Phylogeny of *Neobursaridium* reshapes the systematics of *Paramecium* (Oligohymenophorea, Ciliophora)

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Abstract

The subclass Peniculia (Oligohymenophorea, Intramacronucleata) is one of the most known groups of the phylum Ciliophora, being composed by very notorious representatives, such as *Paramecium* and *Frontonia*. Nevertheless, phylogenetic relationships among genera within this subclass are still far from being resolved. Moreover, for several members of the group the characterization by molecular markers is still lacking, such as for *Wenrichia, Clathrostoma, Paraclathrostoma, Didieria* and, to date, also for *Neobursaridium*. The finding of one strain of *Neobursaridium gigas* from India led to the first molecular characterization of this uncommonly sampled ciliate. The 18S rDNA sequence and the COI sequence were obtained and used for phylogenetic analyses. Moreover, the partial mitochondrial genome of *N. gigas* was sequenced, annotated and employed for phylogenomics analysis. To increase the sampling effort for the *Paramecium* clade, several newly obtained 18S rDNA sequences of parameciids are herein presented. Unexpectedly, the inclusion of *N. gigas's* molecular data in phylogenetics/phylogenomics analyses did not help to solve the complex evolution relationships inside Peniculia. Conversely, it raised new and intriguing questions about *Paramecium* phylogeny, since *N. gigas* clustered inside *Paramecium* clade as sister species of *Paramecium bursaria* in all the performed analyses. A critical revision of past and present data led to rename *N. gigas* as *Paramecium gigas* (Balech, 1941) comb. nov., and triggered the revision of genus *Paramecium*, with the proposal of the new subgenus *Gigaparamecium* subgen. nov. Hypotheses on the evolution of giant morphologies in ciliates are also discussed.

1 INTRODUCTION

The subclass Peniculia (Oligohymenophorea, Intramacronucleata) is one of the most studied groups of the phylum Ciliophora, being composed by very notorious ciliates such as *Paramecium* and *Frontonia*. To date, this subclass includes also less famous members, such as *Apofrontonia, Stokesia, Lembadion, Paranassula, Disematostoma, Marituja, Wenrichia, Clathrostoma, Paraclathrostoma* and *Didieria* (Aescht, 2001; Corliss, 1979; Dragesco, 1970; Dragesco & Dragesco-Kerneis, 1986; Foissner et al., 1994; Jankowski, 2007; Lee et al., 2000; Lynn, 2008; Przybos & Tarcz, 2018; Puytorac et al., 1987), while other genera are not yet formally accepted (Jankowski, 2007) (i.e. *Parastokesia, Frontoniella* and *Parafrontonia*).

Despite extensive studies, the phylogeny of Peniculia is still under debate, mostly because the phylogeny of *Frontonia* is not yet resolved. Indeed, the *Frontonia* monophyly is not supported by phylogenetic reconstructions based on the 18S rDNA (Andreoli et al., 2007; Fokin et al., 2019; Gao

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et al., 2016; Zhao et al., 2016), and based on the mitochondrial COI encoding gene (Zhao et al., 2016).

Recently, some rare members of Peniculia have been molecularly characterized, such as *Disematostoma* (Rossi et al., 2016; Xu et al., 2018) and *Marituja* (Xu et al., 2018). Nevertheless, the obtained phylogenetic reconstructions appeared even more problematic, underlining the paraphyly of *Marituja* and *Disematostoma* (Xu et al., 2018).

As a matter of fact, for several other taxa member of the subclass such as *Wenrichia, Clathrostoma, Paraclathrostoma, Didieria* and *Neobursaridium*, the characterization through molecular markers is still lacking. In the absence of molecular data for these members, the phylogeny of the whole group remains incomplete and liable to inaccuracies.

Neobursaridium gigas, described for the first time by Balech in 1941, is a peniculid not frequently observed. This ciliate was retrieved only few times from distant locations all over the world: Argentina (Balech, 1941), Uganda (Beadle & Nilsson, 1959; Nilsson, 1962, 1969; Thurston, 1964), Gabon, Cameroon (Dragesco, 1970), Central African Republic and some other African countries (Dragesco & Dragesco-Kerneis, 1986), India, nearby Calcutta (Mahajan & Nair, 1971), Brazil (Dias, 2007; Kattar, 1972, 1975), and Thailand (Charubhun & Charubhun, 2000). Being the ciliate prevalently found in tropical regions, its geographical distribution has been defined as 'pan-tropical' (Dragesco & Tuffrau, 1967).

Neobursaridium gigas was described as a huge ciliate (550 × 275 μm on average) (Nilsson, 1962) capable to engulf/feed on a variety of organisms, from other microeukaryotes, including ciliates, to different kind of bacteria (Dragesco & Tuffrau, 1967). It shows a holotrichous ciliature, a dorso-ventrally flattened body, two contractile vacuoles with long collecting channels, one dumbbell-shaped macronucleus, several micronuclei, and trichocysts.

Initially, Balech placed *Neobursaridium* in the Bursariidae family (formerly placed in class Heterotrichea and later moved to the class Colpodea—Lynn, 1980, 2008), based on its large peristome, resembling those of *Bursaria* and *Bursaridium*. Later on, other authors identified in *Neobursaridium* the oral structures typical of Peniculia, namely two peniculi and one quadrulus (Dragesco & Tuffrau, 1967), and the presence of trichocysts similar to those of *Paramecium* (Dragesco, 1968). Accordingly, *Neobursaridium* was assigned to the subclass Peniculia (Oligohymenophorea) in the novel family Neobursariidae (Dragesco & Tuffrau, 1967) (Dragesco, 1970). However, Jankowski (2007) rejected the family Neobursariidae, ranking it as a subfamily within the family Parameciidae (Dujardin, 1840).

To evaluate the phylogenetic relationships of *Neobursaridium* within Peniculia, molecular data, lacking at present, are necessary. The present study fills the gap presenting a new report of *N. gigas* from India, for which both the morphological identification and, for the first time, the characterization of different molecular markers have been carried out. Moreover, following the next-generation taxonomy workflow (Serra et al., 2019), we integrated the ciliate description by providing the mitochondrial genome of such uncommon organism.

The present study also provides the characterization of several other oligohymenophorean representatives, with special attention to *Paramecium* genus. Detailed phylogenetic reconstructions of peniculid relationships based on the 18S rDNA sequence are presented including the novel sequences, in particular the first sequence of *N. gigas*. Additionally, phylogenetic analysis for *N. gigas* based on COI gene sequence has been carried out, as well as a comparative analysis on its mitochondrial genome and the following phylogenomic reconstruction. All the performed analyses

led to a rather unexpected result, indicating that *N. gigas* is phylogenetically nested within genus *Paramecium*. On this basis and according to a critical revision of literature data, we herein propose a new taxonomic rank for the ciliate and provide a revision of genus *Paramecium*.

2 MATERIALS AND METHODS

2.1 Sampling and culturing

2.1.1 Neobursaridium gigas

Specimens of *N. gigas* were sampled on the SH2 site, a freshwater stream nearby Borra Caves located on the East Coast of India, in the Ananthagiri hills of the Araku Valley of Visakhapatnam district, Andhra Pradesh, India (N 18°16′32.3″ E 83°02′15.6″; 27 January 2014). Ciliates were isolated and fed on *Raoultella planticola* in Cherophyll medium. Polyclonal culture survived for some weeks under laboratory conditions, but unfortunately disappeared during transportation from India to Italy. Images and *in vivo* observations were made by using an Axio Lab.A1 (Zeiss) microscope.

2.1.2 Other oligohymenophoreans

Water and sediment samples were collected in some areas of Andhra Pradesh and Odisha states (India) in different periods, from 2013 to 2016. Samples of sediment and water were collected using 50-ml falcon tubes (about 15–20 ml of sediment and water up to a total volume of 50 ml). Aliquots of each sample (about 15–20 ml) were stored in Petri dishes and maintained in humid chambers to avoid dehydration.

To start ciliate cultures, 1–10 cells of each species were collected from the original sample using a micropipette and washed thrice in clean mineral water for several times and finally placed in a depression slide with clean water and a few drops of culture medium containing *R. planticola* as food.

In total, we analysed 30 strains of *Paramecium*, and a single strain of both *Tetrahymena* and *Urocentrum*. Details of strains and sampling sites are shown in Table 1.

TABLE 1. List of Indian strains in analysis and correspondent sampling sites

Strain	Species	Sampling site	Date	Coordinates
B1	*Paramecium* cf. *aurelia*	Boddam pond, Visakhapatnam (AP)	Mar 2013	N 18°0′57.6″, E 83°9′40.319″
BB	*Paramecium* cf. *aurelia*	Boddam pond, Visakhapatnam (AP)	Mar 2013	N 18°0′57.6″, E 83°9′40.319″
C1	*Paramecium* cf. *aurelia*	Chilika Lake (Od)	Feb 2013	N 19°50′21.599″, E 85°24′36.961″
KKV16	*Paramecium* cf. *aurelia*	Kolleru Lake (AP)	Apr 2014	N 16°36′48.4″, E 81°18′32.7″
KKV17A	*Paramecium* cf. *aurelia*	Kolleru Lake (AP)	Apr 2014	N 16°36′48.4″, E 81°18′32.7″
KTC9	*Paramecium* cf. *aurelia*	Kolleru Lake (AP)	Jun 2014	N 16°43′10.2″, E 81°19′34.7″
TP1	*Paramecium* cf. *aurelia*	Kolleru Lake (AP)	Sep 2014	N 16°44′16.0″, E 81°24′18.0″
KU8	*Paramecium* cf. *aurelia*	Kulai Cheruvu (AP)	Dec 2014	N 16°57′41.116″, E 82°13′59.991″
KN1	*Paramecium* cf. *aurelia*	Konam (AP)	May 2015	N 17°58′13.95″, E 82°51′13.34″

Strain	Species	Sampling site	Date	Coordinates
Me4	*Paramecium* sp.	Pond in Meghadri Gedda, Visakhapatnam (AP)	Jan 2013	N 17°50′26.581″, E 83°24′23.04″
BJ4	*Paramecium* sp.	Balugaon Jetty, Pond near Chilika Lake (Od)	Feb 2014	N 19°44′37.021″, E 85°12′44.398″
C18b	*Paramecium* sp.	Station 18 in Chilika Lake (Od)	Feb 2014	N 19°41′40.571″, E 85°25′18.958″
KWBL12	*Paramecium* sp.	Kolleru Lake (AP)	Aug 2014	N 16°36′48.4″, E 81°18′32.7″
TBS17	*Paramecium* cf. *calkinsi*	Kolleru Lake (AP)	Aug 2014	N 16°36′48.4″, E 81°18′32.7″
A522	*Paramecium* cf. *calkinsi*	Kolleru Lake (AP)	Aug 2014	N 16°36′05.0″, E 81°18′47.8″
ML1	*Paramecium* cf. *jenningsi*	Mudasarlova reservoir (AP)	Sep 2014	N 17°45′53.08″, E 83°17′48.03″
HC3	*Paramecium* cf. *jenningsi*	Andhra University Horticulture (AP)	Aug 2014	N 17°43′31.14″, E 83°19′33.52″
JD1	*Paramecium* cf. *jenningsi*	Jagaram (AP)	Sep 2014	N 18°03′48.39″, E 83°12′28.57″
Sm_Pm2	*Paramecium multimicronucleatum*	Pond near Simhachalam temple, Visakhapatnam (AP)	Feb 2013	N 17°46′13.26″, E 83°14′9.121″
OP6-2	*Paramecium multimicronucleatum*	Pond near Chilika Lake (Od)	Feb 2014	N 19°45′21.179″, E 85°12′38.854″
KP	*Paramecium multimicronucleatum*	River in Kasipatnam area on Araku Hills, Visakhapatnam (AP)	Mar 2013	N 18°12′59.825″, E 83°6′19.137″
PC2	*Paramecium multimicronucleatum*	Pedda Cheruvu, Vizianagaram district (AP)	Nov 2014	N 18°6′34.025″, E 83°24′0.835″
OP2-2	*Paramecium multimicronucleatum*	Pond near Chilika Lake (Od)	Feb 2014	N 19°44′0.33″, E 85°11′58.372″
KKV3	*Paramecium multimicronucleatum*	Kolleru Lake (AP)	Apr 2014	N 16°36′48.4″, E 81°18′32.7″
AC2	*Paramecium multimicronucleatum*	Andhra University Audit cell (AP)	Jun 2015	N 17°43′31.47″, E 83°19′27.56″
GD1	*Paramecium polycaryum*	Godavari (AP)	Aug 2015	N 17°00′06.89″, E 81°45′55.00″
DK	*Paramecium polycaryum*	Dharmavaram (AP)	Jun 2015	N 18°07′15.45″, E 83°11′45.36″
TBS3	*Paramecium polycaryum*	Kolleru Lake (AP)	Aug 2014	N 16°36′48.4″, E 81°18′32.7″
PC22	*Paramecium polycaryum*	Pedda Cheruvu, Vizianagaram district (AP)	Nov 2014	N 18°6′34.025″, E 83°24′0.835″
BM	*Paramecium schewiakoffi*	Boddam pond, Visakhapatnam (AP)	Oct 2014	N 18°06′48.72″, E 83°14′30.99″
MU3	*Tetrahymena* sp.	Mudasarlova Reservoir (AP)	Apr 2015	N 17°45′53.08″, E 83°17′48.03″
KP6	*Urocentrum* sp.	River in Kasipatnam area on Araku Hills, Visakhapatnam (AP)	Jan 2014	N 18°12′59.825″, E 83°6′19.137″

Abbreviations: AP, Andhra Pradesh; Od, Odisha.

2.2 Morphological analyses

For *N. gigas*, only live observations were possible due to the complete disappearance of the population during transportation to Italy. Living cells were inspected and photographed using an Axio Lab.A1 (Zeiss) microscope.

For other investigated ciliates, live observations and images were captured with an Axio Lab.A1 microscope and/or by an Orthoplan Leitz microscope equipped with differential interference contrast, as well as a Leica DMR microscope at a ×300–1,250 magnification. Morphological characterization of ciliates was performed by in vivo observation and using the following staining techniques: Chatton-Lwoff silver nitrate method and Feulgen staining. To stain the ciliary pattern, ciliates were treated for silver staining analysis with Champy's solution and then with silver nitrate according to Corliss (1953). Feulgen staining procedure was performed to reveal the nuclear apparatus, using a protocol modified from Dragesco and Dragesco-Kernéis (1986), after cell fixation with celloidin-diethyl ether-alcohol solution.

2.3 Molecular analyses

2.3.1 DNA extraction

In the present work we molecularly characterized a single *N. gigas* strain, 30 *Paramecium* strains, plus two additional strains of oligohymenophoreans from India: MU3 *Tetrahymena* sp. and KP6 *Urocentrum* sp. (Table 1).

For *N. gigas*, five cells were carefully washed few times in sterile distilled water and then stored in 70% Ethanol at −20°C.

For the other oligohymenophoreans, 20–150 cells of each strain were isolated and stored in 70% Ethanol at −20°C. Genomic extractions were performed using a NucleoSpin™ Plant II kit (Macherey-Nagel).

2.3.2 Amplification and sequencing of 18S rDNA gene

The polymerase chain reaction (PCR) was carried out with the following primers: 18S F9 and 18S R1513Hypo. All PCRs were performed in a 40 µl reaction volume with 0.25 µl of each primer (100 µM), TaKaRa PCR reagents and ExTaq polymerase (Takara Bio) using a C1000™ Thermal Cycler (Bio-Rad). The PCR programme used was as follows: initial denaturation step at 94°C for 3 min, followed by 35 cycles (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 2 min), and final elongation step at 72° for 6 min. PCR products were cleaned with the EuroGOLD Cycle-Pure kit (EuroClone) and sent to GATC Biotech Company for sequencing with the following internal primers: 18S R536, 18S R1052 and 18S F783. Details of primers used for 18S rDNA amplification and sequencing are shown in Table 2. Sequencing results have been analysed with specific software (Chromas, Serial Cloner) and compared with sequences already present in online repositories (National Center for Biotechnology Information—NCBI).

TABLE 2. List of primers used for 18S rDNA gene amplification and sequencing

Name	Sequence (5′−3′)	Use	Type	Reference
18S F9	CTG GTT GAT CCT GCC AG	PCR	Forward	Medlin et al. (1988)
18S R1513 Hypo	TGA TCC TTC YGC AGG TTC	PCR	Reverse	Petroni et al. (2002)
18S R536	CTG GAA TTA CCG CGG CTG	SEQ	Reverse	Rosati et al. (2004)
18S R1052	AAC TAA GAA CGG CCA TGC A	SEQ	Reverse	Rosati et al. (2004)
18S F783	GAC GAT CAG ATA CCG TC	SEQ	Forward	Rosati et al. (2004)

Abbreviations: PCR, polymerase chain reaction; SEQ, sequencing.

2.3.3 Whole-genome amplification and genome assembly

The total extracted DNA from *N. gigas* was amplified via whole-genome amplification (WGA) method, using REPLI-g Single Cell Kit (QIAGEN®), following the manufacturer's instructions for purified DNA input material. The amplified DNA was processed with a Nextera XT library and shotgun sequenced at Admera Health, using Illumina HiSeq X technology to generate a total of

65,759,992 reads (paired-ends 2 × 150 bp). Preliminary assembly of the total reads was performed using SPAdes software (v 3.6.0) (Bankevich et al., 2012).

2.3.4 Mitochondrial genome assembly and annotation

The mitochondrial genome of *Paramecium bursaria* was downloaded from BIGD database (He et al., 2019) and annotated using PROKKA 1.10 and blastp searches against NCBI nr database to allow the comparison with the other mitochondrial genomes. Contigs representing the *N. gigas* mitochondrial genome were initially identified by tblastn searches using as queries proteins from reference genomes downloaded from NCBI (i.e. *Paramecium caudatum* (NC_014262), *Paramecium aurelia* (NC_001324) and *Tetrahymena thermophila* (NC_003029)) and the newly annotated *P. bursaria* mitogenome. Selected contigs were then reassembled using the Blobology pipeline (Kumar et al., 2013), and the final output was manually checked. The resulting partial genome sequence was annotated using PROKKA 1.10 (Seemann, 2014), setting the DNA translation codon table '4', and then manually checked. The COI gene sequence of *N. gigas* used for the subsequent phylogenetic analysis was predicted using PROKKA 1.10.

2.3.5 Phylogenetic analyses

The obtained 18S rDNA sequences were aligned with the automatic aligner of the ARB software package version 5.5 (Westram et al., 2011) on the SSU ref NR99 SILVA database (Quast et al., 2012).

For the analysis of the position of *N. gigas* inside Peniculia, 69 18S rDNA sequences of other members of Peniculia plus 12 sequences of other Oligohymenophorea as outgroup were selected (data set 1).

For the analysis of the position of our *Paramecium* sequences inside Peniculia, 108 18S rDNA sequences of other members of Peniculia were selected (data set 2). In this data set were also included four *Paramecium* sequences from India already published in previous work: TP2 *Paramecium multimicronucleatum* (LT549005), PC6 *P. multimicronucleatum* (LT549006), SH2 *P. caudatum* (LT549004) and BJ1 *Paramecium jenningsi* (LT549003) (Serra et al., 2016). Sequences not shown in the resulting tree are listed in Table S1.

After manual editing to optimize base pairing in the predicted rRNA stem regions in each data set, the two alignments were trimmed at both ends to the length of the shortest sequence. Resulting matrices contained 1,088 nucleotide columns (data set 1 and data set 2), which were used for phylogeny and for the identity matrix calculation.

A third data set was obtained using the amino acidic sequence of the COI gene of *N. gigas* (obtained by the annotation of mitochondrial genome sequences), plus other selected COI sequences downloaded from NCBI database. For the analysis, 66 sequences belonging to *Paramecium*, 32 to *Frontonia*, two to *Stokesia*, one to *Lembadion* and five to other Oligohymenophorea as outgroup (data set 3) were selected. Sequences not shown in the resulting tree are listed in Table S1. The sequences were aligned with the automatic aligner of the ARB software package version 5.5 (Westram et al., 2011).

After manual editing to optimize the alignment of translated amino acidic sequences, data set 3 was trimmed at both ends to the length of the shortest sequence. Resulting matrix contained 224 amino acidic columns, which were used for phylogeny.

For data sets 1 and 2, the optimal substitution model was selected with jModelTest 2.1 (Darriba et al., 2012), and for data set 3 with ProtTest3 (Darriba et al., 2011), according to the Akaike

Information Criterion (AIC). Maximum likelihood (ML) trees for data sets 1 and 2 were inferred with the PHYML software version 2.4 (Guindon & Gascuel, 2003) from the ARB package, performing 1,000 pseudo-replicates. ML tree for data set 3 was obtained with the RAxML software version 8.0 (Stamatakis, 2014), performing 100 pseudo-replicates. Bayesian inference (BI) trees were inferred for all data sets with MrBayes 3.2 (Ronquist et al., 2012), using three runs each with one cold and three heated Monte Carlo Markov chains, with a burn-in of 25%, iterating for 1,000,000 generations (data sets 1 and 2) or 2,000,000 generations (data set 3), until reaching convergence, according to the average deviation standard parameter.

2.3.6 Phylogenomic analysis on mitochondrial genomes

For the mitochondrial genome-based phylogenomic analysis, amino acidic sequences of all the 24 available *Paramecium* mitochondrial genomes were downloaded from ParameciumDB database (Arnaiz et al., 2020). To this data set, we added our sequences and the ones belonging to the abovementioned *P. bursaria* (He et al., 2019). As outgroup, we employed the seven available complete mitochondrial genomes belonging to other Oligohymenophorea, downloaded from NCBI. A set of 15 protein coding genes present in all the analysed genomes were selected, and the orthologs were aligned using MAFFT v 7.45 (Katoh & Standley, 2013) and then concatenated together to obtain a final matrix composed by 6,739 sites for each organism. The best substitution model was estimated using ProtTest3 (Darriba et al., 2011), while RAxML (Stamatakis, 2014) was used to estimate ML phylogeny with 1,000 bootstraps. A list of species and protein coding genes employed in the analysis is given in Table S2.

2.3.7 Statistical tests on alternative topologies

Alternative phylogenetic hypotheses were tested placing the *N. gigas* 18S rDNA sequence in random positions inside Peniculia (data set 1). Alternative phylogenomic hypotheses were tested as well on the retrieved mitochondrial-based phylogenomic tree.

Topological tests were performed to assess whether alternative hypotheses significantly differ from our retrieved phylogenetic (18S rDNA-based ML tree; data set 1) and phylogenomic trees (Kishino & Hasegawa, 1989; Shimodaira, 2002; Shimodaira & Hasegawa, 1999; Strimmer & Rambaut, 2002).

Tests were performed on the IQ-TREE software (version 1.6.12) (Nguyen et al., 2015) using RELL method (Kishino et al., 1990) with 10,000 replicates.

3 RESULTS

3.1 *Neobursaridium gigas* strain SH2 from India

3.1.1 Morphology

Neobursaridium gigas from India appeared as a dorso-ventrally flattened, large ciliate, measuring 450–600 µm in length and 200–280 µm in width (live observations). The general body shape was elongated ovoid (oblong) with a truncated anterior part (Figure 1a). The buccal cavity aperture (Figure 1b) presented two peniculi plus a single anarchic ‘quadrulus’ strip; these oral structures were observed in live specimens, but unfortunately are not detectable in provided pictures. Several roundish inclusions (diameter ~12 µm) of unknown nature were visible in the cytoplasm (Figure 1c). *Neobursaridium gigas* cells presented invariably two big contractile vacuoles (CV), with 11–12 long collecting canals and 3–4 pores (CVP) in each CV (Figure 1a,d,e). The macronucleus was elongated and dumbbell shaped. Micronuclei were not detected. Elongated, fusiform trichocysts (length: ~7–14 µm) recalling in morphology those of *Paramecium* in resting state (Figure 1f–h) were present; inserted with their tip under the cell cortex, forming a continuous

layer (Figure 1f,g). The presence of pink inclusions located beneath the trichocyst layer determined the general pink-brownish colour of the cell (Figure 1g). These roundish cortical granule-like structures likely correspond to the 'brown inclusion bodies' reported by Nilsson (1969). However, other vesicles containing liquid pigment were also sparsely distributed in the cytoplasm (Figure 1b). While swimming, the ciliate always rotate in counter-clockwise direction in respect to the antero-posterior body axis. Unfortunately, we cannot present a detailed morphometrical analysis, because the ciliate culture died out before cells could have been processed for staining.

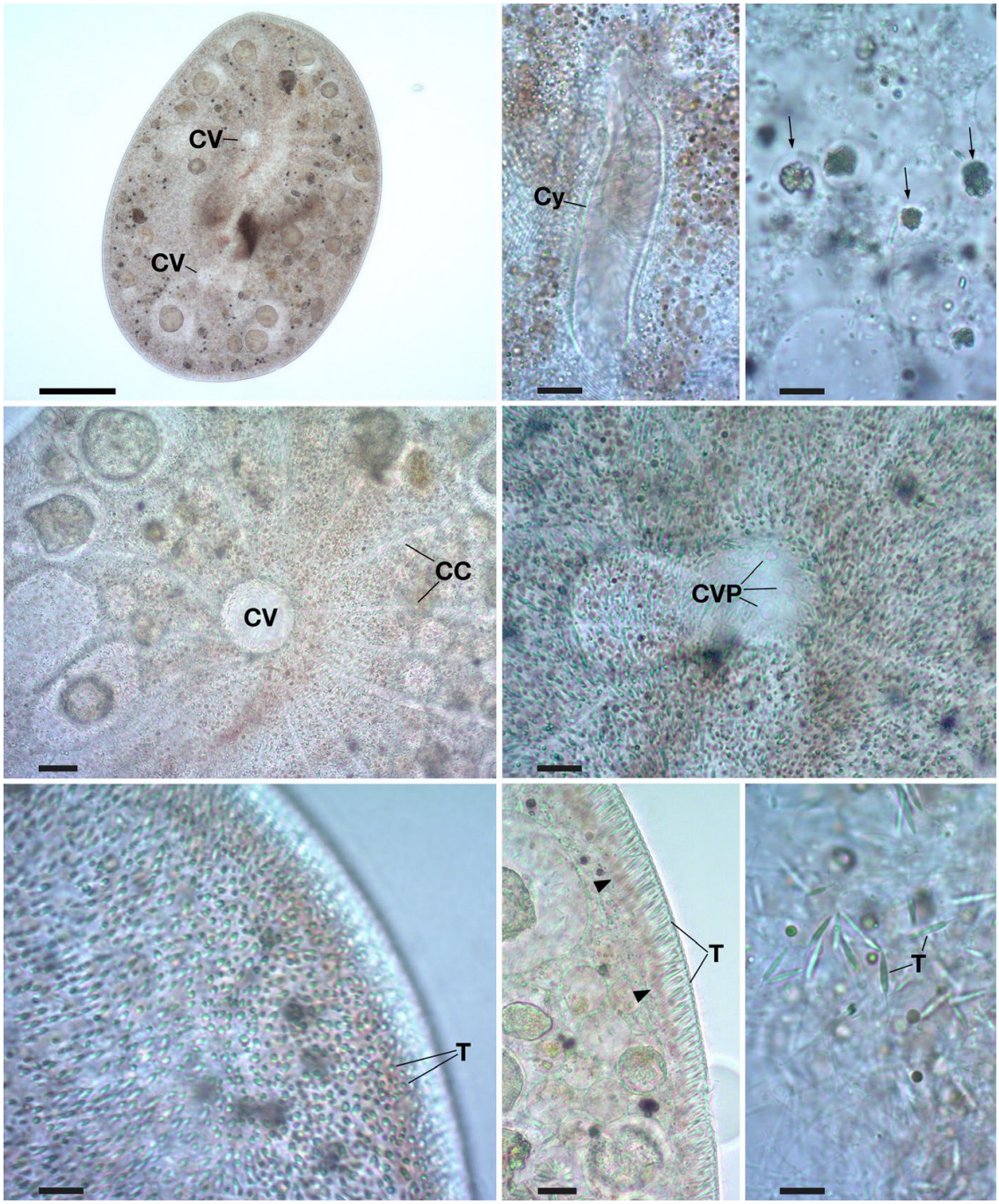


Figure 1. Live images of *Neobursaridium gigas* (*Paramecium gigas* comb. nov.) from India. (a) Whole cell of *N. gigas;* (b) Closer view of the cytostome (Cy); (c) Detail of cytoplasmic inclusions of unknown nature (arrows); (d) Contractile vacuole (CV) with long collecting canals (CC); (e) Detail of contractile vacuole pores (CVP), four are visible; (f) External surface of the cortex, showing trichocysts (T); (g) Longitudinal section of the cell, showing the trichocysts (T) disposition in the cortex and the underlying layer of roundish cortical granule-like structures (arrowhead); (h) Several trichocysts (T) after cell disruption. CC, collecting canal; CV, contractile vacuole; CVP, contractile vacuole pores; Cy, cytostome; T, trichocysts; Arrow, cytoplasmic inclusions; Arrowhead, layer of cortical granule-like structures. Scale bars: 100 µm (a); 20 µm (d); 10 µm (b, c, e, f, g, h)

3.1.2 Gene sequences

The obtained 18S rDNA gene sequence of *N. gigas* resulted 1,704 bp long and was deposited in NCBI GenBank database under the accession number MT066234 (under the name of *Paramecium gigas*; see section 4). On the online BLAST search, it showed the highest identity value with *Paramecium* cf. *bursaria* (LN869940): 94.50% (72 mismatches and 22 gaps). However, in the identity matrix derived from the alignment used for phylogenesis, the identity value slightly lowered to 93.6% (Table 3). On the other side, the 18S rDNA identity values of *N. gigas* with *Paramecium* members ranged between 89.4% and 94.0% (with the highest with *Paramecium putrinum* and *Paramecium buetschlii*) (Table 3).

TABLE 3. Identity values among 18S rDNA sequences of selected *Peniculia* species

	a.	**b.**	**c.**	**d.**	**e.**	**f.**	**g.**	**h.**	**i.**	**j.**	**k.**	**l.**	**m.**	**n.**	**o.**	**p.**	**q.**	**r.**	**s.**	**t.**	**u.**	**v.**	**w.**
a. *Paramecium tetraurelia* AB252008	—																						
b. *Paramecium jenningsi* AF100311	99.8	—																					
c. *Paramecium schewiakoffi* AJ548821	99	99.2	—																				
d. *Paramecium multimicronucleatum* AB252006	96.1	96.1	96.1	—																			
e. *Paramecium chlorelligerum* KX669631	91.5	91.6	91.4	91	—																		
f. *Paramecium calkinsi* AF100301	92.6	92.5	92.3	91.7	90.8	—																	
g. *Paramecium polycaryum* AF100313	91.4	91.3	91.4	91.6	90	92.8	—																
h. *Paramecium nephridiatum* AF100316	93.7	93.6	93.5	93.4	92.1	94	93	—															
i. *Paramecium woodruffi* AF255362	93.6	93.5	93.4	93.4	92.2	94	93.2	99.6	—														
j. *P. bursaria* AB206541	90.3	90.4	90.2	91	89.1	90.2	90.1	90.8	90.6	—													
k. *Paramecium bursaria* KC495068	90.4	90.5	90.3	91.1	88.8	90.3	90.2	90.9	90.7	99.8	—												
l. *Paramecium bursaria* LN869940	90.5	90.6	89.9	91.1	89.1	90.4	89.9	91.1	90.9	98.7	98.4	—											
m. *Paramecium bursaria* AB252001	90.5	90.6	89.9	91.1	89.1	90.4	89.9	91.1	90.9	98.7	98.4	100	—										
n. *Paramecium gigas* comb.nov. MT066234 (formerly *Neobursaridium gigas*)	**91.1**	**91.1**	**91.2**	**91.7**	**90.3**	**91.1**	**89.4**	**91.7**	**91.5**	**93.5**	**93.6**	**93.5**	**93.5**	—									
o. *Paramecium buetschlii* KM091234	92	92	91.8	92	90.1	91.7	90.3	92.1	92.1	93.3	93.6	93.7	93.7	**94**	—								
p. *Paramecium putrinum* AF255360	91.7	91.7	91.6	92.5	90.3	91.5	90.9	92.3	92.1	93.1	93.4	93.1	93.1	**94**	95.4	—							
q. *Paramecium duboscqui* AM236094	92.4	92.4	92	93.2	90.7	92.3	91.9	92.9	92.9	92.8	92.9	93.2	93.2	**92.7**	95.4	94.3	—						
r. *Apofrontonia dohrni* AM072621	89.6	89.6	89.3	89	87.6	90.2	88.2	89.6	89.7	90.4	90.5	90.8	90.8	**89.7**	91.1	91	90.7	—					
s. *Frontonia leucas* AM072622	88.6	88.5	88.5	88.7	87.7	88.9	88.4	88.4	88.3	88.1	88.2	88.2	88.2	**88.6**	88.7	88.9	88.4	90.5	—				
t. *F. paramagna* KJ475304	88.4	88.3	88.3	88.7	87.3	89	88.5	88.1	88.1	87.7	87.8	87.8	87.8	**88.3**	88.2	88.5	88.4	90.5	97.8	—			
u. *Stokesia vernalis* HM030738	89.4	89.5	89.7	88.9	87.3	88.5	88.4	89.2	88.9	88.6	88.7	88.8	88.8	**87.9**	88.9	88.9	88.6	90.6	91.9	91.3	—		
v. *Lembadion bullinum* AF255358	86.9	86.9	86.8	87.4	85.7	88.1	87.8	87	87.1	87.5	87.6	87.8	87.8	**87.4**	87.6	87	87.4	89.9	90.4	90.2	90.5	—	
w. *Paranassula* sp. KC832956	88	88	87.9	88.3	87.3	88.2	88.6	89	88.7	89.3	89.4	89	89	**88.9**	89	89.8	89.3	89.2	89.7	88.8	89.1	88.2	—

Note: Sequences obtained in the present work are shown in *bold*.

The complete COI gene sequence of *N. gigas* annotated on the mitogenome sequence (see below) resulted 2,184 bp long. It showed the highest identity value with the COI gene sequence of *P. caudatum* (FN424190; protein id CAZ66803): 82.69% (alignment length: 1,681; 266 mismatches and 17 gaps).

3.1.3 Mitochondrial genome

The final assembly of the *Neobursaridium's* mitochondrial genome resulted in five linear contigs for a total of 25,329 bp (Figure 2).

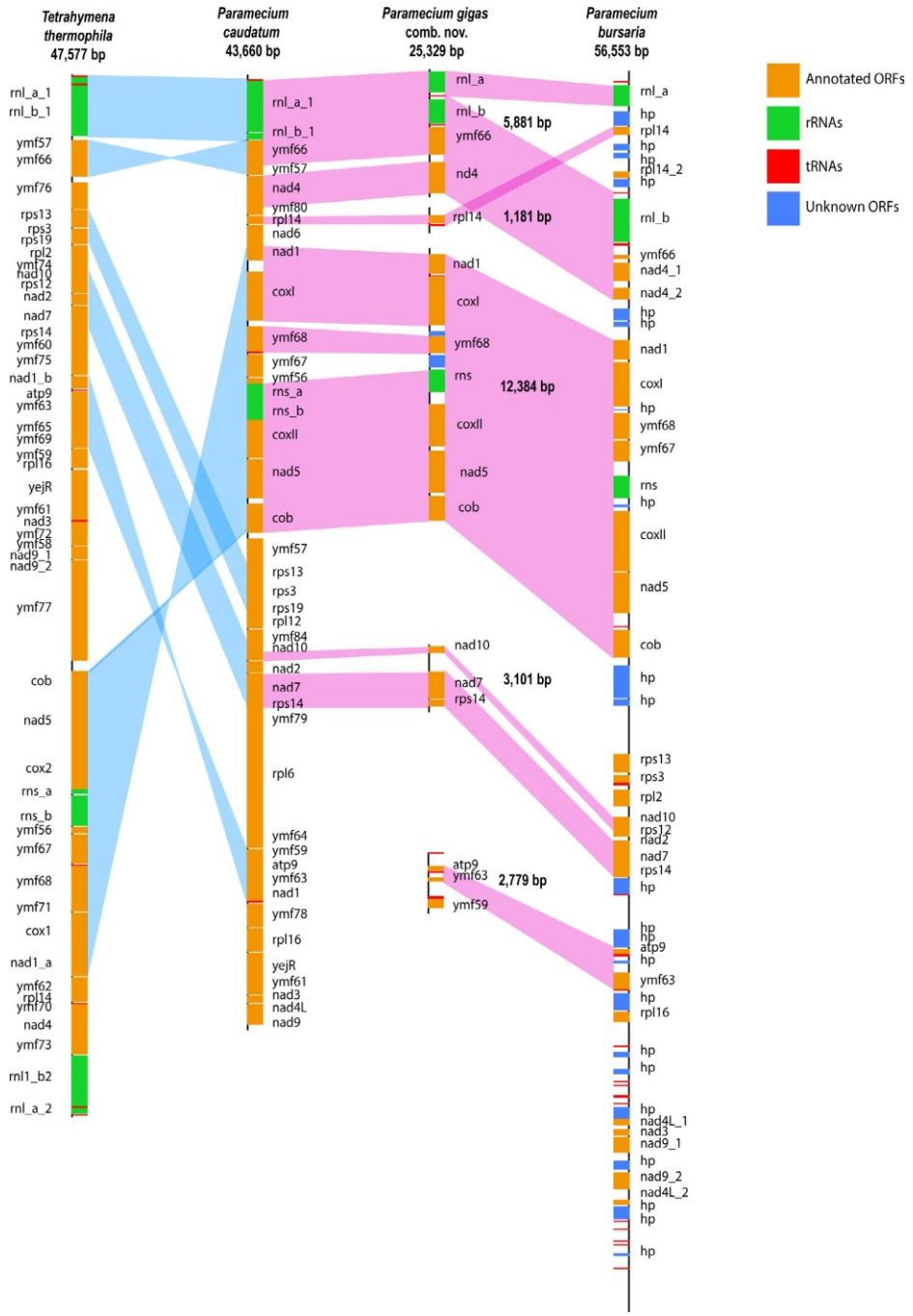


Figure 2. Gene map of the recovered mitochondrial genome of *Paramecium gigas* comb. nov. and comparison with mitogenomes from other Oligohymenophorea. Gene map of the recovered *Paramecium gigas* comb. nov. (formerly *Neobursaridium gigas*) mitochondrial genome in comparison with those belonging to *Paramecium bursaria*, *Paramecium caudatum,* and *Tetrahymena thermophila*. Protein coding split genes are suffixed by an underscore followed by a numerical character, while rRNA split genes are suffixed by an underscore followed by an alphabetical number. Syntenic regions in the analysed mitochondrial genomes are indicated by pink connectors among *Paramecium* species, and by light blue connectors between *T. thermophila* and *P. caudatum*

If compared with the complete available mitochondrial genomes of peniculids (40,469–56,553 bp) on NCBI database, those contigs are estimated to approximately represent the 50% of the whole mitochondrial genome.

The GC content of this partial mitochondrial genome so far retrieved was 23.4%. The five assembled contigs contained 17 ORFs, the 16S rRNA and the 12S rRNA genes (the first split in two separate gene sequences as for other ciliate mitochondrial genomes (Barth and Berendonk, 2011; Pritchard et al., 1990; de Graaf et al., 2009; Serra et al., 2019; Swart et al., 2011)) and five tRNA genes. For 15 out of the 17 predicted ORFs, it was possible to assign an annotation, based on either the automatic prediction made by PROKKA, or on blast analysis against the other peniculids mitochondrial genomes and against NCBI database. The predicted genes were, namely, *cox1, cox2, cob, atp9, nadh1, nadh4, nadh5, nadh7, nadh10, rpL14, rpS14, ymf59, ymf63, ymf66* and *ymf68*. All these proteins were already detected in other mitochondrial genomes from peniculids. For the remaining two predicted ORFs, it was impossible to hypothesize a function.

It has been shown that mitochondrial genomes of *Paramecium* retrieved in previous studies were syntenic (See Figure S1 in Arnaiz et al., 2020); therefore, we selected the mitochondrial genome of *P. caudatum* (Barth and Berendonk, 2011) as reference for comparison in the present analysis.

The retrieved contigs of *N. gigas* showed an overall synteny with the mitochondrion of *P. bursaria* (He et al., 2019) and *P. caudatum* (Berendonk et al., 2011), with few exceptions. One was represented by the contig including the gene homologous to the *ymf59* gene (Figure 2). Indeed, this contig showed a significant difference in gene content and order in comparison with the supposed homologous region in *P. caudatum* and *P. bursaria* mitogenomes.

Another exception was represented by the gene homologous to *rpL14*, which in *P. caudatum* and the hypothesized mitogenome structure of *N. gigas* occupied a different position in comparison with *P. bursaria* (Figure 2). It is worth to notice that the mitochondrial genome of *P. bursaria* seemed to possess a lower degree of synteny with other paramecia and therefore with *N. gigas*.

Although the gene content of the mitochondrial genome of *N. gigas* resulted similar to the one of *T. thermophila*, genes in the mitochondrial genome of *N. gigas* are likely to be organized in a different order. Thus, the degree of synteny between *N. gigas* and *T. thermophila* is lower in respect with the one between *N. gigas* and other parameciids (Figure 2).

The annotated mitogenome of *N. gigas* has been deposited in NCBI database under the name of *P. gigas*: MT622823.

3.1.4 Phylogeny and phylogenomic

Retrieved phylogenies based on the 18S rDNA gene (Figures 3 and 4), on the COI sequence (Figure 5), and the phylogenomic analysis (Figure 6) placed *N. gigas* inside the *Paramecium* clade always with extremely high statistical support: 18S rDNA trees—100/1.00, 99/1.00 (Figures 3 and 4); COI tree—98/1.00 (Figure 5); mitochondrial genome-based tree—100 (Figure 6).

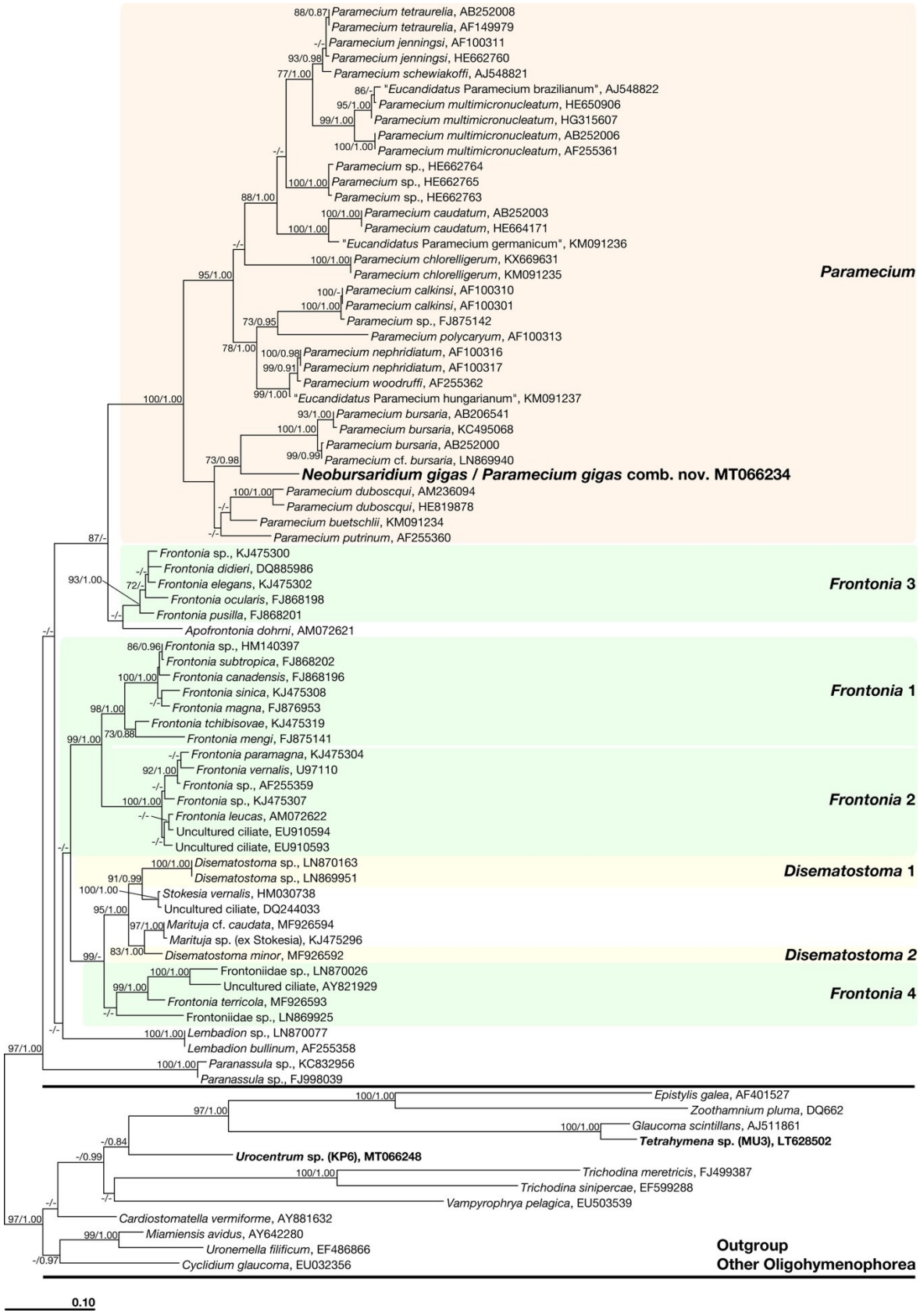


Figure 3. Phylogenetic tree of the subclass Peniculia based on 18S rDNA sequences. Numbers associated to nodes represent bootstrap value from maximum likelihood (ML) and posterior probability from Bayesian inference (BI) analyses, respectively (only values of ML > 70% and BI > 0.80 are shown). The phylogenetic position of *Neobursaridium gigas* lead to propose its attribution to *Paramecium*, as *Paramecium gigas* comb. nov. Particular attention was also given to species selection to investigate the phylogenetic relationships among members of Peniculia. From the present analysis, *Frontonia* and *Disematostoma* resulted paraphyletic. Sequences obtained in the present work are in *bold*

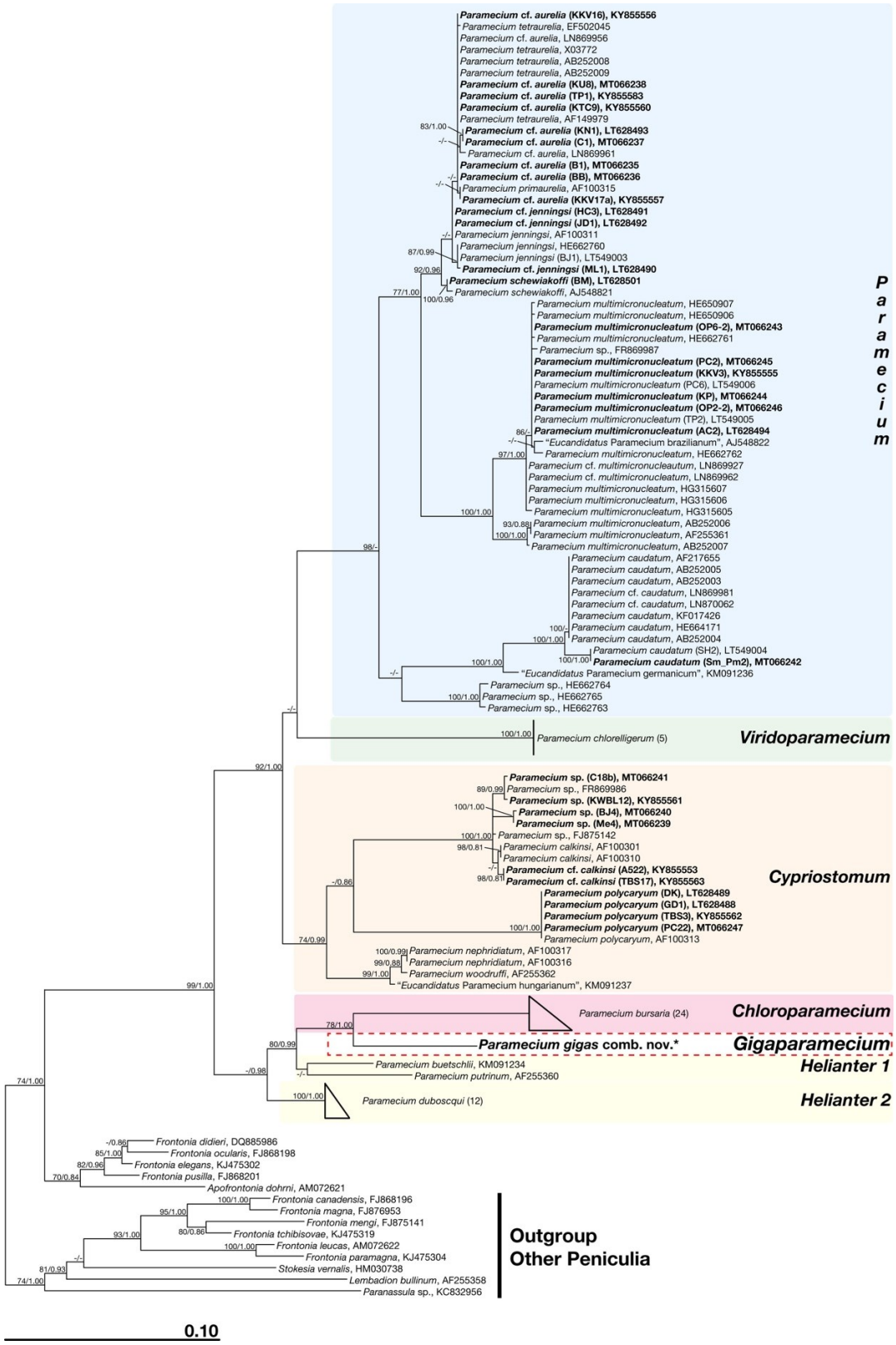


Figure 4. Phylogenetic tree of *Paramecium* based on 18S rDNA sequences. Numbers associated with nodes represent bootstrap value from maximum likelihood (ML) and posterior probability from Bayesian inference (BI) analyses, respectively (only values of ML > 70% and BI > 0.80 are shown). Numbers in brackets, associated with collapsed branches, indicate how many sequences are not shown (list of hidden sequences in Table S1). The phylogenetic positions of 30 strains of *Paramecium* are shown, together with the new proposed subgenus *Gigaparamecium* subgen. nov., including *Paramecium gigas* comb. nov. (former *Neobursaridium gigas*). Particular attention was also given to species selection to investigate the phylogenetic relationships among *Paramecium* subgenera. *Paramecium* strains are indicated in brackets. From the present analysis, *Helianter* resulted paraphyletic. * formerly known as *N. gigas*, accession number MT066234. Sequences obtained in the present work are in *bold*

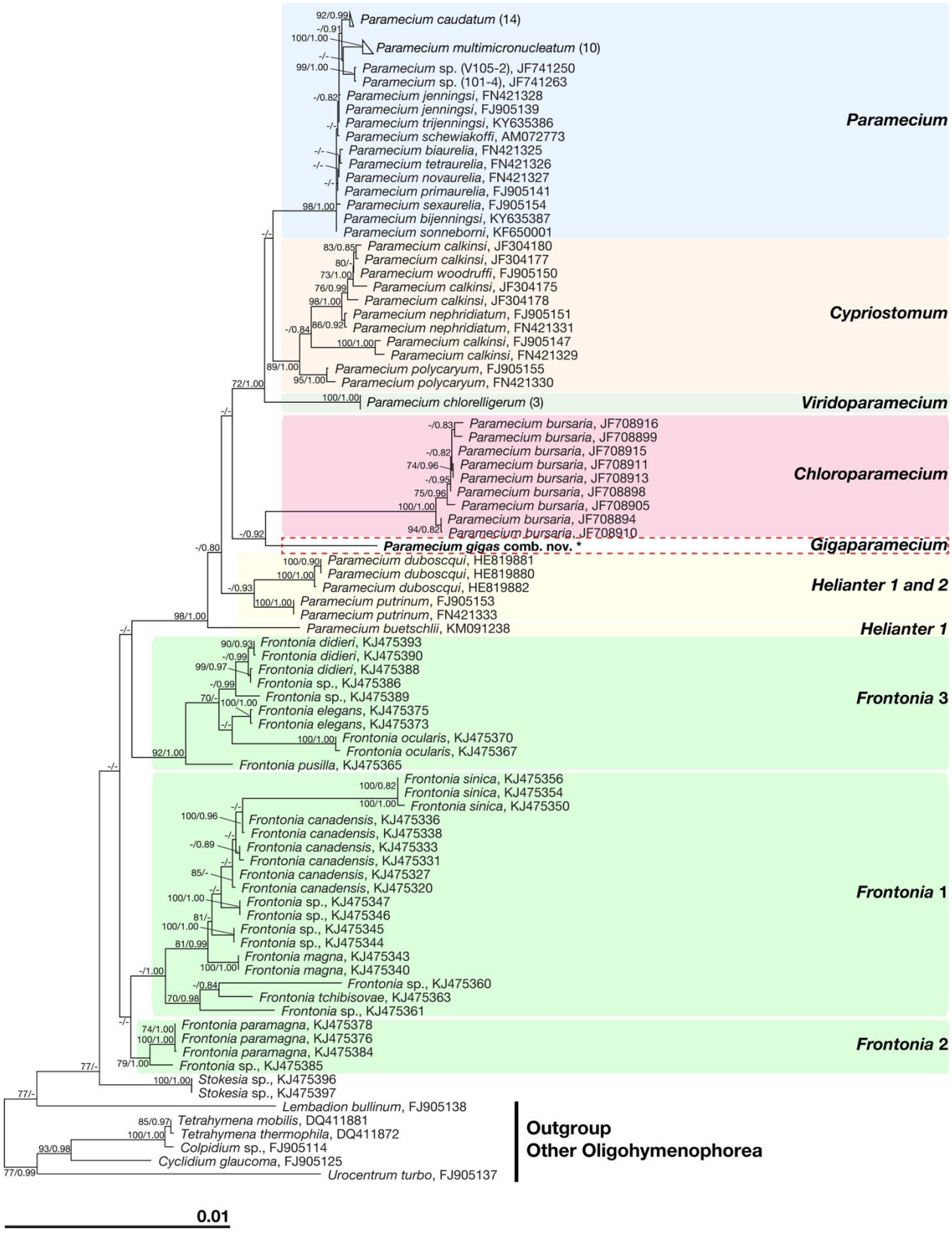


Figure 5. Phylogenetic tree of the subclass Peniculia based on COI mitochondrial gene sequences. Numbers associated to nodes represent bootstrap value from maximum likelihood (ML) and posterior probability from Bayesian inference (BI) analyses, respectively (only values of ML > 70% and BI > 0.80 are shown). Numbers in brackets, associated with collapsed branches, indicate how many sequences are not shown (list of hidden sequences in Table S1). The phylogenetic position of *Neobursaridium gigas* leads to propose its attribution to *Paramecium*, as *Paramecium gigas* comb. nov. Particular attention was also given to species selection to investigate the phylogenetic relationships among *Paramecium* subgenera and other members of Peniculia. From the present analysis, *Frontonia* resulted paraphyletic. *formerly known as *N. gigas*, accession number: MT622823. Sequence obtained in the present work is in *bold*

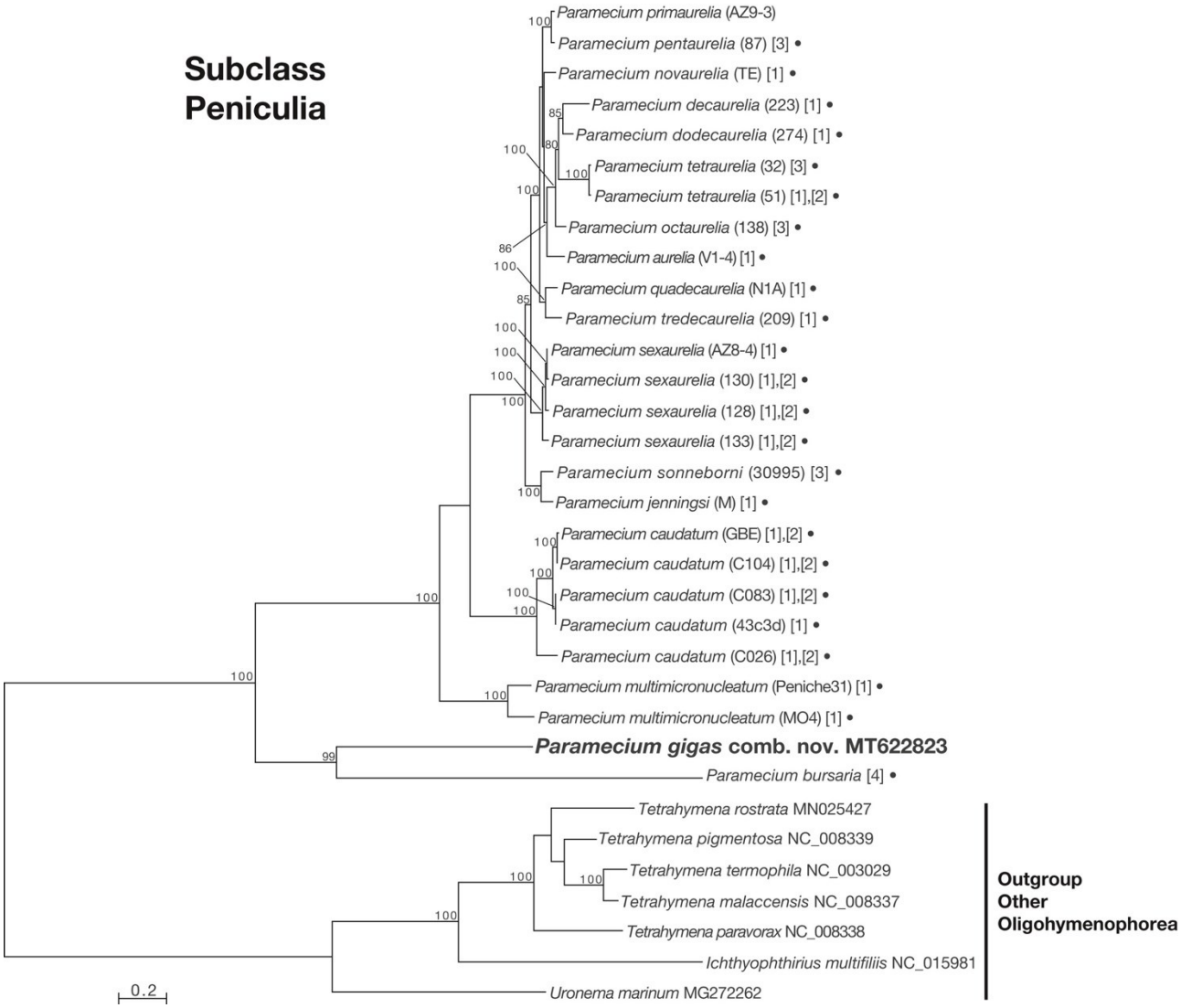
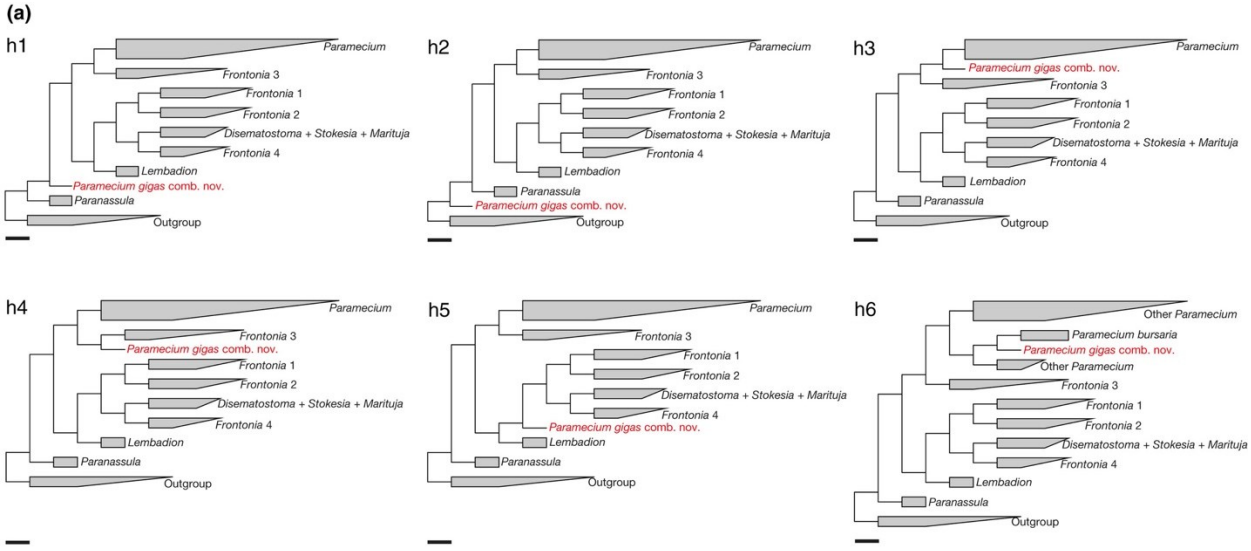


Figure 6. Phylogenomic analysis based on available Oligohymenophorea mitochondrial genomes. Phylogenomic analysis based on 15 concatenated mitochondrial proteins. Numbers associated with nodes represent bootstrap value from maximum likelihood (ML) analysis (only values of ML > 80% are shown). The positioning of the mitochondrial genome sequence of *Paramecium gigas* comb. nov. (formerly *Neobursaridium gigas*) inside the subclass Peniculia is shown. *Paramecium* strains are indicated in brackets. *Black dots* indicate mitochondrial genomes downloaded from ParameciumDB database. [1], Johri et al. (2019); [2], Pritchard et al. (1990); [3], Arnaiz et al. (2020); [4], He et al. (2019)

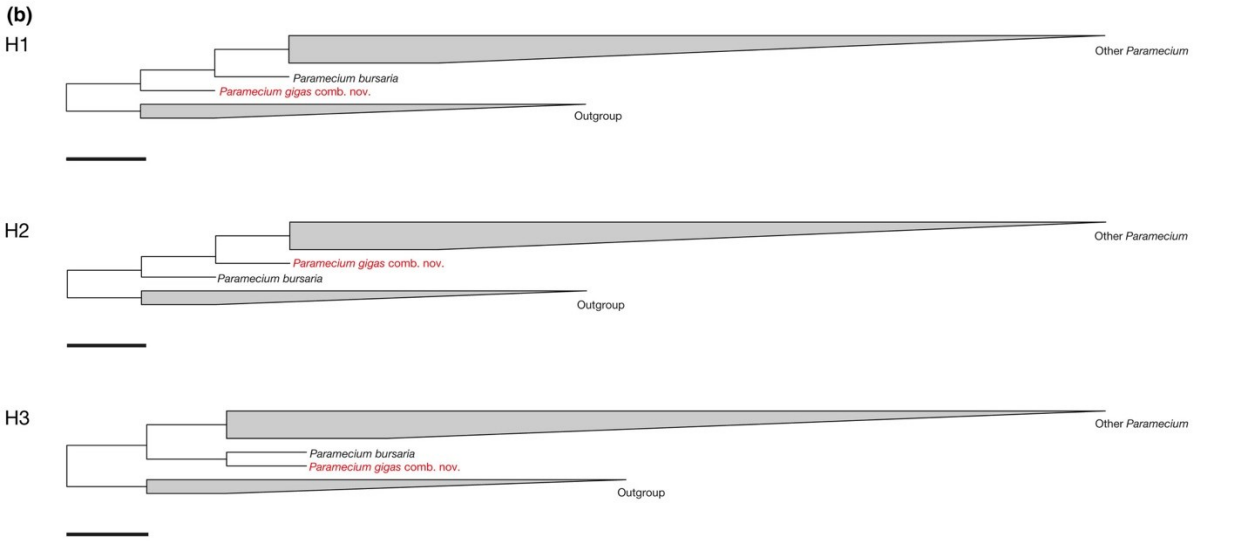
In detail, in our phylogenetic trees, *N. gigas* sequences resulted always sister of *P. bursaria*, with more robust statistical support in the 18S rDNA trees (73/0.98, 78/1.00), (Figures 3 and 4) than in the COI one (−/0.92) (Figure 5).

Also the phylogenomic analysis based on the retrieved mitochondrial genes showed *Neobursaridium* clustering inside the *Paramecium* clade as sister of *P. bursaria* (Figure 6), with high statistical support (99), being in accordance with abovementioned phylogenetic analyses (Figures 3-5).

Moreover, according to tests of alternative phylogenetic/phylogenomic hypotheses, our 18S rDNA-based tree (ML tree obtained from data set 1-h6) and our mitochondrial genome-based tree (H3) are the only statistically supported scenarios (Figure 7) among those tested. Other hypotheses (h1–h5/ H1–H2) were rejected, according to our data sets (Figure 7).



Hypotheses	logL	ΔL	bp-RELL	p-KH	p-SH	p-WKH	p-WSH	c-ELW	p-AU	Conclusion
h1	-12109.68282	101.73	0 -	0 -	0 -	0 -	0 -	1.19e-24 -	4.79e-41 -	Rejected
h2	-12112.80396	104.85	0 -	0 -	0 -	0 -	0 -	6.27e-26 -	2.79e-74 -	Rejected
h3	-12022.95834	15005	0.0117 -	0.0252 -	**0.289 +**	0.0252 -	**0.0558 +**	0.0153 -	0.0084 -	Rejected
h4	-12077.54309	69.59	0 -	0.0001 -	0.0001 -	0.0001 -	0.0001 -	3.01e-09 -	4.89e-12 -	Rejected
h5	-12113.36848	105.41	0 -	0 -	0 -	0 -	0 -	2.12e-26 -	1.14e-33 -	Rejected
h6	-12007.95355	0	**0.988 +**	**0.975 +**	**1 +**	**0.975 +**	**0.998 +**	**0.985 +**	**0.992 +**	Not rejected



Hypotheses	logL	ΔL	bp-RELL	p-KH	p-SH	p-WKH	p-WSH	c-ELW	p-AU	Conclusion
H1	-107348.348	42.483	0.0005 -	0.0024 -	0.0024 -	0.0024 -	0.0043 -	0.000444 -	0.00108 -	Rejected
H2	-107336.2951	30.431	0.0261 -	0.0297 -	0.0302 -	0.0297 -	**0.0518 +**	0.027 -	0.0243 -	Rejected
H3	-107305.8646	0	**0.973 +**	**0.97 +**	**1 +**	**0.97 +**	**0.985 +**	**0.973 +**	**0.981 +**	Not rejected

Figure 7. Alternative phylogenetic and phylogenomic hypotheses based on random positioning of *Paramecium gigas* comb. nov. (formerly *Neobursaridium gigas*) inside Peniculia. (a) Alternative topologies for the 18S rDNA-based tree (data set 1): six alternative phylogenetic hypotheses (h1–h6) are presented, with h6 corresponding to the maximum likelihood (ML) tree retrieved from our analysis (Figure 3) At the bottom are shown the topological tests results of the alternative hypotheses (h1–h6). (b) Alternative topologies for the mitochondrial genome-based tree: three alternative phylogenomic hypotheses (H1–H3) are presented, with H3 corresponding to the ML tree retrieved from our analysis (Figure 6). At the bottom are shown the topological tests results of the alternative hypotheses (H3–H6). The sequence of *P. gigas* is highlighted in red colour. *Bars* stand for 0.10. Plus signs denote the 95% confidence sets (in *bold*). Minus signs denote significant exclusion. logL, log-likelihood; ΔL, logL difference from the maximal logl in the set; bp-RELL: bootstrap proportion using RELL method; *p*-KH, *p*-value of one-sided Kishino-Hasegawa test; *p*-SH, *p*-value of Shimodaira-Hasegawa test; *p*-WKH, *p*-value of weighted KH test; *p*-WSH: *p*-value of weighted SH test; c-ELW, Expected Likelihood Weight; *p*-AU, *p*-value of approximately unbiased test

In accordance with the obtained phylogenetic and phylogenomics results, and based on a new reinterpretation its morphological data (see the 'section 4'), we herein propose to rename *N. gigas* as *P. gigas* comb. nov..

3.2 Phylogeny of Peniculia

In the 18S rDNA-based phylogeny, subclass Peniculia resulted monophyletic (Figure 3): the genera *Paramecium, Neobursaridium Frontonia, Apofrontonia, Disematostoma, Marituja, Stokesia, Lembadion* and *Paranassula* clustered together, supported by significant statistical values (97/1.00).

As already mentioned, in all performed analyses the newly sequenced *N. gigas* was embedded in *Paramecium* clade as sister clade of *P. bursaria* (Figures 3-6) with a rather robust statistical support in the 18S rDNA-based phylogenies (73/0.98—Figure 3; 78/1.00—Figure 4).

The earliest divergent clade of the genus *Paramecium* consisted of subgenera *Helianter* (*P. buetschlii, P. putrinum, Paramecium duboscqui*) and *Chloroparamecium* (*P. bursaria*), along with *N. gigas* (Figures 3 and 4). This branch was indeed the sister clade of all other *Paramecium* sequences, which in turn, are subdivided into two main clades: (a) subgenus *Paramecium* (*P. multimicronucleatum, P. caudatum, P. aurelia* complex*, P jenningsi, Paramecium schewiakoffi*) and subgenus *Viridoparamecium* (*Paramecium chlorelligerum*); (b) subgenus *Cypriostomum* (*Paramecium polycaryum, Paramecium calkinsi, Paramecium woodruffi, Paramecium nephridiatum*). All the subgenera resulted monophyletic except for *Helianter* (Figures 3 and 4).

In the COI-based phylogeny, we retrieved a divergent topology of genus *Paramecium,* with subgenus *Cypriostomum* as sister of subgenus *Paramecium*, although with not significant statistical support (Figure 5). In general, the monophily of the group formed by *Paramecium, Cypriostomum* and *Viridoparamecium* was rather supported in all obtained trees, while the relationships between them were discordant, depending on the particular molecular marker used, and in always showing low statistical support.

In the 18S rDNA-based phylogenies, *Frontonia* resulted paraphyletic (Figures 3 and 4), forming four distinguishable groups composed by (a) *Frontonia canadensis, Frontonia subtropica, Frontonia sinica, Frontonia tchibisovae, Frontonia mengi*; (b) *Frontonia paramagna, Frontonia vernalis, Frontonia leucas*; (c) *Frontonia didieris, Frontonia ocularis, Frontonia elegans, Frontonia pusilla, Frontonia* sp. (KJ475300) and *Apofrontonia dohrni*; (d) *Frontonia terricola* and other uncultured frontoniids (AY821929, LN869925, LN870026) (the numeration of *Frontonia* clades is given accordingly to literature: Fokin et al., 2019). Clade 3 of *Frontonia* resulted sister to *Paramecium* + *Neobursaridium* clades, while clade 4 resulted sister of *Disematostoma* + *Stokesia* + *Marituja* group (Figure 3). Also the COI-based phylogeny confirmed *Frontonia* as a paraphyletic taxon (Figure 5).

Disematostoma minor resulted sister of *Marituja*, although other *Disematostoma* sequences clustered separately, close to *Stokesia vernalis* (Figure 3).

Finally, *Lembadion* branched basally to frontoniids and parameciids, and *Paranassula* was basal to all the other Peniculia in the 18S rDNA-based phylogeny (Figures 3 and 4).

Unfortunately, COI sequences for *Disematostoma, Marituja* and *Paranassula* were not available in online repositories, so we could not provide a COI-based phylogeny for those species. On the other side, *Lembadion bullinum* resulted basal to all other Peniculia (Figure 5).

3.3 Molecular data and phylogeny of other newly characterized ciliates

In the present work, we retrieved and characterized 30 strains of *Paramecium*, belonging to different species (Figure 4): nine *P. aurelia* (B1, BB, C1, KKV16, KKV17a, KN1, KTC9, KU8 and TP1), three *P. jenningsi* (HC3, JD1, ML1), one *P. schewiakoffi* (BM), six *P. multimicronucleatum* (AC2, OP2-2, OP6-2, KKV3, KP, PC2), one *P. caudatum* (Sm_Pm2), two *P. calkinsi* (A522, TBS17), four *P. polycaryum* (DK, GD1, PC22, TBS3) and four strains (BJ4, C18b, KWBL12, Me4) probably belonging to a novel species, sister to *P. calkinsi*. For a more exhaustive report of results, please refer to the Appendix S1 and to Table S3.

It is worth noting that we made the first report of *P. schewiakoffi* from India since this ciliate was only retrieved in Shanghai (China) so far (Fokin, 2001; Fokin et al., 2004).

We also characterized the 18S rDNA gene sequence of MU3 *Tetrahymena* sp. (1,711 bp long) showing 99.9% identity with *Tetrahymena mobilis* (AF364040), and the KP6 *Urocentrum* sp. sequence (1,716 bp long), showing 100% identity with *Urocentrum turbo* (AF255357) (Figure 3).

All the sequences obtained in the present work were deposited in NCBI GenBank database: accession numbers are reported in Figures 3 and 4.

A brief morphological diagnosis of some of the strains in analysis is given in Table S4.

4 DISCUSSION

4.1 *Paramecium gigas* comb. nov.: a member of the newly erected subgenus *Gigaparamecium* subgen. nov. in the genus *Paramecium*

4.1.1 Molecular data

Although the inclusion of the sequence of the peniculid *N. gigas* did not help in resolving the overall complex phylogenetic relationships of Peniculia, it unexpectedly contributed both to raise new and intriguing questions, and to obtain new insights into *Paramecium* evolution.

Indeed, the monophyly of *Paramecium* so far supported by several phylogenetic studies (Barth et al., 2006; Boscaro et al., 2012; Fokin et al., 2004; Krenek et al., 2015; Kreutz et al., 2012; Lanzoni et al., 2016; Castelli et al., 2019; Strüder-Kypke et al., 2000), would be theoretically rejected due to the position occupied by *N. gigas* in all our phylogenetic/phylogenomic reconstructions (Figures 3-6). This, of course, if we still consider the classical view of *N. gigas* as a member of a genus different from *Paramecium*.

Since (a) the same topology has been recovered in four different phylogenetic/phylogenomics analyses, using two different molecular markers and the mitogenome, with high support for the monophyly of *Paramecium* + *N. gigas*, and (b) the range of 18S rDNA identity values of *N. gigas* with *Paramecium* members (89.4%–94%) is comparable to that among representatives of different species of paramecia (e.g. *P. bursaria* showed 88.5%–93.3% identity with congeners), we strongly believe that the genus *Neobursaridium* should be critically revised and *N. gigas* considered, in light of the new findings, as a member of *Paramecium* (i.e. *P. gigas* comb. nov.).

Unfortunately, it was not possible to obtain the complete mitochondrial genome of *Neobursaridium*, probably due to degradation of extracted DNA material. BLAST analysis using the *Paramecium* reference genomes was not able to identify sequence belonging to the missing parts. In our opinion, this was mainly due to the absence of reads corresponding to those regions in the sequencing data. This could be explained by the fact that we only could use the extracted DNA material as template

for the WGA (live cells were not available), and that the reiterated frosting and defrosting of the sample could had damaged the genomic content.

Nevertheless, although partial, also the mitogenome of *N. gigas* indicates a certain similarity with members of *Paramecium*. Indeed, despite the obtained mitogenome of *Neobursaridium* is not complete, the retrieved sequences show a great accordance with available mitogenomes of *Paramecium* (i.e. *P. caudatum*, *Paramecium tetraurelia, P. multimicronucleatum* and *P. bursaria*) regarding gene content and gene order. Comparison with the mitogenome of *T. thermophila*, an oligohymenophorean outside subclass Peniculia, revealed a lower degree of synteny (Burger et al., 2000).

Unfortunately, further comparisons with other Peniculia members are not possible because mitochondrial genomes from this subclass are still missing in online repositories.

Similarly, the lack of complete mitochondrial genomes of other representatives of Peniculia, such as *Lembadion* or *Frontonia*, hampered the resolution ability of the phylogenomic analysis. Nevertheless, the association between *P. gigas* comb. nov. and *P. bursaria* was still retrieved with significant statistical support, further confirming the correct positioning of *P. gigas* inside the genus *Paramecium*. The mitogenome-based phylogenomic tree resulted coherent with the presented 18S rDNA- and COI-based phylogenetic analyses, except for the topology of subgenus *Paramecium*.

It is worth noting that, to date, the present study showed the most comprehensive mitogenome-based phylogenomic analysis so far for peniculines.

4.1.2 Morphology

From a morphological point of view, the main differences between *Neobursaridium* and *Paramecium* concern the cell length (~ 500 µm vs. 80–340 µm), the structure of the 'quadrulus' (a particular kind of third peniculus, see below for a detailed comparison), and the shape of macronucleus (dumbbell vs. roundish/ovoid). Additionally, a large preoral groove, the 'vestibulum', surrounded on the left by a false adoral zone of polykinetids, plus a very deep and large buccal cavity differentiate *N. gigas* from *Paramecium*.

Nevertheless, it is known that all those features, especially size and macronuclear shape, might significantly vary among different species of the same ciliate genus; examples can be found in *Stentor* (Foissner & Wölfl, 1994), *Frontonia* (Long et al., 2008), *Spirostomum* (Boscaro et al., 2014), *Plagiopyla* (Nitla et al., 2019 and *Parablepharisma* (Campello-Nunes et al., 2020).

Regarding the 'quadrulus' of *Neobursaridium*, we believe that it could be considered as a third peniculus showing a scattered pattern of cilia instead of 4 ordered rows of cilia, present in other paramecia. Moreover, such a scattered ciliary distribution is also partly present in the third peniculus of *Paramecium africanum* (Dragesco, 1970), and another different composition of the third peniculus is present in *Paramecium ougandae* (Dragesco & Dragesco-Kernéis, 1986) and *Paramecium jankowski,* which show 5–7 ciliary rows instead of 'classical' four rows (Dragesco & Dragesco-Kernéis, 1986). Therefore, also the oral structure of *Neobursaridium* would include three peniculi as in *Paramecium*. The schematization provided by Dragesco and Tuffrau (1967; p. 139, Figure 3a,c) shows very clearly all similitudes and differences between the two buccal organizations (Figure 8), which are, in our opinion, homologous.

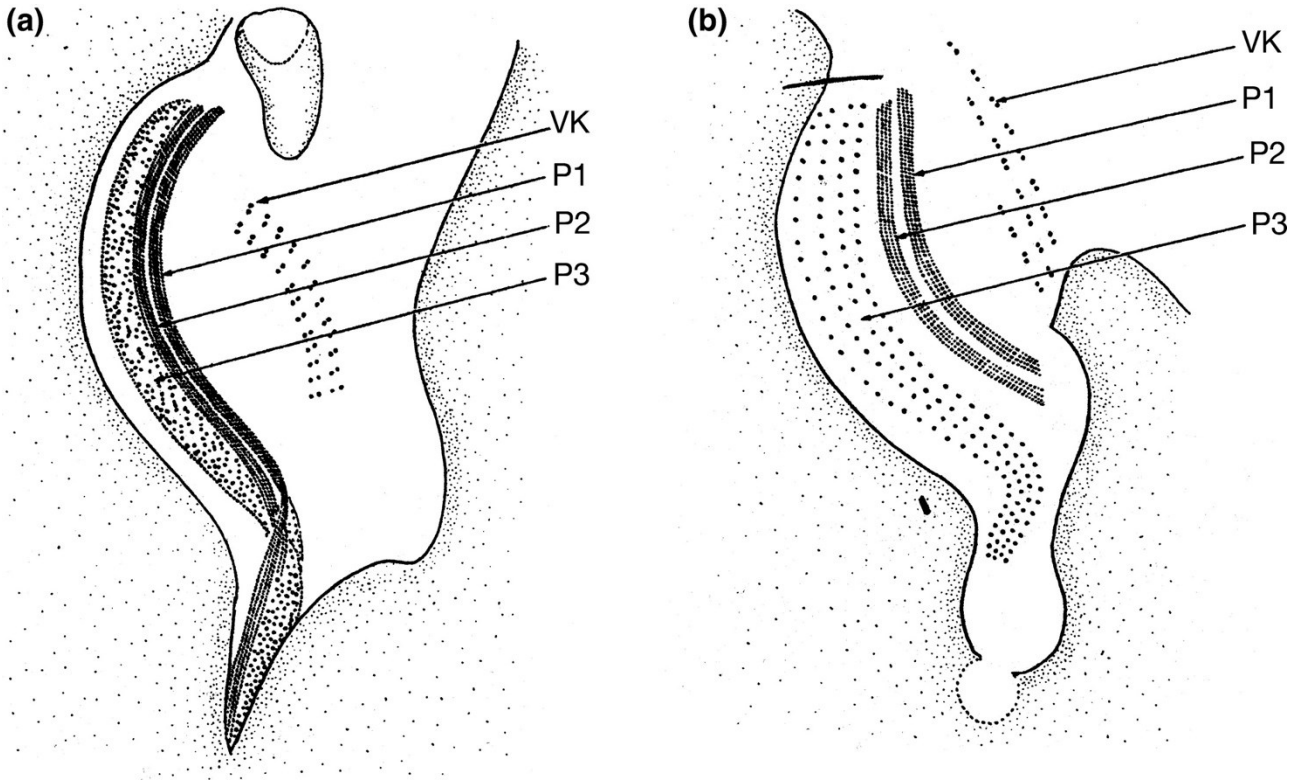


Figure 8. Schematic drawings of oral structures of *Paramecium gigas* comb. nov. and *Paramecium aurelia* (modified from Dragesco & Tuffrau, 1967). (a) *Paramecium gigas* comb. nov. (formerly *Neobursaridium gigas*) oral structures; (b) *Paramecium aurelia* oral structures. VK, vestibular kineties; P1–P3, peniculi. The present schemes were modified from the Figure 3, made by Dragesco & Tuffrau (1967) (p. 139, Figure 3a,c)

Significantly, *N. gigas* and *Paramecium* share some common characters, such as the presence of peniculi, the type of trichocysts, and the presence of two CV with radial canals (usually *Paramecium* species possesses exactly two of these structures, except for *P. africanum* and *P. ougandae* that show more than two CV).

Interestingly, Dragesco and colleagues, describing *N. gigas*, made the following considerations: (a) 'Un examen plus attentive nous montre une invagination péristomienne en "V", associée à une troncature antérieure qui rappelle beaucoup *P. bursaria*' [A closer look shows us a 'V'-shaped peristomal invagination, associated with an anterior truncation that is very reminiscent of *P. bursaria*] (Dragesco, 1966); (b) 'Entre temps, l'un d'entre nous avait retrové ce même Cilié dans le Centre Africain: nous pensions, alors, avoire affaire à une sorte de Paramécie géante' ['Meanwhile, one of us had found this same Ciliate in the African Center: we thought, then, to deal with a sort of giant *Paramecium*'] (Dragesco & Tuffrau, 1967); (c) 'Rappelle un *P. bursaria* géant, un peu aplati et tronqué dans sa région apicale' [Recalls a giant *P. bursaria*, slightly flattened and truncated in its apical region] (Dragesco & Dragesco-Kernéis, 1986).

The resemblance between the morphology of *N. gigas* and that of *Paramecium* was supported also by Jankowski (2007).

Moreover, Dragesco also indicated the presence of *Chlorella*-like endosymbionts in some strains of *N. gigas* (Dragesco & Dragesco-Kernéis, 1986), which is a well known feature of two *Paramecium* spp., that is *P. bursaria* and *P. chlorelligerum*. It should be mentioned that both *N. gigas* and *P. bursaria* possess two CV with collecting canals and several CVP in each of them (Fokin, 2010/11), and, while swimming, they rotate in counter-clockwise direction in respect to the antero-posterior body axis (Fokin, 2010/11; present work).

4.1.3 Hypotheses on the evolution of the giant morphotype

The peculiar gigantic phenotype shown by *P. gigas* is the most striking feature that differentiates this species from other *Paramecia* and is undoubtedly fascinating. Thus, based on molecular data (i.e. *P. gigas* nesting within *Paramecium* genus as sister species of *P. bursaria*) and morphological affinities with *Paramecium* found, we tried somehow to speculate regarding the giant morphotype evolution within *Paramecium* clade. If we consider as plesiomorphic the 'classical' *Paramecium* morphology (with small/medium body size), being the most conservative scenario, we might explain the exceptional size of *P. gigas* hypothesizing a possible process involving the formation of giants in a *P. bursaria*-like ancestor.

It has been shown, indeed, that morphological plasticity, including formation of giants, occurs quite often in many diverse (and phylogenetically distant) ciliates, and it is triggered by different environmental factors (for review, see Kuhlmann, 1993; Wicklow, 1997). For example, the presence of certain predators induces severe morphological alteration in some *Euplotes* (Kuhlmann & Heckmann, 1985; Kusch, 1993), *Onychodromus* (Wicklow, 1988) and *Colpoda* (Fyda, 1998; Fyda & Wiąckowski, 1998) species, and in *Lambornella clarki* (Washburn et al., 1988). These ciliates can enlarge their body size or produce cellular projections, such as wings or spines, to escape predation. Some bacterivorous ciliates, such as *Oxytricha* (Ricci et al., 1989; Ricci & Riggio, 1984), *Euplotes* (Tuffrau, 1959, 2000), *Blepharisma* (Giese, 1938; Schorr & Boggs, 1974), *Tetrahymena* (Williams, 1960, 1961) and others (Janovi, 1963; De Puytorac, 1984; De Puytorac et al., 1992; Wicklow, 1988), can shift their morphology to the giant form or dramatically change their buccal structures after a period of starvation or depending on ciliate culture concentration (e.g. repeated cell-cell contacts among ciliates enhance giant formation in *Oxytricha* (Ricci et al., 1991)), becoming, de facto, carnivorous towards other ciliates or cannibals to conspecifics. Also, the predatory peniculine *L. bullinum* after starvation develops a giant morphotype, able to ingest normal size *Lembadion* (Kuhlmann, 1993). Moreover, it has been demonstrated that *L. bullinum* can adapt its size to that of available preys (Kopp & Tollrian, 2003). Several of these examples of phenotypic plasticity can be interpreted as 'trophic polyphenism', defined as the phenomenon by which two or more morphotypes within a species exploit different food niches (Kopp & Tollrian, 2003).

Intriguingly, *P. gigas* is reminiscent of the giant morphs of the aforementioned ciliates, also considering that it is facultatively carnivorous (Dragesco & Tuffrau, 1967). This might suggest a possible origin of its phenotype, through a hypothetically conserved genetic potential for the development of giant forms. *Paramecium gigas* ancestor would have been able to rely on multiple different food sources (i.e. bacteria and other ciliates/microeukaryotes), exploiting novel niches. Such dietary flexibility could have been advantageous and thus positively selected during the evolutionary course.

On the other side, while in certain cultivation conditions *P. bursaria* and *P. duboscqui* have been observed to double in size, especially if they are somehow 'aged' (Fokin pers. obs.), to our knowledge no trophic polyphenism has ever been found in the genus *Paramecium*.

Thus, it seems premature to draw a defined evolutionary path for the origin of the giant morphotype of *P. gigas* for now. In our opinion, a possible hypothesis could envision a constitutive activation of developmental regulatory patterns at least partly shared with those involved in the trophic polyphenism of other ciliates, in response to environmental or epigenetic factors. In any case, the consistence and stability of the giant phenotype in *P. gigas* both in nature (in different geographic locations) and in laboratory culture strongly suggest that it might have originated and 'fixed' (as compared to observed plasticity in other ciliates) only once and relatively anciently. Whether such fixation was the result of a gradual crystallization from an initial trophic polyphenism of a single

(epi)genetic activation event, or of a more complex scenario, cannot be presently inferred with confidence.

Doubtlessly, further data are needed to address this issue, in particular genetic and phylogenetic characterization of other *P. gigas* strains, as well as molecular genetic and epigenetic data on the molecular bases of the giant phenotype in *P. gigas* and in other ciliates. Indeed, it is worth to notice that, species with extremely large size, far above the average body size of other congenerics, can be commonly found in many ciliate taxa: as examples *Condylostoma patulum* Claparède & Lachmann, 1858 and *Condylostoma reichi* Wilbert & Kahan, 1981 in Condylostomatidae, *Stentor coeruleus* Ehrenberg, 1830 in Stentoridae, *Loxodes rex* Dragesco, 1970, in Loxodidae, but there are many others. This might suggest that also those species might have undergone a sort of 'crystallized gigantism', similarly to *P. gigas*.

Clearly, according to the available data the so-far exposed hypotheses are only speculative, and in-depth inference on such complex evolutionary paths is beyond the aim of the present study, focused on the redescription of a single species. Hopefully, such speculation may at least represent hints for further investigations aimed to shed light on this topic.

4.1.4 Subgenus Gigaparamecium *subgen. nov.*

After a critical analysis of our results and available literature in the field, it is our opinion that *Neobursaridium* is a true *Paramecium* member, presenting an evolutionarily derived morphology. In conclusion, since we are oriented towards an inclusive taxonomic approach and since two different molecular markers, plus a phylogenomic analysis based on mitochondrial genomes, confirmed such an evolutive relationship, together with morphological evidences, we propose to include *N. gigas* in the genus *Paramecium* (see 'section 5' at the end of the section 4) in the subgenus *Gigaparamecium* subgen. nov., renaming it after *P. gigas* comb. nov. The newly erected subgenus would be characterized by the following morphological synapomorphies: cell length over 350–400 μm; dumbbell-shaped macronucleus; false adoral zone of polykinetids; large buccal cavity, midventral, expansive, with inner or right-most oral polykinetid forming the numerous anarchically located rows, that is the strip corresponding to the classical third peniculus.

4.2 Considerations on the Peniculia phylogeny

The monophyly of subclass Peniculia demonstrated by previous works (Chen et al., 2014; Fan et al., 2011; Fan et al., 2013; Fokin et al., 2006; Gao et al., 2008; Pan, Gao, et al., 2013; Pan, Liu, et al., 2013; Xu et al., 2018) was confirmed by our phylogenetic analyses (both 18S rDNA- and COI-based phylogenies). Moreover, coherently with previous studies on Peniculia, they pointed out that a monophyly of *Frontonia* is not supported by molecular data in the 18S rDNA- (Chen et al., 2014; Fan et al., 2011, 2013; Fokin et al., 2006; Gao et al., 2008, 2016; Ong'ondo et al., 2013; Pan, Gao, et al., 2013; Pan, Liu, et al., 2013; Zhao et al., 2016; Xu et al., 2018) and COI-based phylogenies (Zhao et al., 2016).

According to our phylogenetic data, we are prone to consider group 1 and group 2 of *Frontonia* as the 'true *Frontonia*', being the sequence of the type species of the genus, *Frontonia leucas*, embedded in group 2. Therefore, as suggested by other colleagues (Zhao et al., 2016), we strongly point out the need to revise the entire genus, renaming members of group 3 and group 4 (Figure 3).

Similarly, the systematics of *Disematostoma* should be fixed in future, as the sequences included in the clade 'Disematostoma 1' (LN869951, LN870163) would more likely belong to *Stokesia* than to *Disematostoma*.

5 REVISION OF THE FAMILIES NEOBURSARIDIIDAE AND PARAMECIIDAE

After a critical analysis of our results and the available literature, we concluded that *N. gigas* should be considered as a senior synonym of *P. gigas* (Balech, 1941) comb. nov. Therefore, in agreement with Jankowski (2007), we propose the suppression of the family Neobursaridiidae Dragesco & Tuffrau, 1967, and the inclusion of the novel combination in the family Parameciidae Dujardin, 1840, in the genus *Paramecium* O. F. Müller, 1773, subgenus *Gigaparamecium*.

After the last major taxonomic revisions of genus *Paramecium* (Van Wagtendonk, 1974; Wichterman, 1986), some new species have been described (Fokin et al., 2004; Krenek et al., 2015; Paiva et al., 2016) and several others that were considered not valid, were rediscovered and redescribed (e.g. *P. duboscqui*, *P. nephridiatum*, and *P. chlorelligerum*) (Fokin et al., 1999; Kreutz et al., 2012; Lanzoni et al., 2016; Shi et al., 1997). Obviously, this process is far from being concluded. The transfer of *N. gigas* to genus *Paramecium*, as a member of the new subgenus *Gigaparamecium,* demonstrates that this taxon is still largely unexplored.

5.1 FAMILY PARAMECIIDAE DUJARDIN, 1840

Medium to large organisms from brackish and freshwater habitats; distributed into one genus with six subgenera plus one genus *Incertae sedis*:

– *Paramecium* O.F. Müller, 1773

Incertae sedis in the Family Parameciidae Dujardin, 1840:

– *Physanter* Jankowski, 1975

5.2 GENUS *PARAMECIUM* O. F. Müller, 1773

Improved diagnosis

Size, medium to large; shape, elongated ovoid, rounded and/or pointed at either one or both ends; free-swimming; somatic ciliation, holotrichous, dense, with a prebuccal area as a preoral groove (formerly called a ‘vestibulum’) not so extensively expanded, covered by paratenes, and leading to a buccal cavity located around the equatorial line, slightly before or after, with inner or right-most oral polykinetid of four–seven widely spaced rows of cilia (i.e. a quadrulus), sometimes a bit chaotic in one of them, or just presenting a strip of anarchic kinetosomes on the dorsal wall of oral cavity; macronucleus, ellipsoid to elongate ellipsoid or dumbbell-shaped; micronucleus, maybe single, couple or multiple of compact, vesicular, endosomal, and chromosomal types; contractile vacuoles, typically two or sometimes more; cytoproct present; some species containing *Chlorellae*-like endobionts; feeding mainly on bacteria and microalgae, also carnivorous; in brackish and freshwater habitats; six subgenera:

Paramecium Jankowski, 1969

Cypriostomum Jankowski, 1969

Helianter Jankowski, 1969

Chloroparamecium Fokin et al., 2004

Viridoparamecium Kreutz et al., 2012

Gigaparamecium subgen. nov.

5.3 SUBGENUS *GIGAPARAMECIUM* subgen. nov.

Gigaparamecium

[Giga, meaning 'giant' (From Ancient Greek, *gígas*); 'gigas' is the specific epithet of *P. gigas*; paramecium, from the name of genus *Paramecium* (Phylum Ciliophora)].

Diagnosis

Size, large, over 350–400 µm in length; false adoral zone of polykinetids; large buccal cavity, midventral, expansive, with inner or right-most oral polykinetid forming the numerous anarchically located rows, that is the strip corresponding to the classical third peniculus; dumbbell-shaped macronucleus, multiple micronuclei; two contractile vacuoles with several contractile vacuole pores and collecting canals; trichocysts present; feeding on bacteria and microeukaryotes; freshwater habitats; pan-tropical distribution. Includes the new combination *P. gigas* (Balech, 1941) comb. nov.

5.3.1 Paramecium gigas *(Balech 1941) comb. nov.*

1941 ***Neobursaridium gigas* n. gen. n. sp.**—Balech, *Physis*, **19**:29–35 pp. (original description, with illustration; no type material available)

1959 ***Bursaria* sp.**—Beadle & Nilsson, *J. Experimental. Biol*., **36**:583–589 pp. (experimental paper)

1962 ***Neobursaridium gigas*** Balech, 1941—Nilsson, *J. Protozool*. **9**:273–276 pp. (illustrated description, no type material available)

1964 ***Neobursaridium gigas*** Balech, 1941—Thurston, *J. Protozool*. **11**:307–309 pp. (illustrated record, biological study)

1966 ***Neobursaridium gigas*** Balech, 1941—Dragesco, *Biol*. *Gabon*. **2**:91–117 pp. (illustrated record)

1967 ***Neobursaridium gigas*** Balech, 1941—Dragesco & Tuffrau, *Protistologica*. **3**:133–146 pp. (illustrated record, recollocation in Neobursaridiidae family)

1968 ***Neobursaridium gigas*** Balech, 1941—Dragesco, *Protistologica*. **4**:157–167 pp. (biological and ultrastructural study)

1969 ***Neobursaridium gigas*** Balech, 1941—Nilsson*, C. R. Trav. Lab. Carlsberg*. **37**:49–99 pp. figure 1, Pls. I-XVI (illustrated description, ultrastructural study)

1970 ***Neobursaridium gigas*** Balech, 1941—Dragesco, *Ann. Fac. Sci. Yaoundé (Hors série),* 47 p. (record)

1971 ***Neobursaridium gigas*** Balech, 1941—Mahajan & Nair, *Rec. Zool. Surv. India*, **63**:16 p., figure 3f (p. 13) (illustrated record)

1972 ***Neobursaridium gigas*** Balech, 1941—Kattar, *Revta. Bras. Biol*., **32**:499–503 (record)

1975 ***Neobursaridium gigas*** Balech, 1941—Kattar, *Acta Protozool*., **14**:179–184, Pl. I-IX p., (ultrastructural study)

1986 ***Neobursaridium gigas*** Balech, 1941—Dragesco & Dragesco-Kernéis, *Faune tropicale,* **26**:333–335 pp., Pl. 87, figures A-J (improved diagnosis and revision, short taxonomic and ecological monograph)

2000 ***Neobursaridium gigas*** Balech, 1941—Charubhun & Charubhun, *Kasetsart* J., **34**:486–494 pp., figure 1a (illustrated record)

2003 ***Neobursaridium gigas*** Balech, 1941—Nola et al., *Tropicultura*, **21**:73–78 pp. (experimental paper)

2007 ***Neobursaridium gigas*** Balech, 1941—Dias, *Ph.D Thesis—Federal University of Juiz de Fora*, 64–84 pp., Pl. IV-figure 54, Pls. XIV-XVII (illustrated record, revision, scanning electron microscopy)

2007 ***Neobursaridium gigas*** Balech, 1941 –Jankowski. Phylum Ciliophora Doflein, 1901. Review of taxa. In: Protista. Handbook of Zoology. St. Petersburg: 'Nauka'. Part 2:805. figure 359. (illustrated revision, recollocation in Parameciidae family—in Russian).

Improved diagnosis (Table 4)

Body size, large (500 × 270 µm on average); shape, elongated ovoid with a truncated anterior part, typically rounded and broader at posterior end, dorso-ventrally flattened, pink-brownish in colour due to cortical granules-like structures; free-swimming with counter-clockwise rotation; somatic ciliation, holotrichous (300–400 kineties), dense, with a 'heterotrich-looking'; extensive, false adoral zone of polykinetids, actually formed by transverse paratenes of somatic kineties, lying in a prebuccal area as a much expanded preoral groove occupying the anterior half of the body; buccal cavity, midventral, expansive, with inner or right-most oral polykinetid, the strip of anarchic kinetids (i.e. third peniculus or 'quadrulus'); macronucleus, dumbbell-shaped (close to 300 µm in length on average); micronuclei, multiple (3–12); invariably two CV, with 2–4 PCV, long collecting canals (6–12); cytoproct present as a suture on the ventral side; trichocysts (7–14 µm in length *Paramecium*-type); carnivorous on other ciliates, but also bacterivorous; in freshwater habitats only pan-tropical. A single member of subgenus *Gigaparamecium*.

TABLE 4. Comparative morphometrics of *Paramecium gigas* comb. nov. (former *Neobursaridium gigas*) from different authors

Reference	Place	Habitat	Body length (µm)	Body width (µm)	Number of somatic kineties	MA shape	MA length (µm)	Number of MI	MI size (µm)	Number of CVP	Number of CVC	Cytoproct length (µm)	Extrusome size (µm)
Balech (1941) (type description)	Palermo district, Buenos Aires (ARGENTINA)	Freshwater (clear water with *Salvinia* and *Eichornia*)	342–550 (f)	190–380 (f)	ND	Irregular/ 'gymnast's handlebar'	200–220 (f)	ND	ND	ND	6−7[a]	ND	ND
Nilsson (1962)	Kampala (UGANDA)	Freshwater (papyrus swamp)	550 ± 70 (ns)	275 ± 30 (ns)	>300 (f)	Dumbbell[a]	370–430 (f)	Up to 10	ND	ND	~12[a]	30 (f)	7 (f)
Thurston (1964)	Kampala (UGANDA)	Freshwater (pools)	420–710 (l)	180–324 (l)	ND	Dumbbell	ND	11–12	5–6	ND	ND	ND	10–14 (l)
Dragesco (1966)	Ubangi-Shari near Bossembelé (CAR), Makokou (GABON)	Freshwater (pools)	230–710 (l)	~213[a] (l)	400	Dumbbell[a]	~290[a] (l)	3–4	ND	2–3	8–12	ND	10 (f)
Dragesco and Tuffrau (1967)	Kampala (UGANDA); KENYA	ND	350–650 (ns)	ND	ND	Dumbbell/Reniform	ND	5–12	ND	ND	ND	ND	ND
Nilsson (1969)	Kampala (UGANDA)	Freshwater (papyrus swamp)	550 (f)	275 (f)	ND	Dumbbell	ND	~10	3–5 (f)	2	7−8[a]	ND	7 (f)
Mahajan and Nair (1971)	Kolkata (INDIA)	Freshwater (pools)	514 (f)	264 (f)	ND	Dumbbell	353 (f)	ND	ND	2	6–9	ND	ND
Kattar (1975)	Minas Gerais (BRAZIL)	ND	600 (ns)	ND	ND	ND	ND	ND	ND	ND	ND	ND	7[a] (f)
Charubhun and Charubun (2000)	THAILAND	Freshwater	500 (ns)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dias (2007)	Juiz de Fora (BRAZIL)	Freshwater (São Pedro River)	320–590 (l) 280–500 (f)	175–300 (l) 150–300 (f)	400	Dumbbell	200 (f)	ND	ND	2–3	8–12	~96[a] (f)	10
Present study	Araku Hills (INDIA)	Freshwater (anthropized stream)	500–600 (l)	250–280 (l)	ND	ND	ND	ND	ND	3–4	~12	ND	10

Abbreviations: (f), measurements from fixed specimens; (l), measurements from live specimens; (ns), not specified if measurements were from live or fixed specimens; CAR, Central African Republic; CVC, contractile vacuole canals; CVP, contractile vacuole pore; MA, macronucleus; MI, micronucleus; ND, no data.

[a] Trait observed or measured from illustration.

Type locality

Palermo district, Buenos Aires (Argentina) (Balech, 1941): '[…] en aguas bastante claras, con escasez de bacterios, alcalinidad relativamente baja, epipleon bastante denso de *Salvinia* y *Eichornia*; en associatión con algunos Euglenoidíneos, ciliados grandes y otros' ['in quite clear waters, with a shortage of bacteria, relatively low alkalinity, quite dense epipleon of *Salvinia* and *Eichornia*; in association with some Euglenoids, large ciliates and others'].

Ecology and distribution

Isla Santa Fé and Buenos Aires, ARGENTINA (Balech, 1941); Kampala, UGANDA (Beadle & Nilsson, 1959; Dragesco, 1968; Dragesco & Dragesco-Kernéis, 1986; Dragesco & Tuffrau, 1967; Nilsson, 1962, 1969; Thurston, 1964); Ubangi-Shari near Bossembelé, CENTRAL AFRICAN REPUBLIC and Makokou, GABON (Dragesco, 1966, 1970; Dragesco & Dragesco-Kernéis, 1986); KENYA (Dragesco & Tuffrau, 1967); Garoua, Tcholliré, Bénoué (Dragesco, 1970), Yaoundè (Nola et al., 2003), CAMEROON; CONGO, TCHAD, BENIN (Dragesco & Dragesco-Kernéis, 1986); Kolkata (Mahajan & Nair, 1971), Araku hills (present study) INDIA; THAILAND (Charubhun &

Charubhun, 2000); Minas Gerais (Kattar, 1972, 1975) Juiz de Fora (São Pedro River—Dias, 2007), BRAZIL.

Gene sequences

18S rDNA gene sequence (MT066234); partial mitogenome sequence (MT622823), including COI gene.

Remarks

Macronuclear endosymbionts have been observed (Kattar, 1975; Nilsson, 1969). Although all the available publications treat this ciliate as a single species, representatives from different locations strongly differ with respect to several morphological and biological characteristics; this could suggest that they are possibly different species, which was already hypothesized by Dragesco (1968).

6 NOMENCLATURE ACTS

The present work has been registered in ZooBank (code: urn:lsid:zoobank.org:pub:A95F51AC-99F5-4CA5-A544-F1D08BE660FE), as well as *Paramecium gigas* comb. nov. (code: urn:lsid:zoobank.org:act:0ED371B8-0A89-43E0-8B20-374E6A85D18D). The correspondent web pages are available at the following addresses: http://www.zoobank.org/References/a95f51ac-99f5-4ca5-a544-f1d08be660fe and http://www.zoobank.org/NomenclaturalActs/0ED371B8-0A89-43E0-8B20-374E6A85D18D, respectively.

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