DAPI-stained, these food vacuoles are revealed to include the nuclei of glochidia cells (Fig. 1B, Fig. S1 and Video). The macronucleus of the histophage is almost subspherical, ranging from 8–18 µm in length by 6–14 µm in width (Fig. 1B, Table 2). The micronucleus is about 2 µm in diameter (Fig. 1B, Table 2).

The bacterivorous form is slightly more ovoid, ranging in body length from 30–49 µm and body width from 19–27 µm with a length:width ratio of 0.57 (Fig. 1E,F, Table 2). The relatively larger oral apparatus is about 10 µm in length and about 3.7 µm from the anterior end. The paroral or undulating membrane is again prominent, as are the oral ribs extending from it to the cytostome (Figs. 1E,H & 2B). There are 23–26 somatic kineties with 2 of these being postoral (Figs. 1H & 2B, Table 2). While not always obvious, a caudal cilium is sometimes observed at the posterior end (Fig. 1F). The 2 contractile vacuoles are located between the same kineties as in the histophagous

Table 2. Morphometric characterization of *Tetrahymena glochidiophila* n. sp., a histophagous parasite of the freshwater mussel *Lampsilis siliquoidea*; N = sample size. CL: Chatton-Lwoff stained; DAPI: DAPI stained

Character	Mean	SD	Range	N
Body length, µm				
Histophage, CL	50.8	5.6	41–59	33
Bacterivore, CL	39.7	4.9	30–49	33
Body width, µm				
Histophage, CL	25.2	3.0	19–31	33
Bacterivore, CL	22.3	1.9	19–27	33
Body length:body width ratio				
Histophage, CL	0.50	0.04	0.40–0.56	33
Bacterivore, CL	0.57	0.07	0.45–0.72	33
Anterior end to tip of membranelle 1				
Histophage, CL	4.5	0.56	3–6	33
Bacterivore, CL	3.7	0.81	2–5	33
Oral apparatus length, µm				
Histophage, CL	12.4	0.86	10–14	33
Bacterivore, CL	9.8	0.75	8.9–11.1	33
Macronuclear length, µm				
Histophage, DAPI	10.9	1.7	8–18	43
Bacterivore, DAPI	11.9	2.3	9–18	34
Macronuclear width, µm				
Histophage, DAPI	8.7	1.5	6–14	43
Bacterivore, DAPI	9.4	1.5	7–13	34
Micronuclear diameter, µm				
Histophage, DAPI	2.0	0.38	1.4–3.3	43
Bacterivore, DAPI	2.3	0.34	1.8–3.4	32
Somatic kineties				
Histophage, CL	25.0	0.61	24–26	30
Bacterivore, CL	24.3	0.62	23–26	31

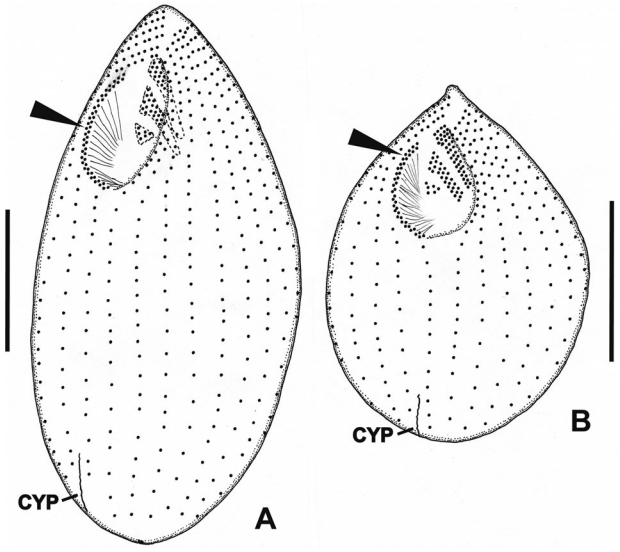

Fig. 2. Schematic drawings of *Tetrahymena glochidiophila* n. sp. (A) Histophagous form isolated from the mantle cavity of glochidia larvae of the freshwater bivalve *Lampsilis siliquoidea*. This is the holotype corresponding to Fig. 1C. The cytoproct (CYP) is indicated. The arrowhead indicates the paroral or undulating membrane along the ciliate's right edge of the oral area while its 3 oral polykinetids or membranelles are along the left side. (B) Bacterivorous form isolated from a bacterial culture. This is the paratype corresponding to Fig. 1H. Labelling of structures as in (A). Scale bars = 20 µm

form. The macronucleus of the bacterivore is almost subspherical, ranging from 9–18 µm in length by 7–13 µm in width (Fig. 1G, Table 2). The micronucleus is about 2 µm in diameter (Fig. 1G, Table 2).

Phylogenetics of *T. glochidiophila* n. sp.

The nearly complete SSU rRNA gene of *T. glochidiophila* n. sp. is 1737 bp in length with a GC content of 43.1%. It is differs in 1 nucleotide, a C to T transition at position 698, from *Tetrahymena* nsp10. Our phylogenetic analyses using NJ (Fig. 3) and maximum likelihood (ML) gave identical topologies. The

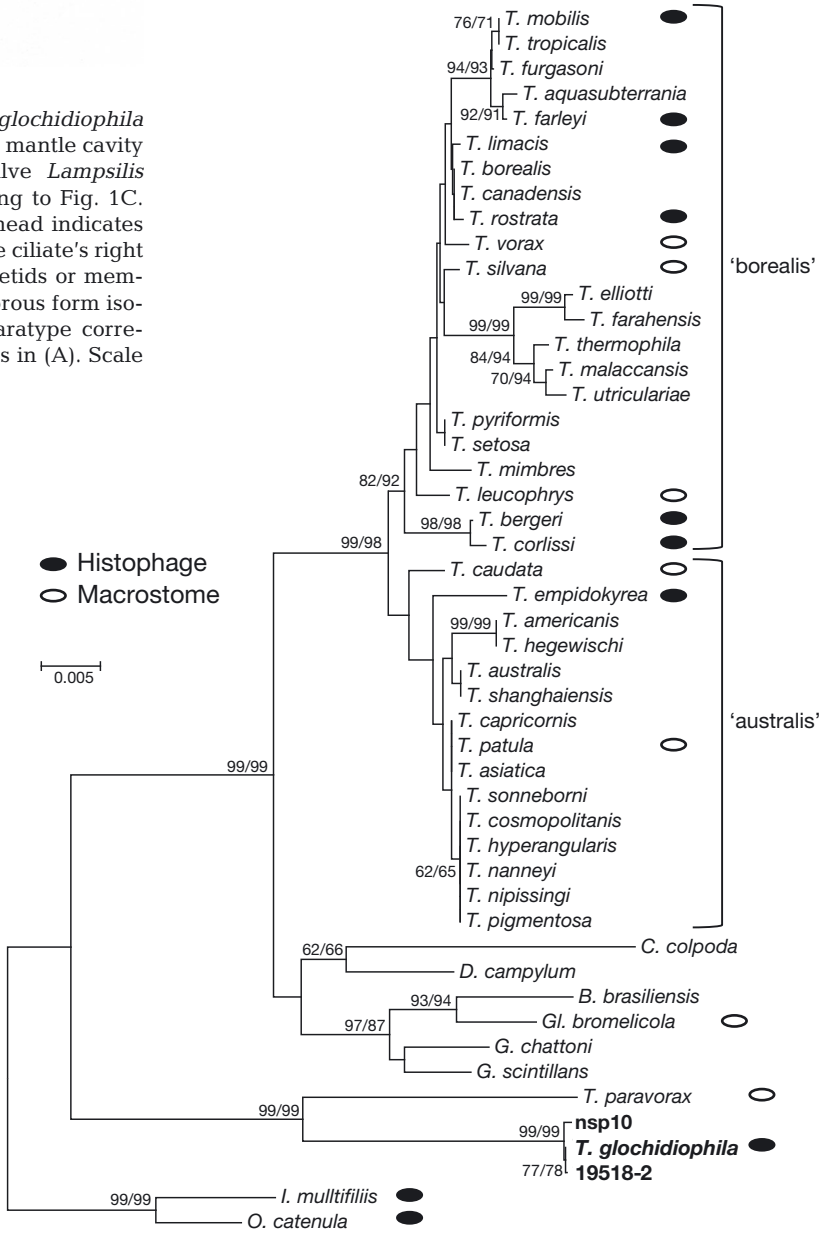

Fig. 3. Small subunit (SSU) rRNA phylogenetic tree of *Tetrahymena* spp. *T. glochidiophila* n. sp., nsp10, and 19518-2 (**bolded**) are related to *T. paravorax*. This neighbor-joining (NJ) tree as shown was based on 1348/1388 positions excluding gaps. The maximum likelihood (ML) tree was essentially identical except for *T. caudata*, which is placed in the '*australis*' clade of the NJ tree (66% bootstrap) and in the '*borealis*' clade of the ML tree (51%) bootstrap. Bootstrap values (NJ/ML) are shown for instances in which they were >50% for both trees. Species recorded as parasites or histophagous are indicated with closed ovals; species in which macrostomes have been observed are indicated by open ovals; all other species are only known to be bacterivores. Key to genera: *T.*: *Tetrahymena*; *C.*: *Colpidium*; *D.*: *Dexiostoma*; *B.*: *Bromeliophrya*; *G.*: *Glaucoma*; *Gl.*: *Glaucomides*; *I.*: *Ichthyophthirius*; *O.*: *Ophyoglena*. Scale bar is 0.5 substitutions in 100

'*australis*' and '*borealis*' clades of *Tetrahymena* formed a robustly supported clade separate from the clade of 4 isolates that included *T. paravorax* and *T. glochidiophila* n. sp. As reported by Strüder-Kypke et al. (2001), this phylogeny is consistent with histophagy as an ancestral feature of tetrahymenines (i.e. the genera *Tetrahymena*, *Dexiostoma*, *Glaucoma*, and *Bromeliophrya*).

The *cox*1 barcode sequences confirm this close relationship between *T. paravorax* and nsp10 and provide some support for monophyly of the genus *Tetrahymena* (Fig. 4). The barcode difference between nsp10 and *T. glochidiophila* is ≤2.0% (range 0.5–2.3%, median 1.7% n = 15; 640/689 nt of barcode). The difference between these 2 isolates, which we consider to belong to the same species, and *T. paravorax* is ~16%, while the '*paravorax*' clade ranges from 17–23% different from the other tetrahymenine subclades (Table 3).

DISCUSSION

Identifying and describing new species of *Tetrahymena*

Warren et al. (2017, p. 245) presented recommendations for the description and naming of new species of ciliates. Among their recommendations is the requirement that detailed morphological descriptions accompany the naming of new species. This requirement is tempered for known complexes of cryptic species, as they stated 'detailed morphological description *should* [italics theirs] be carried out with the aim to search for new taxonomically relevant characters that can be analyzed by multivariate statistics'. Ciliates assigned to the *Paramecium 'aurelia'* and *Tetrahymena 'pyriformis'* complexes were the first recognized cryptic species groups in the phylum Ciliophora, identified by mating compatibility/incompati-

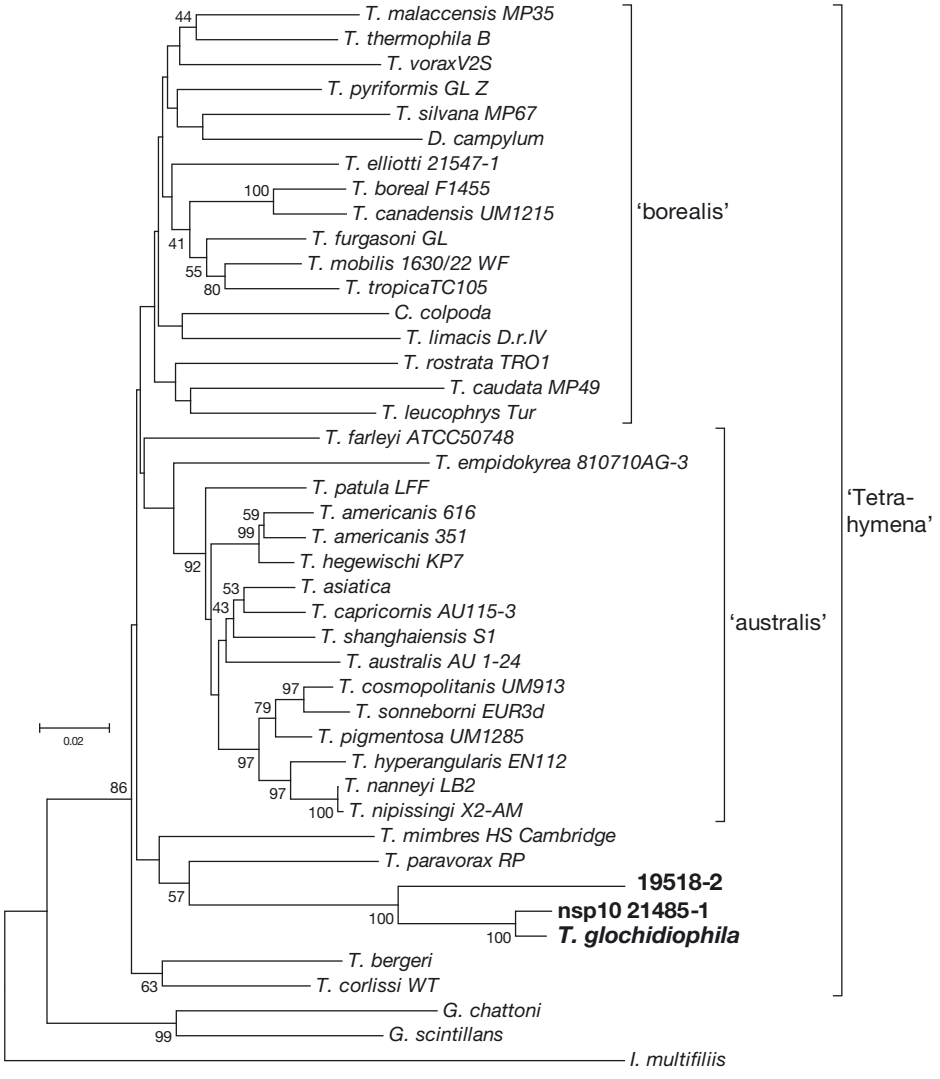

Fig. 4. Cytochrome *c* oxidase subunit 1 (*cox*1) tree of *Tetrahymena* spp. based on a neighbor-joining (NJ) analysis of the 668/689 barcode region. *Tetrahymena* appears to be monophyletic. Bootstrap values are shown for instances in which they were >40%. The '*paravorax*' clade includes *T. glochidiophila* n. sp., nsp10, and 19518-2 (**bolded**). Scale bar is 2 substitutions in 100

Table 3. Pairwise difference (%) in cytochrome *c* oxidase subunit 1 (*cox*1) barcode sequences of the '*paravorax*' clade of *Tetrahymena* species with other selected tetrahymenines. In 3 cases (bold), intraclade distances represent the average pairwise differences among all members of the clade

	Colpidium colpoda	*Dexiostoma campylum*	*Glaucoma chattoni* and *scintillans*	*Tetrahymena*				
				'*borealis*' + '*australis*' clades	'*paravorax*' clade	*T. paravorax*	*T. glochidiophila*	nsp10	19518-2
Ichthyophthirius multifiliis	0.302	0.273	0.294	0.282	0.310	0.293	0.311	0.318	0.325
Colpidium colpoda	–	0.122	0.195	0.124	0.181	0.138	0.190	0.190	0.206
Dexiostoma campylum		–	0.194	0.139	0.187	0.154	0.180	0.188	0.226
Glaucoma chattoni and *G. scintillans*			**0.134**	0.196	0.228	0.213	0.243	0.245	0.261
'*borealis*' + '*australis*' clades				**0.105**	0.171	0.122	0.178	0.180	0.201
'*paravorax*' clade					**0.147**	–	–	–	–
T. paravorax						–	0.160	0.161	0.176
T. glochidiophila							–	0.020	0.105
nsp10								–	0.112
19518-2									–

bility (Sonneborn 1938, Elliott & Nanney 1952). For many years, these biological species were referred to as syngens, biological species without binomial names. Multivariate morphometric approaches could morphologically discriminate some syngens of *P. 'aurelia'* from each other (Gates et al. 1974) but not others (Gates & Berger 1976), and this approach was also successfully applied to discriminating strains of *T. 'pyriformis'* (Gates & Berger 1974). Nevertheless, this morphological approach required highly controlled growth conditions and many measurements of cells that could not be discriminated on the basis of any univariate feature. While we agree that morphological characters are important, the present results once again demonstrate that morphology fails to distinguish among *Tetrahymena* species. The detail required to find new morphological characters is beyond the expertise of most investigators and would be impossible to implement for large-scale studies, such as population and biodiversity surveys.

Warren et al. (2017, p. 544) also stated that

> Genetic data must be evaluated in context of morphological characters to address broad questions about complex processes that involve multiple factors such as evolutionary rates, convergent evolution, population structure, and functional ecology acting in concert. This is also true for relatively narrow avenues of inquiry such as α-taxonomy. For example, the degree of divergence between sequences of a key gene (e.g. SSU rRNA, ITS, CO1), by itself, cannot substitute for actual characters because there is no generally accepted threshold value for the degree of divergence between congeneric species, including cryptic and pseudocryptic species or higher taxa.

For *Tetrahymena*, *Paramecium*, and doubtless many other cryptic ciliate species, it is difficult, if not impossible, to find morphological characters that distinguish among them (see above); it is the very definition of cryptic species. As Corliss & Daggett (1983, p. 315) stated in their review of the taxonomy and nomenclature of cryptic species in the '*pyriformis*' complex, 'In general, morphological features of the various members of the *pyriformis* complex are too similar, or too variable both within and among species, **to serve reliably in diagnosis** of a given species or of a population of unknown tetrahymenas collected from the wild' (bold added). Ultimately biochemical methods were able to identify and discriminate these species. Using these characters, Sonneborn (1975) and Nanney & McCoy (1976) established named species for the 14 syngens/species in the *P. 'aurelia'* complex and the 14 syngens/species of *T. 'pyriformis'* based on the isozyme profiles from starch gel electrophoresis.

What Warren et al. (2017) failed to mention is that many (most) of the cryptic species in these genera are

well characterized as biological species, and as such their genetic divergence can be assessed. DNA sequencing approaches, especially using the *cox*1 barcode for example, can now more easily be applied, and barcodes are available to identify already described species of *Paramecium* (e.g. Barth et al. 2006) and *Tetrahymena* (e.g. Kher et al. 2011). For example, Chantangsi et al. (2007) found that the average genetic divergence among *Tetrahymena* species for *cox*1 is ~10.5%. Doerder (2014) found that a threshold of >4% was sufficient to distinguish new species. In this context, it is relevant to note that asexual *Tetrahymena* species assigned names based on isozyme differences (Nanney & McCoy 1976) were subsequently found to have *cox*1 differences consistent with their assignment as species. This implements the suggestion of Sonneborn (1957) that asexuals, selfers, and inbreeders can be declared species on the basis of 'genetic' divergence. We have used this barcode to establish the genetic distinctness of *T. glochidiophila* n. sp., and the *cox*1 barcode along with the SSU rRNA gene sequence should be required to identify species and to establish any new species of *Tetrahymena*.

Warren et al. (2017) placed considerable emphasis on morphology and the morphospecies concept. They quoted Finlay et al. (1996, cited in Warren et al. 2017), who defined the morphospecies as 'a collection of forms that all fit into a defined range of morphological variation — forms that, so far as we can tell, occupy the same ecological niche'. It is doubtful that members of morphospecies, such as *T. 'pyriformis'* and *P. 'aurelia'*, occupy the same niche. As bacterivores, members of each genus almost certainly split this niche. Parasitic (histophagous) *Tetrahymena* species appear to be mostly host specific, but much more collecting must be done. Because multiple species are often found in the same water source, it is likely that they have different feeding preferences, although again much more study is required.

Liu et al. (2016) argued that the species descriptions of Sonneborn (1975) and Nanney & McCoy (1976) have created nomina nuda, as these descriptions were inadequate because there is neither 'a detailed morphological description based on modern taxonomic methods' nor 'high quality illustrations and photomicrographs' (Liu et al. 2016, p. 761). On the other hand, in their authoritative review of the status of species in the '*aurelia*' and '*pyriformis*' complexes, Corliss & Daggett (1983) had nothing but praise for Sonneborn (1975) and Nanney & McCoy (1976). In this regard, the International Code of Zoological Nomenclature (ICZN) states that a name-bearing type is eligible if it is established on 'any part of an animal' (ICZN Section 72.5.1; http://www.nhm.ac.uk/hosted-sites/iczn/code/). Sonneborn (1975) and Nanney & McCoy (1976) provided clear descriptions or references to clear descriptions of isozyme patterns to identify their proposed species. In this context, we agree with Corliss & Daggett (1983) that isozyme patterns can be considered parts of these 'animals', enabling diagnostic characterization of the species. Further, Sonneborn (1975) designated culture stocks for each *P. 'aurelia'* species, essentially designating a hapantotype, which ICZN Art. 73.3 states can be 'the holotype of the nominal taxon'. Kher et al. (2011), while not designating them as hapantotypes, did provide a list of 'type cultures' (Table 3, p. 9, in Kher et al. 2011) for many species of *Tetrahymena*, including *T. australis*, which Liu et al. (2016) redescribed. Thus, de facto hapantotypes have been designated for many *Tetrahymena* species.

ICZN Art. 13.1.1 states that new names 'be accompanied by a description or definition that states in words characters that are purported to **differentiate** the taxon' (bold added). The isozyme patterns provided by Sonneborn (1975) and Nanney & McCoy (1976) differentiated at that time all species established by them, and now barcodes provide further differentiation of all species (e.g. Barth et al. 2006, Kher et al. 2011, Doerder 2014). Like Corliss & Daggett (1983), we therefore believe that none of these names are nomina nuda, as argued by Liu et al. (2016): they were all adequately described originally and in sufficient detail both to differentiate them from other species and to establish them as valid species. It is ironic that Liu et al. (2016) used the SSU rRNA gene sequence and the *cox*1 barcode to initially assign the *Tetrahymena* morphotype that they isolated to the species *T. australis*. Moreover, Liu et al. (2016), while providing a comprehensive morphological description of *T. australis*, failed to clearly morphologically differentiate *T. australis* from many other *Tetrahymena* species. Its characters overlapped the ranges of characters for most other species (Table 2, p. 767, and Table 3, p. 768, in Liu et al. 2016). Even the caudal cilium of *T. setifera*, which appears to be an unambiguous differentiating feature (Liu et al. 2016), is shared by the bacterivorous form of *T. glochidiophila* n. sp. and also by other species, which, when small in size, might be confused with *T. 'pyriformis'* (Corliss 1973).

Therefore, we conclude that *Tetrahymena* species resembling *T. 'pyriformis'* can only now be properly identified and adequately described by using genetic tools: gene sequences for the *cox*1 barcode and the SSU rRNA gene as a minimum. In our view, these sequences can be interpreted as 'any part of an ani-

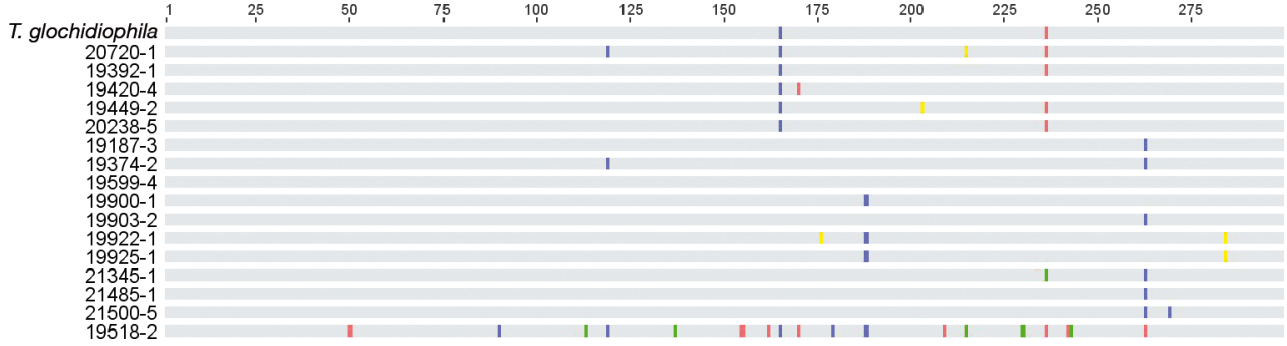

Fig. 5. Alignment of ~640 positions for the cytochrome *c* oxidase subunit 1 (*cox*1) barcode of 15 isolates of *Tetrahymena* nsp10, the new species 19518-2, and *T. glochidiophila* n. sp.. Vertical lines indicate polymorphisms. Note that there are several nsp10 isolates that share the same polymorphism as *T. glochidiophila* n. sp.

(Figure continued on next page)

mal' (ICZN Section 72.5.1), making the name available in the context of the ICZN. If possible, hapantotype cultures should also be submitted to a recognized culture collection as per ICZN Art. 73.3. While a detailed morphological description is of interest, it can rarely provide diagnostic characters that will differentiate a new *Tetrahymena* species from all other described species. Nevertheless, we have provided this for *T. glochidiophila* n. sp., along with its genetic characterization.

Comparison of *T. glochidiophila* n. sp. with other *'paravorax'* species

As discussed above, the first step in differentiating a new *Tetrahymena* species is to provide a genetic characterization. *T. glochidiophila* n. sp. and nsp10 belong to the same 'genetic' species. The SSU rRNA sequences place both, with strong bootstrap support, in the same clade as *T. paravorax* and isolate 19518-2, which is the only isolate of its species (Table 1). Morphologically, *T. glochidiophila* n. sp. can be differentiated from *T. paravorax* as they do not form macrostomes. However, on all other characters listed by Corliss (1973), there is considerable overlap between the 2 species (*T. glochidiophila* n. sp. followed by *T. paravorax*): somatic kineties, 23–26 vs. 22–30; postoral kineties, 2 in both; caudal cilium, present in both; contractile vacuole pores, 1–2 at kineties 7–9 vs. 1–6 at kineties 6–8; body length, 41–59 µm for histophage and 30–49 µm for bacterivore vs. 70–140 µm for macrostome and ~50 µm for microstome; cysts, neither species likely produces either reproductive or resting cysts; micronucleus, single vs. 1–4; micronuclear diameter, 2 µm vs. ~2.5 µm. Thus, we must proceed to distinguish these 2 species on the basis of genetics.

Early molecular studies recognized *T. paravorax* as an outlier, falling into neither the '*borealis*' nor the '*australis*' clades (Williams et al. 1984, Preparata et al. 1989). Consistent with Chantangsi et al. (2007), the SSU rRNA phylogeny (Fig. 3) indicates that the '*paravorax*' clade, containing *T. paravorax* and *T. glochidiophila* n. sp., nsp10, and their relative 19518-2, is more distantly related to *Tetrahymena* than to other genera, such as *Dexiostoma* and *Glaucoma*. However, although *cox*1 pairwise differences are high within the '*paravorax*' clade (14.7%), this clade groups with other *Tetrahymena* species (Fig. 4). Nevertheless, the *cox*1 barcode differences from other genera typically exceed differences between pooled '*borealis*' and '*australis*' clades (Table 3). By these criteria, the '*paravorax*' clade could be designated a new genus. At present, we are reluctant to establish a new genus since our phylogenetic analyses have not included a large selection of oligohymenophorean sequences. Furthermore, phylogenomic analyses are showing that deep branching clades based only on SSU rRNA gene sequences may be misleading (Gentekaki et al. 2017, Lynn & Kolisko 2017).

Within the '*paravorax*' clade, SSU rRNA sequences of *T. glochidiophila* n. sp. and nsp10 differ at a single transition, whereas nsp10 and 19518-2 are identical. Furthermore, *cox*1 barcodes show that *T. glochidiophila* n. sp. and nsp10 belong to the same species: their *cox*1 barcodes differ by an average of only 2% (Table 3), well within the intraspecific range of variation seen in other *Tetrahymena* species (Kher et al. 2011). Several nsp10 isolates shared the same polymorphic sites with the *T. glochidiophila* n. sp. isolate (Fig. 5). The *cox*1 sequences of both nsp10 and *T. glochidiophila* n. sp. differ from 19518-2 at an average of 59 sites; this 9.2% difference is consistent with 19518-2 being a separate species despite its identity with nsp10 on the SSU rRNA gene. Although there

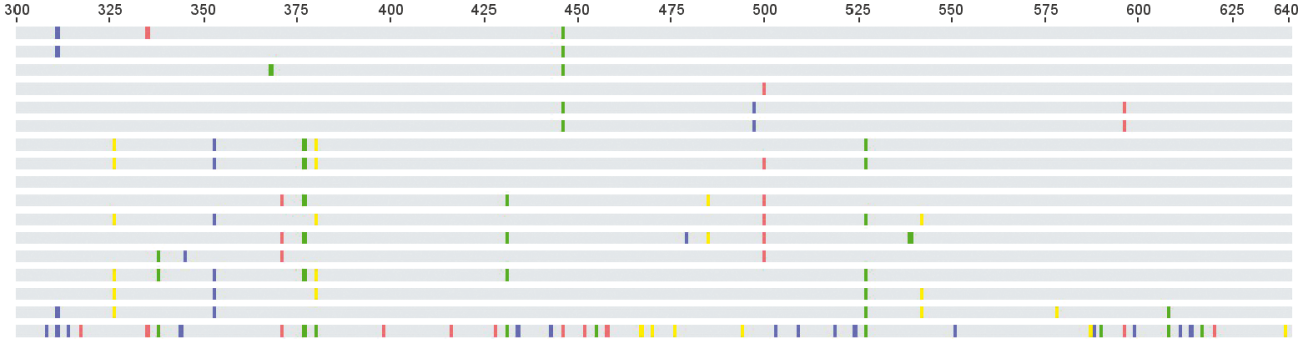

Fig. 5 (continued)

are several bona fide *Tetrahymena* species in both '*borealis*' and '*australis*' clades with identical SSU rRNA sequences, *T. glochidiophila* and nsp10 would be the first instance, to our knowledge, in which the SSU rRNA shows intraspecific polymorphism in a *Tetrahymena* species. Among *Paramecium* species, there are now several examples of intraspecific polymorphism in the SSU rRNA gene associated with identity in *cox*1 and the internal transcribed spacer region (see Lanzoni et al. 2016). For our *Tetrahymena* isolates, the same base was observed in the 2 nsp10 isolates sequenced, and the same transition was observed in multiple sequences of *T. glochidiophila* n. sp., thus eliminating sequencing error. Based on experience with other *Tetrahymena* species (Kher et al. 2011, Doerder 2014), it would be exceptional for 2 species to have *cox*1 differences of 0.5%, the minimum difference observed between *T. glochidiophila* n. sp. and some isolates of nsp10. Therefore, like researchers on *Paramecium* species, we conclude that nsp10 and *T. glochidiophila* n. sp. are conspecific, despite the non-identity of their SSU rRNA gene sequences.

Taxonomic summary

Subclass Hymenostomatia Delage & Hérouard, 1896
Order Tetrahymenida Fauré-Fremiet in Corliss, 1956
Family Tetrahymenidae Corliss, 1952
Tetrahymena Furgason, 1940
Tetrahymena glochidiophila Lynn, Doerder, Gillis and Prosser, 2018

Diagnosis: *Tetrahymena* species with histophagous and bacterivorous forms; pyriform body shape; oral area somewhat more expansive than a typical *Tetrahymena*; bacterivore may have a caudal cilium; body size after Chatton-Lwoff silver staining – histophage about 41–59 µm in length by 19–31 µm in width with a length:width ratio of ~0.50 and bacteriovore about 30–49 µm in length by 19–27 µm in width with a length:width ratio of ~0.57; somatic kineties 23–26 with 2 being postoral; 1–2 contractile vacuole pores between somatic kineties 7 and 8 and 8 and 9; macronucleus, subspheroid, about 8–18 µm by 6–14 µm; single micronucleus.

Type host: *Lampsilis siliquoidea* (Barnes, 1823) (common name: Fatmucket)

Type location: Un-named pond, Missouri, USA (39.004° N, 94.522° W) collected in September 2016.

Type material: A Chatton-Lwoff stain of *T. glochidiophila* n. sp. cells in the histophage stage (Holotype USNM 1437639) was deposited in the International Protozoan Type Slide Collection of the Department of Invertebrate Zoology of the National Museum of Natural History, Smithsonian Institution. The holotype (Figs. 1C & 2A) is circled in black on the underside of the slide. A paratype slide (Paratype USNM 1437640) is also deposited as a Chatton-Lwoff stain of the bacterivore stage of *T. glochidiophila* n. sp.

Gene sequences: Gene sequences of *T. glochidiophila* n. sp. were deposited in GenBank under accession numbers MF693880 (SSU rRNA) and MF693881 (*cox*1).

Etymology: The specific epithet is derived from the habit of this species to attack the glochidia larvae of freshwater mussels.

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