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Phylogenetic relationship analyses of complicated class Spirotrichea based on transcriptomes from three diverse microbial eukaryotes: *Uroleptopsis citrina*, *Euplotes vannus* and *Protocruzia tuzeti*

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ABSTRACT

Ciliates are one of the eukaryotic unicellular organisms which are thought to be the oldest life forms, and widely geographically distributed. For a variety of reasons, some groups of ciliates have attracted more attention than others, such as the class Spirotrichea and related species with its complicated evolutionary relationships. In this study, we obtained the transcriptome data of three typical ciliates, *Uroleptopsis citrina*, *Euplotes vannus*, *Protocruzia tuzeti* using high throughput sequencing. The genetic relationships were revealed by phylogenomic analysis of 109 genes comprising of 34,882 amino acid residues, and analyses based on SSU rDNA of 55 species, as well as the comparison of gene content among spirotricheans and related species. Our phylogenomic analyses show the Spirotrichea is monophyletic when *Protocruzia* is excluded, in which four subclasses: Oligotrichia, Choreotrichia, Hypotrichia and Euplotia also formed momophyletic groups respectively. The Hypotrichia was placed as a sister branch to the assemblage, in which two oligotrichs clustered with two choreotrichs. In addition to this, the Protocruziidia was placed in an independent lineage status out of the Spirotrichea. Together with its high binding-related gene content compared to other species and the significant variation in morphological characters, these findings support the removal of *Protocruzia* from the class Spirotrichea.

1. Introduction

1.1. Spirotrichea: a diverse assemblage of ciliates with complicated evolutionary relationships

Ciliated protozoa are unicellular eukaryotic organisms characterized by their morphological diversity, ubiquitous distribution, and the important roles they play in a wide range of biological and ecological studies (Azovsky and Mazei, 2018; Chen et al., 2016a; Huang et al., 2018; Umar et al., 2018; Wang et al., 2017, 2015; Xiong et al., 2016; Zhao et al., 2015, 2017, 2018). According to Lynn (2008), the phylum Ciliophora is divided into 11 classes, although a further three classes has since been proposed (Chen et al., 2015a; Gao et al., 2016; Orsi et al., 2012). Of these the class Spirotrichea is probably the most diverse and taxonomically difficult group (Chen et al., 2017b; Liu et al., 2017; Yi et al., 2016). As one of the largest classes of ciliates consisting of about 2000 described species, spirotricheans occur in almost every habitat, marine, freshwater and terrestrial, and there are even symbiotic forms (Lobban et al., 2002). Spirotricheans are also commonly known for their diversity and chaotic evolutionary relationships which has been subjected to numerous revisions resulting in inconsistent classification schemes (Adl et al., 2012; Gao et al., 2016; Lynn, 1996; Lynn and Small, 1997; Lynn and Strüder-Kypke, 2002).

In the present study we investigate three species, i.e., *Uroleptopsis citrina*, *Euplotes vannus* and *Protocruzia tuzeti*, representing three diverse groups in order to help clarify the confused phylogenetic relationships within the Spirotrichea. *Uroleptosis* is a core urostyloid of the subclass Hypotrichia which was first established on classification in *Holosticha* (*Keronopsis*) (Kahl, 1932). It was redefined by Berger (Berger, 2004) based on a combination of its morphology and morphogenesis. The main diagnostic character which separates *U. citrina* from its congeners is the absence of transverse cirri. *Euplotes vannus* shows typical Euplotia

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features, the loss of intermediate forms during the long period of time, with respect to its life cycle, and physiological properties (Borror, 1972; Borror and Hill, 1995; Curds, 1983). Unlike these ciliates, the *Protocruzia* has been considered one of the most systematically ambiguous groups within the Spirotrichea sensu lato (Corliss, 1979; Kahl, 1932; Li et al., 2010). This species is known to for its particular morphological and ultrastructural features (e.g. overlapping postciliary ribbons, mitosis-like features, the lack of replication bands) (Corliss, 1979; Gentekaki et al., 2014; Jiang et al., 2017; Kahl, 1932; Levine et al., 1980; Li et al., 2010; Lynn, 2008). A large-scale phylogenomic analysis revealed that *Protocruzia* should be removed from the class Spirotrichea (Gentekaki et al., 2014). Subsequently, based on phylogenetic analysis of multiple genes, Gao et al. (2016) established a separate class, Protocruziea, for this group. Nevertheless, even with the detailed molecular phylogeny and morphological description of some nominal species including *P. tuzeti* (Jiang et al., 2017), the systematics of *Protocruzia* has yet to be fully resolved.

1.2. The omics data restrains help clarify the phylogenetic relationship of ciliates

Phylogenetic analyses mostly rely on critical morphological features, patterns of morphogenesis and, increasingly, SSU rDNA sequences (Chen et al., 2017a; Gao et al., 2017; Huang et al., 2016, 2018; Li et al., 2016; Lu et al., 2017; Wang et al., 2015). More recently, high-throughput sequencing technology has provides a new approach to clarify the complicated evolutionary relationships of ciliates by enabling orthologous genes selected from large datasets to be used for constructing more accurate phylogenetic trees (Chen et al., 2016b; Gentekaki et al., 2014; Lynn and Kolisko, 2017; Sun et al., 2017; Zhang et al., 2014). To date, 12 ciliate genomes have been sequenced including: *Ichthyophthirius multifiliis* (Coyne et al., 2011), *Oxytricha trifallax* (Swart et al., 2013), *Pseudocohnilembus persalinus* (Xiong et al., 2015) and *Stentor coeruleus* (Slabodnick et al., 2017). In addition, the transcriptomes of 22 ciliate species representing six classes have been reported (Table 1) including those of *Strombidium sucaltum* (Chen et al., 2015b) and *Halteria grandinella* (Lynn and Kolisko, 2017). However, these data are far from sufficient, and the lack of genomic and transcriptomic information for ciliates still constrains researchers from elucidating their true phylogenetic relationships.

Compared with genomes, transcriptomes are much smaller and simpler and are enriched with highly expressed and conserved genes that are useful for phylogenetic analyses (Hittinger et al., 2010). Furthermore, eukaryotic mRNA with polyA tails can be amplified using oligo-dT magnetic beads thereby significantly reducing bacterial contaminations (Grant et al., 2012). In this study, we generated RNA-Seq data from three ciliates, *Uroleptopsis citrina*, *Euplotes vannus* and *Protocruzia tuzeti*. Phylogenomic analyses were carried out using 109 single-copy orthologous genes from all 25 ciliates for which genomic and transcriptomic data are available, and their evolutionary relationships were revealed by concordance tree analyses. In addition to enriching the limited ciliate RNA-Seq data, this study also provides insight into evolutionary relationships within the class Spirotrichea and related species.

Table 1
List of the transcriptome data available now.

Species (22)	Taxonomy	PE	Gene
Paralembus digitiformis	Oligohymenophorea	100	12,752
Campanella umbellaria	Oligohymenophorea	81	27,789
Carchesium polypinum	Oligohymenophorea	81	23,899
Paralembus digitiformis	Oligohymenophorea	81	43,528
Halteria grandinella	Spirotrichea	250	25,435
Euplotes focardii	Spirotrichea	50	17,307
Euplotes harpa	Spirotrichea	50	18,712
Protocruzia adherens	Spirotrichea	50	31,274
Pseudokeronopsis riccii	Spirotrichea	50	14,876
Schmidingerella arcuata	Spirotrichea	50	14,736
Strombidinopsis acuminatum	Spirotrichea	50	45,231
Strombidium sucaltum	Spirotrichea	50	22,644
Strombidium inclinatum	Spirotrichea	50	21,441
Strombidium rassoulzadegani	Spirotrichea	50	11,917
Mesodinium pulex	Litostomatea	50	59,182
Myrionecta rubra	Litostomatea	50	16,843
Litonotus pictus	Litostomatea	50	22,760
Platyophrya macrostoma	Colpodida	50	39,863
Aristerostoma sp.	Colpodida	50	13,904
Colpoda aspera	Colpodida	100	32,790
Tiarina fusus	Prostomatea	50	64,134
Condylostoma magnum	Heterotrichea	50	2247

2. Material and methods

2.1. Cultures

The strains of *Uroleptopsis citrina*, *Euplotes vannus*, and *Protocruzia tuzeti* were cultured at room temperature (25 °C) in 75 cm^2 plastic culture flasks with filtered marine water for 7–14 days until they reached a density $\geq 3 \times 10^6$ cells per ml. In each case, the food source was *Escherichia coli* the growth of which was enriched by adding rice grains.

2.2. RNA extraction and Illumina sequencing

Cells of each strain were collected by centrifugation (1700*g*, 10 min) and immediately frozen at −80 °C until further treatment. The RNeasy kit (Qiagen, Hilden, Germany) was designed for purification of total RNA, extra DNase was added for digestion. RNA quality was determined using a BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). 250 ng RNA sample was converted to cDNA according to the standard protocol of Affymetrix 30 IVT Express Kit (Affymetrix Inc., Santa Clara, CA, USA). The resulting high quality cDNA was then ligated to Illumina paired end sequencing.

2.3. Assembly

The sequencing adapter was trimmed and low quality reads were filtered using the Trimmomatic (Bolger et al., 2014) (TruSeq3-PE.fa: 2:30:10, leading: 3, trailing: 3, sliding window: 4:15, minlen: 80). *De novo* assembly of the clean reads was conducted using Trinity (Grabherr et al., 2011) and inGAP-CDG (Peng et al., 2016) with default parameters. CD-HIT (CD-HIT-EST, with 98% sequence identity threshold) was used to reduce the redundancy of assembly (Fu et al., 2012). Non-redundant contigs were searched against the bacterial genomes database downloaded from GenBank using Basic Local Alignment Search Tool (BLAST). Both the identity and coverage more than 50% of the contigs were considered as effective hits and removed by custom Perl script.

2.4. Gene prediction and annotation

Seventeen ciliate transcriptomes and five ciliate genomes are available through the GenBank for gene prediction (accession numbers as shown in Table S1). Contigs derived from the three newly assembled transcriptomes were first analyzed with TransDecoder (http://transdecoder.sourceforge.net: *Euplotes* genetic codes for the spirotricheans, *Tetrahymena* genetic codes for other ciliates), which generated protein predictions longer than 100 amino acids. The translated protein sequences were blasted to the Swiss-Prot database, restricted to the bitscores with E-value $\leq 1.0 \times 10^{-5}$. Proteins were also matched to Pfam-A database using HMMER (Eddy, 2009) (e-value threshold of 1.0×10^{-5}). The resulting file contained both BLAST and Pfam positive hits that were used to retrieve Gene Ontology terms. The remaining proteins were annotated using Interproscan (Jones et al., 2014). Gene

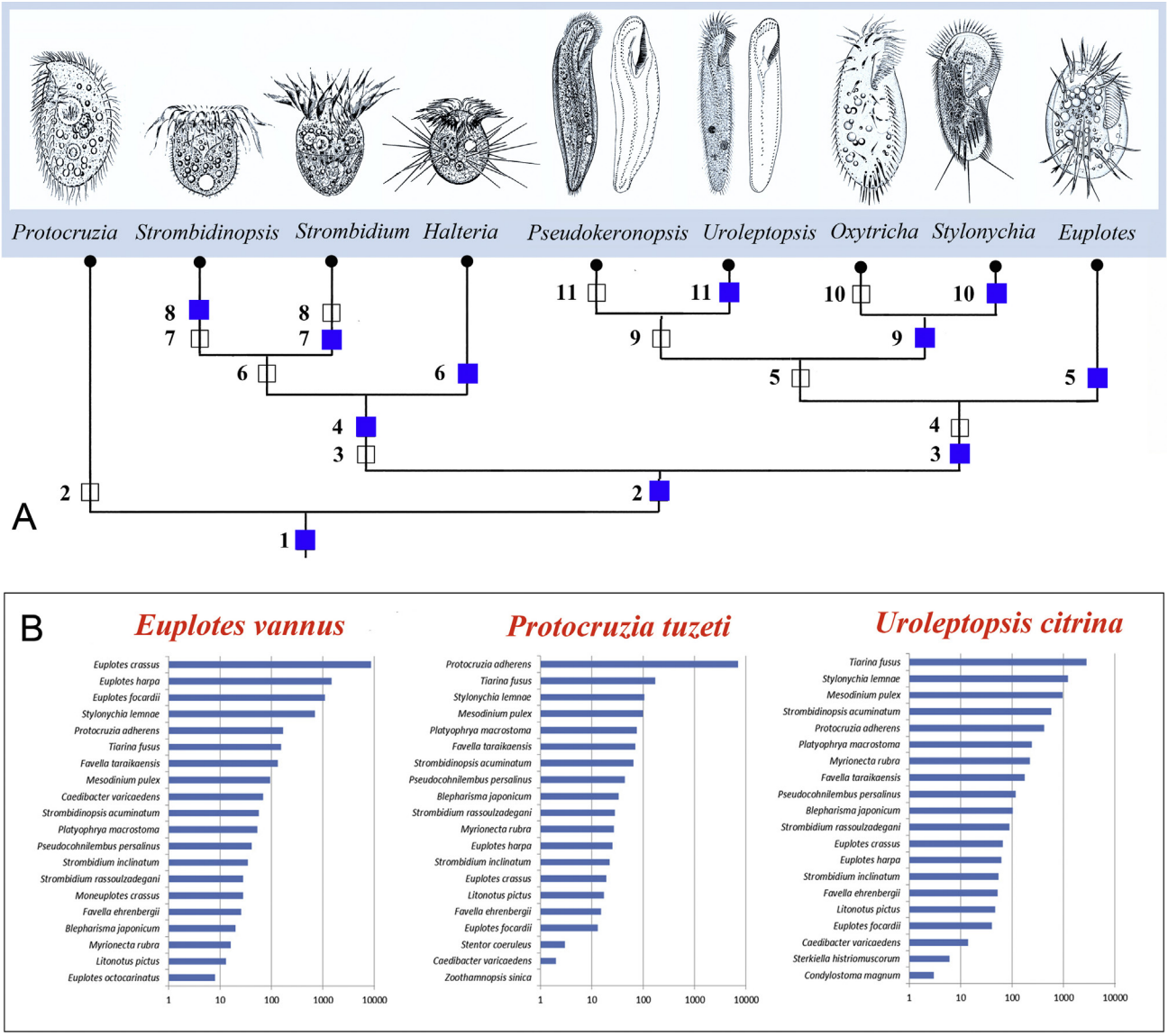

Fig. 1. Morphological and molecular information of Spirotrichea. (A) Assessment of the phylogenetic relationships among five subclasses within the Spirotrichea based on morphological and morphogenetic information (for explanation of numbered characters, see Table 2). Drawings of the representative forms which display in the present work are according to previous studies. (B) Distribution of species with best hits by BLAST. The X-axis represents numbers of contigs or genes.

Ontology (GO) analyses were carried out under WEGO (Ye et al., 2006).

2.5. Orthologs among three ciliates

The Reciprocal Best Hits (RBH) approach was used in BLAST to generate candidate orthologous genes between *Tetrahymena thermophila* (reference species) and each protein-coding set from another twenty-four ciliates, i.e., the three new and 21 published transcriptomes and genomes. The genome of *Uroleptopsis citrina* (Zheng et al., 2018) was also used as a reference during the orthologous genes selection. Only effective hits (E-value $\leq 1.0 \times 10^{-10}$, identity ≥ 50, length ≥ 50) were retained. Twenty-four RBH lists were compared to each other to find intersection as putative orthologous genes group for further analyses. Gene sets including more than 88% of all those available, i.e., at least 22 of the 25 species analyzed, were used for phylogenomic analysis.

2.6. Phylogenetic analyses

One hundred and nine orthologous genes (Table S2) shared by a majority of the 25 ciliates were selected and aligned using MAFFT (Katoh et al., 2002). Ambiguously aligned regions were detected and trimmed using Gblocks (Castresana, 2000) with the following parameters: Maximum number of contiguous non-conserved positions = 8; Minimum length of a block = 10; Allowed gap positions = with half. RAxML (Stamatakis, 2006) was used for Maximum Likelihood (ML) analyses under the LG+I+G+F model selected by ProtTest (Darriba et al., 2011). To obtain statistical support, 100 bootstrap replicates were analyzed for ML tree construction. Bayesian inference (BI) analyses were implemented by the software PhyloBayes (Lartillot et al., 2009). Markov chain Monte Carlo (MCMC) simulations were run with two sets of four chains for 1,000,000 generations with a burn-in of 2500. All remaining trees were used to calculate posterior probabilities (PP) using a majority rule consensus. Tree topologies were visualized using MEGA (Tamura et al., 2013).

For the SSU rRNA gene, 55 sequences of spirotricheans and related species including those of the three species treated here, downloaded from the GenBank database (Table S3), were aligned online by MUSCLE on the GUIDANCE web server (http://guidance.tau.ac.il) with default parameters (Penn et al., 2010). Both ends of the alignments were

trimmed and ambiguous columns were removed with set parameters (scoring below 0.80) resulting in a matrix of 1681 characters. ML analyses with 1000 bootstrap replicates were performed using RAxML-HPC2 on XSEDE v8.2.9 (Stamatakis et al., 2008) on the CIPRES Science Gateway with the GTR + CAT model selected by Modeltest v3.4 (Posada and Crandall, 1998). BI analyses were carried out under MrBayes (Ronquist and Huelsenbeck, 2003) on XSEDE v3.2.6 on the CIPRES Science Gateway using the GTR + I + G model selected by Akaike Information Criterion in MrModeltest v2 (Nylander, 2004). The same parameters described before were used for MCMC simulations and subsequent steps. Split decomposition analysis of phylogenetic networks was performed to reveal all possible relationships among the class Spirotrichea and related species with the computer program SplitsTree (Huson, 1998; Huson and Bryant, 2005). The neighbor-net graphs were calculated based on 19,489 aa from 61 orthologous genes (Table S2) shared by all fourteen ciliates (Table S1), using the neighbornet algorithm with uncorrected distances (Bryant and Moulton, 2004). Bootstrap analyses with 1000 replicates were selected for increasing the credibility of phylogenetic networks.

3. Results

3.1. Morphological description

For the three newly sequenced species, detailed morphological descriptions of *Uroleptopsis citrina*, *Euplotes vannus*, and *Protocruzia tuzeti* have been previously been made (Berger, 2004; Luo et al., 2017; Shao et al., 2014; Song et al., 2009, 2006; Song and Wilbert, 1997; Weibo, 1993). Here we present a summary of the main morphological and morphogenetic features that characterize the five groups (Fig. 1A). In brief, these include, the macronuclear replication band is the main point to distinguish Protocruziea out of the spirotricheans, and the dorsal argyrome plays an important role in the identification of Euplotia. Comparing to other groups, oligotrichs and choreotrichs seems like similarly with some unique features but could also been separated according to “open” and “closed” circle formed by anterior adoral membranelles.

3.2. Transcriptomes assembly of three species

Paired-end reads (average 100 bp for each read) were produced for *U. citrina* (16,887,887 reads), *E. vannus* (18,665,595 reads) and *P. tuzeti* (16,761,513 reads), after filtering low quality reads (6.79% for *U. citrina*, 6.07% for *E. vannus*, 9.61% for *P. tuzeti*), remaining reads were assembled using Trinity and inGAP-CDG. After elimination of rDNA contaminants (two contigs for *U. citrina*, two contigs for *E. vannus*, one contig for *P. tuzeti*), trans chemiras and redundancies were detected and removed. Since *E. coli* was used as the food source during the cultivation, the assembled contigs were searched against the bacterial genomes database. Based on the BLAST result, we determined that some contigs are bacterial and these were discarded (325 for *U. citrina*, 216 for *E. vannus*, one for *P. tuzeti*). By following these steps, raw reads from the three species were assembled into unigenes (Table 3) for the future analysis.

3.3. Gene content in transcriptomes

Considering the close relationships between our three species and Euplotia, all unigenes were used to predict the peptide sequences by TransDecoder with the *Euplotes* genetic codes. The proteins we predicted were conducted a BLAST search to the ciliate protein database in GenBank. A summary of the top hits is shown in Fig. 1B. These species also grouped together in phylogenetic networks (Fig. 2C) representing three groups respectively, agreed with the traditional taxonomy (Adl et al., 2012; Chen and Song, 2002; Huang et al., 2010). To further exploring the relationships between these groups, transcriptomic and genomic data for another 11 ciliates were also used for protein prediction (Table S1). Gene functional enrichment analysis was carried out using the predicted protein based on GO terms (Fig. 2A). Only genes with relatively high proportion (> 1%) are shown in Fig. 2A. After calculating the standard deviation of every group, binding-related (5.206) and catalytic-related (2.339) genes presented significant variations in expression among different groups, which may reflect their diverse morphologies (Fig. 1A). For example, the unique mitosis-like features during macronuclear division in Protocruziea (Ammermann, 1968; Lynn, 2008; Ruthmann and Hauser, 1974) is consistent with the high binding-related gene content in this group, and the abundant catalytic-related genes may help Euplotia survive in sewage sludge is consistent with the high abundance of euplotids in high organic environments such as biological sewage-treatment processes (Madoni, 2011).

Table 2
Morphogenetic and morphological characteristics used for assessment of phylogenetic relationships among five subclasses within the Spirotrichea.[a]

	Character	
	Apomorph ■	Plesiomorph □
1	Paroral membrane present	Without
2	Macronuclear replication band	Without
3	Cirri present	Cilia
4	Morphogenesis enantiotropic	Homeotropic
5	The dorsal argyrome	Without
6	Entire somatic ciliature originates de novo on cell surface between parental ciliature	Entire somatic ciliature originates intrakinetal or in subsurface tub
7	Oral primordium in intracellular tube	In subsurface pouch
8	Anterior adoral membranelles form a “closed” circle	“open” circle
9	18 frontal-ventral-transverse cirri	Not 18 frontal-ventral-transverse cirri
10	Body rigid	Body flexible
11	Transverse cirri lacking	Present

[a] See Fig. 1A.

Table 3
Summary of three Spirotrichea genomes.

Species	Taxonomy	PE	Reads	N50	Gene
Uroleptopsis citrina	Spirotrichea	100	16.9M	1158	32,604
Euplotes vannus	Spirotrichea	100	18.6M	1151	41,204
Protocruzia tuzeti	Spirotrichea	100	16.8M	1939	44,767

3.4. Ortholog detection and phylogenomic analysis

In addition to allowing rough comparisons of gene content, construction of ortholog groups was also used in these newly sequenced transcriptomes. The predicted proteins from *U. citrina*, *E. vannus*, *Strombidium sucaltum* and *Schmidingerella arcuata* representing each of the four subclasses of Spirotrichea and *P. tuzeti*, using a RBH approach. After removing invalid hits (E-value $\geq 1.0 \times 10^{-10}$, identity ≤ 50, length ≤ 50), 767 orthologous genes shared by all five were identified. *Protocruzia tuzeti* shared the fewest orthologous genes with spirotricheans taxa (Fig. 2B), which is consistent with its independent status during the morphological comparison. Among the remaining four species, *U. citrina* and *E. vannus* shared fewer orthologous genes than other two, which means they might have a more distant phylogenetic relationship. These speculations were supported by our phylogenomic analysis (Fig. 3).

Further orthologs were detected among the spirotricheans and related species (Table S1). We selected 61 orthologous genes shared by 14 ciliates using the RBH approach by BLAST (E-value $\leq 10^{-10}$, identity $\geq 50\%$, length ≥ 50). Split decomposition analysis of phylogenetic

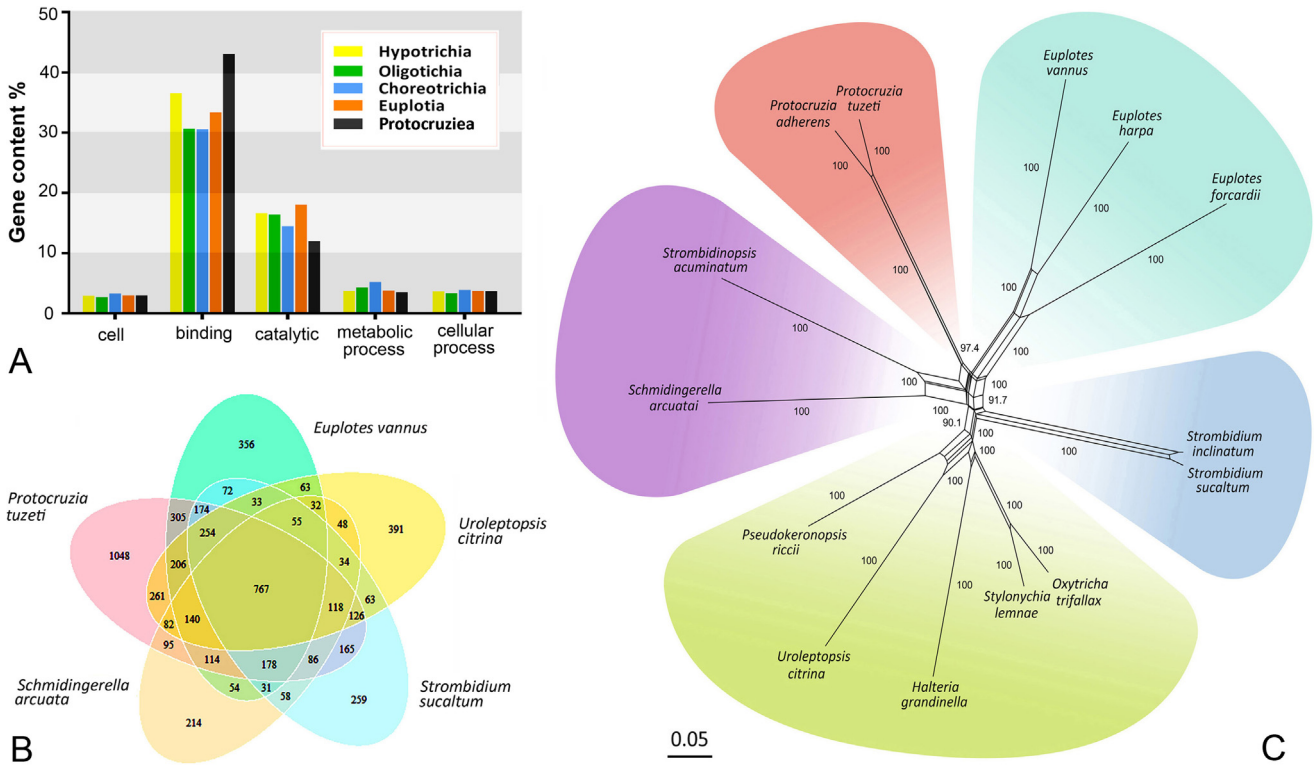

Fig. 2. Analysis of spirotricheans and related ciliates. (A) Gene content of subclasses within the Spirotrichea and related species. (B) Venn diagram showing the comparison of numbers of orthologous genes among *Uroleptopsis citrina*, *Euplotes vannus*, *Protocruzia tuzeti*, *Strombidium sucaltum* and *Schmidingerella arcuata*. (C) Phylogenetic network computed from the orthologous genes alignment dataset among different groups using the neighbornet algorithm with uncorrected distances. Numbers along edges are bootstrap support values coming from 1000 replicates. Only bootstrap support values > 50% are shown. The scale bar indicates five substitutions per one hundred n amino acids.

networks was performed to reveal all possible relationships (Fig. 2C), which were as expected, *E. vannus* was sister to *Euplotes focardii* and *Euplotes harpa*; *U. citrina* fell in the clade Hypotrichia; *P. tuzeti* was in a position combine with *Protocruzia adherens*.

The amount of omics data source has increased into 25 ciliates to find another orthologous groups for further phylogenomic analysis. By using the RBH method as before, more than 88%, i.e., at least 22 of the 25 species for which transcriptomic and genomic data are available possessed the orthologous gene, it was retained to perform phylogenomic analysis. Finally, we established a database with 109 genes comprising of 34,882 amino acid residues from five genomes and 20 transcriptomes. The ML and BI trees had similar topologies therefore only the ML tree is presented with support values of both analyses indicating on branches (Fig. 3). The subclasses Euplotia, Oligotrichia, Choreotrichia and Hypotrichia clustered as a clade with full support. Hypotrichia was sister to the assemblage formed by the other three subclasses, within which the Oligotrichia and Choreotrichia were most closely related followed by Euplotia. The ambiguous genus *Protocruzia*, did not group with any of the major clade, was placed in the earliest diverging position in the ciliate tree. The topology of phylogenetic tree derived from the SSU rRNA genes reconstructed by ML and BI (Fig. 4) was similar to the phylogenomic tree, providing further support for the

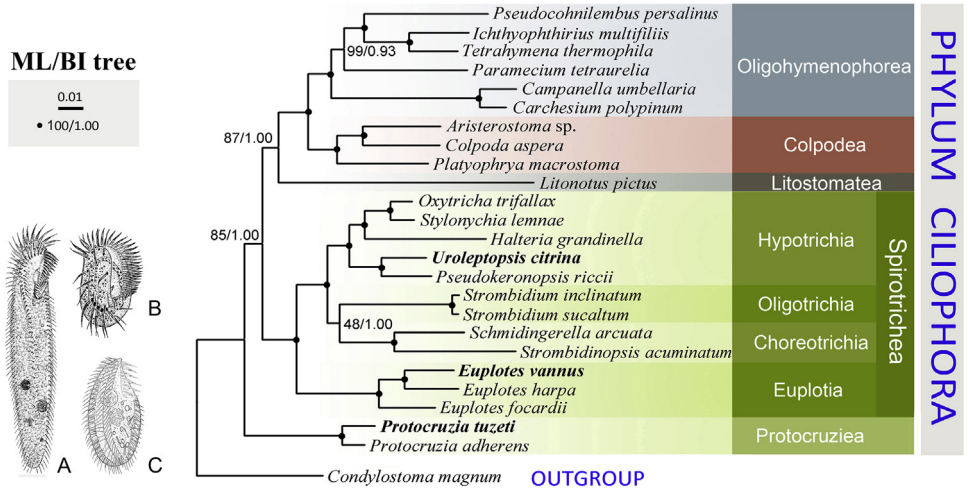

Fig. 3. Phylogenomic relationships of ciliates. New sequencing species in this study is bold with diagrams: (A) *Uroleptopsis citrina*, (B) *Euplotes vannus*, (C) *Protocruzia tuzeti*. Numbers at nodes are BI posterior probability followed by ML bootstrap values. The scale bar corresponds to 0.01 expected substitutions per site.

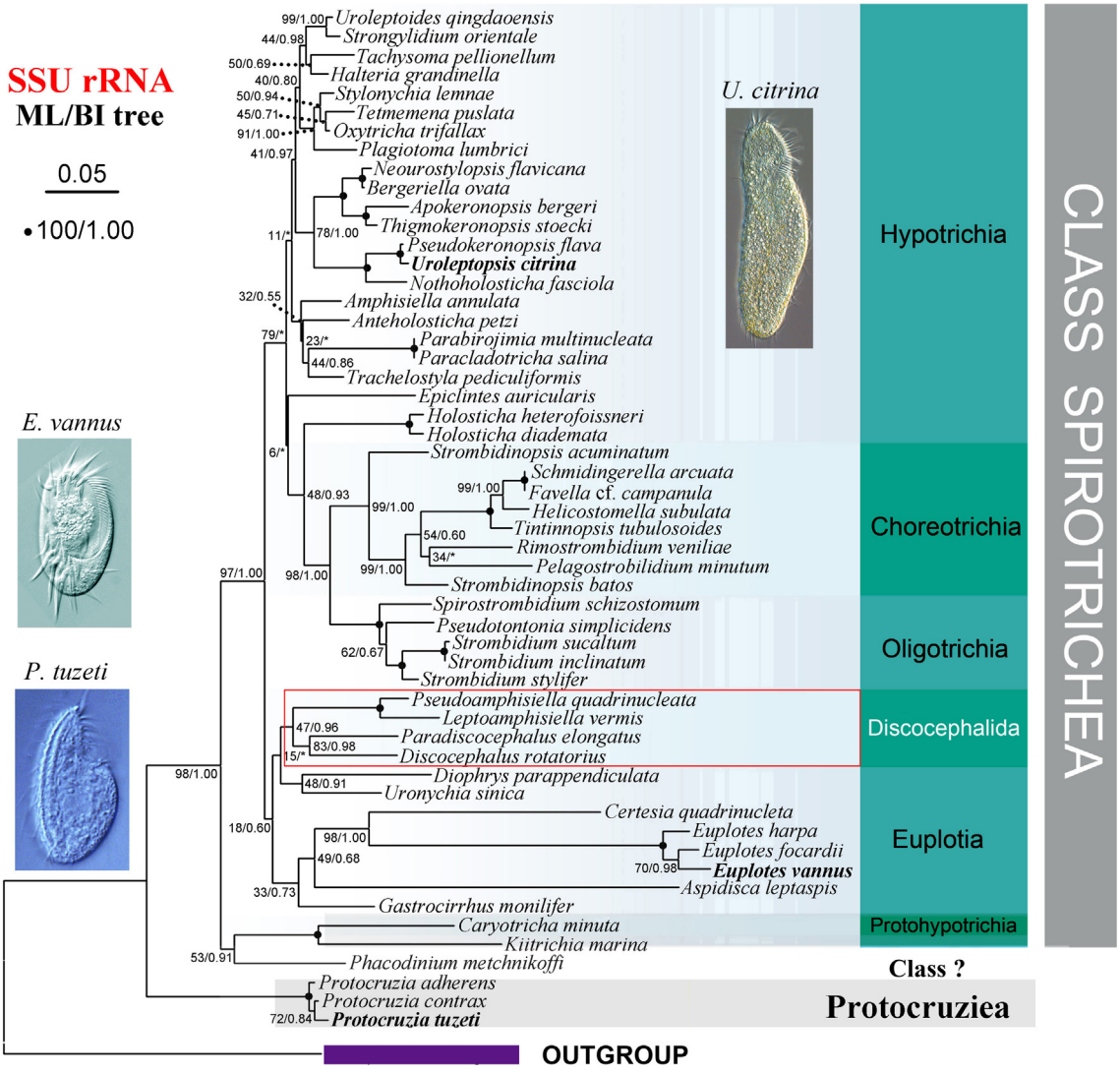

Fig. 4. Phylogenetic tree based on the SSU rRNA gene sequences of ciliates, showing the positions of our three taxa (in bold). Numbers at nodes represent bootstrap values of ML from 1000 replicates and the posterior probability of BI, respectively. Clades with a different topology in the BI tree are indicated by "*". All branches are drawn to the scale bar, which corresponds to five substitutions per 100 nucleotide positions.

placement of *Protocruzia* outside the class Spirotrichea.

4. Discussion

4.1. The diverse and confused Spirotrichea

Spirotrichea has a long tradition of research since it established by Corliss (Corliss, 1979) as a subclass of Polyhymenophora (Lynn, 2008, 1996; Lynn and Small, 1997; Lynn and Strüder-Kypke, 2002). Various taxa were accepted into it constantly resulting in a diverse ciliate group (Chen et al., 2015c; Dong et al., 2016; Park et al., 2017; Singh and Kamra, 2014). This large class currently contains more than 2000 species belongs to seven subclasses: Hypotrichia; Phacodiniidia; Protohypotrichia; Licnophoria; Euplotia; Oligotrichia; Choreotrichia (Gentekaki et al., 2014). The systematics of the Spirotrichea continues to attract the attention of researchers resulting in conflicting classification schemes (Adl et al., 2012; Gao et al., 2016; Lynn, 2008; Paiva et al., 2009; Santoferrara et al., 2017). Traditional taxonomy based on morphological characters reveal fundamental differences among spirotricheans and related ciliates at the rank of subclass (Fig. 1A). The present study seeks to investigate evolutionary relationships within the Spirotrichea by carrying out phylogenomic analyses based on transcriptomic and genomic data. Even a superficial analysis of the transcriptomic data reveals that the content of some special genes in disparate groups which might be linked with their unique morphological characteristics (Fig. 2A). Furthermore, these groups formed independent clades in the phylogenomic trees supporting their separation (Figs. 2C and 3). The Phacodiniidia intermingled with Protohypotrichia placed as an early branching lineage within the main clade (Fig. 4), indicated both of them are basal to Spirotrichea, consistent with the previous results (Li et al., 2009; Shao et al., 2009)

4.2. The independent lineage status of Protocruzia

Unlike other subclasses, the evolutionary lineage status of Protocruziea has always been problematic. Although Lynn (2008) asserted that it belongs to the Spirotrichea, its phylogenetic position remains uncertain, since it is quite different from other spirotricheans (Bernhard and Schlegel, 1998; Shin et al., 2000; Song and Wilbert, 1997). In addition to its unique morphological and morphogenetic characteristics (Fig. 1A; Table 3), the process of macronuclear division shows some mitosis-like features that have not been observed in any other spirotricheans (Ammermann, 1968; Lynn, 2008; Ruthmann and Hauser, 1974). This is consistent with its unusual binding-related gene

content (Fig. 2A). Phylogenetic analyses based on histone (H4 and H3) and SSU rRNA genes have also suggested that *Protocruzia* is independent from the main clade of Spirotrichea (Hammerschmidt et al., 1996; Shin et al., 2000). Considering these analyses were carried out with a limited number of genes, a few discrepancies existed. Thus, we had taken advantage of RNA-Seq method for phylogenomic analyses. The above study clearly demonstrates that Protocruziea is not a member of the class Spirotrichea. Compared with *U. citrina*, *E. vannus*, *S. arcuata* and *S. sucaltum*, the typical ciliates of four subclasses, *P. tuzeti* possessed the fewest common orthologous genes (Fig. 2B), which indicates it shares low homology with the spirotricheans. Likewise, two *Protocruzia* are placed in a deeper and earlier diverging position in the ciliate tree, fully supported by both ML and BI analyses (Gao et al., 2016; Gentekaki et al., 2014) (Fig. 3). Due to those conclusions, we agree that there should be an independent class status for Protocruzia (Gao et al., 2016; Gentekaki et al., 2014; Li et al., 2010).

5. Conclusion

The current study helps clarify the diversity and confused class Spirotrichea in some extent, not merely revealed this group is monophyletic, also the relationships between subclasses have been explored. In phylogenomic analysis, the hypotrichs clustered with oligotrichs and choreotrichs, formed an independent clade separated from the euplotids, and the ancestor position of Phacodiniidia and Protohypotrichia have been proved as they occupied the basal position within the main clade. Our result also support that the ambiguous taxon *Protocruzia* is removed from the class Spirotrichea for its independent lineage status (Figs. 3 and 4). All these conclusion shows highly consistency with the traditional morphology-based taxonomy (Fan et al., 2016; Jiang et al., 2017; Li et al., 2009; Shao et al., 2009; Shin et al., 2000; Zhang et al., 2017), increased their credibility. While considering with the enormous species of spirotricheans, only limited transcriptomes and genomes are available now, for further exploring their real phylogenetic relationships, additional omics data is needed.

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