Molecular Phylogeny of the Cyrtophorid Ciliates (Protozoa, Ciliophora, Phyllopharyngea)

Shan Gao, Jie Huang, Jiamei Li, Weibo Song*
Laboratory of Protozoology, Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao, China

Abstract
Evolutionary relationships of cyrtophorian ciliates are poorly known because molecular data of most groups within this subclass are lacking. In the present work, the SS rRNA genes belonging to 17 genera, 7 families of Cyrtophoria were sequenced and phylogenetic trees were constructed to assess their inter-generic relationships. The results indicated: (1) the assignment of cyrtophorians into two orders is consistently confirmed in all topologies; (2) the order Dysteriida is an outlined monophyletic assemblage while Chlamydomontida is paraphyletic with three separate monophyletic families; (3) Microxysma, which is currently assigned within the family Hartmannulidae, should be transferred to the family Dysteriidae; (4) the systematic position of Plesiotrichopidae remains unclear, yet the two genera that were placed in this family before, Pithites and Trochochilodon, should be transferred to Chlamydomontida; (5) a new family, Pithitidae n. fam., based on the type genus Pithites was suggested; and (6) the sequence of Isochona sp., the only available data of Chonotrichia so far, is probably from a misidentified species. In addition, three group I introns of SS rRNA gene were discovered in Aegyriana oliva, among which Aol.S516 is the first IE group intron reported in ciliates.

Materials and Methods
Source of organisms and morphological identification
Species sequenced in the present study were collected from northern and southern China (Fig. 1, Table S2). Culturing and morphological examination of these species were according to Pan et al. [18]. Species identification was based on the literatures [8,19,20]. Terminology and systematic scheme follow Lynn [1].

DNA extraction, PCR amplification, and sequencing
Cell isolation and genomic DNA extraction were according to Gong et al. [21]. Primers used in the present study were EukA and EukB [22]. The polymerase chain reaction (PCR) followed the protocol of Yi & Song [23].

Secondary structure of intron
Three introns in the SS rRNA sequence of Aegyriana oliva were identified by the alignment of several intron-less cyrtophorian ciliates using CLUSTAL W 1.83 [24]. The secondary structure of introns were predicted by the Group I Intron Sequence and Structure Database (GISSD) [25] by using the covariance model (CM) of the seed alignment of IC1 and IE introns in the package INFERNAL V0.81 (http://infernal.janelia.org/).

Phylogenetic analyses
Sequences newly acquired in this study were deposited in the GenBank database with the accession numbers listed in Table 1. Other sequences used for phylogenetic tree construction were

Introduction
In the system presented by Lynn [1], the subclass Cyrtophoria, a highly divergent ciliate group, embraces 2 orders, 9 families and 46 genera [1–6]. Most schemes depicted this group as a well defined monophyletic assemblage. However, they differ from each other with respect to the relationships and systematic positions among constitute genera, because relatively few morphogenetic criteria can be used in the taxonomy and systematic analyses [7–11].

Compared to the huge number of morphotypes recognized to date, molecular information of Cyrtophoria is relatively rare. For example, only 6 cyrtophorian genera have available SS rRNA sequences in the GenBank database, and there were very few molecular investigations performed concerning the phylogeny of this group, but see [12–17]. Among them, Snoeyenbos-West et al. [13] provided the molecular support for the monophyly of cyrtophorians for the first time, which was again confirmed by Li & Song [15,16]. Nevertheless, the above studies generally focused on the relationship of the higher level taxa based on a very limited species selection, while the systematic arrangements among lower-level groups where most confusions and disputes reside have not been clarified [15,16].

In the current work, we sequenced the SS rRNA gene of 18 species representing 17 genera and subsequently carried out phylogenetic analyses. Our aims are to expand the understanding of the phylogeny of this extremely confusing group, especially focusing on the relationships among genera/families and to supply additional molecular information for future studies on this assemblage.
obtained from the GenBank database (Table 1). Dataset 1 includes representatives from all the Ciliophora classes, and was aligned with the “Ciliophora” model using Hmmer 2.3.2 [26]. Dataset 2 was scaled down to the two classes, Phyllopharygea and Nassophorea, which was aligned with the “Phyllopharyngea” and “Nassophorea” models. The ambiguously aligned sites were refined using Gblocks v.0.91b [27], yielding an alignment of 1557 and 1455 characters for dataset 1 and dataset 2 respectively. Due to the more specific model used for sequence alignment, phylogenetic trees constructed with dataset 2 have the identical topology as those from dataset 1, but with slightly higher bootstrap value/posterior probability (Figs. 2, S1, S2).

A Bayesian inference (BI) was performed with MrBayes 3.1.2 [28] using the GTR+I+G evolutionary model indicated by MrModeltest v.2 [29]. The program was run for 1,000,000 generations with a sample frequency of 100 and a burn-in of 2,500. All trees remaining after discarding the burn-in were used in calculation of posterior probabilities using a majority rule consensus.

The program Modeltest 3.7 [30] selected GTR+I+G (dataset 1: G = 0.5422, I = 0.2922; dataset 2: G = 0.5628, I = 0.2835) under AIC criterion as the best model, which was then used for maximum likelihood (ML) analysis. A ML tree was constructed with the PhyML v2.4.4 program [31]. The reliability of internal branches was assessed using the non-parametric bootstrap method with 1,000 replicates.

A maximum parsimony (MP) tree was produced based on parsimony-informative sites (dataset 1: 655 sites; dataset 2: 648 sites) with PAUP* 4.0b10 [32]. The reliability of internal branches was estimated by bootstrapping with 1,000 replicates.

Seven constrained ML analyses were carried out by PAUP* 4.0b10 [32] according to the constraints listed in Table 2. Resulting constrained topologies were then compared to the non-constrained ML topology using the Approximately Unbiased (AU) test [33] as implemented in CONSEL v0.1 [34]. For all constraints, internal relationships within the constrained groups were unspecified, and relationships among the remaining taxa were unspecified as well.

Nomenclatural acts

The electronic version of this document does not represent a published work according to the International Code of Zoological Nomenclature (ICZN), and hence the nomenclatural acts contained in the electronic version are not available under that Code from the electronic edition. Therefore, a separate edition of this document was produced by a method that assures numerous identical and durable copies, and those copies were simultaneously obtainable (from the publication date noted on the first page of this article) for the purpose of providing a public and permanent scientific record, in accordance with Article 8.1 of the Code. The separate print-only edition is available on request from PLoS by sending a request to PLoS ONE, 1160 Battery Street, Suite 100, San Francisco, CA 94111, USA along with a check for $10 (to cover printing and postage) payable to “Public Library of Science”.

Figure 1. Schematic diagrams of the morphospecies representing genera sequenced in the present study [19]. The cladogram is according to the classification system of Lynn [1]. Arrows indicate the transfer of several species: Microxysma from Hartmannulidae to Dysteriidae; Pithites and Trochochilodon from Dysteriida to Hartmannulida. doi:10.1371/journal.pone.0033198.g001
In addition, this published work and the nomenclatural acts it contains have been registered in ZooBank, the proposed online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: Gao et al article in PLoS ONE: urn:lsid:zoobank.org:act:68A7A13F-341B-4F85-A898-6A30D3391516.

### Results

#### Phylogenetic trees

The topologies of all trees are generally consistent with the classification schemes proposed by previous researchers (Table S1). The class Phyllopharyngea is a monophyletic clade with four distinct groups, Cyrtophoria, Chonotrichia, Suctoria, and Rhynchodia. Cyrtophoria consists of two distinct groups: Dysteriida and Chlamydomonadida, with Chonotrichia nested in Dysteriida (see Discussion below). Suctoria and Rhynchodia are positioned as peripheral branches of Cyrtophoria, while the class Nassoophorea is the nearest “out-group” to the class Phyllopharyngea. These results are also in agreement with previous reports [12,13,15,16].

The order Dysteriida is a monophyletic clade, consisting of two well-separated groups, the families Dysteriidae and Hartmannulidae. Within Dysteriidae, _Mirodysteria_ was always placed within the species of _Dysteria_. _Microxysma_ clustered with _Trochilia_, rather than with species of Chlamydodontida as suggested by previous schemes (Table S1). Within Hartmannulidae, the newly sequenced _Aegyriana_ grouped with _Trichopolliella_, which then clustered with _Hartmannula_ and _Heterohartmannula_. _Trochiliodes_ formed a basal branch out of the above four genera. Unlike the above two families, the branching order of Plesiotorichopidae was not unambiguously resolved in the present topologies. _Trochochilodon_

#### Table 1. Accession numbers of the species used for the phylogenetic tree construction.

| Species name           | GenBank Acc.No. | Species name           | GenBank Acc.No. |
|------------------------|-----------------|------------------------|-----------------|
| Acineta sp.            | AY332718        | Litonotus paracygnus*  | DQ190464       |
| Aegyriana oliva*       | FJ998029        | Loxodes striatus       | U24248         |
| Blepharisma americanum | M97909          | Loxophyllum jini*      | EF123708       |
| Breslaua vorax         | AF006453        | Lycnella nordica*      | FJ998036       |
| Chilodochodiscus excellecatus | AY331790 | Microxysma acutum*     | FJ870069       |
| Chlamydomonadina mmenosyne* | FJ998031 | Mirodysteria decora*   | JN867020       |
| Chlamydomonadina obliquus* | FJ998030 | Nassula sp. OD2*       | EU286810       |
| Chlamydomonadina triquetrus | AY331794 | Nyctotheroides desleri  | AF145353       |
| Chlamydella pseudochilodon* | FJ998032 | Obertrumia georgiana   | X85149         |
| Chlamydonellina calcina* | FJ998033 | Orthodondan apohamatus | DQ232761       |
| Coeloperis sp.*        | FJ998034        | Orthodondan sp. OD1*   | EU286809       |
| Coelops hirtus         | U97109          | Paracyrtophoron tropicum* | FJ998035     |
| Colpoda inflate        | M97908          | Plagiopyla frontata    | Z9440          |
| Colpodidae sp. HBW-2007 | EU264561 | Plagiopyla nasuta      | Z9442          |
| Colpodidium caudatum   | EU264560        | Pithites vorax*        | FJ870070       |
| Cymbodentor auriluca   | DQ445605        | Priscodophraya collini | AY331802       |
| Discophrya collini     | L26446          | Prorodon teres         | X71140         |
| Dysteria brasiliensis* | EU242512        | Prorodon viridis       | U97111         |
| Dysteria deroux*       | AY378112        | Pseudochilodonopsis cf. fluentilis | JN867021 |
| Dysteria procera*      | DQ057347        | Pseudomicrothorax dubius | FM201298     |
| Dysteria sp. 1         | AY331797        | Tokaphrya lemnarum     | AY332720       |
| Dysteria sp. 2         | AY331800        | Tokaphrya quadripinata | AY102174       |
| Ephelota gemmeipara    | DQ834370        | Trichopodiella faurei* | EUS15792      |
| Frontonia lynni*       | DQ190463        | Trithigmostoma cicutulum* | FJ998037     |
| Frontonia tchibisovae* | DQ883820        | Trithigmostoma steini  | X71134         |
| Furgasonia blochmannii | X65150          | Trochilia petrani*     | JN867016       |
| Hartmannula derouxi*   | AY378113        | Trochiloides recta*    | JN867017       |
| Heterohartmannula fang* | FJ868204 | Trochilodon flavus*    | JN867018       |
| Heliothrya erhardt     | AY007445        | Uranochia setigera*    | AF260120       |
| Hypocoma acinetaria*   | JN867019        | Uranochia transfuga*   | EF198669       |
| Isochona sp.           | AY242119        | Zosterodasys transverses | EU286812    |
| Leptopharynx costatus* | EU286811        |                        |                |

Species newly sequenced in the present study are marked in bold. Species sequenced by the authors’ group are marked by sterisks (*).

doi:10.1371/journal.pone.0033198.t001
always appeared as a peripheral branch out of Dysteriida (±Chonotrichia) (Figs. 2, S1, S2). However, the position of Pithites is uncertain; it clustered with species of Lynchellidae in the MP tree from dataset 2 (with low bootstrap value, Fig. S2), but branched outside of and parallel to Chlamydodontida in other trees (Figs. 2, S1).

The order Chlamydodontida was divided into three well-defined families, Chlamydodontidae, Chilodonellidae, and Lynchellidae. In the family of Chlamydodontidae, Paracyrtophoron is nesting within Chlamydon. On the other hand, the topology of the family Chilodonellidae is congruent with previous schemes (Table S1), within which Pseudochilodonopsis formed a clade with Chilodonella and further clustered to two species of Trithigmosta. In the family of Lynchellidae, four genera, Chlamydonella, Chlamydonellopsis, Lynchella, and Coeloperix, were sequenced for the first time and analyzed in the present work. They formed

Table 2. Approximately Unbiased (AU) test results.

| Topology constraints | −Ln likelihood | AU value (p) |
|----------------------|----------------|-------------|
| 1 unconstrained      | 15543.82506    | 0.982       |
| 1 Chlamydon monophyletic | 15561.89373 | 0.169       |
| 2 Chlamydontidae+Chilodonellidae+Lynchellidae monophyletic | 15577.48381 | 0.010       |
| 3 Chlamydontidae+Lynchellidae monophyletic | 15577.74157 | 0.007       |
| 4 Dysteria monophyletic | 15553.84383 | 0.189       |
| 5 Pithites vorax+Trachichlidon flavus monophyletic | 15581.58673 | 0.002       |
| 6 Pithites vorax+Trachichlidon flavus +Hartmannulidae+Dysteridae monophyletic | 15625.36216 | 0.002       |
| 7 Microxysma acutum+Hartmannulidae monophyletic | 15595.58459 | 0.002       |

p<0.05 refute monophyly; p>0.05 do not refute the possibility of monophyly. Results in which p<0.05 are marked in bold and shaded in grey.

doi:10.1371/journal.pone.0033198.t002
consistently a monophyletic clade in all topologies, and thus correspond to the concept of the family Lynchellidae according to Jankowski [35]. Within these four genera, two groups were recognized; one is *Chlamydonella* and *Chlamydomenellopsis*, and the other is *Lynchella* and *Coeloperix*. The close relationship of *Coeloperix* and *Lynchella* is a true reflection of their similar morphology with a slight difference (presence of CSB in *Lynchella* vs. absence in *Coeloperix*) [36].

A species of *Chonotrichia*, *Isochona* sp., grouped with harmanulids, while the only sequenced genera of Rhychoedia, *Hypocoma*, formed a sister clade with the monophyletic clade of Suctoria which branches basally from all caryophorians (Figs. 2, S1, S2).

**Analyses of introns in the SS rRNA gene of Aegyriana oliva**

We discovered three group I introns (376–446 nucleotides) in the SS rRNA gene of *Aegyriana oliva* (Fig. 3). They are at position 516, 943, and 1506 of the SS rRNA gene of *E. coli* (J01695), which are named as AoI.S516, AoI.S943, and AoI.S1506 following Johansen and Haugen [37]. The predicted secondary structure showed that AoI.S516 was affiliated with the IE1 group, while AoI.S943 and AoI.S1506 were affiliated with the IC1 group (Figs. 3B–3D).

**Discussion**

The order Chlamydodontida is a paraphyly

Even though all of the three constituent families were monophyletic groups, our results consistently showed that the order Chlamydodontida was a paraphyletic assemblage. Moreover, the AU test in this study, with an expanded set of sequences (10 genera, 13 species), refuted the possibility that Chlamydodontida is a monophyletic clade (Table 2, constraint 2, p = 0.01) and confirmed the reliability of phylogenetic results. This is in concerto with other studies, even though only four species were included in previous molecular trees [12,13,15,16].

Based on the ciliary patterns and the structure of macronucleus, Gong [19] assigned the families with juxtaposed heteromerous macronucleus, Chilodonellidae and Lynchellidae, into the suborder Chlamydodontina, while placed Chlamydodontidae (+Gastrochilidae) with centric heteromerous macronucleus into Chilodinellina. This assignment agrees with the scheme proposed by de Puytorac [4] (Table S1), but was not supported by our phylogenetic results, in which these three families formed separate monophyletic clades. Accordingly, the AU test rejected the possibility that Chilodonellidae and Lynchellidae belong to a monophyletic group (Table 2, constraint 3, p = 0.007), suggesting...
that the feature of macronucleus may not be a strong diagnostic character to distinguish monophyletic groups.

The relationship between Paracyrtophoron and Chlamydomonad

In our analyses, Paracyrtophoron nested within the species of Chlamydomonad. However, Paracyrtophoron can be easily distinguished from Chlamydomonad by the lack of the cross-striped band (CSB) around the periphery of the somatic field [38]. Such discrepancies could be attributed to an evolutionary scenario that the CSB is a convergent character with some members of the Lynchellidae, which may not be reflected in the SS rRNA sequences. Moreover, the AU test did not refute the possibility that Chlamydomonad is a monophyletic clade (Table 2, constraint 1, p = 0.169). At this point, the available evidence could not support the paraphyly of Chlamydomonad.

Microxysma is a member of the family Dysteriidae. The major features to distinguish Hartmannulidae and Dysteriidae are the body shape and the structure of left ventral kineties [5]. In Hartmannulidae, the body is conspicuously dorsoventrally flattened, and the left ventral kineties are generally developed and continuous with the right ones, whereas in Dysteriidae, the body is mostly highly bilaterally flattened with the left kineties extremely reduced and restricted to the equatorial area [9-11].

In all previous morphology-based classification schemes (Table S1), Microxysma was arranged in the family Hartmannulidae. But this assignment is not supported by our molecular trees, in which Microxysma was placed away from the species of Hartmannulidae. Moreover, the possibility that Microxysma and species of Hartmannulidae are monophyletic was also refuted by the AU test (Table 2, constraint 7, p = 0.002). In fact, there is a large morphological difference between Microxysma and hartmannulids. In Microxysma, the highly shortened left kineties were degenerate to a limited area, which are practically different from those in the typical hartmannulid species, whose kineties cover the majority of the left side. Rather, the bilaterally compressed Microxysma shares the basic pattern of ciliation with the species in Dysteriidae, e.g. right kineties are arranged along the narrow ventral margin with the reduced left field of kineties [19]. Compared with other typical dysteriids, the ciliary pattern of Microxysma is similar to that of the dysteriid Trochilia, which can explain its neighboring position to the latter in all topologies of the molecular trees. Therefore, both morphological and molecular data suggest that Microxysma should be transferred from Hartmannulidae to Dysteriidae.

The paraphyly of the family Plesiotrichopidae and the systematic positions of Trochochilodon and Pithites, with establishment of a new family Pithitidae n. fam.

The family Plesiotrichopidae was erected by Deroux [8], diagnosed roughly by having “Chilodonella-like infraciliature and adhesive apparatus located centrally in ventral depression”. As shown in Table S1, Plesiotrichopidae was tentatively assigned into the order Dysteriida in most classification schemes [1,2,4,5,9], however, up to date, the relationships/systematic positions of taxa in this family have never been investigated using molecular information. We supplemented the knowledge by analyzing the phylogeny of this family based on the SS rRNA gene sequence data of two genera, Trochochilodon and Pithites. It indicates that the two genera are systematically far away from each other, rendering the family Plesiotrichopidae a paraphyletic assemblage. These results correspond well to the morphological and morphogenetic dissimilarities between the two genera: both the structure of buccal apparatus and the formation process during the binary fission are considerably different from each other [8,18,39]. The topology also suggests that neither of them should be placed in the current order Dysteriida, because Trochochilodon grouped outside the order Dysteriida, while Pithites located basally to the other cyrtophorians. Therefore, both the molecular and the morphological/morphogenetic data challenge the scheme to arrange them in the same family.

Unfortunately, the systematic position and the definition of the family Plesiotrichopidae still remain unsolved at the present stage. The problem is that the molecular data for the type genus Plesiotrichopus are totally lacking and not many taxonomic characters can be used to characterize genera within the family. As a result, few pieces of evidence are available to define which one is near to the type genus. Another confusion comes from the presence of a dominant tube-like structure (secretory channels) in Plesiotrichopus, which is absent in Pithites and Trochochilodon. If it is a critical feature of this family, both Pithites and Trochochilodon should be transferred from the current taxon. Currently, the family Plesiotrichopidae is an incertae sedis taxon.

Regarding the phylogeny, no close relationship between Pithites and dysteriids was recovered. Moreover, the possibility that Pithites and Dysteriidae form a monophyletic clade was also rejected by the AU test (Table 2, constraint 6, p = 0.002), which is also supported by the morphological features. For example, taxa in the order Dysteriida are diagnosed by the presence of the adhesive organelle (typically a flexible podie) that is absent in Chlamydomodontida [1,2,5,8,39], whereas Pithites has no such organelle. Even though a filament from the secretory channel (character of Plesiotrichopus) was mentioned in Pithites by Deroux and Dragesco [40], it is not confirmed in the in vivo observations by Pan [18]. In addition, Pithites has separated left and right kineties which is never seen in dysteriids (vs. continuous). Given that Pithites has a peripheral position to Chlamydomodontida in most topologies, lacks the podie and possesses a unique oral structure (apically located, several kinety fragments radiated around the cytosome), it may belong to an isolated taxon at least at family level and should be moved from Dysteriida to the order Chlamydomodontida. Therefore, we suggest a new family here, Pithitidae n. fam. with the type genus Pithites, under the order Chlamydomodontida (urn:lsid:zoobank.org:act:68A7A13F-341B-4F85-A898-6A30D3391516).

The family is characterized by the combination of the following features: (1) pelagic forms with almost non-compressed body shape and apically positioned cytosome; (2) well developed somatic kineties on both left and right fields with a conspicuous cilia-free area between them; (3) oral apparatus consisting of several kinety fragments around the cytosome; and (4) without podite but having a “thigmotactic field” subcaudally near the meridian of ventral side where the thread-like adhesive organelle is located [39].

Meanwhile, it is relatively certain that the genus Trochochilodon should also be transferred from Dysteriida to Chlamydomodontida. According to the observations by Pan [41], this Chilodonella-like taxon is very similar to chlamydomodontid species. The former differs from the latter only by having two preoral kineties (vs. mostly three in chlamydomodontids) and the cilia-free field between left and right somatic kineties is inconspicuous (dominant in some chlamydomodontids; Fig. 1). Regarding the position revealed in our SS rRNA-based topological analyses, it is reasonable to deduce that this organism might represent an intermediate form closer to chlamydomodontids than to dysteriids [8]. However, whether it belongs to the family Plesiotrichopidae still needs further explorations, because the molecular information of the type genus Plesiotrichopus is currently lacking.

In summary, three conclusions can be drawn: (1) the current family Plesiotrichopidae consists of paraphyletic clades and most of
them are systematically unclear; (2) both Pithites and Trochochilodon should be transferred from the order Dysteriida, and they likely belong to Chlamydomontida; and (3) based on both morphological/morphogenetic and molecular information, a new family, Pithitidae n. fam. is suggested for the genus Pithites.

Data of Isochona sp. might come from a misidentified organism

Isochona sp., the only sequenced species of the subclass Chonotrichia, was positioned basally to other hartmannulids in our results. However, morphologically, chonotrichians are a highly specialized group with numerous unique characters, e.g. the attaching living style (or aufwuchs) with flask-shaped body, non-fused conjugation process, and highly reduced infraciliature which is spirally arranged and limited within the choled wall, etc. [2,11]. All the above criteria indicate that they should be clearly distinguished from the taxa of cypertorhians. A reasonable explanation for our phylogenetic result is that the material was misidentified. Species in Chonotrichia are un-cultivatable and, as periphyton forms, they are easily mixed with other attaching ciliates when sampled. Moreover, only one population/species (Isochona sp.) from this subclass has been sequenced so far. Thus, the sequence submitted to the GenBank database is likely from a misidentified organism, that is, a cyrtophorid instead of a chonotrich.

Fine-scale investigation of the order Dysteriida

As stated above, Pithites and Trochochilodon were transferred from the order Dysteriida to Chlamydomontida, and Isochona is likely to be a hartmannulid. This leaves the order Dysteriida as a monophyletic clade, with two well-supported groups, Dysteriidae and Hartmannulidae. The clear separation of these two families was expected on the basis of their distinguished morphology: species in Dysteriidae have “left ventral somatic kineties as midventral postoral field, typically separated from an anterior preoral field”, and those in Hartmannulidae have “left ventral somatic kineties, which may be quite short, as continuous field” [5].

In addition, Dysteriidae and Hartmannulidae are revealed as closely related sister group (Fig. 2, BI/ML:1.00/92), and they both share a very similar secondary structure of the V2 region. This corresponds to the fact that they both embrace the ordinal character such as dorsoventrally compressed body shape, non-thigmotactic ventral cilia, and juxtaposed heteromeric macronucleus [5].

Group I introns in cyrtophorids

Four group I introns have been reported in the SS rRNA gene of three ciliates, with two in Tokophrya lemnarum, and one in Actineta sp. and Trichopodiella faurei each [12,13]. In our current work, Aegyriana oliva is the fourth reported ciliate embracing introns, and is also the first reported ciliate having three introns, namely Aol.S516, Aol.S943, and Aol.S1506. The S943 was first reported in Trichopodiella faurei [12], while the S1506 intron was only described in Tokophrya lemnarum [13]. The Aol.S516, to our knowledge, is the first intron reported at position 516 of the ciliate SS rRNA gene.

On the basis of the conserved secondary structure, conserved core nucleotide regions, and phylogenetic analysis, group I introns have been classified into five major groups: IA, IB, IC, ID, and IE [42]. Aol.S943 and Aol.S1506 belong to the IC group, as well as the four previously reported SS rRNA introns and nine LS rRNA introns. By contrast, Aol.S516 is the only IE group I intron described in ciliates so far (Fig. 3A). Interestingly, all the above species embracing SS rRNA introns belong to the class Phyllopharyngea, while LS rRNA introns were only reported in the tetrathyenid genus Tetrahymena (Fig. 3A), which belongs to the class Oligohymenophorea, a group far away from the cyrtophorians [1].

Supporting Information

Figure S1 Phylogenetic trees inferred from small subunit rRNA gene sequences (dataset 1) with an emphasis on cyrtophorid ciliates. Numbers on branches are the following: bootstrap values from maximum likelihood (ML) analysis, followed by the Bayesian posterior probability value and the bootstrap values of maximum parsimony (MP) analysis. Solid circles represent full bootstrap support in all three algorithms and hyphen (-) represents support values below 0.50/50%. Species sequenced in the present study are shown in bold. (TIF)

Figure S2 A maximum-parsimony tree inferred from the small subunit ribosomal RNA gene sequences (dataset 2). Species sequenced in this work are marked in bold. Numbers at the nodes represent the bootstrap values. (TIF)

Table S1 Taxonomic schemes for the classification of cyrtophorid ciliates. Species newly sequenced in the present study are in grey. (XLS)

Table S2 Sampling sites and habitat information of species sequenced in this study. (XLSX)

Acknowledgments

Our special thanks are given to Dr. Hongbo Pang, OUC, for his kind help in drafting Fig. 1. Many thanks are also due to Ms. Zhou Shen, Xumiao Chen, Jiaome Jiang and Fulan Cui, Mr. Xiangxu Chen, Hongbo Pan, Weixi Liu and Xinpeng Fan, graduate students of our laboratory, for sample collection, gene sequencing and experimental help. Helpful comments on a previous draft were provided by Leiling Tao and two anonymous reviewers.

Author Contributions

Conceived and designed the experiments: SG WS. Performed the experiments: SG JH. Analyzed the data: SG JH JL WS. Contributed reagents/materials/analysis tools: WS. Wrote the paper: SG WS.

References

1. Lynn DH (2008) The ciliated Protozoa: characterization, classification, and guide to the literature. Dordrecht: Springer.
2. Corliss JO (1979) The ciliated Protozoa: characterization, classification and guide to the literature. Oxford and New York: Pergamon Press.
3. Small EB, Lynn DH (1985) Phylum Ciliophora Dollo 1901 in: An Illustrated guide to the Protozoa Lee JJ, Hunter SH, Bovee EC, eds. Lawrence, Kansas: Society of Protozoologists. pp 393-375.
4. Puytorac Pd (1994) Traité de zoologie. Anatomie, systématique et biologie. Tome 2 Fasc. 2 Infusaires ciliés: systématique. Paris: Masson.
5. Lynn DH, Small EB (2002) Phylum Ciliophora Dollo, 1901 in: An Illustrated Guide to the Protozoa Lee JJ, Leedale GF, Bradbury P, eds. Lawrence: Society of Protozoologists. pp 371-656.
6. Jankowski AW (1976) Revision of a system of cyrtophorines. In: Materials of the II All-Union Conference of protozooligists. Part 1 General protozoology, Kiev. pp 167-168. (in Russian. Considered as unpublished work).
7. Chen XR, Gong J, Al-Rasheid KAS, Farraj SA, Song V (2011) New contribution to the morphological taxonomy of three marine cyrtophorid ciliates from the Yellow Sea, China (Ciliophora: Cyrtophorida). Acta Protozoolog 50: 105-119.
Phylogeny of Cyrtophorid Ciliates

8. Deroux G (1976) Phylogenetic position of Dysteria derouxi. Acta Protozool 45: 265–270.

9. Deroux G (1976) Cortical pattern in Cyrtophorida, unity and diversification. Acta Protozool 45: 265–270.

10. Gong J, Song W, Warren A (2009) CYRTOPHORIDS in: Free-living Ciliates in China seas. PhD thesis, Ocean University of China. 141 p. (in Chinese with English).

11. Jankowski AW (2007) Ciliophora Doflein, 1901 in: Protista Alimov AF, ed. St. Petersburg: Nauka. pp 371–993.

12. Gong J, Gao S, Roberts DM, Al-Rasheid KAS, Song WB (2008) Phylogenetic position of Dysteria derouxi. Acta Protozool 47: 197–207.

13. Snoeyenbos-West OLO, Cole J, Campbell A, Coats DW, Katz LA (2004) Phylogeny of the order Urostylida (Protozoa, Ciliophora). J Nat Hist 39: 935–973.

14. Li LF, Song WB (2006) Phylogenetic positions of two cyrtophorid ciliates, Dysteria procera and Hartmannula deroxi. Acta Protozool 41: 23–61.

15. Li LF, Song WB (2006) Phylogenetic position of Dysteria deroxi. Acta Protozool 45: 265–270.

16. Li LF, Song WB (2006) Phylogenetic position of Dysteria procera. Acta Protozool 45: 265–270.

17. Riley JL, Katz LA (2001) Widespread distribution of extensive chromosomal fragmentation in ciliates. Mol Biol Evol 18: 1372–1377.

18. Pan HB (2011) Diversity of marine cyrtophorid and pleurostomatid ciliates in China seas. PhD thesis, Ocean University of China. 141 p. (in Chinese with English).

19. Gong J (2005) Taxonomy of cyrtophorid ciliates (Protozoa, Ciliophora) from coastal waters off Qingdao, with systematic revisions on some families and genera. PhD thesis, Ocean University of China. 164 p. (in Chinese with English).

20. Song W, Wilbert N (2002) Faunistic studies on marine ciliates from the Antarctic area, including description of seven new species (Protozoa, Ciliophora, Cyrtophorida). Eur J Protistol 40: 175–181.

21. Zhou Y, Lu C, Wu QJ, Wang Y, Sun ZT, et al. (2000) GISSD: Group I intron database. Nucleic Acids Res 28: 4673–4680.

22. Eddy SR (2003) HMMER User’s Guide: Biological sequence analysis using profile hidden Markov models 2.3.2.

23. Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17: 540–552.

24. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

25. Jankowski AW (1968) Morphology, phylogeny and position in the system of the order Ciliophora, nov gen., with a description of Coeloperix sleighi nov spec. (Protozoa, Ciliophora, Cyrtophorida). Eur J Protistol 40: 175–181.

26. Johansen S, Haugen P (2001) A new nomenclature of group I introns in ribosomal DNA. J Mol Biol 216: 585–610.

27. Eddy SR (2003) HMMER User’s Guide: Biological sequence analysis using profile hidden Markov models 2.3.2.

28. Ronquist FR, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

29. Nylander JAA (2004) MrModeltest v.2 Evolutionary Biology Centre, Uppsala University.

30. Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

31. Guindon S, Gascuel O (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.

32. Swoford DL (2003) PAUP*: Phylogenetic analysis using parsimony (* and other methods) version 4.0b 10 Sinauer Associates, Sunderland, MA.

33. Shimoda H (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol 51: 492–508.

34. Shimoda H, Hasegawa M (2001) Consel: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17: 1246–1247.

35. Jankowski AW (1968) Morphology, phylogeny and position in the system of the order Ciliophora, nov gen., with a description of Coeloperix sleighi nov spec. (Protozoa, Ciliophora, Cyrtophorida). Eur J Protistol 40: 175–181.

36. Johansen S, Haugen P (2001) A new nomenclature of group I introns in ribosomal DNA. J Mol Biol 216: 585–610.

37. Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17: 540–552.

38. Ronquist FR, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

39. Nylander JAA (2004) MrModeltest v.2 Evolutionary Biology Centre, Uppsala University.

40. Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

41. Guindon S, Gascuel O (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.

42. Swoford DL (2003) PAUP*: Phylogenetic analysis using parsimony (* and other methods) version 4.0b 10 Sinauer Associates, Sunderland, MA.

43. Shimoda H (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol 51: 492–508.

44. Shimoda H, Hasegawa M (2001) Consel: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17: 1246–1247.

45. Jankowski AW (1968) Morphology, phylogeny and position in the system of the order Ciliophora, nov gen., with a description of Coeloperix sleighi nov spec. (Protozoa, Ciliophora, Cyrtophorida). Eur J Protistol 40: 175–181.

46. Johansen S, Haugen P (2001) A new nomenclature of group I introns in ribosomal DNA. J Mol Biol 216: 585–610.

47. Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17: 540–552.

48. Ronquist FR, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

49. Nylander JAA (2004) MrModeltest v.2 Evolutionary Biology Centre, Uppsala University.

50. Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.