The All-Data-Based Evolutionary Hypothesis of Ciliated Protists with a Revised Classification of the Phylum Ciliophora (Eukaryota, Alveolata)

Feng Gao1, Alan Warren2, Qianqian Zhang3,*, Jun Gong3,*, Miao Miao4,*, Ping Sun5,*, Dapeng Xu6,*, Jie Huang1,*, Zhenzhen Yi8 & Weibo Song1

The phylum Ciliophora plays important roles in a wide range of biological studies. However, the evolutionary relationships of many groups remain unclear due to a lack of sufficient molecular data. In this study, molecular dataset was expanded with representatives from 55 orders and all major lineages. The main findings are: (1) 14 classes were recovered including one new class, Protocruziea n. cl.; (2) in addition to the two main branches, Postciliodesmatophora and Intramacronucleata, a third branch, the Mesodiniea, is identified as being basal to the other two subphyla; (3) the newly defined order Discocephalida is revealed to be a sister clade to the euplotids, strongly suggesting the separation of discocephalids from the hypotrichs; (4) the separation of mobilids from the peritrichs is not supported; (5) Loxocephalida is basal to the main scuticociliate assemblage, whereas the thigmotrichs are placed within the order Pleuronematida; (6) the monophyly of classes Phylopharyngea, Karyorelictea, Armophorea, Prostomatea, Plagiopylea, Colpodea and Heterotrichia are confirmed; (7) ambiguous genera Askenasia, Cyclotrichium Paraspathidium and Plagiocampa show close affiliation to the well known plagiopyleans; (8) validity of the subclass Rhynchostomatia is supported, and (9) the systematic positions of Halteriida and Linconophoria remain unresolved and are thus regarded as incertae sedis within Spirotrichea.

The ciliated protists are a large and diverse group of microbial eukaryotes that are of central importance in the functioning of microbial food webs by mediating the transfer of organic matter and energy between different trophic levels1,2. Due to their short lifespan and unique biological characters (e.g., nuclear dimorphism, chromosomal fragmentation), they have attracted the attention of researchers in a wide range of disciplines including cytology, evolutionary biology and genetics3. Despite this attention, however, no broadly accepted hypothesis exists for the phylogenetic relationships within the Ciliophora. For example, two widely used classification systems, i.e. those of Corliss4 based mainly on morphological characters, and Lynn5 inferred mainly from ultrastructural characters and small subunit ribosomal DNA (SSU rDNA) sequences, are broadly consistent at class-level but differ widely at order- or family-levels. In recent years, investigations based on gene trees have been increasingly used to reconstruct ciliate phylogeny and have helped to resolve a number of phylogenetic problems6–11.

Received: 13 November 2015
Accepted: 05 April 2016
Published: 29 April 2016

1Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China. 2Department of Life Sciences, Natural History Museum, London SW7 5BD, UK. 3Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China. 4College of Life Sciences, University of Chinese Academy of Sciences, Beijing 100049, China. 5Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystem, Xiamen University, Xiamen 361102, China. 6State Key Laboratory of Marine Environmental Science, Institute of Marine Microbes and Ecosystems, Xiamen University, Xiamen 361102, China. 7Key Laboratory of Aquatic Biodiversity and Conservation, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. 8Guangzhou Key Laboratory of Subtropical Biodiversity and Biomonitoring, School of Life Science, South China Normal University, Guangzhou 510631, China. 9These authors contributed equally to this work. Correspondence and requests for materials should be addressed to Z.Y. (email: zyi@scnu.edu.cn) or W.S. (email: wsong@ouc.edu.cn)
Unfortunately, most of these investigations are based on sequence data from a single gene, namely SSU rDNA, although a few multi-gene phylogenetic studies have been performed on far more limited numbers of species. To date, no molecular phylogenetic analysis has focused on a full-scale classification of the Ciliophora at the level of order/family.

Comprehensive taxon sampling coupled with gene-rich analyses are critical for resolving accurate phylogenies. However, sampling and identifying targeted ciliate species/groups is very difficult and time-consuming due to their microscopic size. According to Lynn, there are about 300 families and 57 orders of ciliates. In the present study, phylogenetic analyses were carried out based on sequence data from up to four gene markers in a total of 152 species representing 110 families and 55 orders. The main aim of this investigation was to resolve phylogenetic relationships among the principal ciliate groups including all available orders (excluding Cariaciotrichida for which the SSU rDNA sequence is short and in vivo characters have yet to be reported).

**Results**

Concatenated, SSU rDNA, and LSU rDNA trees are topologically similar, and most of their nodes are well-supported (Figs 1 and 2). By contrast, ITS1-5.85-ITS2 rDNA and alpha-tubulin genes produce poorly supported topologies in both deep and crown nodes (Additional file 2: Figs S1–S6), so we do not describe these two trees in detail.

**Concatenated Tree.** Both ML and BI analyses show a similar topology in the concatenated tree, that is, with three main groups are recognizable (Fig. 1A): (1) Mesodinium, represented by the type genus *Mesodinium*; (2) subphylum Postciliodesmatophora, which comprises Karyorelictea and Heterotrichea, and; (3) subphylum Intramacronucleata, which comprises all other classes. Within the Postciliodesmatophora, the classes Heterotrichea and Karyorelictea are each recovered as a monophyletic assemblage. Within the Intramacronucleata, which comprises all other classes, the new order Lynnellida clusters with Choreotrichia, forming a group that is sister to the spirotrichean assemblage. Each of the other major subgroups, i.e. Euplotia, Protohypotrichia, Phacodiniida, Stichotrichida, and Urostylida) are ambiguous. For example, six species of the order Urostylida form a moderately supported clade (89% ML; 1.00 BI), the other five species occupy three separate clades. Within the Euplotia, the order Euplotida is monophyletic whereas the order Discoccephalida clusters with *Pseudoamphisiella*.

As expected, the subclasses Oligotrichia and Choreotrichia, are both monophyletic with high support (Fig. 1A). The newly proposed order Lynnellida clusters with Choreotrichia, forming a group that is sister to the subclass Oligotrichia. The subclass Choreotrichia is comprised of two groups, the tintinnids and the aloricate choreotrichs. Furthermore, groupings within the three hypotrich orders (Sporadotrichida, Stichotrichida, and Urostylida) are ambiguous. For example, six species of the order Stichotrichida occupy five separated clades; seven species of the order Sporadotrichida occupy six different clades; and although eight out of 13 species of Urostylida form a moderately supported clade (89% ML, 1.00 BI), the other five species occupy three separate clades. Within the Euplotia, the order Euplotida is monophyletic whereas the order Discoccephalida clusters with *Pseudoamphisiella*.

Within the class Hypotrichida, the subclass Synhymenia, is both monophyletic and strongly supported (Fig. 1A).

In the class Nassophorea, the order Microthoracida clusters with the order Nassulida in the concatenated tree (61% ML, 1.00 BI) (Fig. 1A). *Discoricha* is consistently placed as an early branching lineage within the ConThreeP superclade with high support (98% ML, 1.00 BI).

Protocruzida, represented by the well-known genus *Protocruzia* and traditionally classified as a member of Spirotrichea, groups with ConThreeP. With *Protocruzia* excluded, the class Spirotrichea is monophyletic (Fig. 1A), albeit with low support (<50% ML, <0.5 BI). Laciniporida occupies the basal position within the spirotrichean clade. Each of the other major subgroups, i.e. Euplotia, Phacodiniida, Oligotrichia and Choreotrichia, forms a separate clade within the spirotrichean assemblage. By contrast, the subclass Hypotrichia is non-monophyletic with *Epiclintes*, *Holosticha* and *Hemigastroxyyla* grouping with the choreotrichs and oligotrichs. Furthermore, groupings within the three hypotrich orders (Sporadotrichida, Stichotrichida, and Urostylida) are ambiguous. For example, six species of the order Stichotrichida occupy five separated clades; seven species of the order Sporadotrichida occupy six different clades; and although eight out of 13 species of Urostylida form a moderately supported clade (89% ML, 1.00 BI), the other five species occupy three separate clades. Within the Euplotia, the order Euplotida is monophyletic whereas the order Discoccephalida clusters with *Pseudoamphisiella*.

As expected, the subclasses Oligotrichia and Choreotrichia, are both monophyletic with high support (Fig. 1A). The newly proposed order Lynnellida clusters with Choreotrichia, forming a group that is sister to the subclass Oligotrichia. The subclass Choreotrichia is comprised of two groups, the tintinnids and the aloricate choreotrichs.

The class Litostomatae consists of three subclasses, Haptoria, Trichostomatia and Rynychostomatia. Haptoria is paraphyletic in all the gene trees (Figs 1 and 2), with the Trichostomatia (represented by *Balantidium* and *Trogloidyta*) nesting within it and *Helicoprorodon* clustering with *Trachelothractus*, forming an early-branching lineage of litostomatesan.

The Oligohymenophorea forms a maximally supported clade and comprises six subclasses (Fig. 1A). Both the subclasses Apostomatia and Astomatia, each of which is represented by a single sequence, nest within the scuticociliate assemblage rendering the Scuticociliata polyphyletic. The scuticociliate order Pleuronematida is monophyletic (Fig. 1A), albeit with low support (<50% ML, >0.5 BI). The order Loxocephalida, represented by five genera and two families, is paraphyletic with *Pseudoplatynematum* and *Satnophilus* clustering with the Philasterida/Pleuronematida + Astomatia assemblage, *Cardiostomatella* and *Paratetrahymena* forming a separate clade that is basal to rest of the scuticociliates (100% ML, 1.00 BI), and *Cinetochilum* clustering with the subclass Apostomatia (100% ML, 1.00 BI).

The subclass Peritrichia comprises two orders, Sessilida and Mobildla. Although each order is monophyletic, the Sessilida groups with the subclass Hymenostomatia and the family Urocentridae (subclass Penicilia), rather than with the Mobildla, rendering the Peritrichia non-monophyletic.

The subclass Penicilia is represented by five families and five genera. Four of these genera (*Paramecium, Frontonia, Lambadion*, and *Paramassula*) form a well-supported clade that is basal to the rest of the Oligohymenophorea (Fig. 1A). The fifth genus, *Urocentrum* (family Urocentridae), occupies a position between the hymenostomes and the peritrich order Mobildla.
A close relationship between the classes Prostomatea and Plagiopylea is well supported (97% ML, 1.00 BI). The Prostomatea-Plagiopylea group (hereafter referred to as the PP clade) is sister to the class Oligohymenophorea with high support (98% ML, 1.00 BI), forming a three-class metaclade (Fig. 1A).

The class Prostomatea is represented by one order, namely Prorodontida, which is paraphyletic. The monophyletic family Colepidae (represented by Apocoleps, Nolandia and Plagiopogon) is closely related to Prorodontidae (represented by Prorodon) with variable support (91% ML, 1.00 BI). The family Placidae (represented by Placus) is basal to the Colepidae-Prorodontidae. The prostome family Plagiocampidae (represented by Plagiocampa), however, clusters with two genera of uncertain taxonomic affiliation, Cyclotrichium and Paraspathidium (see below).
The class Plagiopylea is formed by the well-known plagiopyleans and four ambiguous genera, *Plagiocampa*, *Paraspathidium*, *Cyclotrichium* and *Askenasia*. The former three form a moderately supported sister group (71% ML, 1.00 BI) to *Askenasia* and the well-known plagiopyleans (85% ML, 1.00 BI).

**Topological Difference between SSU rDNA and Concatenated Trees.** Within the class Spirotrichea, the genus *Hemigastrostyla* is not sister to oligotrichs in the SSU rDNA tree (Fig. 2A), whereas this is the case in the concatenated tree (Fig. 1A), and the euplotid family Uronychiidae clusters with Discocephalida rather than Euplotida (Fig. 2A).

Within the class Oligohymenophorea, *Cristigera* groups with other cyclidiids and thigmotrichids with moderate to high support in the SSU rDNA tree (91% ML, 1.00 BI), rather than branching before all the other pleuronematids and thigmotrichids. Furthermore, in contrast to the concatenated tree, the monophyly of the subclass Peritrichia is supported in the SSU rDNA tree, with the sessilids and mobilids clustering together.
The subclass Licnophoria, represented by *Licnophora*, branches relatively early in the SSU rDNA tree and is basal to all groups except the assemblages Litostomatea-Armophorea and the Heterotrichia-Karyorelictea (Fig. 2A) vs. sister to Spirotrichea in the concatenated tree (Fig. 1A).

The two orders, Microthoracida and Nassulida, which are believed to be the members of the class Nassophorea, are placed in separate clades: the former branches in a position that is sister to the class Phyllopharyngea whereas the latter clusters with the colpodean genera *Platyophrides* and *Sorogena* (Fig. 2A) vs. form one clade in the concatenated tree (Fig. 1A).

**Topological Difference between LSU rDNA and Concatenated Trees.** Various taxa are located in different positions in the LSU rDNA tree compared to the concatenated tree (Figs 1A and 2B). These include: *Protocruzia*, which branches within the SAL clade (vs. with the CONthreeP clade); the genera *Amphisiella*, *Trachelostyla*, *Parabirojimia* and *Anteholosticha*, all of which are located outside (vs. within) the main hypotrich clade; *Caryotricha*, which is nested within the Discophoroidea (vs. basal to Spirotrichea); the order Lynnellida, which is more closely related to the subclass Oligotrichia than to the subclass Choreotrichia; *Wilbertia*, which clusters with the thigmotrichids (vs. with the pleuronematid *Hippocoma*); and *Urocentrum*, which clusters with the peniculids (vs. with the peritrichs and hymenostomes). Furthermore, in the SSU rDNA tree the basal clade within the class Oligohymenophorea is that comprising Peritrichia and Hymenostomatia whereas in the concatenated tree the Peniculia is basal.

**Discussion Relationships and Arrangement within the Phylum Ciliophora.** It is widely accepted that the phylum Ciliophora comprises two main groups, the subphyla Intramacronucleata and Postciliodesmatophora (Figs 3 and 4), although relationships among the constituent classes are generally not well resolved due to low support values in gene trees[11,13-15]. In the concatenated and LSU rDNA trees (Figs 1A and 2B), two main groups could be recognized within the subphylum Intramacronucleata: one group has six classes, namely Colpodea, Oligohymenophorea, Nassophorea, Phyllopharyngea, Plagiopylea and Prostomatea (CONthreeP); the other main group comprises three morphologically diverse classes, Spirotrichea, Armphorea and Litostomatea (SAL). It is noteworthy, however, that whereas both Intramacronucleata and Postciliodesmatophora could be defined by morphological differences[1], there are no strong morphological synapomorphies for either CONthreeP or SAL. Nevertheless, the clade comprising Armphorea and Litostomatea is supported by some morphological and morphogenetic synapomorphies, which unite them into a new infraphylum, Lamellicorticata[16].

**Figure 3.** Systematic scheme for the phylum Ciliophora suggested by the present and previous works. The order Discoccephalida was established by Wicklow[47], and revised to contain two suborders Discoccephalina and Pseudoamphisiellina by Miao et al.[45]. The order Discotrichida was established in Fan et al.[103]. The order Loxocephalida was originally proposed by Jankowski[18] and was confirmed by Li et al.[66], Gao et al.[60], Zhang et al.[62], etc. The order Lynnellida was established by Liu et al.[55]. The class Cariacotrichea was established by Orsi et al.[122]. The order Mesodiniida was resurrected and the class Mesodiniea was established by Chen et al.[17]. The classes Protocruzia and Licnophoria are defined in the present study.

The subclass Licnophoria, represented by *Licnophora*, branches relatively early in the SSU rDNA tree and is basal to all groups except the assemblages Litostomatea-Armophorea and the Heterotrichia-Karyorelictea (Fig. 2A) vs. sister to Spirotrichea in the concatenated tree (Fig. 1A).

The two orders, Microthoracida and Nassulida, which are believed to be the members of the class Nassophorea, are placed in separate clades: the former branches in a position that is sister to the class Phyllopharyngea whereas the latter clusters with the colpodean genera *Platyophrides* and *Sorogena* (Fig. 2A) vs. form one clade in the concatenated tree (Fig. 1A).
The subphylum Postciliodesmatophora comprises two classes, the Heterotrichea and Karyorelictea, both of which were recovered as monophyletic groups in our analyses. This is consistent both with previous phylogenetic analyses\textsuperscript{15,17} and with morphological data\textsuperscript{5}.

The systematics of the family Mesodiniidae Jankowski in Small & Lynn, 1985, represented in the present study by the genus *Mesodinium*, has long been problematic. Traditionally it has been assigned to one of two orders within the subclass Haptoria, i.e. Haptorida\textsuperscript{4,5,18–20} or Cyclotrichida\textsuperscript{5}. Puytorac\textsuperscript{19} established the order Mesodiniida for the family Mesodiniidae, however this taxon has not been widely accepted. Nevertheless, the systematic placement of the mesodiniids is ambiguous given that they invariably branch very deep in a range of gene trees and, based on their unique morphological features, they are usually completely isolated from other groups\textsuperscript{17,21,22}. It has recently been suggested that the order Mesodiniida should be resurrected and that the new class Mesodiniea, be established for this taxon\textsuperscript{17}. In the present study, Mesodiniea is consistently basal to all other ciliate lineages thus supporting its validity as a separate class and suggesting that it may even be separated at subphylum level (Figs 1A and 2A). However, the class Mesodiniea was not monophyletic in phylogenomic analyses based on 127 genes\textsuperscript{9}. More data are therefore needed in order to validate this class.

**Relationships within Heterotrichs and Traditionally Related Groups.** Ciliates with a non-specialized somatic ciliature and an adoral zone of membranelles have long been classified as heterotrichs\textsuperscript{18,20}. In recent decades, some “traditional” heterotrichs such as armorphoreans, *Licnophora*, *Phacodinium*, *Protocruzia*, and *Plagiotoma* have been excluded based on ultrastructural and/or molecular data\textsuperscript{23–25}. As a result of these findings, Lynn\textsuperscript{5} assigned the “true” heterotrichs to the class Heterotrichea, a decision that is firmly supported by the current analyses.

According to Lynn\textsuperscript{5}, Phacodiniidia Small and Lynn, 1985 and Protocruziidia Puytorac et al.\textsuperscript{25} are subclasses of the class Spirotrichea. Each contains only a single genus. Hitherto, the systematic positions of these two subclasses have never been satisfactorily resolved. Both are usually placed within the class Spirotrichea despite the fact that neither has a replication band during macronuclear DNA replication, an important apomorphy for the Spirotrichea\textsuperscript{5,20,26,27}. In the case of Protocruziidia, other evidence casting doubt on its affiliation to the Spirotrichea includes its infraciliature (non-specialized somatic ciliature, non-differentiation of the ventral-dorsal sides, etc)\textsuperscript{27} and its process of ontogenesis which is a mixture of parakinetal and scuticokinetal modes\textsuperscript{28}. Although some early molecular studies suggested that *Protocruzia* could be related to Spirotrichea\textsuperscript{24,29,30}, this finding has been repeatedly rejected by recent studies which invariably conclude that it is not a member of the class Spirotrichea\textsuperscript{11,31}. Li et al.\textsuperscript{31} suggested that *Protocruzia* represents a separate class, although they failed to define this taxon. Thus, the new class Protocruzia n. cl. is formally established here, based on the subclass Protocruziidia which contains...
a single order, Protocruziida Jankowski in Small & Lynn, 1985, a single family, Protocruziidae Jankowski, 1980, and a single genus, Protocruzia de Faria, da Cunha and Pinto, 1922, with the characters diagnosed for its subclass and the order3. The current analyses reveal that Protocruzia n. cl belongs to neither of the main sub-groups of Intramacronucleata (CONthreeP or SAL) but instead occupies a position between the two as sister to the CONthreeP/Discotrichida assemblage (Figs 1A and 2A).

Protocruzia de Puytorac et al. 1987 n. cl. Diagnosis. Body small-sized, bilaterally flattened; somatic ciliature typically with dikenoids on both left and right sides; extrusomes present; adoral zone with several membranelles on left of dominant oral region; paroral membrane composed of dikenoids; stomatogenesis in mixokinetal mode; nuclear apparatus as a cluster of similar-sized nuclei with paradiploid macronuclei surrounding one or more micronuclei; free-swimming in marine and brackish water habitats; one order.

Type order. Protocruziida Jankowski, 1980 The genus Phacodinium is another highly questionable taxon regarding its phylogenetic position having long been regarded as a heterotrich, then as having affiliations to the hypotrichs and finally as being an intermediate form between the heterotrichs and hypotrichs32. In Lynn & Small’s system33, it was treated as a sedis mutabilis and assigned to the order Phacodiniida. The first molecular investigation based on LSU rDNA sequence data concluded that Phacodiniida is basal within Spirotrichea and suggested the establishment of a new subclass Phacodiniidina34. The present study supports this finding with Phacodiniidina occupying a basal position within the Spirotrichea.

Licnophora, for which the subclass Licnophoria Corliss, 1957 was established, is characterized by its highly specialized ciliature, unique morphology (e.g. presence of the adhesive disc with concentric kinetal rings) and the unusual pattern in ontogenesis with the cell undergoing a highly modified form of homothetogenic fission, i.e., a type of parallel division producing two daughter cells that develop alongside each other, similar to that in peritrichs4,19,20,33. In Lynn et al.’s system4,28, Licnophoria was assigned Licnophoria to the class Spirotrichea, although this placement has been repeatedly contradicted by molecular studies which indicate that it does not consistently cluster with spirotricheans13,17,34.

Regarding its unique morphology that is unlike that of any spirotrichean, Licnophoria may represent a separate class that is sister to Spirotrichea. However, as molecular data are available for only a few species and complete information on morphogenesis remain unclear, we treat Licnophoria as incertae sedis within the SAL group.

Phylogeny of Hypotrichia sensu str. The subclass Hypotrichia s.l. (formerly Stichotrichia) is one of the most morphologically diverse group of ciliates and has been subject to numerous phylogenetic analyses35–38. This has resulted in the development of at least ten systematic systems4,5,19,20,36. In the Lynn’s system5, three orders of hypotrichs were recognized, namely Stichotrichida, Urostylida and Sporadotrichida, based mainly on the ventral ciliary pattern and partly on morphogenetic features. Until now, however, classification within this subclass remained unclear35–38.

In common with previous investigations, the multi-gene analyses reported here failed to separate the Stichotrichida and Sporadotrichida, the clustering patterns of these two orders being ambiguous due to low support values in all trees. This indicates that the pattern of the ventral ciliation, i.e. in specific, localized frontal and ventral groups in Sporadotrichida vs. in one or more linear longitudinal files in Stichotrichida, which is used as a main apomorphy at order level7, might be a result of convergent evolution. As in previous phylogenetic analyses6,35–38, the order Urostylida is non-monophyletic, consisting of monophyletic “core urostylids” and others (Figs 1 and 2). It is also noteworthy that classifications of hypotrichous orders based mainly on morphological characters are distinct from each other and none is completely consistent with trees based on molecular data4,5,39,40. Thus, it seems likely that some morphological characters regarded as apomorphies at order level might be plesiomorphies.

Systematic Position of the Subclass Protohypotrichia. The subclass Protohypotrichia, which contains a single order Kiitrichida, was established in 2009 based on both ontogenetic and molecular information41. Historically, the members of this group were believed to be a primordial assemblage within the Hypotrichia or ancestral forms of euplotids4,19,20,33. This hypothesis has received increasing support, both by the addition of more gene sequence data34,41 and by the recognition of various unique morphological and morphogenetic characters32,42. In the present study, the addition of newly sequenced LSU rDNA, 5.8S rDNA, and alpha-tubulin genes invariably resulted in the protohypotrichians clustering in a well-supported clade that is basal to the hypotrichs and euplotids (Figs 1A and 2). Thus all available evidence, molecular, morphological and ontogenetic, indicates that the Protohypotrichia should be recognized as a distinct group at subclass rank that is ancestral both to the hypotrichs and to the euplotids.

Systematics of the Subclass Euplotia and Related Groups. Based on previous studies and the results of the present work, we accept the system proposed by Adl et al.44 that the assemblage comprising the euplotids and discocephalids represents a distinct taxon at subclass level, namely Euplotia. Previously these two subgroups were treated as two orders (Euplotida and Discocephalida) in different subclasses33, or as suborders within the order Euplotida3. In the present study, the monophyly of Euplotida is recovered in trees inferred both from LSU rDNA gene sequence data alone (Fig. 2B) and from concatenated data of four genes (Fig. 1A), although the support values are not high. In the SSU rDNA tree the discocephalids nest within the Euplotida assemblage. The discocephalids are found only in marine biotopes and are characterized by their cephalized body shape. The group most closely related to Discocephalida is generally thought to be Pseudoamphisiellina, this conclusion being based both on morphological characters (e.g., cephalized body shape, highly developed fiber system connecting the cirri, generally two clearly separated ventral rows, and highly developed transverse cirri) and ontogenetic characters (e.g., the unique formation of the ventral rows during morphogenesis)45–48. This finding is supported by the present study, the sister relationship between these two groups being consistently recovered in all trees.
New Understanding of the Oligotrichs s.l. Traditionally, the loricate (tintinnids) and non-loricate (oligotrichids) oligotrichs were considered to be sister groups. Only in last three decades have they been assigned to separate subclasses, the Choreotrichia and Oligotrichia, respectively. Choreotrichians are characterized by having a closed AZM and the group includes both tintinnids and some non-loricate forms, e.g. the strobilids and related taxa. By contrast, oligotrichians have an open AZM. The separation of these two subclasses is supported by the present study.

The family Linnellidae was established by Liu et al. for the genus Linnella, which shares some morphological features with both oligotrichians and choreotrichians. In a previous study based on analyses of all available data, i.e. morphological and molecular characters, Li et al. concluded that members of the Linnellidae are intermediate forms between the Choreotrichia and Oligotrichia. Recently, Liu et al. established the order, Linnellidae for the Linnellidae. The present study broadly supports these findings with Linnella either sister to the oligotrichians (Figs 2A) or sister to choreotrichians (Figs 1A and 2B).

The order Halteriida, represented by the genus Halteria, is a unique group that has long puzzled taxonomists. Based on their morphology and pattern of morphogenesis, halteriids share similarities with the oligotrichs sensu lato but differ from the hypotrichs. However, the results of gene sequence data suggest that Halteriida might be a member of the hypotrichs/stichotrichs, possibly belonging to the oxystrichids, a highly specialized group of hypotrichs with very conservative modes of stomatogenesis. Given this conflicting evidence, we believe that the Halteriida should be regarded as incertae sedis within Spirotrichea.

Phylogeny of Scuticociliates and Closely Related Taxa. Due to their small size and similar morphologies and ciliary patterns, scuticociliates are one of the most ambiguous groups of ciliates. According to Lynn, the subclass Scuticociliatia contains three orders: Philasterida, Pleuronematida, and Thigmotrichida. Although Philasterida is a well-outlined lineage, analyses of gene sequences data have challenged the monophyly of both Pleuronematida and Thigmotrichida. Indeed as more data have accumulated, the thigmotrichids are well-defined group comprising two orders: Sessilida and Mobilida. Recent molecular analyses, however, have challenged this arrangement suggesting that the molecular and morphological information are not always consistent with previous studies.

Phylogeny of Peniculia and Hymenostomatia. The peniculians are characterized by their three oral polynucleated oral apparatus. The Astomatia and Apostomatia are two specialized subclasses within the class Oligohymenophorea. Astomes are endosymbionts typically found in the digestive tract of annelids, especially oligochaetes, and entirely lack an oral apparatus. By contrast, apostomes are usually found as epibionts of marine and brackish water crustaceans, and have highly modified oral structures and polymorphic life cycles. Analyses based on molecular data consistently reveal both groups to be closely related to the scuticociliates. Present analyses show that either astomes or apostomes are more closely related to the loxocephalids than to the core scuticociliates, which is consistent with previous studies. A reasonable hypothesis could be that they may be derived from loxocephalid-like ancestral lineages, their highly specialized morphologies being a result of adaptation to their symbiotic life styles.

The Monophyly of the Traditional Peritrichs. Historically, peritrich ciliates were considered to be a well-defined group comprising two orders: Sessilida and Mobilida. Recent molecular analyses, however, have challenged this arrangement suggesting that the molecular and morphological information are not always consistent with previous studies.
Phylogenetic Relationship within Litostomatea. The class Litostomatea has been traditionally rather poorly defined as having an apically positioned cytostome, uniform somatic ciliation and a non-distinct oral apparatus.\(^5,33,92\) Lynn\(^2\) recognized two subclasses, Haptoria and Trichostomatia. A third subclass, Rhynchozostomatia, was recently established by Vd’Ačny\ et al.\(^3\). Recent molecular phylogenetic analyses, however, do not provide unambiguous support for the monophyly of Haptoria, with several of its members grouping with Trichostomatia, and the haptorid genera Helicoprorodon and/or Trachelotractus occupying a basal position within the class Litostomatea.\(^4\) Our analyses with additional sequences also failed to recover the subclass Haptoria as a monophyletic group. In addition, for the first time, we reveal the close phylogenetic relationship between Helicoprorodon and Trachelotractus, and confirm their basal position within the Litostomatea. Our findings support a previous suggestion based on morphological features that the helicoprorodonids represent an independent group, possibly at the rank of order within the subclass Haptoria or even as a subclass within the class Litostomatea.\(^5,96\). Helicoprorodon and Trachelotractus differ from other haptorid genera in having a peribuccal ridge with extrusomes, and specialized ciliary rows curving around the pharyngeal opening.\(^98\).

Further Insights into Phyllopharyngea and Nassophorea. Members of classes Phyllopharyngean and Nassophorean have a basket-like, ventrally opening oral apparatus or cytostome as result of which it has long been assumed that these two groups are closely related.\(^5,33,92\) The class Nassophorea sensu Lynn, 2008 comprises three orders, Synhymeniida, Nassulida and Microthoracida. However, SSU rDNA-based phylogenies have shown that the order Synhymeniida clusters strongly with the class Phyllopharyngea rather than with the other two orders of Nassophorea. Based on these findings, Gong \et al.\(^100\) revised the higher classification of these groups, regarding the synhymeniids as a subclass of the class Phyllopharyngea. In the present study, trees based on multi-gene data (Fig. 1A) also recover the synhymeniids as a distinct lineage within the Phyllopharyngea.

The group-name “Subkinetalia” was coined for phyllopharyngean super-clade comprising the subclasses Cyrtophoria, Chonotrichia, Rhynchodia and Suctoria, the synapomorphy of which is the possession of subkinetal microtubules.\(^100\) The multi-gene based phylogeny in the present study is consistent with this finding and reveals a highly supported monophyletic Phyllopharyngea comprising two subgroups, the Subkinetalia (cyrtophorians, rhynchodians, chonotrichians, suctorians) and Synhymenia. Since the Subkinetalia represents a taxon sharing the same synapomorphic character, a new class name Phyllopharyngea is proposed.

Discotrichids are traditionally classified as a family within the nassophorean order Microthoracida.\(^5\) However, Fan \et al.\(^103\) established the order Discotrichida since discotrichids do not group with other microthoracids and are even distinct from all other nassophorean lineages. The present study also recovers the discotrichids (represented by Discotricha) as a distinct lineage that occupies a basal position within CONthreeP. However, evolutionary relationships between the discotrichids and other nassophoreans (microthoracids and nassulids) remain uncertain, probably due to undersampling within these groups.

The genus Paranassula in the nassophorean order Nassulida possesses distinct morphological characters that separate it from other nassulids, e.g. two polykinetids that are restricted to a shallow oral cavity and the presence of a paroral kinety.\(^72\) Furthermore, phylogenetic analyses based on SSU rDNA and LSU rDNA sequence data suggest that Paranassula is related to Peniculida (class Oligohymenophorea)\(^102\). Our analyses with newly added gene sequence data support the most recent assignment for this taxon, i.e. Paranassula should be assigned to subclass Peniculida, class Oligohymenophorea. However, in contrast to the conclusion of Zhang \et al.\(^102\) which suggested a resurrection of order Paranassulida, Deroux \et de Puytorac\ et al.\ (1993), we propose that Paranassula should be assigned to the order Peniculida, as a member of the family Paranassulidae Fauré-Fremiet, 1962. The phylogenetic positions of other genera in the family Paranassulidae, e.g. Enneameron, Gullmarella, remain uncertain due to undersampling.

Systematically Ambiguous Taxa Find Their Close Relatives in the Class Plagiopylea. The systematic positions of the litostomatean genera Askenasia, Cycлотrichium, Parapathidium and the prostone genus Plagiocampa have long been ambiguous. The former three have been variously assigned to the Cyclotrichida and Haptorida, or even as incertae sedis in the phylum Ciliophora.\(^9,33,95\) Jankowski\(^104\) established the family Cyclotrichidae and the order Cyclotrichida for Cycлотrichium which he assigned to the class Litostomatea, but failed to define these taxa. In the absence of any molecular data, Lynn\(^5,33\) regarded Cyclotrichidae as a junior synonym of Didiniidae within the order Haptorida while retaining the order Cyclotrichida to include Mesodiniidae, e.g. Askenasia, Mesodinium and Myronecta. Recent studies based on rDNA and the alpha-tubulin protein gene sequence data suggested that the two cyclotrichid genera Askenasia, Cycлотrichium, and the
prostome genus *Paraspathidium*, should be removed from the class Litostomatea but failed to give their exact positions within the classes Plagiopylea and Prostomatea, respectively. Lynn assigned the prostome genus *Plagiocampa* to the order Prorodontida. However, recent phylogenetic analyses recognized a distinct clade comprising Plagiocampidae and two closely related genera *Urotricha* and *Cryptocaryon*, which have a closer relationship to *Cyclotrichium* and *Paraspathidium* than to the prostomes.

In the present study, based on multi-gene analyses, the concatenated gene trees robustly show that these four genera with uncertain taxonomic affiliations, i.e., *Askenasia*, *Cyclotrichium*, *Paraspathidium* and *Plagiocampa*, are most closely related to the plagiopyleans. Because of the highly specialized infraciliature and anaerobic life style of the plagiopyleans, their morphological synapomorphies are not well understood. It is possible, for example, that a combination of the following features could define this clade (class Plagiopylea + *Askenasia + Paraspathidium-Cyclotrichium*) + various prostomatean morphospecies represented by *Plagiocampa*: somatic monokinetids and an oralized somatic ciliature around a dominant cytostome consisting of densely ciliated dikinetids. In addition, a brosse structure composed of dikinetids on the border of the oral slit has been commonly observed in *Paraspathidium*, *Urotricha* and *Plagiocampa*. Based on the information above, we tentatively place *Askenasia Paraspathidium*, *Cyclotrichium* and various prostomatean morphospecies represented by *Plagiocampa* in the class Plagiopylea, pending further evidence including increased taxon sampling, ultrastructural studies and phylogenomic analyses.

**A Highly Supported Metaclade of Oligohymenophorea, Prostomatea and Plagiopylea within the CONthreeP.** Several studies based on SSU rDNA sequence data have revealed a close evolutionary relationship among the classes Oligohymenophorea, Prostomatea and Plagiopylea. Following the inclusion of sequences of additional genes and increased taxon sampling, the metaclade containing Oligohymenophorea, Prostomatea and Plagiopylea was recovered with high support. A close relationship between prostomes and oligohymenophorans has been suggested based on similarities of their patterns of morphogenesis revealed by electron microscopy. However, the phenotypic features that unite the riboclass Plagiopylea with Oligohymenophorea and Prostomatea remain unknown.

**Material and Methods**

**DNA Extraction, Gene Sequencing, Dataset Assembly and Alignments.** Gene sequence data were obtained for a total of 104 species representing almost all the main ciliate lineages. Genomic DNA extraction, PCR amplifications and sequencing were performed as described in previous studies for the following genes: completed sequence (~1800 bp) of the SSU rDNA; a partial sequence (~500 bp) of the ITS1-5.8S-ITS2; a partial sequence (~1800 bp) of the LSU rDNA; and, a partial sequence (~1000 bp) of the alpha-tubulin gene.

In total, 232 sequences were submitted to the GenBank database (Additional file 1: Table S1). In order to maximize the taxonomic diversity of ciliates included in our analyses, newly characterized sequences were combined with relevant sequences obtained from GenBank (Additional file 1: Table S2). Six datasets were evaluated: (1) concatenation of the aligned SSU rDNA, 5.8S rDNA, LSU rDNA and alpha-tubulin amino acid sequences from datasets 2–5; (2) SSU rDNA sequences including all 152 group representatives; (3) 5.8S rDNA sequences of 113 taxa; (4) LSU rDNA sequences of 118 taxa; (5) alpha-tubulin amino acid sequences of 116 taxa; (6) alpha-tubulin nucleotide sequences, including the first two codon positions, of 116 taxa. Orthologs of alpha-tubulin for concatenations were selected according to Gao and Katz. Sequences were aligned using the GUIDANCE algorithm with default parameters in GUIDANCE web server. Regions that could not be unambiguously aligned were excluded from the phylogenetic analyses.

| Topology constraints | AU test |
|----------------------|---------|
| Climacostomidae      | 0.011   |
| Urostyliida + Pseudoamphisiellidae | 0.007 |
| Oligotrichia + *Lynnella* | 0.682 |
| Choreotrichia + *Lynnella* | 0.397 |
| Scuticociliatia      | 0.065   |
| Philasterida + loxocephalids | 0.028 |
| Loxocephalida        | 0.021   |
| Pleuronemastisita    | 7.00E-06 |
| Penicula             | 0.218   |
| Peritrichia          | 0.657   |
| Colpodea             | 0.365   |
| Nassophorea          | 0.069   |
| Cyclotrichida        | 0.088   |
| Plagiopylea          | 0.695   |
| Prostomatea          | 0.253   |

**Table 1. Approximately Unbiased test (AU) results based on SSU rDNA data.** Notes: The topology constraints column refers to proposed taxonomic groups that were tested for monophyly through the approximately unbiased test (AU). Rejected monophyly ($p < 0.05$) is highlighted in gray.

...
Phylogenetic Analyses. Three apicomplexans and three dinoflagellates were used as outgroups (Additional file 1: Table S2)44. Maximum likelihood (ML) analyses were carried out using RAxML-HPC2 v7.6.6114 on CIPRES Science Gateway115. The DNA partition was analyzed with GTR + gamma. ProtTest 2.4.116 selected the MtArt + I + G + F amino acid replacement matrix as the best-fitting model for alpha-tubulin amino acid sequences. The alpha-tubulin amino acid partition was run under the MtArt + gamma model as this was the best-fitting model available in RAxML. Support for the best-scoring ML tree came from 1000 bootstrap replicates. Bayesian inference (BI) analysis was performed with MrBayes 3.2.2117 on CIPRES Science Gateway using the GTR + I + G model for the DNA partition as selected by MrModeltest v.2.218 and using mixed model for the alpha-tubulin amino acid partition. Markov chain Monte Carlo (MCMC) simulations were run with two sets of four chains for 4,000,000 generations with a sample frequency of 100 generations. The first 10,000 trees were discarded as burn-in. All remaining trees were used to calculate posterior probabilities using a majority rule consensus. Systematic schemes are mainly based on Lynn and Adl et al., except for some revisions made in the present study.

The approximately unbiased (AU) test119 was used to test the monophyly of the focal group against competing phylogenetic hypotheses (Table 1). Constrained ML trees enforcing the monophyly of the respective focal groups were generated based on SSU rDNA data. For all constraints, internal relationships within the constrained groups and among the remaining taxa were unspecified. The site-wise likelihoods for the resulting constrained topologies were generated based on SSU rDNA data. For all constraints, internal relationships within the constrained groups and among the remaining taxa were unspecified. The site-wise likelihoods for the resulting constrained topologies and the non-constrained ML topology were calculated using PAUP120 and were then analyzed in CONSEL121 with standard parameters to obtain p-values.

References
1. Kathol, M., Norf, H., Arndt, H. & Weitere, M. Effects of temperature increase on the grazing of planktonic bacteria by biofilm-dwelling consumers. Aquat. Microb. Ecol. 55, 65–79 (2009).
2. Xu, H., Zhang, W. & Jiang, Y. Do early colonization patterns of periphytic ciliate fauna reveal environmental quality status in coastal waters? Environ. Sci. Pollut. Res. Int. 21, 7097–7112 (2014).
3. Hausmann, K. & Bradbury, P. Ciliates: cells as organisms. (Gustav Fischer Verlag, 1996).
4. Corliss, J. O. The ciliated protozoa: characterization, classification and guide to the literature. (Pergamon Press, 1979).
5. Lynn, D. H. The ciliated protozoa: characterization, classification and guide to the literature. 3rd edition edn. (Springer Verlag, 2008).
6. Huang, J., Chen, Z., Song, W. & Berger, H. Three-gene based phylogeny of the Urostyloidea (Protiasta, Ciliophora, Hypotricha), with notes on classification of some core taxa. Mol. Phylogen. Evol. 70, 337–347 (2014).
7. Yi, Z., Dunthorn, M., Song, W. & Stoeck, T. Increasing taxon sampling using both unidentified environmental sequences and identified cultures improves phylogenetic inference in the Prorodontida (Ciliophora, Protostomatea). Mol. Phylogen. Evol. 57, 937–941 (2010).
8. Foissner, W., Kussuoka, Y. & Shimano, S. Morphology and gene sequence of Leviceolposes bisvae n. gen., n. sp. (Ciliophora, Protostomatea), a proposed endemic from the ancient Lake Biwa, Japan. J. Eukaryot. Microbiol. 55, 185–200 (2008).
9. Chen, X. et al. Phylogenomics of non-model ciliates based on transcriptomic analyses. Protein Cell 6, 373–385 (2015).
10. Feng, J. M. et al. Phylogenomic analyses reveal subclass Scuticociliatia as the sister group of subclass Hymenostomatia within class Oligohymenophorea. Mol. Phylogen. Evol. 90, 114–115 (2015).
11. Gentekaki, E. et al. Large-scale phylogenomic analysis reveals the phylogenetic position of the problematic taxon Protocruzia and unravels the deep phylogenetic affinities of the ciliate lineages. Mol. Phylogen. Evol. 78, 36–42 (2014).
12. Parfrey, L. W. et al. Evaluating support for the current classification of eukaryotic diversity. PloS Genet. 2, e220 (2006).
13. Dunthorn, M., Hall, M., Foissner, W., Stoeck, T. & Katz, L. A. Broad taxon sampling of ciliates using mitochondrial small subunit ribosomal DNA. Acta Protozool. 53, 207–213 (2014).
14. Dunthorn, M. et al. Placing environmental next-generation sequencing amplicons from microbial eukaryotes into a phylogenetic context. Mol. Biol. Evol. 31, 993–1009 (2014).
15. Gao, F. & Katz, L. A. Phylogenomic analyses support the bifurcation of ciliates into two major clades that differ in properties of nuclear division. Mol. Phylogen. Evol. 70, 240–243 (2014).
16. Václavc, P., Orsi, W. & Foissner, W. Molecular and morphological evidence for a sister group relationship of the classes Armophorea and Litotostomea (Ciliophora, Intramembranaucleata, Lamellilicorticata infraphyl. nov.), with an account on basal litotostomeans. Eur. J. Protistol. 46, 298–309 (2010).
17. Chen, X., Ma, H., Al-Rasheid, K. A. S. & Miao, M. Molecular data suggests the ciliate Mesodinium (Protiasta: Ciliophora) might represent an undescribed taxon at class level. Zool. Syst. 40, 31–40 (2015).
18. Jankowski, A. W. Conspectus of a new system of the phylum Ciliophora. Trudy Zool. Inst., Leningr. 94, 103–121 (1980).
19. Puytorac, P. D. In Traité de zoologie, infusoires ciliés (ed de Puytorac, P.) 621–679 (Masson, 1994).
20. Johnson, M. D., Tengs, T., Oldach, D. W., Delwiche, C. F. & Stoecker, D. K. Highly divergent SSU rRNA genes found in the marine ciliates Myrionecta rubra and Mesodinium pulex. Protist 155, 347–359 (2004).
21. Sröder-Kypke, M. C., Wright, A. D., Foissner, W., Chatzinotas, A. & Lynn, D. H. Molecular phylogeny of litostome ciliates (Ciliophora, Litotostomea) with emphasis on free-living haptorian genera. Protist 157, 261–278 (2006).
22. Affaã, F. M., Hickey, D. A., Struder-Kypke, M. & Lynn, D. H. Phylogenetic position of species in the genera Anoplophrya, Plagiostoma, and Nyctotheroides (Phylum Ciliophora), endosymbiotic ciliates of anemids and anurans. J. Eukaryot. Microbiol. 51, 301–306 (2004).
23. Shin, M. K. et al. Phylogenetic position of the ciliates Phacodinium (Order Phacodiniida) and Protocruzia (Subclass Protocruzida) and systematics of the spirotrich ciliates examined by small subunit ribosomal RNA gene sequences. Eur. J. Protistol. 36, 293–302 (2000).
24. Puytorac, P., Grain, J. & Mignot, J. P. Précis de protistologie. Boubèe èd. (1987).
25. Grolière, C. A., de Puytorac, P. & Grain, J. Observations de quelques espèces de ciliés endocommensaux d’échinides du Golfe du Mexique et de la Mer des Antilles. Protistologica 16, 233–239 (1980).
26. Song, W. & Wilbert, N. Morphological investigation on some free living ciliates (Protocozoa, Ciliophora) from China sea with description of a new hypotrichous genus, Hemigastrostyla nov. gen. Arch. Protistenkd 148, 413–444 (1997).
27. Foissner, W. In Ciliates cell as organisms. (eds Hausmann, K. & Bradbury, P. C.) (Gustav Fischer, 1996).
29. Hammerschmidt, B. et al. Insights into the evolution of nuclear dualism in the ciliates revealed by phylogenetic analysis of rRNA sequences. J. Eukaryot. Microbiol. 43, 225–230 (1996).
30. Lynn, D. H. My journey in ciliate systematics. J. Eukaryot. Microbiol. 43, 253–260 (1996).
31. Li, L. et al. Protocruzia, a highly ambiguous ciliate (Protozoa; Ciliophora): very likely an ancestral form for Heterotricha, Colpodaea or Spirotricha? With re-evaluation of its evolutionary position based on multigene analyses. Sci. China Life Sci. 53, 131–138 (2010).
32. da Silva Neto, I. D. Structural and ultrastructural observations of the ciliate Placidochilus metchnikoffii Cercel, 1891 (Heterotricha, Phacodinium). Eur. J. Protistol. 29, 209–218 (1993).
33. Lynn, D. H. & Small, E. P. In An illustrated guide to the protozoa (eds Lee, J. J., Leedale, G. F. & Bradbury, P.) (Allen Press Inc., 2002).
34. Miao, M. et al. Caryotricha minuta (Xu et al., 2008) nov. comb., a unique marine ciliate (Protista, Ciliophora, Spirotricha), with phylogenetic analysis of the ambiguous genus Caryotricha inferred from the small-subunit rRNA gene sequence. Int. J. Syst. Evol. Microbiol. 59, 430–438 (2009).
35. Schmidt, S. L., Bernhard, D., Schlegel, M. & Foissner, W. Phylogeny of the Stichotrichia (Ciliophora; Spirotricha) reconstructed with nuclear small subunit rRNA gene sequences: discrepancies and accordances with morphological data. J. Eukaryot. Microbiol. 54, 201–209 (2007).
36. Yi, Z. & Song, W. Evolution of the order Urostylida (Protozoa, Ciliophora): new hypotheses based on multi-ge information and identification of localized incongruence. PloS one 6, e17471 (2011).
37. Hewitt, E. A. et al. Phylogenetic relationships among 28 spirotrichaceous ciliates documented by rDNA. Mol. Phylogenet. Evol. 29, 258–267 (2003).
38. Foissner, W. et al. Reconciling classical and molecular phylogenies in the stichotrichines (Ciliophora, Spirotricha), including new sequences from some rare species. Eur. J. Protistol. 4, 265–281 (2004).
39. Berger, H. Monograph of the Urostylidea (Ciliophora, Hypotricha). Monographiae Biol. 85, 1–1304 (2006).
40. Song, W. et al. Progress in Protozoology. (Qingdao Ocean University Press, 1999).
41. Li, L. et al. Does Kitrichia (Prostoma, Ciliophora, Spirotricha) belong to Euplotida or represent a primordial spirotrichous taxon? With suggestion to establish a new subclass Protolyphiotrichia. Int. J. Syst. Evol. Microbiol. 59, 439–446 (2009).
42. Jiang, J. et al. Two new marine ciliates, Caryotricha rarietae n. sp. and Discocellulus paratloratus n. sp. (Ciliophora, Spirotricha), with phylogenetic analyses inferred from the small subunit rRNA gene sequences. J. Eukaryot. Microbiol. 60, 388–398 (2013).
43. Shao, C., Song, W., Warren, A. & Al-Rasheid, K. A. Morphogenesis of the ciliate Loxocephalum exornatum Gao, 2004 (Ciliophora, Spirotricha), a possible model for the ancestor of hypotrichs s. l. J. Eukaryot. Microbiol. 45, 292–304 (2009).
44. Adl, S. M. et al. The revised classification of eukaryotes. J. Eukaryot. Microbiol. 59, 429–493 (2012).
45. Miao, M., Shao, C., Chen, X. & Song, W. Evolution of discocephalid ciliates: molecular, morphological and ontogenetic data support a sister group of discocephalids and pseudoamphistellids (Protozoa, Ciliophora) with establishment of a new suborder Pseudoamphistellina subord. n. Sci. China Life Sci. 54, 634–641 (2011).
46. Shao, C. et al. Systematic position of Discocellulus-like ciliates (Ciliophora: Spirotricha) inferred from SSU rDNA and ontogenetic information. Int. J. Syst. Evol. Microbiol. 58, 2962–2972 (2008).
47. Wicklow, B. J. The Discocelida (n. subord.): ultrastructure, morphogenesis and evolutionary implications of a group of endemic marine interstitial hypotrichs (Ciliophora, Ciliophora, Prototistologia) 18, 299–330 (1982).
48. Shao, C. et al. Morphogenesis of the marine ciliate Pseudoamphistella alveolata (Kahl, 1932) Song & Warren, 2000 (Ciliophora, Stichotrichia, Urostylida) during binary fission. J. Eukaryot. Microbiol. 53, 388–396 (2006).
49. Yi, Z. et al. A molecular phylogenetic investigation of Pseudoamphistella and Parabirojinia (Protozoa, Ciliophora, Spirotricha), two genera with ambiguous systematic positions. Eur. J. Protistol. 44, 53–58 (2008).
50. Agatha, S. Global diversity of alorate Oligotrichia (Protista, Ciliophora, Spirotricha) in marine and brackish water. PloS one 6, (2011).
51. Gao, S., Gong, J., Lynn, D., Lin, X. F. & Song, W. B. An updated phylogeny of oligotrich and choreotrich ciliates (Protozoa, Ciliophora, Spirotricha) with representative taxa collected from Chinese coastal waters. Syst. Biodivers. 7, 235–242 (2009).
52. Snoeyenbos-West, O. L., Salcedo, T., McManus, G. B. & Katz, L. A. Insights into the diversity of choreotrich and oligotrich ciliates (Class: Spirotrichea) based on genealogical analyses of multiple loci. Int. J. Syst. Evol. Microbiol. 52, 1901–1903 (2002).
53. Liu, W., Yi, Z., Lin, X. & Al-Rasheid, K. A. Morphologic and molecular data suggest that Lynnellia semiglobulosa n. g., n. sp. represents a new family within the subclass Choreotrichia (Ciliophora, Ciliophora, Spirotricha). J. Eukaryot. Microbiol. 58, 43–49 (2011).
54. Li, J., Liu, W., Gao, S., Warren, A. & Song, W. Multigene-based analyses of the phylogenetic evolution of oligotrich ciliates, with consideration of the internal transcribed spacer 2 secondary structure of three systematically ambiguous genera. Eukaryot. Cell 12, 430–437 (2013).
55. Liu, W. W. et al. Two new genera of planktonic ciliates and insights into the evolution of the family Strombidiidae (Protista, Ciliophora, Oligohymenophorea). Sci. China Life Sci. 56, 296–307 (2013).
56. Agatha, S. & Foissner, W. Phylogeny of the Nitidocladia endosymbionts of echinoids (phylum Echinodermata): phylogenetic relationships among species in the genera Entodiscus, Plagiopyliella, Thyphrylas, and Entorhidiopsis (phylum Ciliophora). J. Eukaryot. Microbiol. 59, 429–438 (2012).
57. Monographiae Biol. (eds Lee, J. J., Leedale, G. F. & Bradbury, P.) (Allen Press Inc., 2002).
58. Lynn, D. H. & Strüder-Kypke, M. Scuticociliate endosymbionts of echinoids (phylum Echinodermata): phylogenetic relationships inferred from sequences of the small-subunit rRNA gene. Int. J. Syst. Evol. Microbiol. 60, 460–468 (2010).
59. Gao, F., Gao, S., Wang, P., Katz, L. A. & Song, W. Phylogenetic analyses of cyclidiids (Protista, Ciliophora, Scuticociliatia) based on multiple genes suggest their close relationship with flagellomicrotichs. Mol. Phylogenet. Evol. 75, 219–226 (2014).
60. Li, L. et al. Phylogenetic position of the marine ciliate, Cardiostomatella vermiforme (Kahl, 1938) Corliss, 1960 inferred from the complete SSrRNA gene sequence, with establishment of a new order Loxocephalida n. ord. (Ciliophora, Oligohymenophorea). Eur. J. Protistol. 42, 107–114 (2006).
67. Fokam, Z., Ngassam, P., Strüder-Kypke, M. & Lynn, D. Genetic diversity and phylogenetic position of the subclass Astomatia (Ciliophora) based on a sampling of six genera from West African oligochaetes (Glossoscolecidae, Megascolecidae), including a description of the new genus Panachauliicola n. gen. Eur. J. Protistol. 47, 161–171 (2011).

68. Grain, J. & Puytorac, P.D. Particularités ultrastructurales des cœlomés et de leurs annexes fibrilaires chez certains ciliés astomes Hoplophryidés. J. Microsc. 19, 231–246 (1974).

69. Bradbury, P. C., Song, W. & Zhang, L. Stomatogenesis during the formation of the tomites of Hyalophyza chattoni (Haptorida; Vorticellida, Ciliophora). Eur. J. Protistol. 33, 409–419 (1997).

70. Zhang, Q., Fan, X., Clamp, J. C., Al-Rasheid, K. A. S. & Song, W. Description of Paratetrahyrmena parassisi n. sp. using morphological and molecular evidence and a phylogenetic analysis of Paratetrahyrmena and other taxonomically ambiguous genera in the order Loxocephalida (Ciliophora, Oligohymenophorea). J. Eukaryot. Microbiol. 57, 483–493 (2010).

71. Fan, X., Chen, X., Song, W., Al-Rasheid, K. A. S. & Warren, A. Two new marine Frontonia species, *F. mengi* spec. nov. and *F. magna* spec. nov. (Protozoa; Ciliophora), with notes on their phylogeny based on SSU rRNA gene sequence data. Int. J. Syst. Evol. Microbiol. 61, 1476–1486 (2011).

72. Sun, P. Two new marine species of the genus *Paratetrahyrmena* (Ciliophora: Oligohymenophorea) inferred from small subunit rRNA gene sequences. J. Eukaryot. Microbiol. 47, 419–429 (2000).

73. Yi, Z. et al. Phylogeny of six oligohymenophorean ciliates (Protozoa; Ciliophora) inferred from small subunit rRNA gene sequences. Zool. Scr. 38, 323–331 (2009).

74. Strüder-Kypke, M. C., Wright, A. D., Fokin, S. I. & Lynn, D. H. Phylogenetic relationships of the Subclass Peniculida (Ciliophora, Oligohymenophorea). Eur. J. Protistol. 46, 221–242 (2010).

75. Strüder-Kypke, M. C. & Lynn, D. H. Comparative analysis of the mitochondrial cytochrome c oxidase subunit I (COI) gene in ciliates (Alveolata, Ciliophora) and evaluation of its suitability as a biodiversity marker. Syst. Biodivers. 8, 131–148 (2010).

76. Miao, W., Tu, X. & Shen, Y. Phylogenetic relationships of the subclass Peritrichia (Ciliophora, Oligohymenophorea) with emphasis on the genus *Epistylis*, inferred from small subunit rRNA gene sequences. J. Eukaryot. Microbiol. 48, 583–587 (2001).

77. Miao, W., Yu, Y., Shen, Y. & Zhang, X. Intraspecific phylogeography of *Carchesium polyptum* (Peritrichia, Ciliophora) from China, inferred from 18S-ITS1:5.8S ribosomal DNA. Sci. China 47, 11–17 (2004).

78. Sun, P., Fan, J., Xu, D., Kusuoka, Y. & Miao, W. Reevaluation of the phylogenetic relationship between mobilids and sessilids (Ciliophora, Oligohymenophorea) using molecular characters derived from the gene coding for small subunit rRNA. Protist 163, 129–142 (2012).

79. Utz, L. R. & Eizirik, E. Molecular phylogenetics of subclass Peritrichia (Ciliophora: Oligohymenophorea) based on expanded analyses of 185 rRNA sequences. J. Eukaryot. Microbiol. 54, 303–307 (2007).

80. Williams, D. & Clamp, J. C. A molecular phylogenetic investigation of *Opisthionema* and related genera (Ciliophora, Peritrichia, Sessilida). J. Eukaryot. Microbiol. 54, 317–323 (2007).

81. Sun, P., Clamp, J. C. & Xu, D. P. Analysis of the secondary structure of ITS transcripts in peritrich ciliates (Ciliophora, Oligohymenophorea): Implications for structural evolution and phylogenetic reconstruction. Mol. Phylogenet. Evol. 56, 242–251 (2010).

82. Sun, P., Clamp, J. C., Xu, D. P., Kusuoka, Y. & Horii, M. Molecular phylogeny of the family Vorticellidae (Ciliophora, Peritrichia) using combined datasets with a special emphasis on the three morphologically similar genera *Carchesium*, *Epicarchesium* and *Apocarchesium*. Int. J. Syst. Evol. Microbiol. 61, 1001–1010 (2011).

83. Sun, P. et al. An ITS-based phylogenetic framework for the genus *Vorticella*: finding the molecular and morphological gaps in a taxonomically difficult group. Proc. R. Soc. B 280(1771), 20131177 (2013).

84. Gong, Y. et al. Alpha-tubulin and small subunit rRNA phylogenies of peritrichs are congruent and do not support the clustering of mobilids and sessilids (Ciliophora, Oligohymenophorea). J. Eukaryot. Microbiol. 57, 265–272 (2010).

85. Zhan, Z., Xu, K. & Dunthorn, M. Evaluating molecular support for and against the monophyly of the Peritrichia and phylogenetic relationships within the Mobiliida (Ciliophora, Oligohymenophorea). Zool. Scr. 42, 213–226 (2013).

86. Gong, Y., Yu, Y., Villalobo, E., Zhu, F. & Miao, W. Reevaluation of the phylogenetic relationship between mobilid and sessilid peritrichs (Ciliophora, Oligohymenophorea) based on small subunit rRNA genes sequences. J. Eukaryot. Microbiol. 53, 397–403 (2006).

87. Zhan, Z., Xu, K., Warren, A. & Gong, Y. Reconsideration of phylogenetic relationships of the subclass Peritrichia (Ciliophora, Oligohymenophorea) based on small subunit ribosomal RNA gene sequences, with the establishment of a new subclass Mobilia Kahl, 1933. J. Eukaryot. Microbiol. 56, 552–558 (2009).

88. Wang, H., Clamp, J. C., Shi, X., Utz, L. R. & Liu, G. Evolution of variations in the common pattern of stomatogenesis in peritrich ciliates: evidence from a comparative study including a new description of stomatogenesis in *Pseudepistylis songi* Peng et al., 2007. J. Eukaryot. Microbiol. 59, 300–324 (2012).

89. Xu, K. & Song, W. In *Pathogenic Protozoa in Mariculture* (eds Song, W. et al.) 291–314 (Science Press, 2003).

90. Carey, P. G. Marine interstitial ciliates. An illustrated key. (Chapman and Hall, 1992).

91. Vďačný, P., Bourland, W. A., Orsi, W., Epstein, S. S. & Foisner, W. Phylogeny and classification of the Litostomata (Prostist, Ciliophora), with emphasis on free-living taxa and the 18S rRNA gene. Mol. Phylogenet. Evol. 59, 510–522 (2011).

92. Zhang, Q., Simpson, A. & Song, W. Insights into the phylogeny of systematically controversial haptorian ciliates (Ciliophora, Haptoridea) based on multigene analyses. Proc. R. Soc. B 279, 2625–2635 (2012).

93. Foisner, W. & Foisner, I. The fine structure of *Fuscheria terricola* Berger et al., 1983 and a proposed new classification of the subclass Haptoria Corliss, 1974 (Ciliophora, Litostomata). Arch. Protistenkd. 135, 213–235 (1988).

94. Gréau, J. In *Infrutesores Ciliés - Systématique. Traité de Zoologie. II Fasc. 2.* (ed de Puytorac, P.) 267–310 (Masson, 1994).

95. Kahl, A. *Urtiere oder Protozoa I: Urtiere oder Protozoa I* (Infusoria). 2. *Holotrichia. Tierverf. Bd 21*, 181–398 (1931).

96. Gong, J. et al. Small subunit rRNA phylogenies show that the class Nassophorea is not monophyletic (Phylum Ciliophora). J. Eukaryot. Microbiol. 56, 339–347 (2009).

97. Kivimaki, K. L., Bowditch, B. M., Riordan, G. P. & Lipscomb, D. L. Phylogeny and systematic position of *Zosterolasys* (Ciliophora, Synhymenida): a combined analysis of ciliate relationships using morphological and molecular data. J. Eukaryot. Microbiol. 56, 325–338 (2009).

98. Zhang, Q. et al. Further insights into the phylogeny of two ciliate classes Nassophorea and Prostomata (Prostista, Ciliophora). Mol. Phylogenet. Evol. 70, 162–170 (2014).

99. Fan, X. et al. Phylogeny of the poorly known ciliates, microthoracida, a systematically confused taxon (ciliophora), with morphological reports of three species. J. Eukaryot. Microbiol. 61, 227–237 (2014).
104. Jankowski, A. W. In Protista: Handbook on Zoology, Part 2. (eds Krylow, M. V. & Frolov, A. O.) 415–976 (St. Petersburg, 2007).
105. Diggles, B. K. Some information on the morphology of Cryptocaryon irritans from south-east Queensland, Australia. *Eur. J. Protistol.* **33**, 200–210 (1997).
106. Foissner, W. Infraciliature and systematic position of the marine interstitial ciliates (Protozoa, Ciliophora) *Lopezozentrenia torpens* (Kahl, 1931) nov. gen., nov. comb., *Discotiaria pupillifera* Tuffrau, 1954, and *Paraspasilidium fuscum* (Kahl, 1928) Field, 1955. *Rev. Soc. Mex. Hist. Nat.* **47**, 41–63 (1997).
107. Foissner, W. A compilation of soil and moss ciliates (Protozoa, Ciliophora) from Germany, with new records and descriptions of new and insufficiently known species. *Eur. J. Protistol.* **36**, 253–283 (2000).
108. Lipscomb, D. L. & Riordan, G. The ultrastructure of *Placus striatus* and a revision of the family Placidae (Ciliophora). *J. Eukaryot. Microbiol.* **59**, 407–422 (2012).
109. Fleury, A., Delgado, P., Ilorde, E. & Adoutte, A. A molecular phylogeny of ciliates: What does it tell us about the evolutionary of the cytoskeleton and of developmental strategies? *Dev. Genet.* **15**, 247–254 (1992).
110. Baroin-Tourancheau, A., Delgado, P., Perasso, R. & Adoutte, A. A broad molecular phylogeny of ciliates: identification of major evolutionary trends and radiations within the phylum. *Proc. Natl. Acad. Sci. USA* **89**, 9764–9768 (1992).
111. Bardele, C. F. Ultrastructure, morphogenesis, and a discussion of the phylogeny of the prosoome ciliate *Balantion planctonicum*. *Eur. J. Protistol.* **35**, 17–33 (1999).
112. Yi, Z., Katz, L. A. & Song, W. Assessing whether alpha-tubulin sequences are suitable for phylogenetic reconstruction of Ciliophora with insights into its evolution in eutardigrades. *PloS one* **7**, e40635 (2012).
113. Penn, O. et al. GUIDANCE: a web server for assessing alignment confidence scores. *Nucleic Acids Res.* **38**, W23–28 (2010).
114. Stamatakis, A., Hoover, P. & Rougemont, J. A rapid bootstrap algorithm for the RAxML web servers. *Syst. Biol.* **57**, 758–771 (2008).
115. Miller, M. A., Pfeiffer, W. & Schwartz, T. *In Proceedings of the Gateway Computing Environments Workshop (GCE)* 1–8 (New Orleans, LA, 2010).
116. Abascal, F., Zardoya, R. & Posada, D. ProtTest: Selection of best-fit models of protein evolution. *Bioinformatics* **21**, 2104–2105 (2005).
117. Ronquist, F. & Huelsenbeck, J. P. *MrBayes 3*: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574 (2003).
118. Nylander, J. A. (2004). *MrModeltest v2*. Uppsala University.
119. Shimodaira, H. An approximately unbiased test of phylogenetic tree selection. *Syst. Biol.* **51**, 492–508 (2002).
120. Swofford, D. L. (2002) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. URL http://www.paup.csit.fsu.edu/.
121. Shimodaira, H. & Hasegawa, M. Consel: for assessing the confidence of phylogenetic tree selection. *Bioinformatics* **17**, 1246–1247 (2001).
122. Orsi, W. et al. Class Cariacotrichnea, a novel ciliate taxon from the anoxic Cariaco Basin, Venezuela. *Int J Syst Evol Microbiol* **62**, 1425–1433 (2012).
123. Bardele, C. F., Foissner, W. & Blanton, R. L. Morphology, morphogenesis and systematic position of the sorocarp forming ciliate *Sorogena stoianovitchae* (Kahl, 1928) Tuffrau, 1954. *J. Protozool.* **38**, 7–17 (1991).
124. Foissner, W., Skogstad, A. & Purr, J. R. Morphology and infraciliature of *Trichilopsis australis* n. sp., *Pelagohaltera viridis* (Fromentel, 1876) n. g., n. comb., and *Sorobulbul us fascinatus* n. sp. (Protozoa, Ciliophora). *J. Protozool.* **35**, 489–497 (1988).
125. Foissner, W., Agatha, S. & Berger, H. Soil ciliates (Protozoa, Ciliophora) from Namibia (Southwest Africa), with emphasis on two new and insufficiently known species. *Eur. J. Protistol.* **35**, 17–33 (1999).
126. Dehontky, B. A. A new family of entodinomorph protozoa from the marsupial forestomach, with descriptions of a new genus and five new species. *J. Eukaryot. Microbiol.* **43**, 285–295 (1996).

**Acknowledgements**

This work is supported by the Natural Science Foundation of China (grant numbers 31430077 to W. Song, and 31471973 to Z. Yi), the Applied Basic Research Plan of Qingdao (15-12-1-1-jch). International Exchanges Scheme supported by the Royal Society/Natural Science Foundation of China (31411130122), and the BBSRC China Partnering Award Scheme, provided support for training in data analyses. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Many thanks are due to Dr. Yan Zhao, Dr. Jiamei Li, Ms. Lijuan Huang, Pu Wang, and Mr. Weibo Zheng, graduate students at OUC or SCNU, for their help with gene sequencing.

**Author Contributions**

W.S., F.G. and Z.Y. designed the study, F.G. and Z.Y. undertook experiments and performed all analysis; F.G., A.W., Q.Z., J.G., M.M., P.S., D.X., J.H., Z.Y. and W.S. drafted and wrote the manuscript. All authors contributed to and approved the final manuscript.

**Additional Information**

Supplementary information accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Gao, F. et al. *The All-Data-Based Evolutionary Hypothesis of Ciliated Protists with a Revised Classification of the Phylum Ciliophora (Eukaryota, Alveolata).* *Sci. Rep.* **6**, 24874; doi: 10.1038/srep24874 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/