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New considerations on the phylogeny of cyrtophorian ciliates (Protozoa, Ciliophora): expanded sampling to understand their evolutionary relationships

Xiao Chen*, Hongbo Pan*, Jie Huang, Alan Warren, Saleh A. Al-Farraj & Shan Gao

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To rationalize the confusing relationships among the cyrtophorian ciliates, we expanded the taxon sampling by sequencing the small subunit ribosomal RNA (SSU rRNA) gene of representatives of 12 genera (20 species, 23 new sequences). The SSU rRNA sequences of *Spirodysteria*, *Agnathodysteria*, *Brooklynella* and *Odontochlamys* are reported for the first time. Phylogenetic trees were constructed, and secondary structures of variable region 4 (V4) of all genera for which SSU rRNA gene sequence data are available were predicted. The results indicate that (i) *Brooklynella* is likely an intermediate taxon between Dysteriidae and Hartmannulidae; (ii) the genus *Dysteria* is paraphyletic with *Spirodysteria* and *Mirodysteria* nested within it; (iii) the genus *Agnathodysteria* is well separated from *Dysteria* based on both molecular and morphological data; *and* (iv) *Trithigmostoma* is a basal genus of Chilodonellidae, based on both the morphological and molecular data.

Corresponding author: *Shan Gao, Laboratory of Protozoology, Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China and Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266003, China. E-mail: shangao@ouc.edu.cn*

Xiao Chen, Laboratory of Protozoology, Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China. E-mail: seanchen607@gmail.com

Hongbo Pan, College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China. E-mail: panhongbobio@sina.com

Jie Huang, Laboratory of Protozoology, Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China. E-mail: hjbx152@163.com

Alan Warren, Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK. E-mail: a.warren@nhm.ac.uk

Saleh A. Al-Farraj, Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia. E-mail: salfarraj@hotmail.com

Shan Gao, Laboratory of Protozoology, Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China and Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266003, China. E-mail: shangao@ouc.edu.cn

**These authors contributed equally to the study.*

Introduction

As the main component of the class Phyllopharyngea de Puytorac *et al*., 1974, the subclass Cyrtophoria Fauré-Fremiet in Corliss, 1956 is a highly divergent ciliate group with numerous morphotypes (Figs 1 and 2) (Corliss 1979; Small & Lynn 1985; de Puytorac 1994; Lynn & Small 2002; Gong 2005; Lynn 2008). The membership of the Cyrtophoria has experienced several changes since its establishment. It contained one order, eight families and 43 genera in Corliss (1979). De Puytorac (1994) recognized three orders, that is Chilodonellida Deroux, 1970, Chlamydodontida Deroux, 1976 and Dysteriida Deroux, 1976. Lynn & Small (2002) assigned phyllopharyngeans with subclass rank and recognized two orders, namely Chlamydodontida and Dysteriida, with the order Chilodonellida being assigned to the order Chlamydodontida as the family

Chilodonellidae Deroux, 1970. The latter classification was retained by Lynn (2008) who also resurrected the subclass name Cyrtophoria, added five genera (*Lynchellodon* Jankowski, 1980, *Paragastronauta* Foissner, 2001, *Planilamina* Ma *et al.*, 2006, *Talitrochilodon* Jankowski, 1980, and *Wilbertella* Gong & Song, 2006) to this subclass and transferred *Allosphaerium* Kidder & Summers, 1935 from Dysteriidae Claparède & Lachmann, 1858 to Hartmannulidae Poche, 1913 and *Orthotrochilia* Song, 2003 from Hartmannulidae to Dysteriidae. Currently, it is accepted that cyrtophorians are divided into two orders, Chlamydodontida and Dysteriida. The former is characterized by its typically dorsoventrally compressed body, somatic kineties ventrally arranged in two equal fields and lacking both a non-ciliated adhesive region and a podite. On the other hand, the second order is identified by its laterally compressed body, non-thigmotactic ventral cilia and with either a non-ciliated adhesive region or a podite. However, interrelationships among lower ranked taxa, such as genera/families, are not well resolved because of the paucity of morphogenetic criteria that can be used for analysing the systematics of cyrtophorians, and the large number of morphotypes that have been described (Fig. 2) (Deroux 1976, 1994; Jankowski 2007; Gong *et al.* 2009; Chen *et al.* 2011).

The first published gene sequence of a cyrtophorian ciliate was the small subunit ribosomal RNA (SSU rRNA) gene of *Trithigmostoma steini* (Blochmann, 1895) Foissner, 1988, by Leipe *et al.* (1994). Several molecular investigations have since been performed, and these have consistently supported the monophyly of the cyrtophorians (Snoeyenbos-West *et al.* 2004; Li & Song 2006; Gong *et al.* 2008). These studies, however, mainly focused on relationships among higher-level taxa (e.g. class, subclass and order), whereas the phylogeny of lower-rank groups (e.g. family, genus), where most confusions and ambiguity reside, has not been clarified. Moreover, these studies were based on a very limited species sampling, which limits the reliability and resolution of the phylogenetic inference. Recently, Gao *et al.* (2012) sequenced the SSU rRNA gene of 18 species representing 17 genera of cyrtophorians and explored the phylogenetic relationships among certain taxa, mostly genera and families, which have long been unresolved. As a result of this study, *Pithites* Deroux & Dragesco, 1968 and *Trochochilodon* Deroux, 1976 were transferred from Dysteriida to Chlamydodontida, the family Pithitidae Gao *et al.*, 2012 was established, *Microxysma* Deroux, 1977 was transferred from Hartmannulidae to Dysteriidae, and the order Chlamydodontida was found to be non-monophyletic. Generic relationships within the family Chlamydodontidae Stein, 1859 have since been further investigated (Pan *et al.* 2013). However, the number of taxa with available molecular information remains low considering the large number of described morphospecies. Furthermore, some long-standing confusion, such as the monophyly of *Dysteria* Huxley, 1857, and the systematic position of *Brooklynella* Lom & Nigrelli, 1970, remains unclarified.

In this study, the SSU rRNA gene was sequenced for 20 species belonging to 12 cyrtophorian genera, including four genera whose molecular phylogeny has been investigated for the first time. In addition, the predicted secondary structures of the V4 region of the SSU rRNA gene were

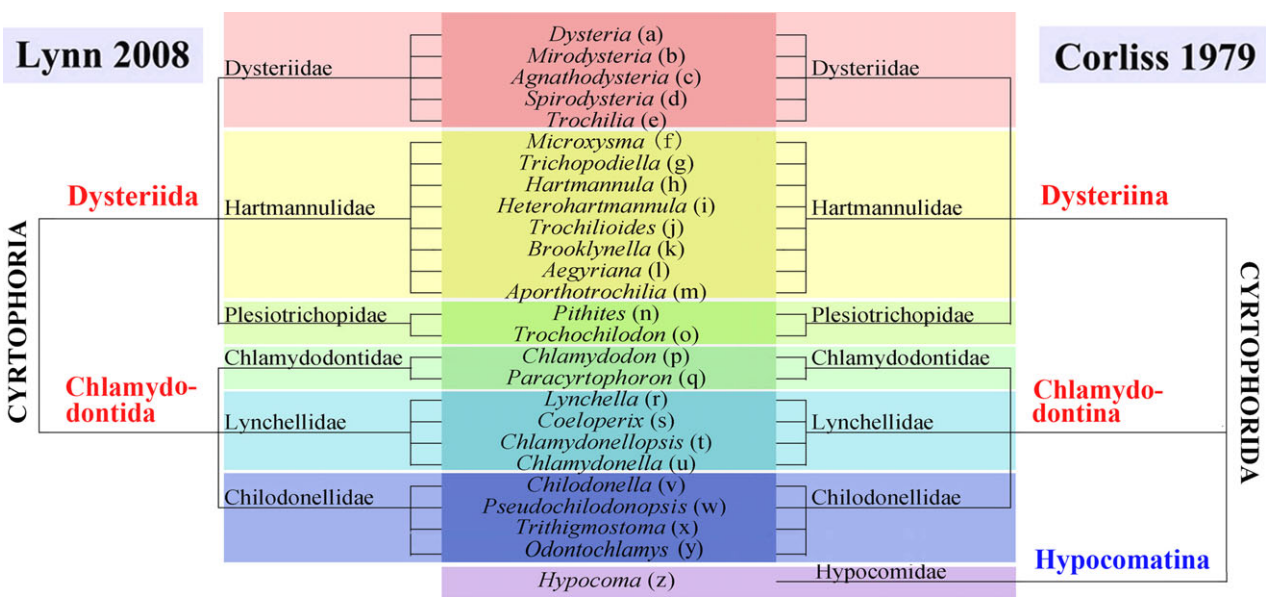

Fig. 1 Systematic arrangements of the order Cyrtophorida (Corliss 1979) and the subclass Cyrtophoria (Lynn 2008).

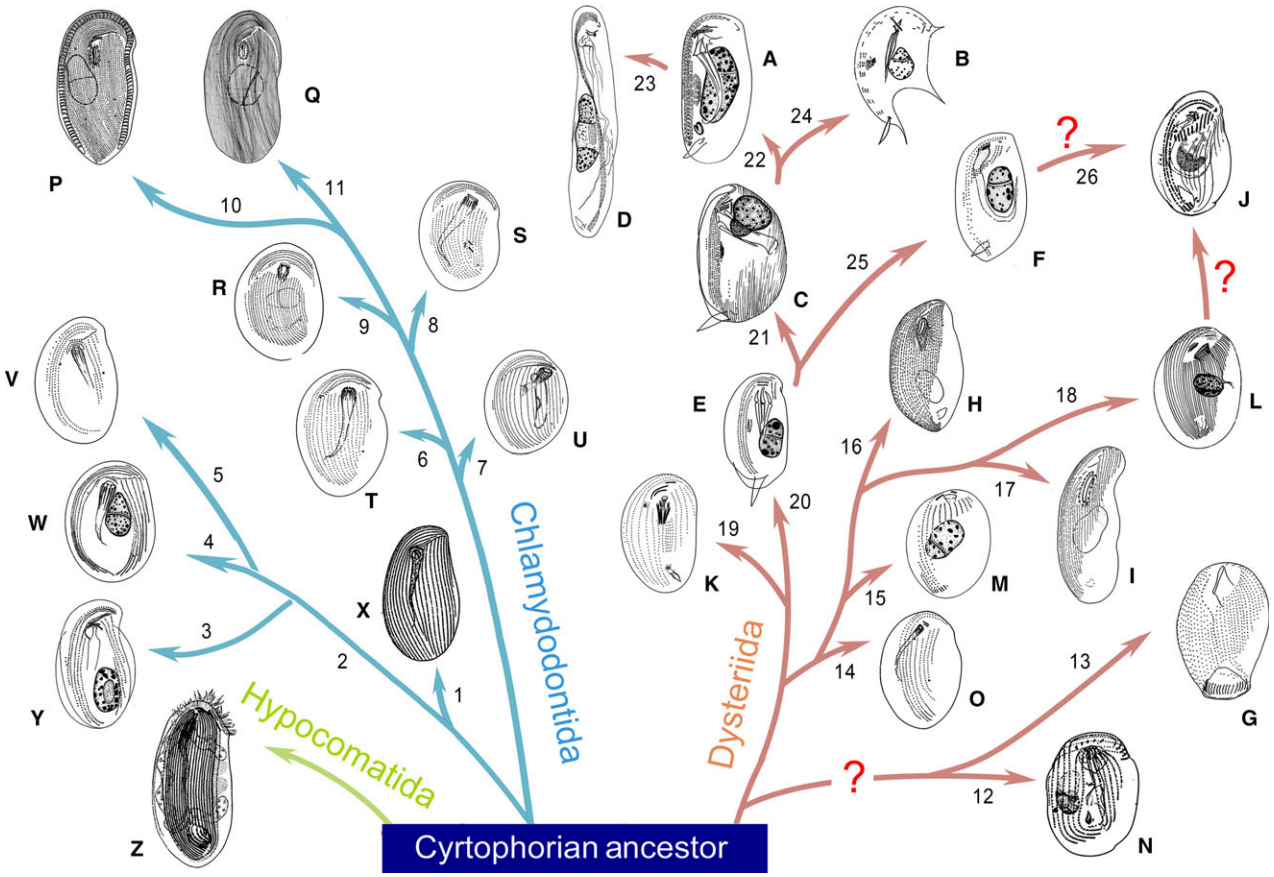

Fig. 2 Diagrammatic representations and suggested evolutionary relationships among the currently recognized cyrtophorian genera. Roman letters in the diagrams correspond to those in parentheses in Fig. 1, referring to each genus. Numbers label the morphological generic features: 1. cell usually with pronounced anterior projection or 'beak' to left; macronucleus centric and heteromerous; all ventral kineties continuous (*Trithigmostoma*); 2. right kineties separated from left kineties; the presence of glabrous region in the middle of ventral side; 3. preoral kinety continuous; terminal fragment apically positioned (*Odontochlamys*); 4. preoral kinety segmented (*Pseudochilodonopsis*); 5. preoral kinety continuous; terminal fragment subapically positioned (*Chilodonella*); 6. oral ciliature comprises only one kinety (*Chlamydonellopsis*); 7. three oral kineties forming a Y-shape (*Chlamydonella*); 8. the presence of cross-striated band around perimeter between ventral and dorsal surfaces; pre- and postoral kineties completely separated; perioral kineties consisting one continuous anterior and two detached posterior rows (*Coeloperix*); 9. the presence of non-cross-striated grooves around perimeter between ventral and dorsal surfaces; pre- and postoral kineties completely separated; perioral kineties consisting one continuous anterior and two detached posterior rows (*Lynchella*); 10. the presence of cross-striated band; oral ciliature consisting two circumoral kineties and one preoral kinety (*Chlamydodon*); 11. the absence of cross-striated band; oral ciliature consisting two circumoral kineties and one preoral kinety (*Paracyrtophoron*); 12. right kineties separated from left kineties; oral kinety segmented; with non-ciliated adhesive region (*Pithites*); 13. right and left kineties continuous with each other; oral kinety segmented; posterior glandular region conspicuously depressed (*Trichopodiella*); 14. right kineties separated from left kineties posteriorly; oral ciliature consisting only two circumoral kineties; podite absent (*Trochochilodon*); 15. oral kineties consisting two fragments; postoral kineties strongly shortened posteriad; terminal fragments consisting several parallel arranged fragments; podite present (*Aporthotrochilia*); 16. left kineties shortened posteriad; podite present; oral kineties consisting two circumoral kineties and one preoral kinety (*Harmannula*); 17. oral kineties consisting obliquely arranged fragments (*Heterohartmannula*); 18. podite surrounded by kineties; circumoral kineties consisting more than two parallel fragments (*Aegyriana*); 19. postoral kineties considerably shorter than right kineties and terminating at the same postequatorial level; podite present (*Brooklynella*); 20. body laterally compressed but no ventral groove; two nematodesmal rods; constantly two left frontal kineties (*Trochilia*); 21. six nematodesmal rods; three left frontal kineties (*Agnathodysteria*); 22. body laterally compressed with ventral groove; two nematodesmal rods (*Dysteria*); 23. body twisted; right kineties shortened posteriad (*Spirotdysteria*); 24. right kineties reduced to several sparsely arranged fragments; spines present on dorsal margin (*Mirodysteria*); 25. four nematodesmal rods; postoral and left kineties extremely short, positioned anterior of equator (*Microxysma*); 26. six nematodesmal rods; postoral and left kineties short, positioned anterior of equator (*Trochilioides*).

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used to explore its variance and to better understand evolutionary relationships among cyrtophorian genera.

Materials and methods

Source of organisms and morphological identification

Species sequenced in this study were collected from both northern and southern regions of China (Table 1). Culturing and morphological examination of these species were according to Qu *et al.* (2015a,b). Species identification was based on published guides and descriptions (Deroux 1976; Song & Wilbert 2002; Gong 2005). Terminology and systematics follow Lynn (2008).

DNA extraction, PCR amplification and sequencing

Cell isolation and genomic DNA extraction were according to the study of Gong *et al.* (2008). Primers used in this study were EukA and EukB (Medlin *et al.* 1988). The polymerase chain reaction (PCR) followed the protocol of Yi & Song (2011).

Alignments

Twenty-three newly acquired sequences were deposited in the GenBank database with the accession numbers listed in Table 2. Other sequences used for phylogenetic tree construction were obtained from GenBank (Table 2). The main data set, which includes 59 SSU rRNA sequences of the subclass Cyrtophoria, plus 7 representatives of the subclass Suctoria Claparède & Lachmann, 1858, and one of the subclass Rhynchodia Chatton & Lwoff, 1939, serving as the outgroup taxa, was aligned using MUSCLE (Edgar 2004). Sequence identities were calculated by BIOEDIT v. 7.2.0 (Hall 1999). One-factor analysis of variance (one-way ANOVA) was applied to compare means of sequence identities among taxa employing SPSS v. 16.0 (Norusis 2008). The ambiguously aligned sites were masked using GBLOCKS v. 0.91b (Castresana 2000) yielding the data set 1, an alignment of 1786 characters. Parsimony-informative sites in 19 SSU rRNA sequences of all 17 dysteriids were picked out from the main data set, yielding the data set 2.

Phylogenetic analyses

Bayesian inference analysis was performed with MRBAYES on XSEDE v. 3.1.2 (Ronquist & Huelsenbeck 2003) using the GTR+I+G evolutionary model indicated by MRMODELTEST v. 2.2 (Nylander 2004). The program was run for one million generations with a sample frequency of 100 and a burn-in of 2500 (Gao & Katz 2014). All trees remaining after discarding the burn-in were used to calculate posterior probabilities of the 50% majority rule consensus tree.

The program MODELTEST v. 3.4 (Posada & Crandall 1998) selected GTR+I+G ($G = 0.4831$, $I = 0.2167$) under the AIC criterion as the best model, which was then used for ML analysis. The ML tree was constructed with the RAXML-HPC2 on XSEDE v. 7.6.3 (Guindon & Gascuel 2003).

Table 1 Sampling sites and habitat information of species sequenced in this study

Species name	Sampling site	Latitude/longitude	Habitat description	GB. Acc. No.
Agnathodysteria littoralis	Techeng Island, Zhanjiang	21.16°N 110.43°E	Mangrove, T = 28.4 °C, S = 25.8‰, pH = 7.3	KC753482
Brooklynella sinensis	Donghai Island, Zhanjiang	21.02°N 110.52°E	Sand beach, T = 24.7 °C, S = 15.5‰, pH = 9.3	KC753483
Chlamydodon sp.	Changyi, China	36.25°N 119.13°E	Shrimp pond, T = 21 °C	KC753485
Chlamydonella sp.	Aoshanwei, Qingdao	36.37°N 120.69°E	Sea cucumber pond, water sample	KC753486
Chlamydonellopsis calkinsi	First Bathing Beach, Qingdao,	36.05°N 120.30°E	Intertidal, sandy sediment, T = 10 °C, S = 12‰.	KC753487
Coeloperix sleighi	Zhanjiang	21.03°N 110.50°E	T = 23.8 °C, S = 28.6‰, pH = 8.6	KC753489
Dysteria cristata	–	–	–	KC753488
Dysteria pectinata	Xiaogang Port, Qingdao, China	36.07°N 120.31°E	Water sample	FJ870068
Dysteria lanceolata	First Bathing Beach, Qingdao,	36.05°N 120.30°E	Intertidal, sandy sediment, T = 10 °C, S = 12‰.	KC753490
Dysteria compressa	Donghai Island, Zhanjiang	21.02°N 110.52°E	Intertidal, T = 26 °C, S = 25.9‰, pH = 8.9	KC753491
Dysteria crassipes pop. 1	Zhuhai	22.27°N 113.58°E	Oyster pond, water sample, T = 20 °C, S = 15‰	FJ868206
Dysteria crassipes pop. 2	Shenzhen	22.52°N 114.01°E	Mangrove, T = 20.7 °C, S = 13.8‰	KC753492
Dysteria crassipes pop. 3	Daya Bay	22.70°N 114.53°E	T = 19.4 °C, S = 32.6‰, pH = 8.2	KC753493
Dysteria reesi	Daya Bay	22.70°N 114.53°E	Intertidal, water sample T = 25 °C, S = 30‰	FJ868205
Hartmannula sinica	–	–	S = 30‰	EF623827
Odontochlamys alpestris biciliata	The estuary of the Pearl River	21.15°N 110.62°E	T = 25.8 °C, S = 12.2‰, pH = 8.9	KC753484
Pseudochilodonopsis sp. 1	First Bathing Beach, Qingdao,	36.05°N 120.30°E	Sand beach, T = 22 °C, S = 30‰	KC753495
Pseudochilodonopsis sp. 2	Shenzhen	22.52°N 114.01°E	Mangrove, T = 20.7 °C, S = 13.8‰	KC753497
Pseudochilodonopsis sp. 3	Shenzhen	22.52°N 114.01°E	Mangrove, T = 20.7 °C, S = 13.8‰	KC753496
Pseudochilodonopsis mutabilis	Shenzhen	22.52°N 114.01°E	Mangrove, T = 20.7 °C, S = 13.8‰	KC753498
Spirodysteria kahli	Changyi	37.72°N 119.31°E	Shrimp pond, T = 23 °C, S = 87‰	KC753499
Trichopodiella faurei pop. 1	Xiaogang Port, Qingdao, China	36.07°N 120.31°E	Marine, periphytic, T = 13 °C, S = 30‰	FJ870071
Trichopodiella faurei pop. 2	Dameisha, Shenzhen	22.59°N 114.31°E	Stone, T = 22.8 °C, S = 31.9‰, pH = 8.5	KC753500

–, data lacking.

Table 2 Accession numbers of the species used for the phylogenetic tree construction. Species newly sequenced in this study are marked in bold. Species sequenced by the authors' group are marked by asterisks (*)

Species name	GenBank Acc. No.	Species name	GenBank Acc. No.
Acineta sp.*	AY332718	*Ephelota gemmeipara*	DQ834370
*Aegyriana oliva**	FJ998029	*Hartmannula derouxi**	AY378113
Agnathodysteria littoralis*	KC753482	***Hartmannula sinica****	EF623827
Brooklynella sinensis*	KC753483	*Heliophrya erhardi*	AY007445
Chilodonella uncinata	AF300281	*Heterohartmannula fangi**	FJ868204
*Chlamydodon caudatus**	JQ904059	*Hyocoma acinetarum**	JN867019
Chlamydodon excocellatus	AY331790	*Isochona* sp. OOSW-1*	AY242116
Chlamydodon* sp.	KC753485	*Isochona* sp. OOSW-2*	AY242117
Chlamydodon mnemosyne pop.1*	FJ998031	*Isochona* sp. OOSW-3*	AY242118
*Chlamydodon obliquus**	FJ998030	*Isochona* sp. OOSW-4*	AY242119
*Chlamydodon paramnemosyne **	JQ904058	*Lynchella* sp.*	FJ998036
*Chlamydodon salinus **	JQ904057	*Microxysma acutum**	FJ870069
Chlamydodon triquetrus	AY331794	*Mirodysteria decora**	JN867020
*Chlamydonella pseudochilodon**	FJ998032	***Odontochlamys alpestris biciliata****	KC753484
Chlamydonella* sp.	KC753486	*Paracyrtophoron tropicum**	FJ998035
Chlamydonellopsis calkinsi*	KC753487	*Pithites vorax**	FJ870070
Chlamydonellopsis sp.*	FJ998033	*Prodiscophrya collini*	AY331802
Coeloperix sleighi*	KC753489	***Pseudochilodonopsis mutabilis****	KC753498
Coeloperix sp.*	FJ998034	***Pseudochilodonopsis* sp. 1***	KC753495
Discophrya collini	L26446	***Pseudochilodonopsis* sp. 2***	KC753497
*Dysteria brasiliensis**	EU242512	***Pseudochilodonopsis* sp. 3***	KC753496
***Dysteria compressa **	KC753491	*Pseudochilonopsis fluviatilis**	JN867021
Dysteria crassipes* pop. 1	FJ868206	***Spirodysteria kahli****	KC753499
Dysteria crassipes* pop. 2	KC753492	*Tokophrya lemnarum*	AY332721
Dysteria crassipes* pop. 3	KC753493	*Tokophrya quadripartita*	AY102174
Dysteria cristata*	KC753488	*Trichopodiella faurei**	EU515792
*Dysteria derouxi**	AY378112	***Trichopodiella faurei* pop. 1***	FJ870071
Dysteria lanceolata*	KC753490	***Trichopodiella faurei* pop. 2***	KC753500
Dysteria pectinata*	FJ870068	*Trithigmostoma cucullulus**	FJ998037
*Dysteria subtropica**	KC753494	*Trithigmostoma steini*	X71134
*Dysteria procera**	DQ057347	*Trochilia petrani**	JN867016
Dysteria reesi*	FJ868205	*Trochilioides recta**	JN867017
Dysteria sp. 1	AY331797	*Trochochilodon flavus**	JN867018
Dysteria sp. 2	AY331800		

Table 3 Approximately unbiased (AU) test results

No.	Topology constraints	-lnL	AU value (*P*)
1	*Microxysma acutum* + Hartmannulidae	23859.77	**0.045**
2	*Dysteria*	23898.26	**4e-4**
3	*Brooklynella sinensis* + Hartmannulidae	23838.61	0.175
4	*Dysteria* + *Agnathodysteria*	23985.55	**2e-51**
5	*Dysteria* + *Agnathodysteria* + *Mirodysteria* + *Spirodysteria*	23825.19	0.765

$P < 0.05$ refutes monophyly; $P > 0.05$ does not refute the possibility of monophyly. Results in which $P < 0.05$ are marked in bold.

The reliability of internal branches was assessed using the nonparametric bootstrap method with 1000 replicates.

A neighbor-joining tree was produced with MEGA v. 5.05 (Tamura *et al.* 2011). The reliability of internal branches was assessed using the bootstrap method with 1000 replicates.

A maximum parsimony tree was calculated according to the parsimony-informative sites (832 sites) with PAUP* v. 4.0b10 (Swofford 2002). The reliability of its internal branches was estimated by bootstrapping with 1000 replicates.

Five constrained ML analyses were carried out by PAUP* v. 4.0b10 according to the constraints listed in Table 3. Resulting constrained topologies were then compared to the non-constrained ML topology using the approximately unbiased (AU) test (Shimodaira 2002) as implemented in CONSEL v. 0.1 (Shimodaira & Hasegawa 2001). For all constraints, internal relationships within the constrained groups were unspecified, and relationships among the remaining taxa were also unspecified (Zhang *et al.* 2014; Zhao *et al.* 2014).

Secondary structure predictions

RNA structures of representatives of each cyrtophorian genus were decomposed into substructural components and

their features characterized and coded using an alphanumerical format, based on the model proposed by Van de Peer & de Wachter (1997). Preliminary modelling of blocks of high positional variation by energy minimization was carried out using MFOLD (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) (Zuker 2003). The sequences at the beginning and the end of the V4 region are highly conserved among ciliates. The SSU rRNA gene sequences of other ciliates were compared with the SSU rRNA secondary structure model of *Tetrahymena canadensis* in the European ribosomal RNA database (http://bioinformatics.psb.ugent.be/webtools/rRNA/secmodel/) (Wuyts *et al.* 2004) and manually adjusted to ensure retention of conserved core elements as well as the beginning and the end of the V4 region, taking into account predicted tertiary interaction (Alkemar & Nygard 2004). Folding results were displayed using RNAVIZ2 (de Rijk *et al.* 2003). One-factor analysis of variance (one-way ANOVA) was applied to compare means of nucleotide numbers of E23_7 regions among taxa employing SPSS v. 16.0 (Norusis 2008).

Results

SSU rRNA gene sequence comparison among cyrtophorians

The SSU rRNA sequence identities among species within the family Dysteriidae varied from 79.9% to 99.2%, the lowest sequence identity being between *Agnathodysteria littoralis* Deroux, 1976 and *Dysteria* sp.1 (79.9%). Sequence identities between *Spirodysteria* Gong *et al.*, 2007 and other genera of Dysteriidae varied from 81.5% to 97.5%, whereas for *Mirodysteria* Kahl, 1933, it was 87.2% to 90.9%. Within the family Hartmannulidae, sequence identities among species varied from 76.4% to 95.3%. Sequence identities between *Brooklynella* and other genera of Hartmannulidae varied from 78.7% to 82.7%. Within the family Chlamydodontidae, sequence identities among species varied from 82.3% to 96.5%; within the family Chilodonellidae, it was 82.9% to 99.9%; and within the family Lynchellidae Jankowski, 1968, it was 76.6% to 93.8% (Table S1).

Two morphospecies (*Dysteria crassipes* Claparède & Lachmann, 1859 and *Trichopodiella faurei* Gong *et al.*, 2008) were isolated more than once. For the SSU rRNA sequence of *D. crassipes*, the three populations were 0.7%–0.9% divergent. For *T. faurei*, the SSU rRNA sequences of Shenzhen (pop. 2) and Daya Bay (Gong *et al.* 2008) populations were identical, whereas the Qingdao population (pop. 1) differed from the other two by 17 base pairs (Gong *et al.* 2008). No morphological differences were detected among these three populations both *in vivo* and after protargol preparation. Considering that morphological characters used for identification of cyrtophorians are limited, *T. faurei* might be a complex with cryptic species.

Phylogenetic analyses

Based on 23 new SSU rRNA gene sequences and 40 SSU rRNA sequences of phyllopharyngeans obtained from GenBank, phylogenetic trees were constructed using maximum likelihood (ML), Bayesian inference (BI), neighbor-joining (NJ) and maximum parsimony (MP) methods. All four algorithms generated trees with a similar topology; therefore, only the ML tree is shown here (Fig. 3). Most of the newly sequenced species appeared in expected positions, grouping with their closest relatives (see below for exceptions).

The order Dysteriida comprises two large families, that is Dysteriidae and Hartmannulidae, plus two much smaller families, that is Kyaroikeidae Sniezek & Coats, 1996 (for which SSU rRNA gene sequence data are not available), and Plesiotrichopidae Deroux, 1976. Dysteriidae was monophyletic, whereas *Dysteria* was paraphyletic because it contained *Mirodysteria* and the newly sequenced *Spirodysteria* (ML/BI: 100/1.00). *Spirodysteria kahli* (Tucolesco, 1962) Gong *et al.*, 2007 formed a clade with *D. procera* Kahl, 1931 and *D. subtropica* Qu *et al.*, 2015, which is in part consistent with their similar morphologies, that is all three species have a slender and elongated body shape which is unusual in *Dysteria* (Gong *et al.* 2007). *Dysteria compressa* (Gourret & Roeser, 1888) Kahl, 1931 is most closely related to *D. brasiliensis* Faria *et al.*, 1922, and *D. crassipes* in our trees. A single spine is present caudally in both *D. compressa* and *D. brasiliensis* and subcaudally in some individuals of *D. crassipes* (Kahl 1931; Gong *et al.* 2009). Therefore, we concluded that body shape and the presence of a spine are important characters for inferring phylogenetic relationships within the genus *Dysteria*. *Microxysma* and *Trochilia* Dujardin, 1841, grouped together with low support (ML/BI: 58/67). Another newly sequenced genus, *Agnathodysteria* Deroux, 1977, occupied the basal position within the Dysteriidae clade with low support in the ML tree (bootstrap value 28). Within Hartmannulidae, the newly sequenced populations of *Trichopodiella faurei* grouped with *T. faurei* EU515792 with full support, whereas *Heterohartmannula* Pan, 2012 (represented by *H. fangi* Pan, 2012), nested within the *Hartmannula* Poche, 1913 clade. *Aegyriana* Song & Wilbert, 2002 and *Trichopodiella* Corliss, 1960, formed a clade which then clustered with *Hartmannula–Heterohartmannula*. *Trochilioides* Kahl, 1931 was a relatively long branch that was sister to all other hartmannulids. The newly sequenced *Brooklynella* was positioned outside the family Hartmannulidae, clustering with Dysteriidae with high support (ML/BI: 96/1.00) and hence rendering Hartmannulidae paraphyletic. Plesiotrichopidae, represented by *Trochochilodon flavus* Deroux, 1976, occupied a sister position to the Dysteriidae–Hartmannulidae–Chonotrichia clade with low support (ML/BI: 50/0.66). Pithitidae Gao *et al.*, 2012, clustered with Lynchellidae with low support (ML/BI: 51/0.55).

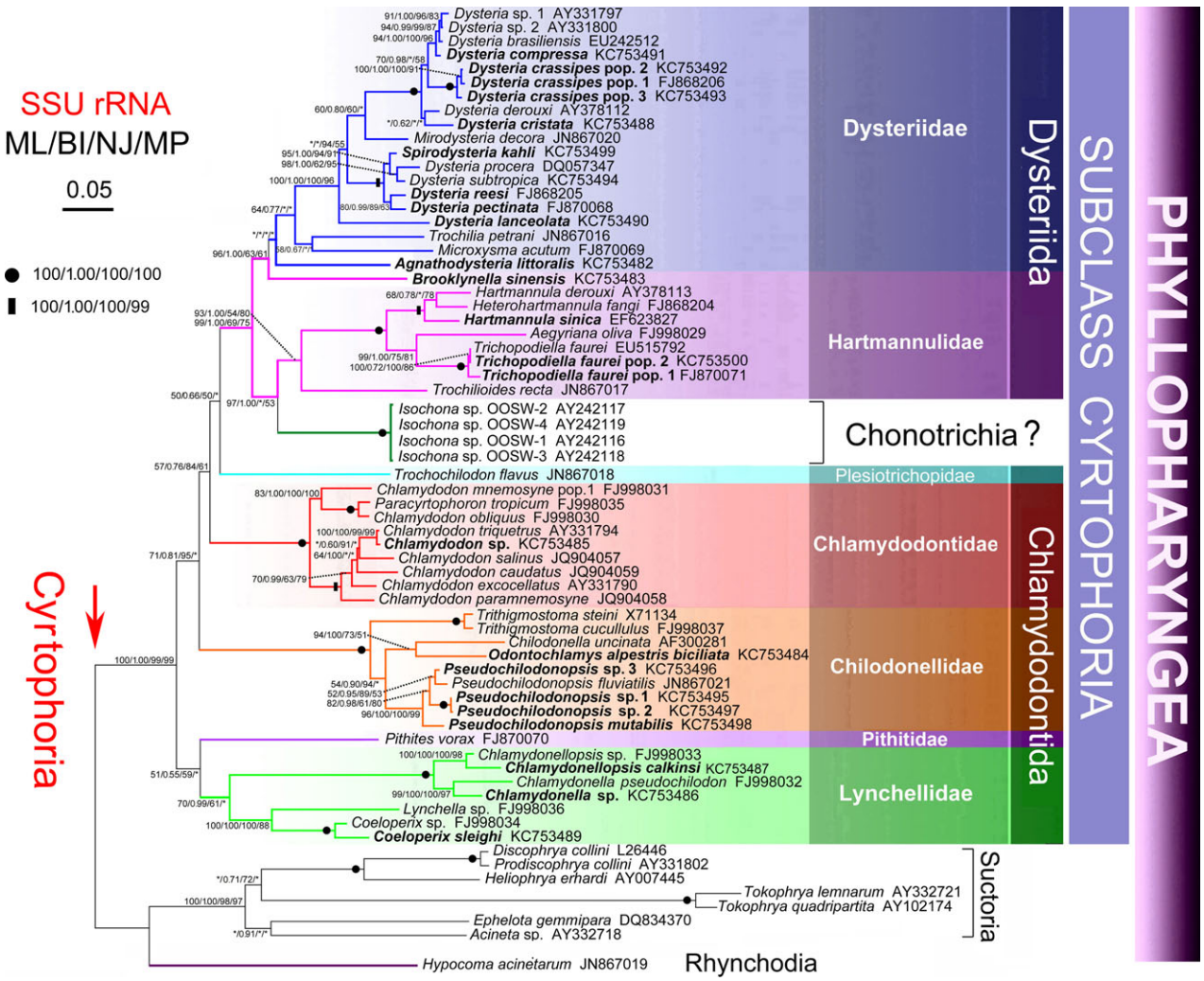

Fig. 3 The maximum likelihood phylogenetic tree inferred from small subunit rRNA (SSU rRNA) gene sequences. Support values at the nodes represent the bootstrap or posterior possibility values from ML/BI/NJ/MP analyses, respectively. Asterisks indicate bootstrap values <50%. Evolutionary distance is represented by the branch length separating the species in the figure. The scale bar corresponds to 5 substitutions per 100 nucleotide positions. Newly sequenced species are in bold.

The order Chlamydodontida was paraphyletic because the three well-defined monophyletic families, Chlamydodontidae, Chilodonellidae and Lynchellidae, did not group together (Fig. 3). In the family Chilodonellidae, the newly sequenced *Odontochlamys* Certes, 1891, clustered with *Chilodonella* Strand, 1928 (ML/BI: 94/1.00), which then grouped with species of *Pseudochilodonopsis* Foissner, 1979 (ML/BI: 96/1.00). This arrangement is consistent with their morphology, both having separated right and left kineties and non-fragmented preoral kineties, although they differ in the location of the terminal fragment (apical vs. subapical), and both have a distinct oral ciliary pattern that differs from *Pseudochilodonopsis* (preoral kineties non-fragmented vs. fragmented) (Foissner *et al.*, 1991). Two species of *Trithigmostoma* Jankowski, 1967 appeared as a peripheral branch of the three above-mentioned genera with full support. In the family Lynchellidae, three species were newly sequenced, namely *Chlamydonellopsis calkinsi* Kahl, 1928, *Coeloperix sleighi* Gong & Song, 2004; and *Chlamydonella* sp., and each clustered with its congeners with strong support (ML/BI: 100/1.00 or 99/1.00). Two fully supported groups were recovered within the Lynchellidae: (i) *Chlamydonella* Petz *et al.*, 1995, and *Chlamydonellopsis* Blatterer & Foissner, 1990, and (ii) *Lynchella* Kahl in Jankowski, 1968, and *Coeloperix* Gong & Song, 2004.

Secondary structure of the hypervariable region

The predicted secondary structure of the SSU rRNA genes of cyrtophorians corresponds to that of *Tetrahymena canadensis*, which is a widely accepted eukaryotic SSU

rRNA secondary structure model (Neefs *et al.* 1993) (Fig. 4). Fifty universal helices were distinguished in SSU rRNA secondary structures and were numbered according to their order of occurrence on the 5'-proximal strand. Several hypervariable regions were also recognized and numbered in this model. Length and structural differences occurred mainly in the variable region 4 (V4). Within the subclass Cyrtophoria, variations occurred in hypervariable region E23_7: (i) species in the family Dysteriidae had more nucleotides (39 bases on average, Table 4B), which is significantly different from that of other families (Fig. 4A–F, Table 4D); (ii) the average length of E23_7 in the family Hartmannulidae was much shorter (35 bases, Table 4B) and differed significantly from that of other families (Fig. 4G–K, Table 4D); (iii) *Isochona* spp. had the same length of E23_7 region (39 bases, Fig. 4L, Table 4B); and (iv) families in the order Chlamydodontida did not differ significantly from one another in length of E23_7 (37 bases on average, Table 4B), but differed significantly from families in the order Dysteriida (Fig. 4M–W, Table 4D).

Discussion

Systematic arrangement review

The present study is consistent with previous findings in that (i) Chlamydodontida is paraphyletic with three well-defined monophyletic families, namely Chlamydodontidae, Chilodonellidae and Lynchellidae; (ii) the systematic position of Plesiotrichopidae remains unclear; and (iii) *Chlamydodon* is monophyletic (Gao *et al.* 2012).

The present study also supports the assignments of *Pithites*, *Trochochilodon* and *Microxysma* suggested by Gao *et al.* (2012).

Pithites. After addition of 23 newly sequenced species/population into phylogenetic analyses, *Pithites*, which used to be a member of Plesiotrichopidae, remains separated from another plesiotrichopid genus, *Trochochilodon*, in all trees (Fig. 3). Considering that it has separated right and left kineties, lacks the podite and is topologically located basally to Lynchellidae, we agree with Gao *et al.* (2012) that it should be removed from Dysteriida and be assigned to Chlamydodontida. Moreover, most of its characteristics, that is apically positioned cytostome, body shape not dorsoventrally compressed and oral ciliature consisting several kinety fragments, are rather different from those of all other chlamydodontid families (Deroux & Dragesco 1968); thus, it should represent a distinct family.

Trochochilodon. This genus clustered outside Dysteriida and occupied an intermediate position between Chlamydodontidae and Hartmannulidae (Fig. 3). A closer relationship of *Trochochilodon* to the order Chlamydodontida than to the order Dysteriida is supported also morphologically in that *Trochochilodon* does not display a podite or adhesive apparatus, that is diagnostic features of Dysteriida (Pan *et al.* 2012). Therefore, we agree with Gao *et al.* (2012) that *Trochochilodon* should be transferred from Dysteriida to Chlamydodontida.

Microxysma. Regarding the highly laterally compressed body and reduced left kineties, which is the characteristic of Dysteriidae rather than Hartmannulidae, Gao *et al.* (2012) transferred *Microxysma* from Hartmannulidae to Dysteriidae. In the current work, *Microxysma acutum* Deroux, 1976 nested within the Dysteriidae as a sister clade of *Trochilia petrani* Dragesco, 1966 (Fig. 3), and the monophyly of *Microxysma* + Hartmannulidae is refuted by the AU test (Table 3, constraint 1, $P = 0.045$). Therefore, we support the assignment of *Microxysma* to Dysteriidae.

The genus Dysteria is paraphyletic

The closest relatives of *Dysteria* are thought to be *Spirodysteria* Gong *et al.*, 2007 and *Mirodysteria* Kahl, 1933. *Spirodysteria* differs from *Dysteria* mainly in its spirally twisted body shape (Gong *et al.* 2007), whereas *Mirodysteria* is distinguished by its conspicuous spines and loosely spaced kinetosomes in the right kineties (Pan *et al.* 2011). Previous phylogenetic studies have depicted *Dysteria* as being paraphyletic, although this finding was not supported statistically (Gao *et al.* 2012). In the present study, with expanded taxon sampling and the inclusion of molecular data for *Spirodysteria*, the paraphyly of *Dysteria* was confirmed and monophyly of the genus *Dysteria* was also excluded by the AU test at a significance level of 0.001 (Table 3, constraint 2). This supports the contention that the genus *Dysteria* is genetically diverse and should be split into several morphologically and ontogenetically defined genera (Gao *et al.* 2012).

Paraphyly of *Dysteria* might have also resulted from an evolutionary process known as budding that led to the emergence of new lineages during the phylogenetic history of *Dysteria*. Budding is usually caused by the development of new characters with a separate evolution in a new niche (Mayr & Bock 2002). When a new taxon originates and diverges while the parental taxon is extant, this results in paraphyly (Hörandl 2006). Within Ciliophora, examples of paraphyly caused by budding have been posited for taxa from the class Colpodea Small & Lynn, 1981, and among dileptids (Foissner *et al.* 2011; Vďačný & Rajter 2015). *Dysteria* is probably another example, whereby it is the stem lineage of Dysteriidae. Budding from this stem lineage has probably occurred at least twice resulting in the formation of two genera: (i) *Mirodysteria* whose right kineties consist of few kinetosomes and form several cirrus-

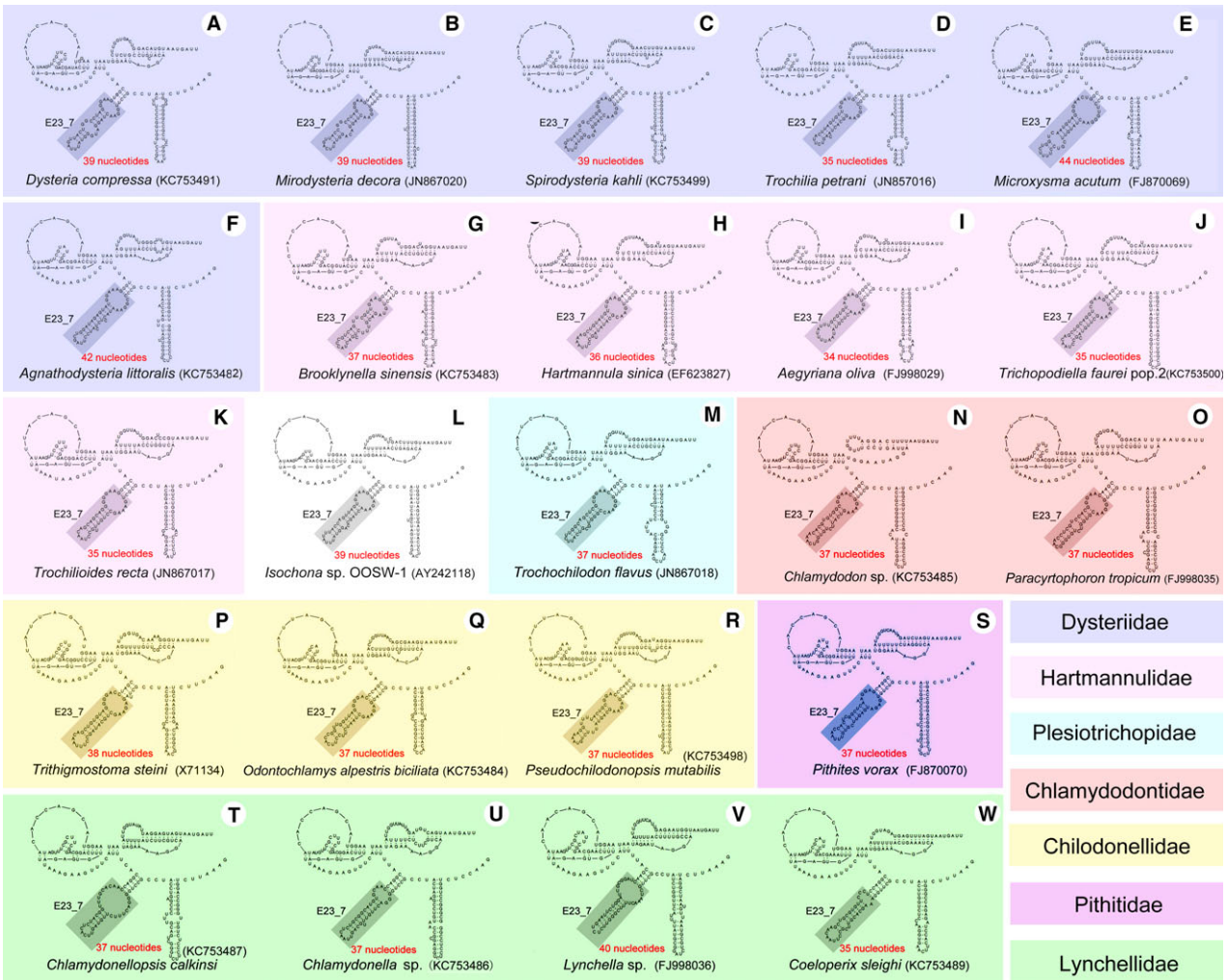

Fig. 4 Predicted secondary structures of variable region 4 (V4) of the small subunit rRNA of representatives of each cyrtophorian genus, comparing the nucleotide numbers of helix 23_7 (shaded) in the genera of Dysteriidae (A–F: *Dysteria*, *Mirodysteria*, *Spirodysteria*, *Trochilia*, *Microxysma*, *Agnathodysteria*); Hartmannulidae (G–K: *Brooklynella*, *Hartmannula*, *Aegyriana*, *Trichopodiella*, *Trochilioides*); Chonotrichia (L: *Isochona*); Plesiotrichopidae (M: *Trochochilodon*); Chlamydodontidae (N, O: *Chlamydodon*, *Paracyrtophoron*); Chilodonellidae (P–R: *Trithigmostoma*, *Chilodonella*, *Pseudochilodonopsis*); Pithitidae (S: *Pithites*); Lynchellidae (T–W: *Chlamydonellopsis*, *Chlamydonella*, *Lynchella*, *Coeloperix*). GenBank/EMBL accession numbers are given in parentheses. The number of nucleotides in helix E23_7 for each species is given beneath the helix.

like fragments, probably as an adaptation for crawling among sand grains and (ii) *Spirodysteria* whose body is conspicuously twisted, possibly as an adaptation to its pelagic lifestyle.

Brooklynella is an intermediate taxon between Hartmannulidae and Dysteriidae

It has previously been suggested that *Brooklynella sinensis* is an intermediate form between the hartmannulids and dysteriids because it possesses characters in common with both (Gong & Song 2006). On the one hand, it has unciliated postoral kineties and about six nematodesmal rods, which are typical features of dysteriids. On the other hand, it is hartmannulid-like in having a dorsoventrally compressed body and continuous kineties in the left field. In the present study, molecular evidence supporting this inference was as follows: (i) *Brooklynella* occupies a peripheral position outside Dysteriidae with high support (ML/BI: 96/1.00), and AU tests do not refute the possibility of *Brooklynella* being a hartmannulid (Table 3, constraint 3, $P = 0.175$); (ii) average sequence identity between *Brooklynella* and Dysteriidae (0.805) is significantly lower than that among dysteriid species (0.903). Likewise, that between *Brooklynella* and Hartmannulidae (0.809) is lower than that among hart-

Table 4 Statistics of E23_7 region of Cyrtophoria species and multiple comparisons among families

Order	Family	Species	E23_7 Count
Dysteriida	Dysteriidae	***Agnathodysteria littoralis****	42
		*Microxysma acutum**	44
		*Trochilia petrani**	35
		Dysteria lanceolata*	40
		Dysteria cristata*	39
		Dysteria crassipes* pop. 3	39
		Dysteria crassipes* pop. 1	39
		Dysteria crassipes* pop. 2	39
		*Dysteria derouxi**	39
		Dysteria compressa*	39
		Dysteria sp.1	39
		*Dysteria brasiliensis**	39
		Dysteria sp.2	39
		*Mirodysteria decora**	39
		Dysteria pectinata*	39
		Dysteria reesi*	39
		*Dysteria subtropica**	40
		*Dysteria procera**	39
		Spirodysteria kahli*	39
	Hartmannulidae	*Aegyriana oliva**	34
		Trichopodiella faurei* pop. 1	35
		*Trichopodiella faurei**	35
		Trichopodiella faurei* pop. 2	35
		*Heterohartmannula fangi**	36
		*Hartmannula derouxi**	36
		Hartmannula sinica*	36
		Brooklynella sinensis*	37
		*Trochilioides recta**	35
	Isochona	*Isochona* sp. OOSW-2*	39
		Isochona sp. OOSW-3*	39
		Isochona sp. OOSW-1*	39
		Isochona sp. OOSW-4*	39

(B) Descriptives

Family or Genus	*n*	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Dysteriidae	19	39.32	1.668	0.383	35	44
Hartmannulidae	9	35.44	0.882	0.294	34	37
Isochona	4	39	0	0	39	39
Plesiotrichopidae	1	37	–	–	–	–
Chlamydodontidae	9	37	0	0	37	37
Chilodonellidae	9	37.22	0.441	0.147	37	38
Pithitidae	1	37	–	–	–	–
Lynchellidae	7	37	1.732	0.655	35	40

(C) ANOVA

Group	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	112.047	5	22.409	15.061	0
Within Groups	75.883	51	1.488		
Total	187.93	56			

(D) Multiple comparisons

(I) group	(J) group	Mean Difference (I–J)	Std. Error	Sig.
Dysteriidae	Hartmannulidae	3.871**	0.494	0
	Isochona	0.316	0.671	0.64
	Chlamydodontidae	2.316**	0.494	0
	Chilodonellidae	2.094**	0.494	0
	Lynchellidae	2.316**	0.539	0
Hartmannulidae	Dysteriidae	−3.871**	0.494	0
	Isochona	−3.556**	0.733	0
	Chlamydodontidae	−1.556**	0.575	0.009
	Chilodonellidae	−1.778**	0.575	0.003
	Lynchellidae	−1.556**	0.615	0.015
Isochona	Dysteriidae	−0.316	0.671	0.64
	Hartmannulidae	3.556**	0.733	0
	Chlamydodontidae	2.000**	0.733	0.009

Table 4 Continued

Order	Family	Species	E23_7 Count	(D) Multiple comparisons				
				(I) group	(J) group	Mean Difference (I–J)	Std. Error	Sig.
Chlamydodontida	Plesiotrichopidae	*Trochochilodon flavus**	37		Chilodonellidae	1.778**	0.733	0.019
					Lynchellidae	2.000**	0.765	0.012
	Chlamydodontidae	*Chlamydodon salinus**	37	Chlamydodontidae	Dysteriidae	−2.316**	0.494	0
		Chlamydodon excocellatus	37		Hartmannulidae	1.556**	0.575	0.009
		*Chlamydodon caudatus**	37		*Isochona*	−2.000**	0.733	0.009
		*Chlamydodon paramnemosyne**	37		Chilodonellidae	−0.222	0.575	0.701
		Chlamydodon triquetrus	37		Lynchellidae	0	0.615	1
		Chlamydodon* sp.	37	Chilodonellidae	Dysteriidae	−2.094**	0.494	0
		Chlamydodon mnemosyne pop.1*	37		Hartmannulidae	1.778**	0.575	0.003
		*Chlamydodon obliquus**	37		*Isochona*	−1.778**	0.733	0.019
		*Paracyrtophoron tropicum**	37		Chlamydodontidae	0.222	0.575	0.701
					Lynchellidae	0.222	0.615	0.719
	Chilodonellidae	*Trithigmostoma steini*	38	Lynchellidae	Dysteriidae	−2.316**	0.539	0
		Trithigmostoma sp.*	38		Hartmannulidae	1.556**	0.615	0.015
		Chilodonella uncinata	37		*Isochona*	−2.000**	0.765	0.012
		Odontochlamys alpestris biciliata*	37		Chlamydodontidae	0	0.615	1
		Pseudochilodonopsis mutabilis*	37		Chilodonellidae	−0.222	0.615	0.719
		Pseudochilodonopsis* sp. 1	37					
		Pseudochilodonopsis* sp. 3	37					
		*Pseudochilonopsis fluviatilis**	37					
		Pseudochilodonopsis* sp. 2	37					
	Pithitidae	*Pithites vorax**	37					
	Lynchellidae	*Chlamydonella pseudochilodon**	38					
		Chlamydonella* sp.	37					
		Chlamydonellopsis sp.*	37					
		Chlamydonellopsis calkinsi*	37					
		Lynchella sp.*	40					
		Coeloperix sp.*	35					
		Coeloperix sleighi*	35					

Species newly sequenced in this study are marked in bold. Species sequenced by the authors' group are marked by asterisks (*).

**The mean difference is significant at the 0.05 level.

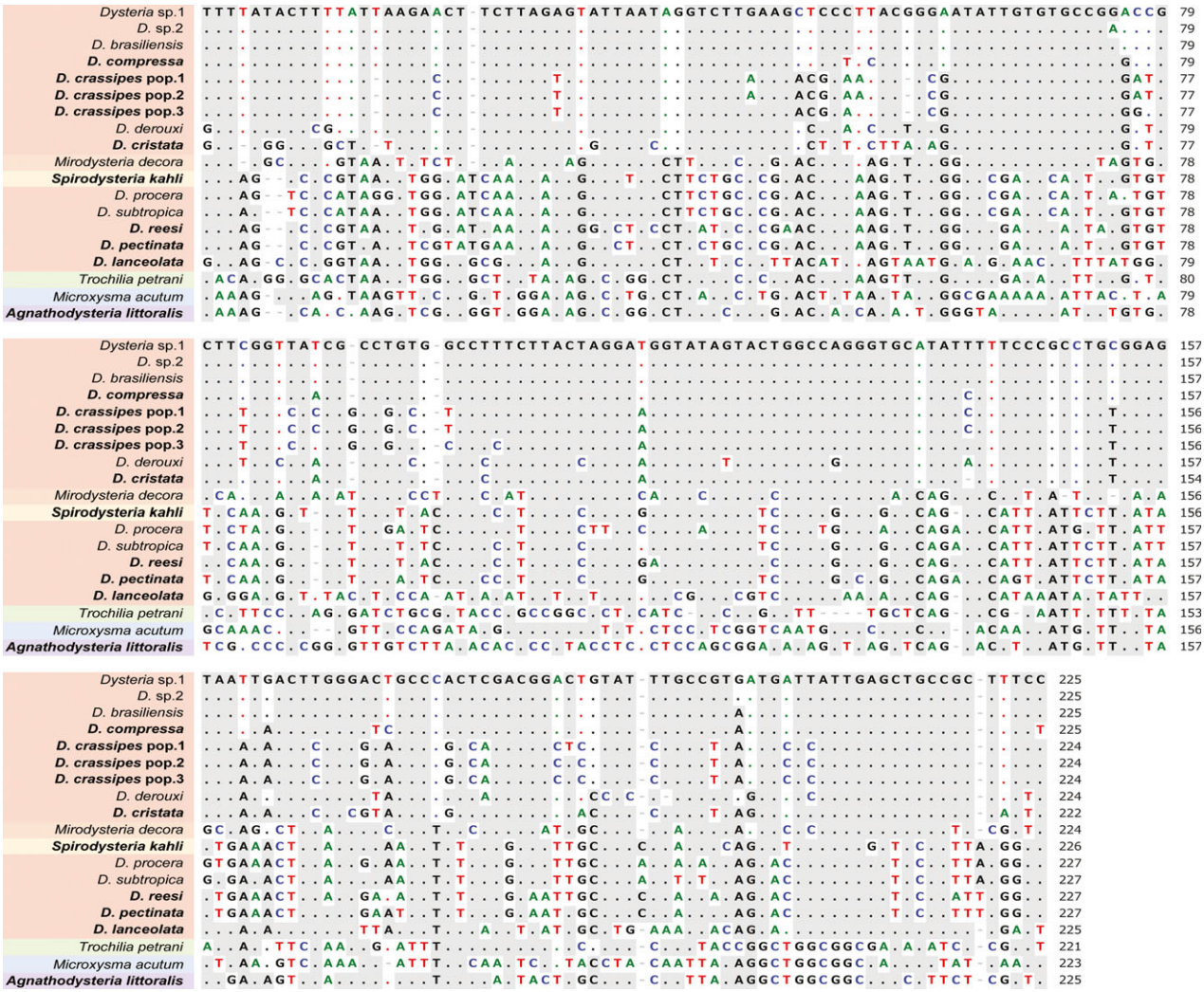

Fig. 5 SSU rRNA sequence alignment of all 17 dysteriids included in the present study; only parsimony-informative sites are displayed. Numbers in the right margin indicate the number of nucleotides. Gaps (-) represent insertion or deletion sites, and dots (.) stand for the matched sites. Sites that are ≥50% similarity are shaded in grey. Newly sequenced species are in bold.

mannulid species (0.882); and (iii) the length of E23_7 region of *Brooklynella* is 37 bp, which is between the mean values of Hartmannulidae (35.44 bp) and Dysteriidae (39.32 bp).

Molecular data are lacking for the type species of *Brooklynella*, *B. hostilis* Lom & Nigrelli, 1970. However, like *B. hostilis*, *B. sinensis* Gong & Song, 2006, possesses morphological characters that are typical of both hartmannulids and dysteriids. Therefore, both the morphological and the molecular data suggest that *Brooklynella* occupies an intermediate position between Hartmannulidae and Dysteriidae.

Is the genus Agnathodysteria valid?

Agnathodysteria littoralis Deroux, 1976 which is distinguished by its laterally flattened body and the possession of nine somatic kineties and six nematodesmal rods, was designated as the type species of *Agnathodysteria* by Deroux (1976). In Deroux's interpretation of protargol-stained specimens, *Agnathodysteria* can be distinguished from *Dysteria* by: (i) the number of nematodesmal rods (six in *Agnathodysteria* vs. only two in *Dysteria*) and (ii) body shape (slightly vs. highly laterally compressed). Deroux (1976) hypothesized that Dysteriina diversified in the evolution of three main independent morphological features, that is the shape of the cyrtopharyngeal apparatus, the reduction of the number of kineties and the relative extension of both the 'tectal' and the ciliated cortex. It was also inferred that the cyrtopharyngeal apparatus of *Dysteria* might have evolved from that of *Agnathodysteria* (Deroux 1976), which is consistent with the topology of the SSU rRNA gene tree (Fig. 3).

In the present study, *Agnathodysteria* groups outside the *Dysteria* clade. More importantly, the hypothesis that the group *Agnathodysteria* + *Dysteria* is monophyletic is rejected by the AU test (Table 3, constraint 4, $P = 2e\text{-}51$), even though the monophyly of the group comprising *Agnathodysteria* + *Dysteria* + *Spirodysteria* + *Mirodysteria* is still possible (Table 3, constraint 5, $P = 0.765$) (see Discussion above). Other evidence supporting the separation of *Agnathodysteria* from *Dysteria* include (i) unlike *Agnathodysteria*, the molecular biologically investigated species of *Dysteria* share unique nucleotides in 36 sites in semi-conserved, parsimony-informative regions of the SSU rRNA alignment (Fig. 5); (ii) the sequence similarities between *Agnathodysteria* and *Dysteria* spp. are significantly lower than those within the genus *Dysteria* (Table S1, $P < 0.05$); and (iii) *Agnathodysteria* has more nucleotides in E23_7 than *Dysteria* spp. (42 vs. 39/40). Thus, our results support the separation of *Agnathodysteria* from *Dysteria* as suggested by Deroux (1976).

Trithigmostoma is a basal genus of Chilodonellidae

Our phylogenetic analyses agree with previous schemes that *Trithigmostoma* is a member of Chilodonellidae (Corliss 1979; Small & Lynn 1985; de Puytorac 1994; Lynn & Small 2002; Gong 2005; Lynn 2008). Similar to other members of Chilodonellidae, *Trithigmostoma* possesses three oral kineties (two circumoral kineties and one preoral kinety), one centrally located heteromeric macronucleus, and all somatic kineties are restricted to the ventral side (Foissner *et al.* 1991). Unlike other chilodonellid genera, *Trithigmostoma* has continuous ventral somatic kineties and most of its right kineties extend to the posterior end of the cell (Fig. 2). These features are the characteristics for Chlamydodontidae and Lyncheliidae and hence can be considered as apomorphies of the order Chlamydodontida, but as plesiomorphies for *Trithigmostoma*. Therefore, based on the chilodonellid apomorphies and chlamydodontid plesiomorphies as well as molecular trees, we propose *Trithigmostoma* as a basal genus of Chilodonellidae.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The sequence identity (below) / variation (upper) matrices among SSU rRNA sequences of species from each family