



Review

## Systematic studies on ciliates (Alveolata, Ciliophora) in China: Progress and achievements based on molecular information

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Available online 6 May 2017

### Abstract

Due to complex morphological and convergent morphogenetic characters, the systematics of ciliates has long been ambiguous. Since 1990, the Laboratory of Protozoology, Ocean University of China, in collaboration with other research groups worldwide, has carried out a series of integrative investigations on ciliate systematics. To date, genomic DNA has been extracted from about 1700 ciliate strains, and phylogenetic analyses have been performed for two-thirds of orders. Main findings are: (1) Classifications of about 50 hypotrichous species have been resolved, although the monophylies of three hypotrichous orders remain unconfirmed; (2) Euplotia and two orders and all seven families within them are monophyletic assemblages; (3) *Lynnella* represents an order-level taxon, and is separated from two sister monophyletic subclasses Oligotrichia and Choreotrichia; (4) the peritrich families Zoothamniidae and Vorticellidae are separated from each other, and *Zoothamnium* exhibits a high genetic diversity; (5) the scuticociliate order Philasterida is monophyletic and separated from loxocephalids, and the thigmotrichids is a suborder within Pleuronematida; (6) 14 classes were recovered including one new class Protocruzidea, and Mesodinina is basal to subphyla Intramacronucleata and Postciliodesmatophora; (7) mitochondrial cytochrome *c* oxidase subunit I heteroplasmy was reported in ciliates for the first time, and candidate barcoding genes for *Frontonia* species identification were identified.

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**Keywords:** Ciliates; Molecular systematics; Phylogeny; Barcoding

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## Introduction

Ciliated protists (ciliates) are a diverse clade of microbial eukaryotes that are the most morphologically complex and highly differentiated taxa among single-celled organisms. They exhibit a combination of unique characters, such as nuclear dimorphism (the presence of a somatic macronucleus and a germline micronucleus), sexual reproduction by conjugation, highly specialized organelles and complex cytoskeletal structures, that make them important model organisms in studies of cell biology, molecular biology, genetics, ecology, and evolution (Hausmann and Bradbury 1996). Traditionally, the taxonomy and systematics of ciliates were mainly based on morphogenetic and morphological characteristics, e.g. ciliature, mouth structure, nuclear apparatus and ultrastructure (Corliss 1979). Over the last three decades, phylogenetic analyses based on molecular data have become increasingly important in determining evolutionary relationships among ciliates (Chen et al. 2015, 2016; Gentekaki et al. 2014; Luo et al. 2015; Wang et al. 2015; Zhang et al. 2014). Considering their high species diversity, however, molecular phylogenetic studies have been performed on only a small proportion of ciliates. This is mainly due to the initial technical difficulties of obtaining and extracting DNA, a problem that has largely been overcome, and a lack of accurate, verifiable morphological and biogeographical data upon which to build molecular studies. Therefore, phylogenetic relationships among many ciliate groups remain unknown, even those which are morphologically and/or morphogenetically well characterized (Xu et al. 2015; Yan et al. 2015, 2016a, 2016b; Zhao et al. 2014).

Since 1990, the research group of the Laboratory of Protozoology in the Institute of Evolution and Marine Biodiversity, Ocean University of China, has carried out a series of investigations on the ciliate fauna in the temperate-tropical seas in China. These studies are supported by a variety of programmes and funding organizations, both national and international, including: the “National Basic Research Program of China (973 Program)”, the “Cheung Kong Scholars Programme”, the National Natural Science Foundation of China, Deutscher Akademischer Austausch Dienst (Germany), the Royal Society (UK), the Darwin Initiative (UK) and the Center of Excellence in Biodiversity Research (Saudi Arabia). Much of this work has been carried in collaboration with institutions overseas, in particular the University of Bonn, Germany, the Natural History Museum, UK, and the King Saud University, Saudi Arabia. More than 800 morphospecies have been isolated and identified (Song et al. 2003, 2009). Following detailed and accurate taxonomic identification, genomic DNA has been extracted from more than 1700 ciliate species/strains and marker genes have been sequenced e.g. small subunit ribosomal RNA (SSU rRNA) gene (Table 1). Phylogenetic analyses based mainly on gene sequence data have been integrated with the study of morphological and morphogenetic characters for several major

ciliate groups including hypotrichs, euplotids, scuticociliates, oligotrichs, peritrichs, heterotrichs etc., covering about two-thirds of ciliate orders (Gao et al. 2012; Huang et al. 2012, 2016; Zhang et al. 2012). Additionally, theoretical research focusing on gene marker selection and topological optimization have also been performed in order to provide guidance for future studies (Yi et al. 2010, 2014). The present paper summarizes the main findings of phylogenetic studies performed by our group on five major ciliate lineages, namely hypotrichs, euplotids, oligotrichs, peritrichs and scuticociliates.

## Phylogenetic Studies of Hypotrichia

The subclass Hypotrichia sensu str. is a diverse and cosmopolitan assemblage of ciliates. To date, about 600 valid species have been reported (Berger 1999, 2006, 2008, 2011). The phylogeny of hypotrichs is one of the most frequently discussed topics within Ciliophora. Ventral cirral patterns are the most important characters for dividing orders of this group. However, these patterns are susceptible to convergent evolution, which greatly reduces their reliability as phylogenetic markers (Berger 2006; Lynn 2008). In addition, phylogenetic trees based on SSU rRNA gene sequences produce poor resolution with low support values on many nodes (Schmidt et al. 2007).

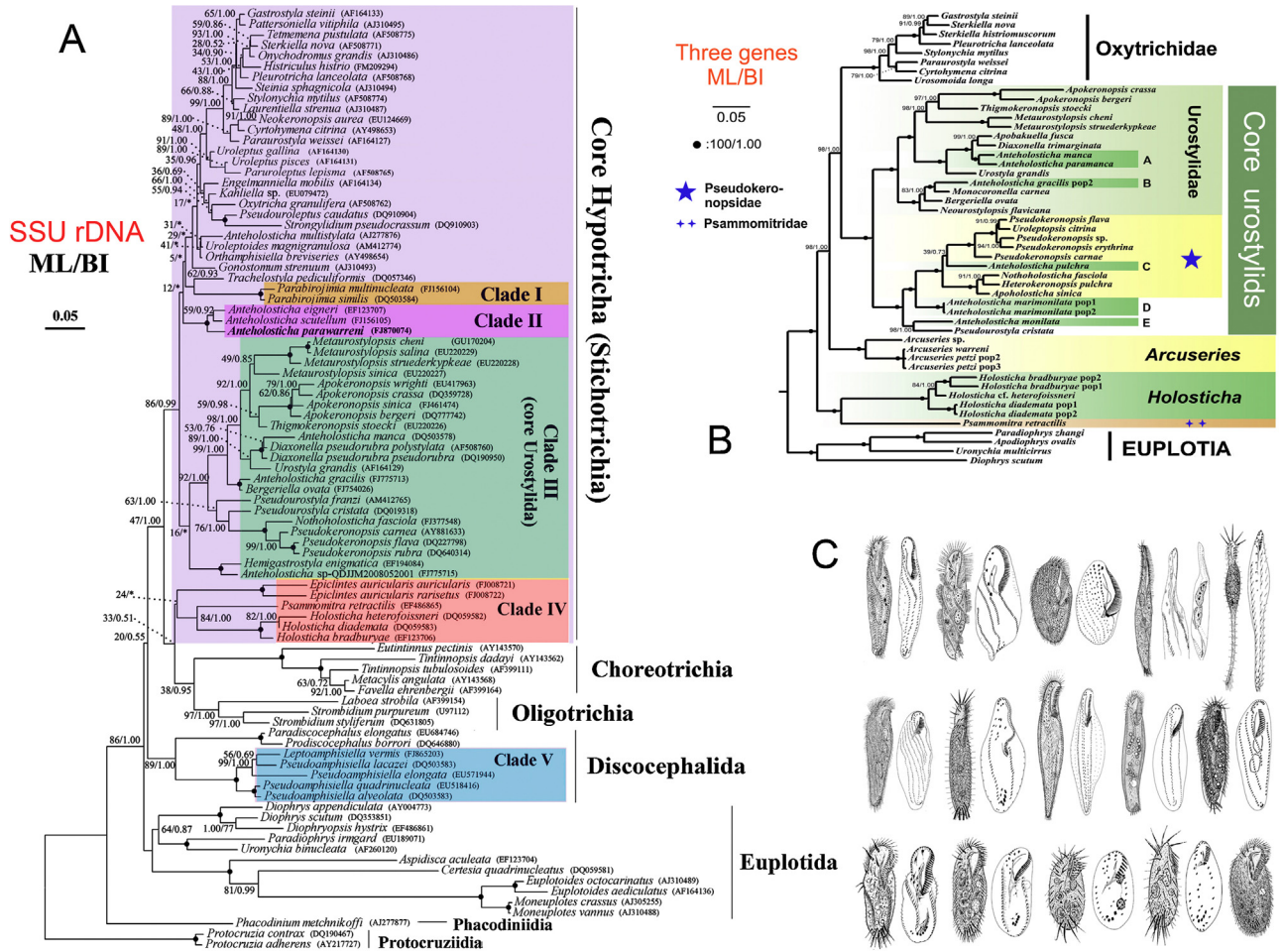
Since 2006, we have submitted to GenBank 207 SSU rRNA gene sequences, 97 internal transcribed spacer (ITS)-5.8S rRNA gene sequences and 87 large subunit ribosomal RNA (LSU rRNA) gene sequences of hypotrichs. A total of 48 papers focused mainly or partly on the phylogeny of hypotrichs have been published based on these sequences. Several recent papers have reported on analyses of multi-gene sequence data integrated with morphological and morphogenetic characters (Fig. 1).

Most papers focused on phylogenetic assignments of species with ambiguous positions. To date, classifications of about 50 species have been resolved. For instance, morphologically *Parabirojimia* was thought to be a urostylid (Berger 2006; Hu et al. 2002). However, it possesses some unique morphogenetic features, such as the origin of the transverse cirri (Hu et al. 2002). Our molecular phylogenetic study revealed that *Parabirojimia* represents a unique taxon that is related to but separate from the typical urostylids, and for which the suborder Parabirojimina was established (Yi et al. 2008). In addition, some integrative studies have included both morphological descriptions and molecular phylogenies when new species or genera were reported (e.g. Fan et al. 2014, 2015). This involves a total of 23 species and eight genera (*Apobakuella*, *Apogastrostyla*, *Apourostylopsis*, *Bergeriella*, *Heterokeronopsis*, *Monocoronella*, *Parabistichella* and *Paracladotricha*).

Some papers aimed at resolving within-group or species-complex relationships. An example is the *Holosticha-*

**Table 1.** Ciliate sequences: contribution to the GenBank rRNA gene database by authors' groups (from Yi et al. 2016).

Groups	No. of SSU rDNA sequences			No. of ITS-5.8SrDNA sequences			No. of LSU rDNA sequences		
	GenBank	Authors' groups	Ratio of our sequences in GenBank	GenBank	Authors' groups	Ratio of our sequences in GenBank	GenBank	Authors' groups	Ratio of our sequences in GenBank
Litostomatea	358	54	15%	127	6	5%	17	14	82%
Hypotrichia & Euplotia	705	207	29%	286	97	34%	113	87	77%
Oligotrichia s.l.	933	43	5%	528	39	7%	24	24	100%
Phyllopharyngea	140	76	54%	9	4	44%	7	6	86%
Nassophorea	18	10	56%	4	4	100%	5	5	100%
Prostomatea	54	10	19%	4	4	100%	5	5	100%
Karyorelictea	59	27	46%	3	2	67%	1	1	100%
Heterotricha	179	24	13%	83	7	8%	8	8	100%
Peritrichia	261	20	8%	230	14	6%	40	6	15%
Scuticociliatia	206	78	38%	89	49	55%	35	35	100%
Peniculia	154	20	13%	507	3	1%	3	2	67%

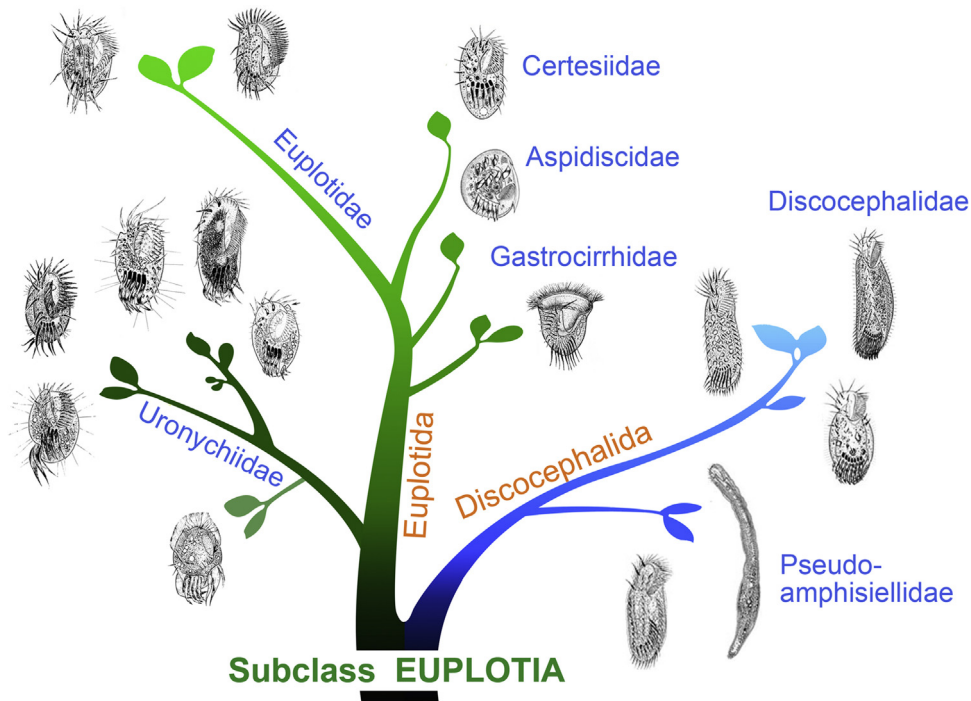


**Fig. 1.** Phylogenetic studies of hypotrichs. (A) Phylogenetic relationships focusing on the order Urostylida inferred from SSU rRNA gene sequences (from Yi and Song 2011); (B) Phylogenetic relationships focusing on *Holosticha*-complex inferred from SSU rRNA, 5.8S rRNA and LSU rRNA gene sequences (from Zhao et al. 2015); (C) Morphology of known genera in subclass Hypotrichia.

complex. Historically, all hypotrichs with three frontal cirri, transverse cirri, and a zigzag midventral complex were assigned to the genus *Holosticha* (Hu and Song 2001;

Berger 2006). In 2003, Berger divided this genus into four genera: *Holosticha*, *Anteholosticha*, *Biholosticha* and *Caudiholosticha*, having concluded that *Holosticha*-complex was a





**Fig. 2.** Hypothetical evolution based on morphological and molecular data to show the relationships of orders and families within subclass Euplotia. Insets show the morphology of representative genera of seven families.

poorly characterized taxon based on an unspecified combination of plesiomorphies (Berger 2003). Over a period of several years we collected representative species of *Holosticha* and *Anteholosticha* and confirmed the validity of these genera based on their molecular phylogeny (Lv et al. 2015; Yi et al. 2010; Zhao et al. 2015). Additionally, two new genera, i.e. *Nothoholosticha* and *Arcuseries*, which have similar morphological characters to other members of the *Holosticha*-complex species but could not be assigned into any of the four constituent genera, were established (Huang et al. 2014).

The increasing availability of sequences in GenBank provided the opportunity to investigate evolutionary relationships among and within higher level taxa, for instance, monophylies of the orders Urostylida, Stichotrichida and Sporadotrichida (Yi and Song 2011; Huang et al. 2014). Phylogenetic investigations of urostylids indicated that the order Urostylida is not monophyletic, although a core group was always robustly recovered (Yi and Song 2011; Huang et al. 2014). The genera *Uroleptus* and *Paruroleptus*, which are traditionally classified as urostylids, formed a sister group with the oxytrichids (Yi and Song 2011; Huang et al. 2014). Similarly, the genera *Leptoamphisiella* and *Pseudoamphisiella* should be assigned to the order Discocephalida rather than the order Urostylida (Yi and Song 2011).

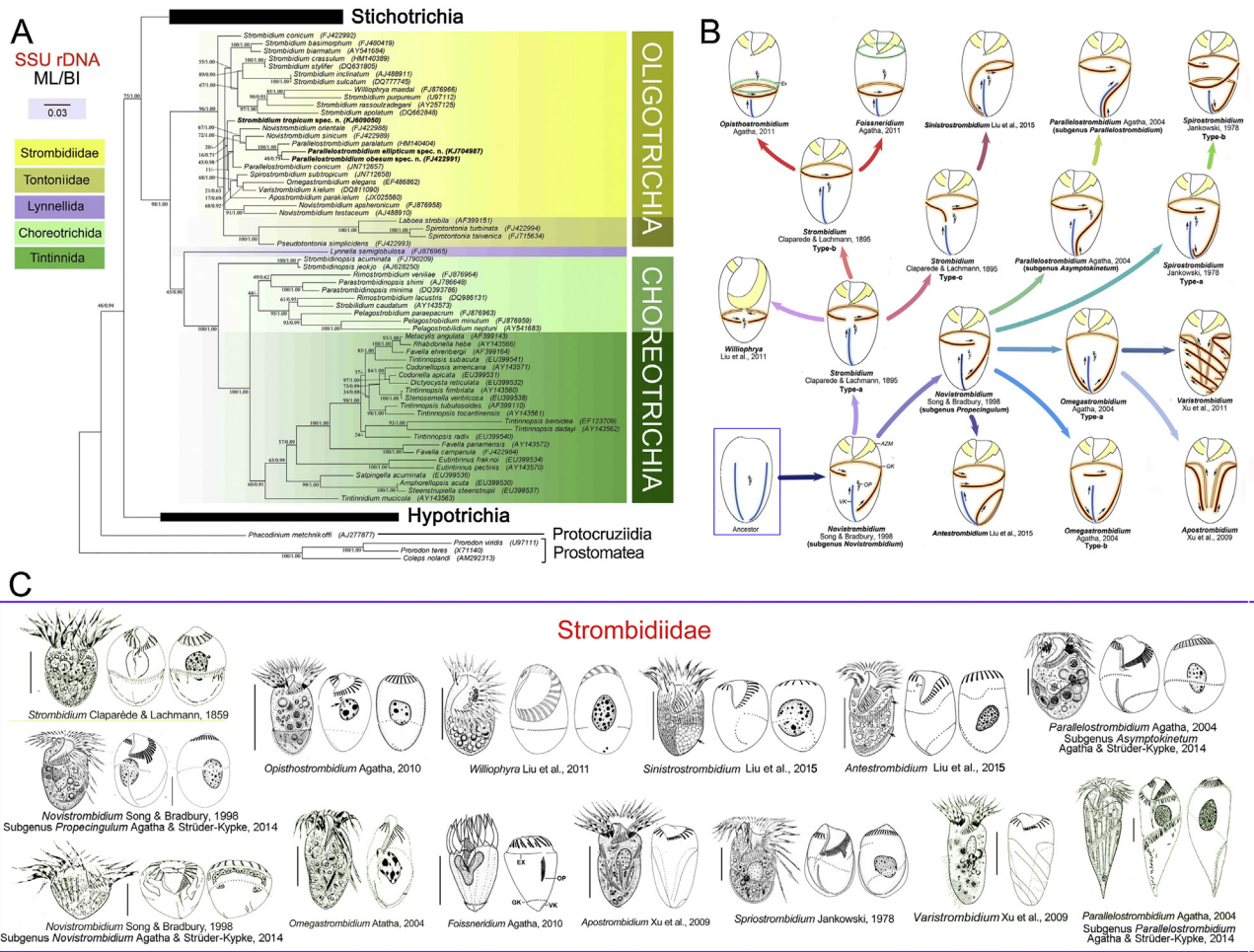
Studies on sporadotrichids have revealed that the order Sporadotrichida and the family Oxytrichidae are both polyphyletic, supported the monophyly of the family Trachelostylidae, and confirmed the validity of the family Gonostomatidae (Hu et al. 2011; Huang et al. 2016). Stud-

ies on the order Stichotrichida have dealt with the familial placement of various stichotrichid genera and the systematics of the families Amphisiellidae and Trachelostylidae (Yi and Song 2011; Huang et al. 2014). We have also demonstrated that members of the genus *Amphisiella* are separated into two clades in phylogenetic trees, corroborated by differences in cortical granule distribution (Huang et al. 2016).

Numerous unresolved phylogenetic problems, for example, how to outline orders and families better, are still present in Hypotrichia, even though this subclass is one of best-studied groups within the Ciliophora. Future integrative studies, combining phylogenomic data, multiple gene sequences and detailed morphological and morphogenetic characters are expected to help resolve such problems.

## Phylogenetic Studies of Euplotia

The subclass Euplotia (syn. Hypotrichia sensu Lynn 2008) was resurrected in the classification system of Adl et al. (2012). It currently comprises two orders, Euplotida and Discocephalida, and seven families, Aspidiscidae, Certesiidae, Euplotidae, Gastrocirrhidae, Uronychiidae, Pseudoamphisiellidae and Discocephalidae (Gao et al. 2016a; Li et al. 2009a; Lynn 2008). This is considered to be one of the most confused assemblages of ciliates with a high degree of species diversity and wide distribution in freshwater, terrestrial and marine habitats. In the genus *Euplotes*, almost 150 species and sub-species have been recognized (Berger 2001; Jiang et al. 2010). Meanwhile, novel



**Fig. 3.** Phylogenetic studies of oligotrichs and choreotrichs. (A) Phylogenetic tree inferred from SSU rDNA gene sequences of subclasses Oligotrichia and Choreotrichia (from Liu et al. 2015c). (B) Hypothetical evolution of Strombidiidae (from Liu et al. 2015b). (C) Morphology of known genera in family Strombidiidae.

species and genera are being described at a brisk rate (e.g. Hu 2008; Jiang and Song 2010; Lobban et al. 2005; Luo et al. 2014). However, phylogenetic relationships among euplotians are largely unresolved mainly due to the unavailability of gene sequences of the majority of morphospecies and disagreements among conclusions based on morphological, morphogenetic and single-gene data (Miao et al. 2007; Yi et al. 2009).

During the last two decades, we have published more than 130 multiple gene sequences of identified species with detailed morphological information (e.g. Liu et al. 2015a; Shen et al. 2009; Song et al. 2004). This accounts for more than 60% of SSU rDNA gene records and 80% of ITS-5.8S rDNA, LSU rDNA gene, and alpha-tubulin gene sequences of euplotians in the GenBank database. Nevertheless, molecular data are still available for less than 30% of euplotian morphospecies. Based on concatenated data from the authors' groups, the main findings concerning the systematics of the Euplotia are as follows (see also Fig. 2): (1) the subclass Euplotia and its two orders, Euplotida and Discocephalida, are all monophyletic based on phylogenetic analyses of con-

catenated data of four genes (Gao et al. 2016a); (2) all seven families are well-supported clades in the SSU rDNA gene tree, whereas the monophyly of Uronychiidae is not recovered in trees inferred from limited sampling of other gene markers (Huang et al. 2012; Liu et al. 2015a; Miao et al. 2011; Yi et al. 2009); (3) LSU rDNA gene data and concatenated data of three rDNA genes fully support the monophyly of *Paradiophrys* and the establishment of *Apodiophrys* and *Diophryopsis*, although none of these genera are monophyletic in the SSU rDNA gene tree (Huang et al. 2012; Jiang and Song 2010; Jiang et al. 2011); (4) the validity of the genera *Euplotopsis*, *Euplotoides*, and *Moneuplotes* (Borror and Hill 1995; Jankowski 1978) is not supported by molecular data and we therefore proposed to remerge them into a single genus *Euplotes* (Liu et al. 2015a; Yi et al. 2009); (5) the hypothesis based on morphological data that discocephalids and pseudoamphisiellids are closely related was consistently supported in both single-gene and concatenated trees (Gao et al. 2016a; Miao et al. 2011).

It is noteworthy that the present molecular phylogeny should not be over interpreted as some gene sequences

in GenBank are likely to be from misidentified morphospecies. Therefore, additional integrative information of phylogenomic data or multiple gene markers from the same DNA source, and detailed morphological and morphogenetic characters, will help in understanding the phylogenetic relationships within Euplotia.

## Phylogenetic Studies of Oligotrichia and Choreotrichia

The oligotrichs and choreotrichs are ecologically important components of the marine plankton as together they graze ~70% of the primary production and transfer the energy in the phytoplankton-based food web (Pierce and Turner 1992). Based on both morphological and genetic data the subclasses Oligotrichia and Choreotrichia, along with the monospecific order Lynnellida, comprise a total of at least 1687 species, including 133 species of oligotrichs, over 1500 species of loricate choreotrichs (tintinnids) and 53 species of aloricate choreotrichs (Agatha 2011; Agatha and Strüder-Kypke 2014; Gao et al. 2016a, 2016b). The molecular phylogenies often reveal unexpected relationships that do not map onto cladograms of these groups based on morphological data, especially at genus level (Agatha and Strüder-Kypke, 2012; McManus and Katz 2009). These inconclusive results are probably caused by the considerable morphological convergence among these forms, although other possible explanations are limited taxon representation in the datasets and high genetic variation among the genera.

Over the last decade, genomic DNA of 157 oligotrichous species/populations has been sequenced by our group, including that of 27 new species. About 43 sequences of the SSU rRNA gene, 39 sequences of the ITS-5.8S rRNA gene and 24 sequences of the LSU rRNA gene have been submitted to the GenBank database. Based on these and other published data, we have carried out numerous molecular phylogenetic studies on oligotrichs and choreotrichs (Gao et al. 2009; Liu et al. 2015b; Song et al. 2015a). Main conclusions include that the subclasses Oligotrichia and Choreotrichia are both strongly supported monophylies and are sister groups, whereas the systematic position of *Lynnella* is ambiguous and unstable (Liu et al. 2011). Recent phylogenetic analyses integrating both the molecular and morphological data indicated that *Lynnella* might represent a new order separated from both the subclasses Oligotrichia and Choreotrichia (Liu et al. 2015b).

Within the subclass Oligotrichia, the systematic relationships of the families Tontoniidae and Strombidiidae were investigated. The main findings were: (1) the strombidiid genus *Laboea* should be transferred into the family Tontoniidae, suggesting that the spiralled girdle kinety is probably a synapomorphy rather than a convergent feature as previously speculated for tontoniids and *Laboea* (Agatha 2004), and the tail was likely lost during the evolution of *Laboea* species (Gao et al. 2009); (2) the family Strombidiidae is

polyphyletic and the genus *Strombidium* is so genetically diverse that should probably be split into several yet to be defined genera (Liu et al. 2015c; Song et al. 2015a, 2015b); (3) our phylogenetic analyses support a new evolutionary hypothesis of the 12 known strombidiid genera, i.e. that the ciliary pattern of the subgenus *Novistrombidium* probably represents the ancestral state in the evolution of strombidiids, which then gave rise to two main groups, one composed of *Strombidium*-like genera and the other non-*Strombidium*-like genera (Fig. 3; Liu et al. 2015b).

Within the subclass Choreotrichia, the monophyly of the order Choreotrichida was not recovered because the family Strobilidiidae groups with the tintinnids rather than with strombidiid genera. Other main findings were that: (1) the order Tintinnida is monophyletic, while the family Tintinnidae might be paraphyletic; (2) the lorica-based definition for the genus *Tintinnopsis* is not consistent with the molecular phylogeny; (3) the codonellopsid genera *Stenosemella* and *Codonellopsis* might need to be merged into a single genus based on molecular analyses and the possession by both of a hyaline collar (Li et al. 2009b).

Despite the recent advances in the use of molecular tools for studying oligotrich and choreotrich phylogeny, the topology of the gene trees remains unstable because genetic data are unavailable for several key taxa such as *Leegaardiella*, *Lohmanniella*, and *Parastrombidium*. Additionally, molecular information of tintinnids is still scant relative to the large number of morphotypes currently recognized. Extensive sequence sampling of these taxa along with increases in knowledge of their taxonomy based on ultrastructure and infraciliature characters, are expected to significantly enhance our future understanding of the phylogeny of oligotrichs and choreotrichs (Agatha 2011; Santoferrara et al. 2016).

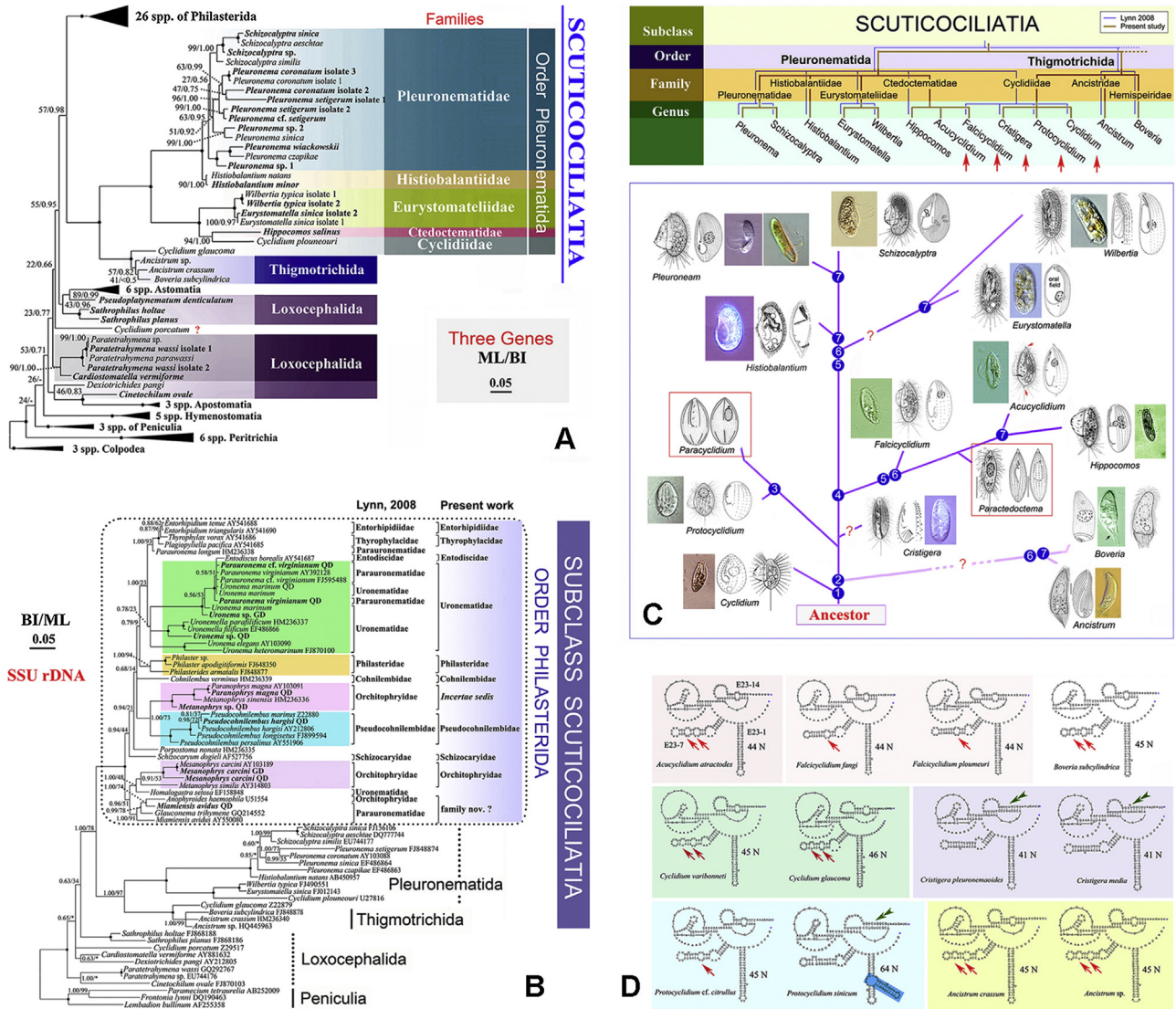
## Phylogenetic Studies of Peritrichia

The peritrichs are among the most diverse and commonly observed ciliates with more than 105 genera representing over 1000 species (Foissner et al. 2010; Lynn 2008). Systematically, peritrich ciliates are generally treated as a subclass, Peritrichia, within the class Oligohymenophorea (Lynn 2008). However, phylogenetic relationships among many peritrichs remain confused because molecular, especially multi-gene, data are available for relatively few representatives.

In order to improve this situation, we have submitted to NCBI/GenBank gene sequences of numerous well-characterized and accurately identified peritrichs, including 20 SSU rRNA, 14 ITS1-5.8S-ITS2, and six LSU rRNA. While several of our molecular studies have provided support for some hypotheses based on morphological and/or ontogenetic characters, others have yielded radically different results. Our main results can be summarized as follows (see also Fig. 4): (1) sessilids which have stalks with con-







**Fig. 5.** Phylogenetic studies of scuticociliates. (A) Phylogenetic tree focusing on pleuronematids and loxocephalids inferred from the concatenated data of SSU rRNA, 5.8S rRNA and LSU rRNA gene sequences (from Gao et al. 2013); (B) Phylogenetic tree focusing on philasterids inferred from SSU rRNA gene sequences (from Gao et al. 2012); (C) Classification and cladogram to show the possible evolution of pleuronematid and thigmotrichid genera (from Gao et al. 2014); (D) Secondary structures of variable region 4 (V4) of the SSU rRNA of 12 scuticociliates (from Gao et al. 2014).

phylogenetic relationships within the subclass Scuticociliata were largely unresolved (Foissner et al. 2009; Lynn and Strüder-Kypke 2005).

Over the past decade, we have expanded the taxonomic sampling of scuticociliates having isolated and characterized 61 morphospecies and provided 162 new gene sequences, including 78 SSU rRNA, 49 ITS-5.8S rRNA and 35 LSU rRNA. This includes the first molecular data of any kind for two orders, namely Thigmotrichida and Loxocephalida, and 25 genera. Phylogenetic analyses based on single-gene and concatenated data, secondary structural analyses and predicted topology tests have been carried out to expand our understanding of phylogeny within the subclass Scuticociliata. The main results are summarized as follows (Fig. 5): (1)

The order Philasterida is a well-supported lineage although the evolutionary relationships among most of its families remain unresolved (Gao et al. 2012; Pan et al. 2013); (2) in all analyses the thigmotrichids are nested within the order Pleuronematida and so should be regarded as a suborder within Pleuronematida (Gao et al. 2014); (3) the loxocephalids are polyphyletic, being basal to the core scuticociliates and most closely related to the subclasses Astomatia and Apostomatia, and should be separated from the order Philasterida (Gao et al. 2013; Li et al. 2006; Zhang et al. 2010, 2011); (4) *Parauronema* is a junior synonym of *Uronema*; consequently, Parauronematidae becomes a junior synonym of Uronematidae (Gao et al. 2012); (5) the families Orchitophryidae, Uronematidae, Cyclidiidae, Loxocephalidae and



Cinetochilidae are all non-monophyletic (Gao et al. 2012, 2013, 2014); (6) the genera *Metanophrys*, *Uronema*, *Cyclidium*, and *Protocyclidium* are all non-monophyletic (Gao et al. 2012, 2014); (7) four new genera were established, *Eurystomatella*, *Wilbertia*, *Acucyclidium*, and *Paramesanophrys* (Fan et al. 2011; Gao et al. 2014; Miao et al. 2010).

There are, however, still many significant challenges with the phylogeny and systematics of the scuticociliates. For example, family assignments in the order Philasterida are still uncertain, the phylogenetic positions of thigmotrichids and cyclidiids need to be revised with more evidence, loxocephalids are dispersed in different groups and need to be defined, etc. There are two main reasons for these ongoing problems: firstly, scuticociliates are generally small in size and share similar infraciliature and characters *in vivo*, resulting in many misidentifications that lead to errors in phylogenetic analyses; secondly, detailed morphological/morphogenetic/molecular data from some key taxa are still missing, including for many type species, which hinders the revision of the existing classification and systematics. To solve these problems, more taxa need to be sampled and studied in detail at the morphological, morphogenetic, and molecular levels. Moreover, the application of new technologies such as DNA-barcoding for accurate species identification and phylogenomic analyses for expanding the number of informative gene markers, are also likely to help resolve these problems.

## Phylogenetic Studies of the Phylum Ciliophora

Although numerous studies on the taxonomy and systematics of ciliates have been performed and published, the vast majority of these are focused on one or a few specific groups and/or based on sequence data for a single gene, the SSU rRNA. In two recent studies, we investigated evolutionary relationships within the entire phylum Ciliophora using a taxon-rich strategy based on multiple gene loci (Gao and Katz 2014; Gao et al. 2016b).

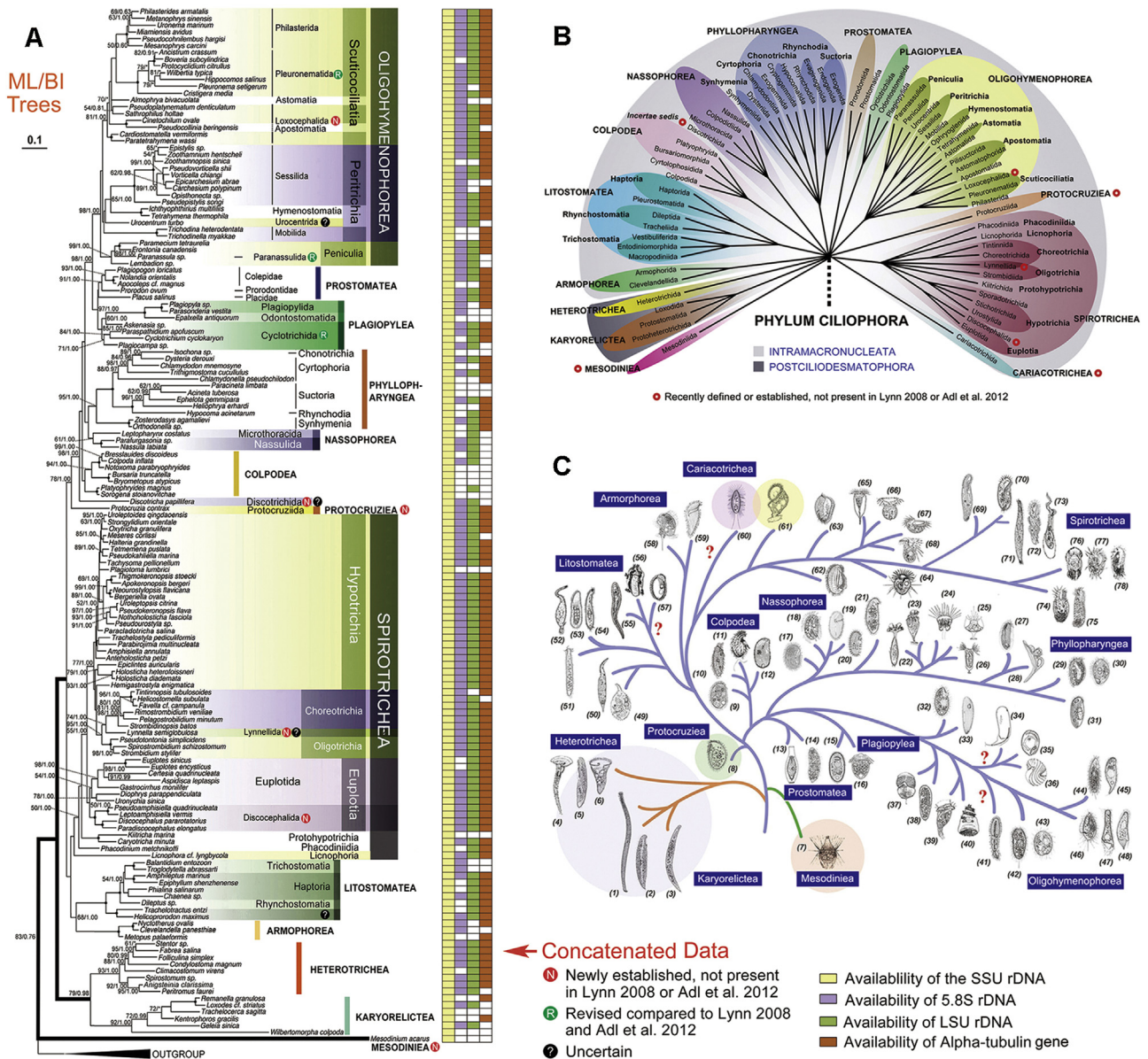
The first of these studies included phylogenomic analyses of 537 ciliate taxa, all of which have been sampled for the SSU rRNA gene sequence and a subset of which included LSU rRNA gene and up to seven protein-coding sequences. The full data matrix and six submatrices were analysed in order to assess the impact of taxon sampling and missing data. These analyses support the bifurcation of ciliates into two major clades defined by differences in division of the somatic macronuclei: the subphyla Intramacronucleata and Postciliodesmatophora (Gao and Katz 2014; Lynn 2008). The second study focused on a full-scale classification of the Ciliophora at the level of order/family. Phylogenetic analyses were carried out based on up to four gene markers (SSU rRNA, 5.8S rRNA, LSU rRNA, alpha-tubulin) from a total of 152 species representing 110 families and 55 orders. The

main findings were as follows (see also Fig. 6): (1) 14 classes were recovered including one new class, Protocruzia; (2) in addition to the two main branches (Postciliodesmatophora and Intramacronucleata), a third branch, the Mesodininea, was identified as being basal to the other two; (3) the monophyly of the subclass Hypotrichia was supported and the newly defined order Discocephalida was revealed to be a sister clade to the euplotids, strongly suggesting the separation of discocephalids from the hypotrichs; (4) Phacodiniida and Protohypotrichia were consistently recovered as a clade that is basal to the core spirotricheans; (5) the newly established order Lynnellida is placed between the Oligotrichia and Choreotrichia; (6) the separation of the mobilids from the sessilid peritrichs was not supported by evidence from morphology, ontogenesis or molecular analyses; (7) within the subclass Scuticociliatia, the paraphyletic order Loxocephalida is basal to the main scuticociliate assemblage, whereas the thigmotrichs are placed within the order Pleuronematida; (8) the monophyly of classes Phyllopharyngea, Karyorelictea, Armophorea, Prostomatea, Plagiopylea, Colpodea and Heterotrichea were confirmed; (9) the ambiguous genera *Askenasia*, *Cyclotrichium*, *Paraspathidium* and *Plagiocampa* showed close affiliation to the well-known plagiopyleans; (10) the validity of the subclass Rhynchostomatia, which is basal to the Litostomatea, was supported; and (11) the systematic positions of *Halteria* and *Licnonophoria* (represented by *Licnophora*) remain unresolved due to extremely contradictory information from morphology, ontogenesis and SSU rRNA gene sequence data, and are thus regarded as *incertae sedis* within Spirotrichea (Gao et al. 2016b).

Numerous phylogenetic and systematic problems within the ciliates are still largely unresolved. Notable among these are the systematic positions of genera such as *Halteria*, *Licnophora*, *Protocruzia*, *Mesodinium*, and *Urocentrum*, phylogenetic relationships within the Prostomatea-Plagiopylea group, the assignments of the taxa in Nassophorea, the evolutionary relationships among some classes, etc. Part of the reason for this is that molecular information of many taxa is unavailable and phylogenetic analyses have mainly focused on few genes. Phylogenomic analyses are now increasingly applied for investigating evolutionary relationships among ciliates (Feng et al. 2015; Gao et al. 2014; Gentekaki et al. 2014, 2017). This will help significantly to resolve most if not all of the questions mentioned above as genome/transcriptome data become available for more taxa.

## Barcoding Ciliates: Exploring Potential Candidate Genes

The use of DNA barcoding, as a molecular tool to aid in the discrimination of species, was introduced by Hebert et al. (2003). The mitochondrial cytochrome c oxidase subunit I (*cox 1*) gene was chosen as the preferred marker because of its central role in the metabolism of aerobic eukaryotic organ-



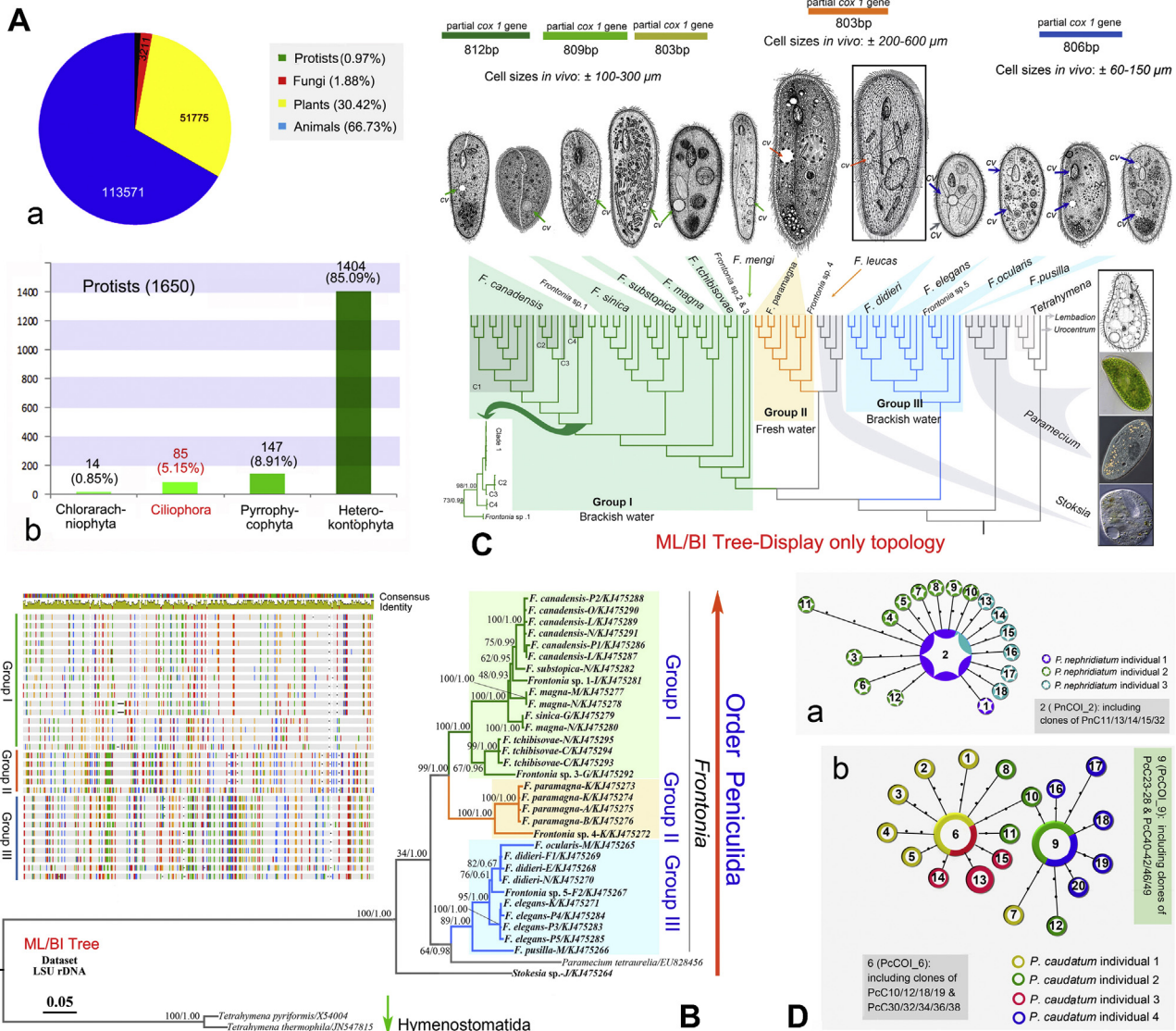
**Fig. 6.** Molecular phylogeny of the phylum Ciliophora (from Gao et al. 2016b). (A) Maximum likelihood (ML) tree reconstructed using 152 ciliates and concatenated gene sequence data, the SSU rRNA gene sequence is available for all the taxa whereas the 5.8S rRNA, LSU rRNA and alpha-tubulin gene sequences are available for only a subset of these taxa. (B) Systematic scheme for the phylum Ciliophora at order level. (C) Hypothetical evolution of ciliated protozoa based on both morphological and molecular data to show the relationship and the positions of the taxa at order level.

isms and therefore its presence in almost all eukaryotes. The *cox 1* gene is now extensively used as the standard barcode gene for almost all animal groups, although other genes are preferred for plants (*rbcl*) and fungi (ITS region). There is, however, no standard barcode for the protists, largely due to their extreme diversity and the levels of genetic divergence between and within major protistan groups (Pawlowski et al. 2012). Barcoding work on ciliates is still at the initial stage even though there are some pioneering works that evaluate the utility of mitochondrial candidate genes (e.g. Barth et al. 2006; Chantangsi et al. 2007; Kher et al. 2011; Strüder-Kypke

and Lynn 2010). To date, however, no single set of molecular markers has been confirmed to work in all ciliate lineages.

In a pilot study, we focused on assessing genetic divergence of the *cox 1* gene within individuals and how its presence might affect species identification and population structure analyses (Fig. 7). The results demonstrate that although heteroplasmy exists in *Paramecium*, *cox 1* is nevertheless a suitable marker for resolving inter- and intra-specific relationships among *Paramecium* species (Barth et al. 2006; Zhao et al. 2013). Due to its high divergence even within members of the same taxon, however, the difficulty of designing universal *cox1* gene primers hampers its utility for all cil-





**Fig. 7.** Barcoding research on ciliates. (A) Current state of barcoding research on protists (a) and ciliates (b). (B) Evidence that D1–D2 region of LSU rRNA gene shows promising results for species delineation (from Zhao et al. 2016). (C) Integrated information suggests that *cox 1* is suitable for *Frontonia* species delineation (from Zhao et al. 2016). (D) Evidence that *cox 1* heteroplasmy exists in *Paramecium* species (from Zhao et al. 2013).

iates. Consequently, we tested various other gene markers in an effort to identify the ideal barcoding candidate for ciliate species identification. For this, we investigated well-characterized members of the species-rich genus *Frontonia* in order to test the accuracy and efficacy of different candidates. These studies revealed that the mitochondrial *cox1* gene, the nuclear ITS1 and ITS2 regions and the hypervariable D1–D2 region of the LSU rRNA gene are promising candidates for species delineation. Of these, the D1–D2 region of the LSU rRNA gene has the greatest potential to build a barcoding reference database since it can be obtained using universal PCR primers (Zhao et al. 2016). Group-specific barcodes are still being validated among other protist lineages (Pawlowski et al. 2012), and our case studies in ciliates provide valuable

data on the appropriateness of different genetic markers to the barcoding enterprise.

At present, the construction of a DNA barcode library and its performance for identifying cryptic species is still at the exploratory stage. Besides, we also have to take into consideration that the accuracy of barcoding datasets is dependent on recovery of as many taxonomically verified specimens as possible. Therefore, we can foresee daunting challenges, such as universal primer design and voucher specimen identification, when accomplishing the Ciliate Barcoding Project. Here, we recommend the development of group-specific primers, which may help resolve failed amplification of particular taxa. Last but not least, a solid taxonomic foundation is prerequisite in barcoding ciliates and new barcoding data are



reliable only when the species is identified accurately based on traditional taxonomic methods.

## Acknowledgements

The IRCN-BC program supported by the NSF (USA) and NSFC (China) is highly acknowledged. This work is also supported by the Natural Science Foundation of China (31430077, 31572230, 31401963, 31471973, 41576124), International Exchanges Scheme supported by the Royal Society/Natural Science Foundation of China (31411130122), a BBSRC China Partnering Award, the Applied Basic Research Plan of Qingdao (15-12-1-1-jch), the Fundamental Research Funds for the Central Universities (201564022), and AoShan Talents Program Supported by Qingdao National Laboratory for Marine Science and Technology (No.2015ASTP). The authors extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this work through Research Group (RGP-242).

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