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Phylogenetic relationships within the genus *Tetrahymena* inferred from the cytochrome *c* oxidase subunit 1 and the small subunit ribosomal RNA genes

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ABSTRACT

Details of the phylogenetic relationships among tetrahymenine ciliates remain unresolved despite a rich history of investigation with nuclear gene sequences and other characters. We examined all available species of *Tetrahymena* and three other tetrahymenine ciliates, and inferred their phylogenetic relationships using nearly complete mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and small subunit (SSU) rRNA gene sequences. The inferred phylogenies showed the genus *Tetrahymena* to be monophyletic. The three "classical" morphology-and-ecology-based groupings are paraphyletic. The SSUrRNA phylogeny confirmed the previously established *australis* and *borealis* groupings, and nine ribosets. However, these nine ribosets were not well supported. Using *cox1* gene, the deduced phylogenies based on this gene revealed 12 well supported groupings, called coxisets, which mostly corresponded to the nine ribosets. This study demonstrated the utility of *cox1* for resolving the recent phylogeny of *Tetrahymena*, whereas the SSU rRNA gene provided resolution of deeper phylogenetic relationships within the genus.

1. Introduction

The ciliate genus *Tetrahymena* has long been used as a model organism for the investigation of ultrastructure, physiology, biochemistry, genetics, pharmacology, and toxicology. The versatility of this ciliate allows scientists to use it to identify several important factors regarding biological processes in other eukaryotes. Understanding the genealogical relationships of this ciliate genus is therefore important, since it will not only shed the light on its biology and ecology but also provide better insight into research in other fields in which *Tetrahymena* species are used.

Phylogenetic relationships among *Tetrahymena* species have been inferred from various characters (Corliss, 1973; Nanney et al., 1989; Preparata et al., 1989; Sadler and Brunk, 1992; Sogin et al., 1986; Strüder-Kypke et al., 2001; Ye and Romero, 2002). Based on morphology and ecology, *Tetrahymena* species were divided into three complexes: *pyriformis*, *rostrata*, and *patula*. The *pyriformis*-complex members are microstomatous bacterivores, being small cells with fewer somatic ciliary rows. The *rostrata* complex includes parasites of animal hosts, feeding as histophages; they are larger cells with more somatic ciliary rows. Furthermore, some *rostrata* complex species have the ability to form a resting cyst. Species in the third group, the *patula* complex, are capable of transforming between a microstome and macrostome stage; they typically have only a reproductive cyst in their life cycle and are never found as parasites. Nevertheless, these morphology–ecology based complexes have been shown to be paraphyletic based on molecular data (Nanney et al., 1989; Preparata et al., 1989; Strüder-Kypke et al., 2001).

Phylogenies of tetrahymenine ciliates, including *Colpidium*, *Glaucoma*, and *Tetrahymena*, as inferred from isozyme variations and molecular markers, such as nuclear genes, including 5S, 5.8S, and a part of the large subunit rRNA (LSUrRNA) genes, divided the organisms into nine groups, the so-called ribosets (Nanney et al., 1989; Preparata et al., 1989). In addition, some molecular data, such as histones, small subunit rRNA (SSUrRNA), and telomerase RNA (TER) sequences, divided *Tetrahymena* species into two major groups, the *australis* and *borealis* groups (Sadler and Brunk, 1992; Sogin et al., 1986; Strüder-Kypke et al., 2001; Ye and Romero, 2002). However, several of these nuclear genes have long been known to be too conserved to provide unambiguous delineation of genealogical relationships of closely related species. In addition, since no confidence method has been performed to test support for the nine ribosets, the establishment of these groups is open to question.

It is known that mitochondrial genes generally evolve at a faster rate (5–10 times) than nuclear genes in mammalian mitochondrial DNA (mtDNA) (Morin and Cech, 1988). This attribute of faster nucleotide substitution has led scientists to further refine evolutionary relationships among closely related organisms. Among

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the mitochondrial genes, the cytochrome *c* oxidase subunit 1 (*cox1*) gene is among the most conserved mitochondrial protein-coding genes in animals (Folmer et al., 1994). Therefore, this gene has been extensively used in evolutionary studies and proven to be a very good phylogenetic marker (Miya and Nishida, 2000). In addition, some regions of this gene are highly conserved, enabling design of universal primers to PCR amplify the gene from various animal phyla (Folmer et al., 1994). Besides the conserved regions of the *cox1* gene, other parts of the gene are variable, enabling discrimination of animal species (Hebert et al., 2003) and protist species (Barth et al., 2006; Chantangsi et al., 2007; Lynn and Strüder-Kypke, 2006). These benefits form the basis of a system called the Barcode of Life Database (BoLD), which is rapidly becoming a new taxonomic tool for the biological identification of species (Hebert et al., 2003).

Most molecular evolutionary studies of *Tetrahymena* species have used various nuclear genes and only a few have used a mitochondrial gene. The earliest such study used the mitochondrial large subunit α (LSUα) rRNA (5.8S-like RNA) gene sequences (Morin and Cech, 1988). However, only six species of *Tetrahymena* were included in that study. Therefore, we undertook the current study of mitochondrial *cox1* gene sequences: (1) to delineate phylogenetic relationships of 36 of the 42 closely allied species of the genus *Tetrahymena*, those for which cultures are available; and (2) to evaluate and compare *Tetrahymena* phylogenies deduced from mitochondrial *cox1* gene with those derived from the nuclear SSUrRNA gene with emphasis on well supported groupings. Comprehensive coverage of all available valid species of *Tetrahymena* and use of a more variable phylogenetic marker enabled us to better elucidate genealogical relationships of *Tetrahymena*, which have so far remained unresolved.

2. Materials and methods

Forty five isolates of tetrahymenine ciliates were examined (Table 1). Of 42 taxonomically valid species of *Tetrahymena* (Fenchel and Finlay, 2004), 36 are available in cultures and at least one isolate of each of these species was selected as a species representative, mostly using cultures from the American Type Culture Collection (Table 1). The remaining six valid *Tetrahymena* species—*Tetrahymena chironomi*, *T. dimorpha*, *T. edaphoni*, *T. rotunda*, *T. sialidos*, and *T. stegomyiae*—were not examined due to the unavailability of their cultures. In addition, six isolates of undescribed *Tetrahymena* species and three other tetrahymenine species, *Colpidium campylum*, *C. colpoda*, and *Glaucoma chattoni*, were also investigated. Sequences of cytochrome *c* oxidase subunit 1 (*cox1*) gene and the SSUrRNA gene of these 45 tetrahymenine ciliates were retrieved from GenBank (Table 1), many of them derived from the recent study by Chantangsi et al. (2007) on *cox1* species barcoding.

2.1. Dataset constructions

The *cox1* and SSUrRNA gene sequences were first aligned automatically by ClustalW (Thompson et al., 1994) using the MEGA (Molecular Evolutionary Genetics Analysis) program version 3.1 (Kumar et al., 2004) and then further refined by eye.

Except for the *cox1* gene sequence of *G. chattoni*, which is 1785 bp long, the *cox1* gene sequences used in this study are 1821 bp in length. Thirty six positions from 1786–1821 at the 3′-end of the *cox1* gene sequence of *G. chattoni* were treated as missing data. The 1821 nucleotides start at position 52–1872 with reference to the complete length of the *cox1* genes of *Tetrahymena pyriformis* and *T. thermophila* published in GenBank (Brunk et al., 2003; Burger et al., 2000).

Aligned SSUrRNA gene sequences of all 45 tetrahymenine ciliates were constructed as a dataset, which was 1650 positions in length and included a few gaps and some ambiguous characters. Exclusion of these gaps and ambiguous positions gave a total of 1639 positions, which were used in sequence and phylogenetic analyses. Sequence alignments for *cox1* and SSUrRNA genes are available from the authors upon request.

Sequences of about 1821 bp of the *cox1* gene and of about 1639 bp of the SSUrRNA gene for all 45 species were combined to one dataset. A total of 3460 bp of this dataset was divided into two partitions according to nucleotide numbers of each gene. The partition homogeneity test with 1000 replications implemented in PAUP* 4.0 beta 10 (Phylogenetic Analysis Using Parsimony) (Swofford, 2003) was performed to determine whether these two genes can be combined into one dataset for further phylogenetic analyses. The result indicated that the *cox1* and SSUrRNA genes were incongruent and could not be combined into one dataset for further analysis.

2.2. Sequence and phylogenetic analyses

Sequence divergences were calculated for each dataset using the Kimura two parameter (K2P) distance model (Kimura, 1980). Neighbour-joining (NJ) phylogenetic trees were inferred from genetic distances of *cox1* and SSUrRNA gene sequences calculated by DNADIST with the K2P model of sequence evolution using the *PHYL*ogeny *I*nference *P*ackage (PHYLIP) version 3.65 (Felsenstein, 2004; Saitou and Nei, 1987). *G. chattoni* was used as the outgroup species in all analyses. In addition, 1000 bootstrap resamplings were carried out using SEQBOOT to determine confidence levels of deduced relationships. CONSENSE within the PHYLIP package was used to construct consensus trees.

Maximum parsimony (MP) approach was carried out using PAUP* 4.0 beta 10 (Swofford, 2003). Heuristic searching was set with the tree bisection-reconnection (TBR) branch-swapping algorithm in effect. All characters were equally weighted and of unordered type. Sequences were added randomly. Starting tree(s) were obtained via stepwise addition. This phylogenetic approach was performed on the datasets of *cox1* and the SSUrRNA gene sequences. Since a dataset of SSUrRNA gene sequences gave a total of 330 most parsimonious trees, bootstrap resampling was only carried out 1000 times to provide confidence levels of deduced relationships for the *cox1* gene sequences.

MrBayes (MB) program version 3.1.1 was chosen to perform the Bayesian estimation of phylogeny (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). MrModeltest version 2.2 was first performed to select the most suitable nucleotide substitution models for the data to be used in a Bayesian inference (Nylander, 2004). The chosen best-fit model was implemented and then Bayesian analysis was performed. Four Markov Chain Monte Carlo (MCMC) chains—one cold chain and three heated chains—were run for 2,000,000 generations, sampling every 50th generation (tree). The first 4000 trees were discarded as burn-in. The remaining trees were used to compute the 50% majority-rule consensus tree. Branch lengths of the trees were saved. Bayesian analysis was performed on the datasets of *cox1* and the SSUrRNA gene sequences.

Maximum likelihood (ML) phylogenetic tree construction was carried out by using PAUP* 4.0 beta 10 (Swofford, 2003). Modeltest version 3.7 was first performed to select the most suitable nucleotide substitution models for the data (Posada and Crandall, 1998). MTgui version 1.5 was used as a simple interface to Modeltest (Nuin, 2004). The chosen best-fit model was implemented using the maximum likelihood optimality criterion. This phylogenetic approach was performed on the datasets of *cox1* and the SSUrRNA gene sequences. In addition, 100 bootstrap resamplings were car-

Table 1
List of species of *Tetrahymena* and three other tetrahymenine ciliates that were examined in this study

No.	Species name	ATCC number[a]		GenBank Accession No.		Groupings of tetrahymenines		
		cox1	SSUrRNA	cox1	SSUrRNA	CME	Riboset	Coxiset
1.	*T. americanis*	205052	205052	EF070267	EF070242	*pyriformis*	C	C
2.	*T. asiatica*	205167	205167	EF070268	EF070243	*pyriformis*	C	C
3.	*T. australis*	30271	30831	EF070269	X56167	*pyriformis*	C	C
4.	*T. bergeri*	50985	—	EF070270	AF364039	*rostrata*	A2	A4
5.	*T. borealis*	30317	205012	EF070271	M98020	*pyriformis*	A2	A2
6.	*T. canadensis*	30368	30368	EF070276	X56170	*pyriformis*	A2	A2
7.	*T. capricornis*	30290	30291	EF070277	X56172	*pyriformis*	C	C
8.	*T. caudata*	50087	50087	EF070278	EF070244	*patula*	H	H
9.	*T. corlissi*	50086	50086	EF070279	U17356	*rostrata*	A2	A4
10.	*T. cosmopolitanis*	30324	30324	EF070280	EF070245	*pyriformis*	C	C
11.	*T. elliotti*	205065	205065	EF070281	EF070246	*pyriformis*	A2	A5
12.	*T. empidokyrea*	50595	—	EF070282	U36222	*pyriformis*	C	H
13.	*T. farleyi*	50748	50748	EF070283	AF184665	*pyriformis*	A2	A3
14.	*T. furgasoni*	30006	30006	EF070284	EF070247	*pyriformis*	A2	A3
15.	*T. hegewischi*	30354	30823	EF070285	X56166	*pyriformis*	C	C
16.	*T. hyperangularis*	30273	30273	EF070286	X56173	*pyriformis*	C	C
17.	*T. leucophrys*	50069	50069	EF070287	EF070248	*patula*	B	B1
18.	*T. limacis*	30771	30771	EF070288	EF070249	*rostrata*	A2	A4
19.	*T. lwoffi*	1630/1G[b]	1630/1K[b]	EF070289	EF070250	*pyriformis*	A2	A3
20.	*T. malaccensis*	50065	—	EF070291	M26360	*pyriformis*	A1	A1
21.	*T. mimbres*	30330	30330	EF070292	EF070251	*pyriformis*	A2	A5
22.	*T. mobilis*	PRA-174	PRA-174	EF070293	AF364040	*rostrata*	A2	A3
23.	*T. nanneyi*	50071	30840	EF070294	X56169	*pyriformis*	C	C
24.	*T. nipissingi*	30837	30837	EF070295	EF070252	*pyriformis*	C	C
25.	*T. paravorax*	205177	205177	EF070296	EF070253	*patula*	D	C
26.	*T. patula*	50064	50064	EF070297	X56174	*patula*	C	C
27.	*T. pigmentosa*	30278	—	EF070299	M26358	*pyriformis*	C	C
28.	*T. pyriformis*	30005	—	EF070300	X56171	*pyriformis*	B	B1
29.	*T. rostrata*	30770	30770	EF070305	AF364042	*rostrata*	A2	A2
30.	*T. setosa*	30782	30782	EF070306	AF364041	*pyriformis*	B	B1
31.	*T. shanghaiensis*	205039	205039	EF070307	EF070256	*pyriformis*	C	C
32.	*T. silvana*	50084	50084	EF070308	EF070257	*patula*	B	B2
33.	*T. sonneborni*	205040	205040	EF070309	EF070258	*pyriformis*	C	C
34.	*T. thermophila*	—	—	EF070310	M10932	*pyriformis*	A1	A1
35.	*T. tropicalis*	30276	30352	EF070314	X56168	*pyriformis*	A2	A3
36.	*T. vorax*	30421	30421	EF070319	AF364038	*patula*	B	B2
37.	*Tetrahymena* sp.1 (Foissner)	—	—	EF070320	EF070263	ND[c]	B	B1
38.	*Tetrahymena* sp.2 (CO)	—	—	EF070321	—[d]	ND	A2	A3
39.	*Tetrahymena* sp.3 (RA9)	—	—	EF070322	EF070264	ND	A2	A3
40.	*Tetrahymena* sp.4 (SIN)	—	—	EF070323	—[d]	ND	C	C
41.	*Tetrahymena* sp.5 (NI)	—	—	EF070324	EF070265	ND	A2	A3
42.	*Tetrahymena* sp.6 (Brandl)	—	—	EF070325	AY755629	ND	A2	A3
43.	*C. campylum*	—	—	EF070326	X56532	—	G	G
44.	*C. colpoda*	—	—	EF070327	EF070266	—	F	F
45.	*G. chattoni*	—	—	EF070328	X56533	—	E	E

Sources of cultures and GenBank Accession Nos. (if available) for cytochrome *c* oxidase subunit 1 (*cox1*) and small subunit rRNA (SSUrRNA) genes are listed. Groupings of tetrahymenines based on different data, including the "classical" morphology-and-ecology-based (CME), the ribosomal genes (Riboset), and the *cox1* gene (Coxiset) are also provided.

[a] ATCC number = American type culture collection number.
[b] CCAP number = culture collection of algae and protozoa number.
[c] ND = not determined.
[d] Their SSUrRNA gene sequences were kindly provided by Dr. Michaela C. Strüder-Kypke.

ried out using phyML to determine confidence levels of deduced relationships (Guindon and Gascuel, 2003).

The CONSEL program by Shimodaira and Hasegawa (2001) was used to assess the confidence in the tree selection. This program was employed to calculate the *p*-value based on the approximately unbiased (AU) test (Shimodaira, 2002; Shimodaira and Hasegawa, 1999) to determine the probabilities of having tree topologies between the original and the alternative ones both inferred from 1821 bp of the *cox1* gene when the latter was modified to have a clade of the two *Colpidium* species as a sister group to the assemblage of all *Tetrahymena* species. This topology was similar to that derived from 1639 bp of SSUrRNA gene sequences. The analysis was conducted to determine the potential of *cox1* gene as a phylogenetic marker for delineating ancient history of the examined ciliates when conflicting topologies were generated using different tree construction methods.

3. Results

3.1. Phylogenetic trees based on cox1 gene sequences

Phylogenetic trees based on Bayesian (MB) and maximum likelihood (ML) approaches showed identical topologies. All 42 *Tetrahymena* spp. were clustered together with 1.00 posterior probability in the MB tree. In addition, the MB and ML phylogenies showed *C. colpoda* as the sister taxon of the *Tetrahymena* clade with 0.97 posterior probability (Fig. 1). Deep phylogenetic relationships within the genus *Tetrahymena* inferred from neighbour-joining (NJ) and maximum parsimony (MP) approaches remained unresolved as indicated by low percent bootstrap values (Supplementary Figs. 1 and 2). However, some groups of species were well supported with high percent bootstrap values, confirming relationships demonstrated by MB and ML analyses (Table 2). Bayesian inference and

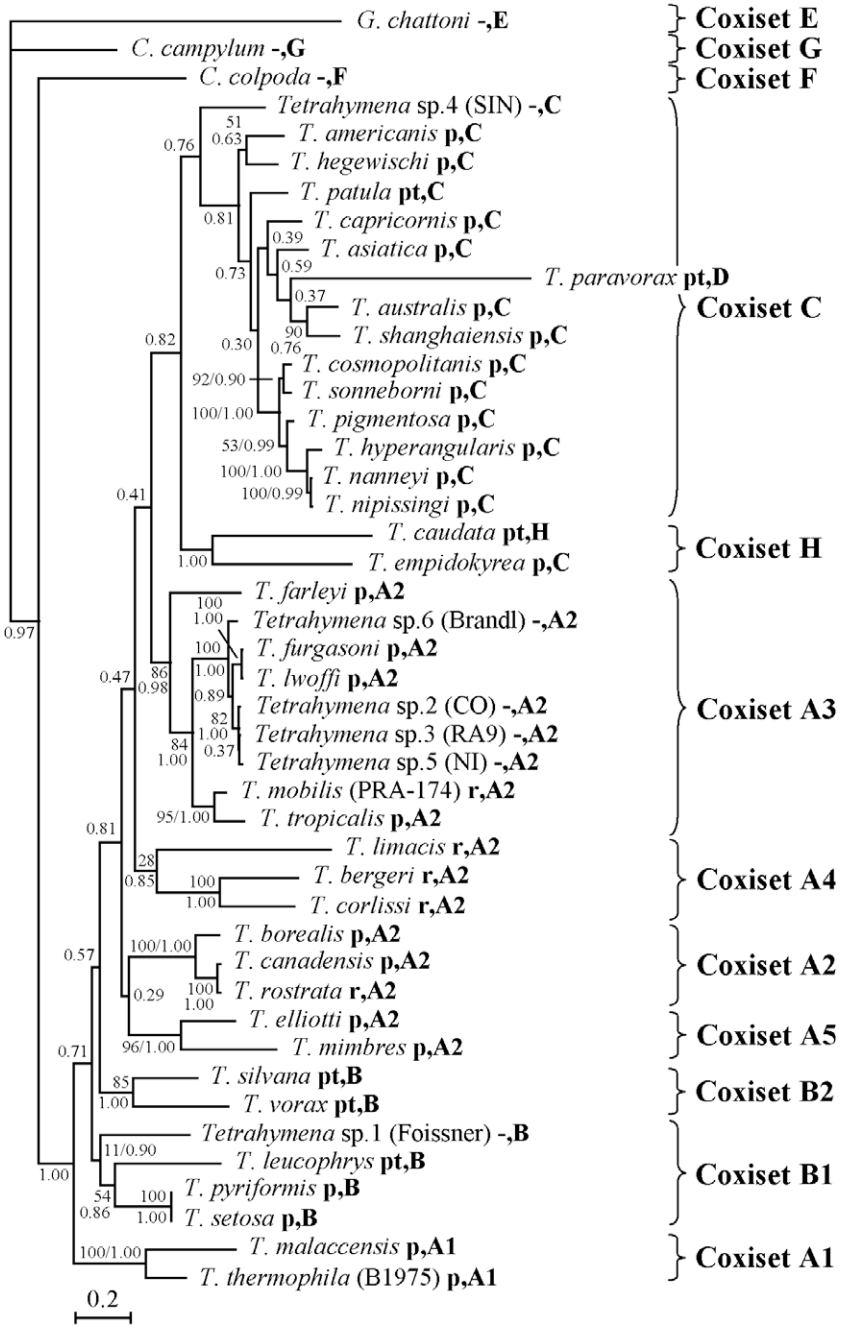

Fig. 1. Bayesian phylogeny deduced from 1821 bp of *cox1* gene sequences of 45 tetrahymenine species illustrating 12 groupings designated as coxisets. Designations of the "classical" morphology-and-ecology-based grouping [p = *pyriformis*; pt = *patula*; r = *rostrata*] and the riboset for each taxon are provided in bold after species names, respectively. The best-fit model selected by the hierarchical likelihood ratio test (hLRT) in MrModeltest 2.2 was the General-Time-Reversible model with invariable sites and gamma distribution (GTR + I + G). The tree is a consensus of 36,002 trees (mean ln $L = -16631.43$) with the scale bar corresponding to 0.2 expected changes per site. The numbers indicate posterior probabilities and phyML bootstrap values at the nodes. PhyML bootstrap values are provided only at the nodes of the same relationships found in MB, ML, and phyML analyses. The ML tree was retained by heuristic searching with 10 replicates. The best-fit model selected by hLRT in Modeltest 3.7 was the GTR + I + G with Lset Base = (0.3936, 0.0528, 0.0617); Nst = 6; Rmat = (74.0751, 64.2377, 4.6806, 76.4430, 2079.4424); Rates = gamma; Shape = 0.2256; and Pinvar = 0.4486. Due to the identical topologies of MB and ML trees, only the MB tree is shown.

ML, among various tree construction approaches, have been recognized for their power and robustness in modeling phylogenetic relationships (Huelsenbeck et al., 2001). Due to these features, groupings of tetrahymenine species revealed by these two methods were selected and designated as 12 cytochrome *c* oxidase sets (coxisets), based on the criterion that posterior probability exceeded 0.75. In fact, posterior probability always exceeded 0.90, except for coxisets A4 and C (Fig. 1; Table 2). The species component and designations of coxisets basically corresponded to those of ribosets (Preparata et al., 1989). For example, *C. colpoda* belonged to riboset F and therefore was assigned to coxiset F.

3.2. Phylogenetic trees based on small subunit rRNA gene sequences

Phylogenetic trees, based on NJ, MP (data not shown), MB, and ML and with *G. chattoni* as the outgroup species, showed similar topologies in terms of grouping of *C. campylum* and *C. colpoda* separately from the *Tetrahymena* clade (Fig. 2; Supplementary Figs. 3

Table 2
Nine coxisets (CS) of *Tetrahymena* species were inferred from two different datasets—the 1821 bp of the mitochondrial *cox1* gene and 1639 bp including gaps of the small subunit rRNA (SSUrRNA) gene

CS	Species	*cox1* 1821 bp				SSUrRNA gene 1639 bp			
		NJ	MP	MB	ML[a]	NJ	MP	MB	ML[a]
A1	*T. malaccensis* *T. thermophila* (B1975)	99.8	98.9	1.00	100	100	—[b]	0.99	100
A2	*T. borealis* *T. canadensis* *T. rostrata*	100	100	1.00	100	72.8	—	NF[c]	S[d]
A3	*T. farleyi* *T. furgasoni* *T. lwoffi* *T. mobilis* *T. tropicalis* *Tetrahymena* sp.2 (CO) *Tetrahymena* sp.3 (RA9) *Tetrahymena* sp.5 (NI) *Tetrahymena* sp.6 (Brandl)	94.1	52.6	0.98	86	93.7	—	0.97	91
A4	*T. bergeri* *T. corlissi* *T. limacis*	12.6[e]	NF	0.85	28	NF	—	NF	NF
A5	*T. elliotti* *T. mimbres*	96.9	86.8	1.00	96	91.8	—	0.92	87
B1	*T. leucophrys* *T. pyriformis* *T. setosa* *Tetrahymena* sp.1 (Foissner)	4.2[e]	NF	0.90	11	NF	—	0.59	27
B2	*T. silvana* *T. vorax*	87.5	76.3	1.00	85	26.7	—	0.18[e]	S[d]
C	*T. americanis* *T. asiatica* *T. australis* *T. capricornis* *T. cosmopolitanis* *T. hegewischi* *T. hyperangularis* *T. nanneyi* *T. nipissingi* *T. paravorax* *T. patula* *T. pigmentosa* *T. shanghaiensis* *T. sonneborni* *Tetrahymena* sp.4 (SIN)	10.8[e]	NF	0.76	S[f]	NF	—	NF	NF
H	*T. caudata* *T. empidokyrea*	11.6[e]	24.4	1.00	S[f]	NF	—	NF	NF

These datasets were analyzed by four different phylogenetic approaches—neighbour-joining (NJ), maximum parsimony (MP), MrBayes (MB), and maximum likelihood (ML). Support values provided in the table.

[a] Bootstrap values obtained from phyML analysis.

[b] Support for the clades was not presented by maximum parsimony analysis of 1639 positions of SSUrRNA gene as 330 most parsimonious trees were obtained from this analysis.

[c] NF = the relationship was not found.

[d] One or more additional species were part of this clade (S = support).

[e] These support values were obtained from output files of the performed analyses and were not shown on the presented trees.

[f] This clade was supported by maximum likelihood analysis (S = support).

and 4). In addition, these two *Colpidium* species were grouped together with 81.4% bootstrap support in NJ (Supplementary Fig. 3) and 0.94 posterior probability in MB (Fig. 2), in contrast to *cox1* phylogenies, which grouped the *Colpidium* spp. with lower NJ and MP support values and placed them within the *Tetrahymena* clade (Supplementary Figs. 1 and 2). In MB and ML trees, all *Tetrahymena* species were grouped with 0.85 posterior probability in MB and 76% phyML bootstrap value (Fig. 2), and 61.3% bootstrap value in the NJ tree (Supplementary Fig. 3). However, all *Tetrahymena* species were grouped together and supported by a 95% NJ bootstrap value when excluding *T. paravorax*, which was placed next to the remaining *Tetrahymena* taxa (Supplementary Fig. 3).

The phylogenetic trees deduced from the four different methods showed quite similar topologies. *Tetrahymena* species were clustered into two major clades—the so-called *australis* and *borealis* (Fig. 2) with the exception of *T. paravorax* in the NJ tree which branched prior to formation of the two clades (Supplementary Fig. 3). These clades were not well supported by posterior probability in MB, but obtained at least 83% bootstrap values in the NJ (Supplementary Fig. 3). Each clade comprised identical members except for *T. caudata*, which was placed differently in the trees inferred by the different methods: this species was placed in the *australis* group by NJ but in the *borealis* group by MB and ML (Fig. 2; Supplementary Figs. 3 and 4). The genetic distances within the genus *Tetrahymena* were extremely low with many of them at 0%. Only *T. paravorax* showed a high level of sequence divergence (Fig. 2; Supplementary Fig. 3).

The phylogenetic trees inferred from SSUrRNA gene sequences remained fundamentally unresolved. There were low support values in both NJ and MB trees (Fig. 2; Supplementary Fig. 3), while the ML tree showed an unresolved polytomic branching of several taxa (Supplementary Fig. 4). Only a few groups identified by *cox1* sequences were recovered and well supported, such as coxisets A1, A3, and A5 (Table 2). The nine previously established ribosets

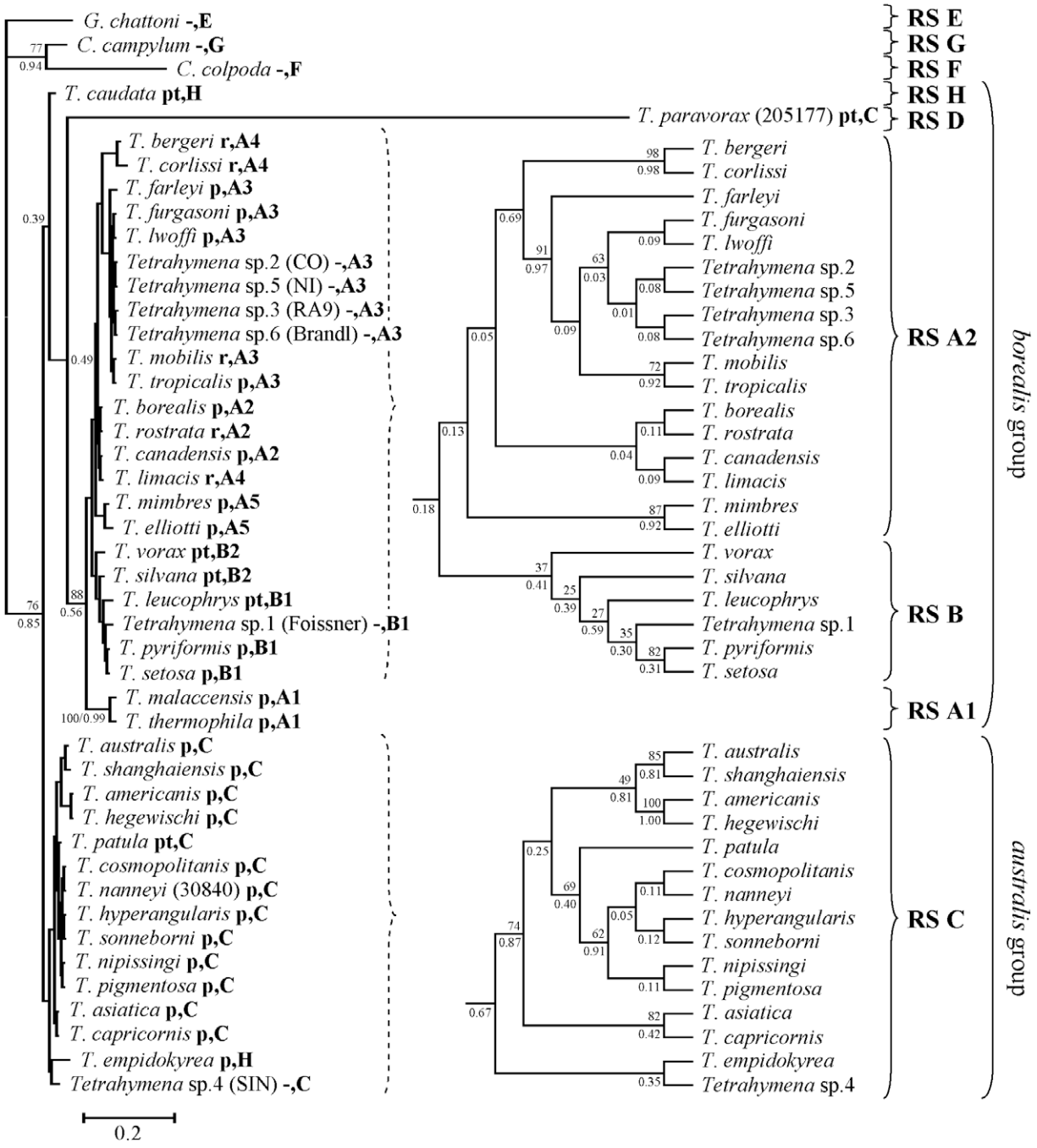

Fig. 2. Bayesian phylogeny deduced from 1639 bp of SSUrRNA gene sequences of 45 tetrahymenine species illustrating nine ribosets (RS). Designations of the "classical" morphology-and-ecology-based grouping [p = *pyriformis*; pt = *patula*; r = *rostrata*] and the coxiset for each taxon are provided in bold after species names, respectively. *Tetrahymena* members of *australis* and *borealis* groups are also labeled. The best-fit model selected by the hierarchical likelihood ratio test (hLRT) in MrModeltest 2.2 was the General-Time-Reversible (GTR) model with invariable sites and gamma distribution (GTR + I + G). The tree is a consensus of 36,002 trees (mean ln $L = -4383.80$) with the scale bar corresponding to 0.2 expected changes per site. The numbers indicate posterior probabilities and phyML bootstrap values at the nodes both in the consensus tree and the magnified clades. PhyML bootstrap values are provided only at the nodes of the same relationships found in MB and phyML analyses.

constructed from 5S, 5.8S, and LSUrRNA gene sequences were also recovered from the SSUrRNA gene sequences, but not with strong support (Fig. 2).

4. Discussion

4.1. Comparative phylogenetic relationships of tetrahymenine ciliates

Phylogenetic relationships of the genus *Tetrahymena* as well as some tetrahymenines have been inferred from various nuclear genes, such as the 5S and 5.8S rRNA genes (Preparata et al., 1989), the SSUrRNA gene (Sogin et al., 1986; Strüder-Kypke et al., 2001), the LSUrRNA gene (Nanney et al., 1989, 1998; Preparata et al., 1989), the histone H3II/H4II genes (Brunk et al., 1990; Sadler and Brunk, 1992), and telomerase RNA (TER) (Ye and Romero, 2002) and also from a mitochondrial marker—the large subunit α (LSUα) rRNA (5.8S-like RNA) (Morin and Cech, 1988).

Using *G. chattoni* as the outgroup species, our study of evolutionary relationships inferred from nuclear SSUrRNA and mitochondrial *cox1* gene sequences showed the entire genus

Tetrahymena to be a monophyletic assemblage using Bayesian and ML analyses, confirming previous studies (Strüder-Kypke et al., 2001; Wright and Lynn, 1995; Ye and Romero, 2002). In contrast, the *cox1* analysis using NJ and MP approaches showed a paraphyletic assemblage of the genus *Tetrahymena*: *C. campylum* and *C. colpoda* were placed within the *Tetrahymena* clade but with low bootstrap support. Nevertheless, analyses of alternative tree topologies using AU tests demonstrated that the NJ and MP tree topologies with the two species of *Colpidium* placed outside the *Tetrahymena* clade could not be rejected (*p*-values of 0.51 and 0.42, respectively).

4.2. Tetrahymena and the two "classical" groupings

Tree topologies deduced from nuclear genes showed similar results with division of *Tetrahymena* species into two major groups, the *australis* and *borealis* groups. In addition, phylogenetic relationships established using rRNA gene sequences revealed nine groupings, the so-called ribosets (Nanney et al., 1989; Preparata et al., 1989). A major subgroup of the *australis* group comprises riboset C, whereas the *borealis* group is composed of members of ribosets A1, A2, B, D, and H.

The earliest phylogeny inferred from the SSUrRNA gene of the genus *Tetrahymena* was presented by Sogin et al. (1986). The result obtained from this study was identical to the tree inferred from a mitochondrial gene, large subunit α (LSUα) rRNA (5.8S-like RNA), which was used to deduce the evolutionary relationships of six *Tetrahymena* species—*T. hegewischi*, *T. hyperangularis*, *T. malaccensis*, *T. pigmentosa*, *T. pyriformis*, and *T. thermophila* when the same set of species was considered (cf. Morin and Cech, 1988; Sogin et al., 1986). Subsequently, SSUrRNA gene sequences of several additional *Tetrahymena* species and other *Tetrahymena*-related taxa have been included to resolve phylogenetic relationships (Brandl et al., 2005; Greenwood et al., 1991; Strüder-Kypke et al., 2001; Wright and Lynn, 1995).

Our study based on SSUrRNA gene confirmed the *australis* and *borealis* groups (Brunk et al., 1990; Sadler and Brunk, 1992; Sogin et al., 1986; Strüder-Kypke et al., 2001; Wright and Lynn, 1995; Ye and Romero, 2002) although the *cox1* trees did not show these two major classical groups. Nevertheless, these two groups are well supported by high bootstrap values in the SSUrRNA gene NJ but not in the MB tree. Species compositions of these two groups differ slightly between our study and previously published phylogenies. In our SSUrRNA gene NJ tree, *T. caudata* groups with the *australis* species with 83% bootstrap support. In addition to *T. caudata*, *T. empidokyrea* was placed as the closest relative of *Tetrahymena* sp.4 (SIN) and was placed in the *australis* group, similar to a previously inferred SSUrRNA gene phylogeny (Strüder-Kypke et al., 2001). In contrast, *T. caudata* clustered with the *borealis* group in the H3II/H4II phylogenies, although this relationship had low bootstrap support (Sadler and Brunk, 1992).

4.3. Riboset and coxiset groupings of tetrahymenine ciliates

The nine previously established ribosets were recovered in our SSUrRNA phylogenies with some slightly different groupings and placement of taxa. Furthermore, 12 groupings, designated here as coxisets, could be defined from the phylogenetic relationships inferred from *cox1* gene sequences, by Bayesian and maximum likelihood approaches, which provided identical topologies. The coxisets mostly correspond to subgroups of the nine tetrahymenine ribosets derived from nuclear rRNA gene sequences (Preparata et al., 1989).

Using the species of riboset E—*G. chattoni*—as the outgroup, *T. caudata* (riboset H) and *T. paravorax* (riboset D) were distantly related to the other ribosets, which is consistent with 5S and 5.8S rRNA phylogenies (Preparata et al., 1989), LSUrRNA phylogenies (Nanney et al., 1989, 1998), and isozyme variation (Meyer and Nanney, 1987; Nanney et al., 1989). In addition, *C. colpoda* (riboset F) and *C. campylum* (riboset G) were placed together but separately from the *Tetrahymena* spp., which is consistent with the 5S and 5.8S rRNA phylogenies (Preparata et al., 1989). Similar relationships were recovered from *cox1* phylogenies: coxisets E (*Glaucoma*), F (*C. colpoda*), and G (*C. campylum*) were placed separately from the *Tetrahymena* spp. and are each composed of a single species.

4.3.1. Phylogenetic relationships within coxiset A

Coxisets A1, A2, A3, A4, and A5 were derived from the original species of ribosets A1 and A2 with other species added. The original riboset A1, based on LSUrRNA and H3II/H4II region, included *T. elliotti*, *T. malaccensis*, and *T. thermophila* (Preparata et al., 1989; Sadler and Brunk, 1992). The complete SSUrRNA sequence of *T. elliotti* newly placed this species within riboset A2 with *T. mimbres* as a sister taxon, constituting coxiset A5. Species of coxiset A5, which are both amicronucleate, were grouped as sister taxa with high support values. However, this coxiset was not supported by the histone H3II/H4II sequences (Sadler and Brunk, 1992) and 23S rRNA (Nanney et al., 1998) phylogenies. The two remaining species in riboset A1—*T. malaccensis* and *T. thermophila*—were recovered in *cox1* analyses with 99–100% support values and 1.00 posterior probability; coxiset A1 was observed in the mitochondrial LSUα rRNA phylogeny, as well as several nuclear gene phylogenies, such as the histone H3II/H4II (Brunk et al., 1990; Sadler and Brunk, 1992), the SSUrRNA (Results; Brandl et al., 2005; Sogin et al., 1986; Strüder-Kypke et al., 2001; Wright and Lynn, 1995), and the TER sequences (Ye and Romero, 2002). However, the relationships of *T. malaccensis* and *T. thermophila* (coxiset A1) and *T. elliotti* and *T. mimbres* (coxiset A5) were not supported by isozyme analysis, as these pairs of species showed low isozyme similarity coefficients: 39% for the former pair and 15% for the latter pair (Meyer and Nanney, 1987).

Riboset A2 has increased in size to include several additional taxa, including *T. bergeri*, *T. farleyi*, *T. lwoffi*, *T. mobilis*, *Tetrahymena* sp.2 (CO), *Tetrahymena* sp.3 (RA9), *Tetrahymena* sp.5 (NI), and *Tetrahymena* sp.6 (Brandl). *T. elliotti* of riboset A1 and the original members of riboset A2 are interspersed among four different coxisets—A2, A3, A4, and A5. Coxiset A2—*T. borealis*, *T. canadensis*, and *T. rostrata*—was supported by high isozyme similarity coefficients and also recovered in the histone H3II/H4II phylogeny (Brunk et al., 1990; Sadler and Brunk, 1992). In addition to *T. furgasoni* and *T. tropicalis*, seven additional species are included in coxiset A3: *T. farleyi*, *T. lwoffi*, *T. mobilis*, *Tetrahymena* sp.2 (CO), *Tetrahymena* sp.3 (RA9), *Tetrahymena* sp.5 (NI), and *Tetrahymena* sp.6 (Brandl). The SSUrRNA gene tree shows a similar result in grouping *T. farleyi* and *T. mobilis* together with species of this coxiset (Brandl et al., 2005; Strüder-Kypke et al., 2001). *T. mobilis* and *T. tropicalis* were grouped together as sister taxa as reported in previous SSUrRNA gene phylogenies (Brandl et al., 2005; Strüder-Kypke et al., 2001). Furthermore, Meyer and Nanney (1987) indicated some similarity between *T. furgasoni* and *T. lwoffi* based on their indistinguishable isozyme patterns.

It is claimed that amicronucleate *Tetrahymena* species have been repeatedly derived from micronucleate sexual species (McCoy, 1975; Meyer and Nanney, 1987; Preparata et al., 1989). Analysis of the histone H3II/H4II sequences of *T. furgasoni* (amicronucleate) and *T. tropicalis* (micronucleate), which show a unique 11-bp deletion, led Brunk et al. (1990) to support this hypothesis and suggest that the former species was derived from the latter by the loss of the micronucleus some time during its evolutionary history. Our results based on SSUrRNA and *cox1* analyses showed the clustering between *T. tropicalis* and another micronucleate spe-

cies, *T. mobilis* with high support values. Except for the amicronucleate *T. pyriformis* (isozyme phenoset A), which grouped with the micronucleate species, *T. setosa*, all other amicronucleate "species" first grouped most closely with another amicronucleate species: *T. elliotti* grouped with *T. mimbres* and *T. furgasoni* grouped with *T. lwoffi*. It is noteworthy that these amicronucleate species, *T. elliotti*, *T. furgasoni*, *T. lwoffi*, and *T. mimbres*, were placed in different isozyme phenosets—B, C, E, and G, respectively, (Nanney, 1989; Nanney and McCoy, 1976). Nevertheless, these "amicronucleate clades" are embedded in clades with micronucleate members, consistent with the hypothesis that amicronucleate *Tetrahymena* have been repeatedly derived from micronucleates.

Tetrahymena bergeri, *T. corlissi*, and *T. limacis* constitute coxiset A4 with 0.85 posterior probability support. They are all parasites of various animals and have been assigned to the *rostrata* complex (Corliss, 1979). Although they exhibit a parasitic mode of life, they differ from each other by several morphological and biological characteristics (Corliss, 1979; Strüder-Kypke et al., 2001). Furthermore, *T. corlissi* and *T. limacis* were ambiguously and inconsistently positioned at several different places in the equally parsimonious trees inferred from histone H3II/H4II (Sadler and Brunk, 1992). However, the sister grouping of *T. bergeri* and *T. corlissi* was observed in previous SSUrRNA phylogenies (Brandl et al., 2005; Strüder-Kypke et al., 2001).

4.3.2. Phylogenetic relationships within coxiset B

The present study recovered riboset B and also included a new species *Tetrahymena* sp.1 (Foissner). Species of riboset B are divided into coxisets B1 and B2. Coxiset B1 includes the three original riboset B species—*T. leucophrys*, *T. pyriformis*, and *T. setosa*—plus one additional species, *Tetrahymena* sp.1 (Foissner). Coxiset B1 was also found in the histone H3II/H4II phylogeny although the grouping was not supported by isozyme analysis as shown by low similarity coefficients among them (Meyer and Nanney, 1987; Sadler and Brunk, 1992). However, the grouping of *T. pyriformis* and *T. setosa* was recovered with high support values in previous SSUrRNA phylogenies (Brandl et al., 2005; Strüder-Kypke et al., 2001). Coxiset B2 species—*T. silvana* and *T. vorax*—were clustered with 1.00 posterior probability; they were adjacent species, but not sister species, in our SSUrRNA analyses. These two species belong to the *patula* complex of macrostome and microstome transformers and show some degree of isozyme similarity (54%) (Meyer and Nanney, 1987). Furthermore, histone H3II/H4II and TER phylogenies also support the coxiset B2 grouping (Sadler and Brunk, 1992; Ye and Romero, 2002). This grouping is yet another example of convergent life cycles of the macrostome and microstome transforming capabilities: these two species are distantly related to other members of the former *patula* complex—*T. patula* (coxiset C) and *T. paravorax* (coxiset C).

4.3.3. Phylogenetic relationships within coxiset C

The species composition of riboset C is identical to the previously published ones, but with the addition of 4 recently sequenced species—*T. cosmopolitanis*, *T. empidokyrea*, *T. shanghaiensis*, and *Tetrahymena* sp.4 (SIN). The riboset C members, excluding *T. empidokyrea*, form the largest group in coxiset C whose members are derived mostly from the *australis* group with the exception of *T. paravorax*, which belongs to riboset D. Although the posterior probability for this coxiset at 0.76 is not high, several groupings within the coxiset are highly supported in our study. The original 11 riboset C species were recovered and supported with 100% bootstrap value by the histone H3II/H4II sequences (Sadler and Brunk, 1992). A subset of species of coxiset C were placed within the same cluster with high support values in several phylogenies inferred from various molecules, such as TER (Ye and Romero, 2002), SSUrRNA (Brandl et al., 2005; Sogin et al., 1986; Strüder-Kypke et al., 2001; Wright and Lynn, 1995), and isozymes (Nanney et al., 1989). For example, *T. hegewischi* has been placed as a sister taxon of *T. americanis* within riboset C with 1.00% and 100% support values as confirmed by the lack of nucleotide difference in the SSUrRNA and LSUrRNA gene sequences and a high isozyme similarity coefficient (87%) (Meyer and Nanney, 1987; Nanney et al., 1998).

4.3.4. Tetrahymena paravorax: riboset D or coxiset C

Tetrahymena paravorax, which is very divergent from other *Tetrahymena* species, has always been considered as an aberrant *Tetrahymena* (Nanney et al., 1998; Preparata et al., 1989). This species was placed separately from the two major groups in the SSUrRNA gene NJ tree as it was in the phylogenies deduced from the histone H3II/H4II (Brunk et al., 1990; Sadler and Brunk, 1992) and the TER sequences (Ye and Romero, 2002). However, *T. paravorax* was placed within the *borealis* group in the ML tree, or close to the base of this group and close to *T. caudata* in the MB tree. In contrast to the histone H3II/H4II sequence data (Brunk et al., 1990), the sequence divergence based on the SSUrRNA gene indicates that *T. paravorax* is more closely related to *G. chattoni* (3.36%) than it is to the other species of *Tetrahymena* (7.32%). This distant relationship between *T. paravorax* and other *Tetrahymena* species is similar to the result using LSUrRNA sequence data (Nanney et al., 1998; Preparata et al., 1989). However, this is not in agreement with the morphological data, which clearly show that *T. paravorax* is a *Tetrahymena* species (Brunk et al., 1990; Corliss, 1957). Consistent with this, our *cox1* analyses clustered *T. paravorax* within the *Tetrahymena* clade.

5. Conclusions

Although the two major clades—*australis* and *borealis*—as well as the nine ribosets were recovered by our SSUrRNA gene phylogeny, several genealogical relationships were not well supported as indicated by low support values. These uncertainties in relationships are caused by the highly conserved nature of the rRNA genes (Nanney et al., 1989, 1998; Preparata et al., 1989; Strüder-Kypke et al., 2001). In the present study, only 100 sites out of 1,639 in the SSUrRNA gene were parsimony-informative whereas 578 characters out of 1821 in the *cox1* gene were parsimony-informative. However, some consistent relationships could be drawn from this SSUrRNA gene phylogeny. For example, the sister taxon groupings between *T. bergeri* and *T. corlissi*, *T. elliotti* and *T. mimbres*, *T. americanis* and *T. hegewischi*, and *T. malaccensis* and *T. thermophila* were recovered with at least or higher than 0.92 posterior probabilities and 87% bootstrap values. Several of these relationships were also found by others: Brandl et al. (2005) and Strüder-Kypke et al. (2001) demonstrated the sister taxon status between *T. bergeri* and *T. corlissi*. Furthermore, in our study, the three classical species complexes—*pyriformis*, *rostrata*, and *patula*—have again proven to be paraphyletic as previously shown by others (Nanney et al., 1989, 1998; Preparata et al., 1989; Strüder-Kypke et al., 2001). Finally, our study demonstrated the potential usefulnesses of *cox1*, which is being widely used both as a species diagnostic marker and for inferring phylogenetic relatednesses of organisms, in particular closely allied species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.09.017.

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