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Further analyses on the evolutionary "key-protist" *Halteria* (Protista, Ciliophora) based on transcriptomic data

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Abstract

Considerable discordance between morphologies and molecules, which can be caused by convergent morphologies, cryptic species or plastic phenotypes, has been frequently detected, especially in microbes. One of the best examples could be the well-known halteriid ciliates, whose systematic position remains highly unresolved, because it resembles oligotrichs in terms of morphology while associates with hypotrichs in phylogeny based on maker genes. In the present work, we report the deep sequencing and analyses of RNA-seq data from the representative halteriid, *Halteria grandinella.* The results indicate that: 1) the transcriptome includes 92,114 genes (aa ≥ 50) with the N50 of 957 bp, which is much better than the single-cell transcriptome; 2) *H. grandinella* shares more homologous genes in higher sequence identity with hypotrichous oxytrichids than with oligotrichs; 3) the codon usage bias of *H. grandinella* is much more similar with that of oligotrich *Strombidium sulcatum*, and UAA and UAG are reassigned to encode glutamine, which is a common feature of oligotrichs and hypotrichs; and 4) phylogenomic analyses based on 132 orthologs and 47,263 amino acid sites place *H. grandinella* as a sister to hypotrichs while other oligotrichs cluster together. Based on all the information so far available, we thus suggest that *H. grandinella* could be an extremely specialized "oligotrich-like" hypotrich. The similar morphology between halteriids and oligotrichs results from convergent evolution to adapt to the planktonic lifestyle. We hypothesize the evolutionary relationship of the core spirotrichs that halteriids and oxytrichids share the most common ancestor, followed successively by other hypotrichs, oligotrichs/choreotrichs, and euplotids.

KEYWORDS
ciliated protist, *Halteria grandinella*, phylogenomics, stop codon reassignment, transcriptome

1 | INTRODUCTION

Free-living ciliated protists constitute a large group of single-celled eukaryotes and play important roles in microbial food webs and energy flow processes (Kathol, Norf, Arndt, & Weitere, 2009; Liu et al., 2017; Xu, Zhang, & Jiang, 2014). Their highly diverse morphology, unique nuclear dimorphism, distinctive chromosomal fragmentation and special sexual reproduction process (conjugation), have attracted many researchers' attention in the field of taxonomy, ecology, evolution, genomics, epigenetics, etc. (Chen et al., 2016; Gao et al., 2013; Huang et al., 2018; Wang, Sheng, et al.,

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2017; Wang, Chen, Sheng, Liu, & Gao, 2017; Wang, Zhang, et al., 2017; Xiong et al., 2016; Yan et al., 2018; Yan, Rogers, Gao, & Katz, 2017; Zhang, Wang, Katz, & Gao, 2018; Zhao, Wang, Wang, Liu, & Gao, 2017; Zhao, Yi, Warren, & Song, 2018). Although these topics have been studied for hundreds of years, there are still many unresolved issues, such as the considerable discordance between morphologies and molecules, which can be caused by convergent morphologies, cryptic species, plastic phenotypes or life-cycle stage variation (reviewed in Lahr, Laughinghouse, Oliverio, Gao, & Katz, 2014). One of these examples could be halteriids, a unique group in the phylum Ciliophora, whose systematic position has puzzled taxonomists for decades (Agatha, 2004; Foissner, Muller, & Agatha, 2007; Gao et al., 2016; Hu, Hu, Al-Rasheid, Al-Farraj, & Song, 2011).

Represented by the well-known species, *Halteria grandinella*, halteriids are a cosmopolitan group that lives predominantly in freshwater habitats and is typically planktonic (Foissner et al., 2004; Šimek, Jürgens, Nedoma, Comerma, & Armengol, 2000). They are characterized mainly by the globular body shape, reduced somatic ciliature (cirrus-like "bristles" used for performing conspicuous jumps), apical adoral structure (with the "collar" as an "open" circle) and enantiotropic cell division mode (the cell axes of proter and opisthe not oriented in the same direction, but in opposite orientation) (Figure 1) (Fauré-Fremiet, 1953; Foissner et al., 2007; Petz & Foissner, 1992; Song, 1992). All these characteristics are shared with high similarity with these in the oligotrichs (mostly globular, reduced somatic ciliature and enantiotropic cell division) (Agatha, 2004; Liu et al., 2016; Petz & Foissner, 1992; Song et al., 2018), but differ from these in the hypotrichs (mostly dorsoventrally compressed, ventral cirri and synclastic cell division) (Figure 1) (Chen, Zhao, Shao, Miao, & Clamp, 2017; Lu, Huang, Chen, & Berger, 2018; Luo et al., 2017, 2018). However, phylogenetic analyses based on small subunit ribosomal DNA (SSU rDNA), internal transcribed spacer (ITS), 5.8S rDNA, large subunit ribosomal DNA (LSU rDNA), α-tubulin and actin I genes have suggested that halteriids are closely related to oxytrichids, a highly specialized group of hypotrichs (Gao et al., 2016; Hu et al., 2011; Lynn & Sogin, 1988; Paiva, Borges, Harada, & Silva-Neto, 2009). However, these phylogenetic analyses are based on single or a few genes/loci, which is not convincing enough to address conclusively any controversy.

With the rapid development of the high-throughput sequencing techniques, genomic or transcriptomic analyses can provide comprehensive insights into the evolution of many organisms (Farlow et al., 2015; Kapusta, Suh, & Feschotte, 2017; Shubha et al., 2016). Besides, phylogenomic investigation has become an effective approach to study the evolutionary relationships of ciliates (eg: Chen, Wang, Sheng, Warren, & Gao, 2018; Chen et al., 2015; Feng et al., 2015; Gentekaki et al., 2014; Gentekaki, Kolisko, Gong, & Lynn, 2017; Sheng, He, Zhao, Shao, & Miao, 2018). In order to investigate the systematic position of halteriids, Lynn and Kolisko (2017) sequenced the transcriptome of *H. grandinella* based on single-cell sequencing techniques and found *H. grandinella* clustered with hypotrichs in multigene trees. Here, we sequenced and analysed the culture-based transcriptome of *H. grandinella*, including assembly, annotation, and comparison with the single-cell transcriptome data downloaded from National Center for Biotechnology Information (NCBI, Lynn & Kolisko, 2017). The amino acid codon usage and stop codon reassignment of *H. grandinella* were also analysed and compared with other ciliates. Phylogenomic analysis based on 47,263 amino acid sites from 132 orthologous proteins of 43 taxa was performed to infer the evolutionary relationships among halteriids, hypotrichs and oligotrichs.

2 | MATERIAL AND METHODS

2.1 | Ciliate culture

Halteria grandinella, collected from a pond in Baihuayuan Park (36°04'N, 120°22'E) in Qingdao, China, was identified based on the morphological traits observed both in vivo and via protargol staining (Song, 1992). Its SSU rDNA sequence was amplified and characterized according to Wang, Zhang, et al. (2017), which is 100% identical to the published SSU rDNA sequence of *H. grandinella* (acc. no. MF002423). Cells were isolated, cleaned (i.e. diluted the surroundings multiple times) and cultured in filtered and autoclaved in situ water (from the pond where we found *H. grandinella*) with the monoclonal bacteria *Escherichia coli* as the food source. Cultures were maintained in 600 ml cell culture flasks at 25°C in the incubator.

2.2 | RNA extraction and Illumina sequencing

When the cultures reaching ca. 10^5 cells, the cultures were starved and treated with ampicillin (Antibiotic-Antimycotic, ThermoFisher Scientific, Cat No. 15,240,062, 1ml for 1L) for 24 hr before RNA extraction. Cells were collected by centrifugation at 300g for 5 min, and total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Hiden, Germany; Lot No. 74,136) according to the kit's instructions.

Poly-A mRNAs were isolated using Sera-Mag Magnetic Oligo (dT) beads from Illumina, and then fragmented with divalent cations. Double-stranded cDNA was synthesized using the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen, Camarillo, USA; Lot No. 11,917,020) with random hexamer primers in Illumina. Thereafter, the cDNA was subjected to end-repair, phosphorylation, 3' adenylation and adapter ligation. After these steps, cDNA fragments with an insert size of approximately 250 bp were purified and

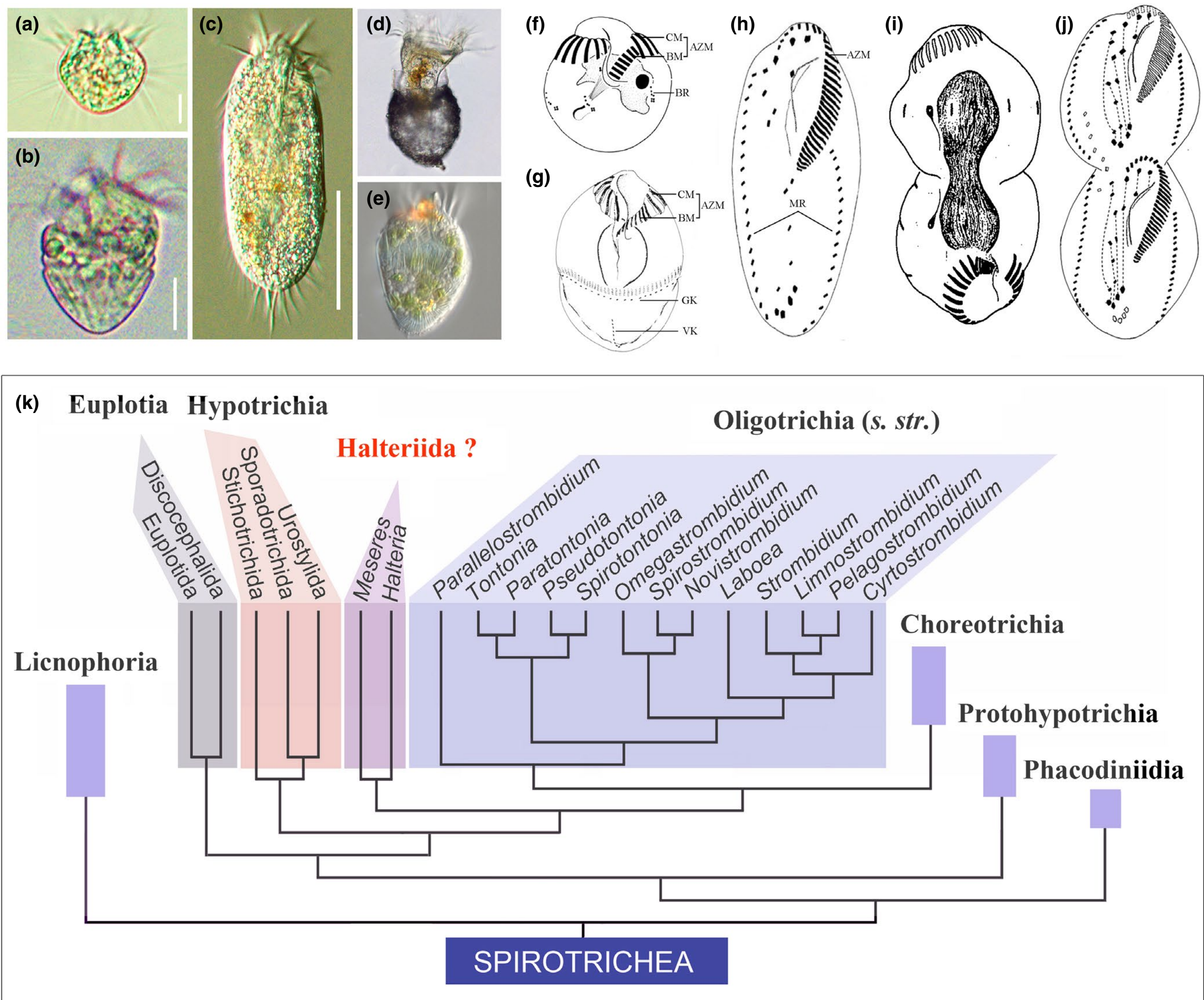


FIGURE 1 Morphology and ontogenesis of oligotrichs (sensu lato) and hypotrichs (a–j) and the relationships within the class Spirotrichea based on morphological data (k). (a, f, i), *Halteria grandinella*; b, (g), *Strombidium sulcatum*; (c, h, j), *Oxytricha trifallax*; (d), *Codonellopsis mobilis*, (e), *Strombidium* sp. (f) is from Agatha and Foissner (2009); (g) is from Song, Wang, and Warren (2000); (h and j) are from Jiang, Ma, and Shao (2013); (i) is from Song (1992). (k) is modified from Agatha (2004) and Foissner et al. (2007). The scale bar in (a), (b) and (c) represent 10μm, 20μm and 50μm, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

enriched to construct the library, which was sequenced for 125 bp at both ends (paired-end) using an Illumina HiSeq 2,500 sequencer (Novogene, Beijing, China).

2.3 | Assembly and gene annotation

The quality of reads was assessed using FastQC v.0.11.4 (Andrews, 2010), and the low-quality reads were removed with the FASTX-Toolkit for both the mass-culture and single-cell RNA-seq reads (acc. no. SRX2788015 and SRX2788016) with the parameters of -q 20 -p 80 (Gordon & Hannon, 2010). The rRNA reads were removed by mapping to *H. grandinella's* rRNA gene sequences (acc. no. AF508759) using Bowtie v.1.0.0 with the parameters of –a, –n 2 and –p 8 (Langmead, Trapnell, Pop, & Salzberg, 2009). The transcriptome was assembled de novo using Trinity v.2.2.0 (Grabherr et al., 2011) with default parameters. Redundancy of contigs was eliminated by CD-HIT v.4.6.1 (Fu, Niu, Zhu, Wu, & Li, 2012) with a 98% sequence identity threshold (-c 0.98, -n 10). The mitochondrial genomes of *Oxytricha trifallax* (acc. no. JN383843), *Tetrahymena pyriformis* (acc. no. AF160864), *Paramecium aurelia* (acc. no. NC001324), *Euplotes minuta* (acc. no. GQ903130) and *Nyctotherus ovalis* (acc. no. GU057832) as well as the bacterial nucleotide database were downloaded from GenBank as mapping references to remove contaminations of mitochondria or bacteria using blastn with the e-value of 1e-2. The regions of coding sequences (CDS) of *H. grandinella* were predicted using Augustus v.3.2.3 with *Tetrahymena thermophila* as model

species (Stanke, Diekhans, Baertsch, & Haussler, 2008) and predicted peptides longer than 50 amino acids were retained. The predicted peptides were annotated using InterProScan v.5.2–45.0 with the parameters of -iprlookup and -goterms (Jones et al., 2014). The Gene Ontology (GO) analyses were carried out with Web Gene Ontology Annotation Plotting v.2.0 (WEGO, http://wego.genomics.org.cn/). The gene clusters were performed by MCL v.14–137 with --abc and -I 1.5 (Enright, Van Dongen, & Ouzounis, 2002). Subsequently, the predicted peptides were blasted with the protein sequences of ciliates downloaded from NCBI and The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) to investigate the sequence similarity between *H. grandinella* and other ciliates (e-value: 1e-5). The orthologs among *H. grandinella*, *O. trifallax* and *Strombidium sulcatum* were detected via OrthoFinder v.2.2.7 with default parameters (Emms & Kelly, 2015), and the Venn diagram was generated by R v.3.4.0.

2.4 | Codon usage analysis

To analyse the amino acid codon usage of *H. grandinella*, the contigs from the mass-culture and single-cell-combined transcriptome data of *H. grandinella* were blasted against all the ciliate protein sequences in NCBI and MMETSP and the contigs with the e-value ≤ 1e-10, sequence identity ≥ 50% and alignment length ≥ 300 amino acids were retained for analyses. The codon and its corresponding amino acid were extracted by a custom Perl script. The codon usage of *S. sulcatum* was evaluated with the same method as *H. grandinella*. The codon usages of *O. trifallax*, *Stylonychia lemnae*, *Euplotes octocarinatus*, *Tetrahymena thermophila* and *Paramecium tetraurelia* were assessed using their coding sequences published in NCBI.

The coding regions of the ATP-dependent 26S proteasome regulatory subunit and actin II genes were amplified and sequenced according to Wang, Zhang, et al. (2017). The primers are 26S_regulator_F: 5’-ATG CCG TAA TTT TTG GTT AAT-3’, 26S_regulator_R: 5’-CCC TTA CTG ACA AGA GAG TTG-3’ and Actin_II-F: 5’-ATG CGT ATC AGT GAT AAA GC-3’, Actin_II-R: 5’-TCA AAT ATG CAG TTA GAA TAA TT-3’, positioned outside the coding regions.

2.5 | Phylogenomic analyses

For the phylogenomic analyses, except for the transcriptome of *H. grandinella*, 10 ciliate genomes, 30 ciliate transcriptomes and two *Plasmodium* genomes as outgroups were included. The sources of the omics data used in the present work are listed in Table S1.

Orthologs were detected employing the reciprocal BLAST hit (RBH) approach according to Chen et al. (2015). The predicted peptides of *H. grandinella* were blasted to the peptide dataset of other species by blastp with the parameters of e-value ≤ 1e-10, identity ≥ 30%, length ≥ 50 aa, and vice versa. Only the proteins present in more than 90% of the taxa (at least 39 different species of our 43 species) were retained for further analyses. The protein sequences were aligned using ClustalW v.2.1 with the bootstrap value of 1,000 (Sievers et al., 2011). The ambiguous alignment sites were masked using Gblocks v.0.91b (-b2 = 0.65, -b3 = 10, -b4 = 5, -b5 = a) (Castresana, 2000). A final dataset containing 47,263 amino acid sites from 132 proteins was used for maximum-likelihood (ML) and Bayesian inference (BI) analyses. The ML analysis was conducted in CIPRES Science Gateway with RAxML-HPC v.8 on XSEDE (Stamatakis, 2014) with the model of LG + F + Γ4 and 1,000 replicates. BI analysis was implemented by PhyloBayes v.4.1 (Lartillot, Lepage, & Blanquart, 2009) under a mixture model of CAT + POI + Γ4. Four Markov chain Monte Carlo (MCMC) chains were run for 1,000 generations. The bpcomp programme within PhyloBayes was applied to compare the discrepancy of bipartition frequencies and to yield a consensus tree (convergence Maxdiff = 0.00722892). The ML tree based on single gene was built in CIPRES Science Gateway using RAxML-HPC v.8 on XSEDE with the same parameters of the concatenated tree. MEGA v.6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) was utilized to visualize tree topologies.

As a comparison, phylogenetic analyses based on SSU rDNA sequences (accession numbers as in Figure 4b) were also performed according to Wang, Zhang, et al. (2017). Briefly, the 44 SSU rDNA sequences were aligned with the GUIDANCE Server (http://guidance.tau.ac.il/ver2/) with default parameters (Sela, Ashkenazy, Katoh, & Pupko, 2015) and then manually modified using BioEdit v.7.0.1 (Hall, 1999) to generate a matrix of 44 taxa with 1,669 nucleotide sites. The ML analysis was performed in CIPRES Science Gateway using RAxML-HPC2 on XSEDE v.8.1.24 with the model of GTR + I + G (Stamatakis, Hoover, & Rougemont, 2008). The reliability of internal branches was assessed with a non-parametric bootstrap method with 1,000 replicates. The BI tree was constructed using MrBayes on XSEDE v.3.2.6 tool in CIPRES Science Gateway with the model of GTR + I + G, which was selected by MrModeltest v.2.0 (Nylander, 2004). Markov chain Monte Carlo simulations were run with two sets of four chains for 6,000,000 generations with a sampling frequency of 100 generations, and the first 10% of generations were discarded as burn-in.

3 | RESULTS

3.1 | Comparisons between single-cell and culture-based datasets of *Halteria grandinella* transcriptome

The high-throughput RNA-seq data of *H. grandinella* from mass-culture (present study) and single-cell downloaded

from NCBI were assembled via Trinity (Table 1). For the RNA-seq data from mass-culture of *H. grandinella* in the current work, a total of 31,412,761 paired-end reads (125 bp for each read) were produced (7,853,190,250 bases, GC: 49%). After removing low-quality reads, 90.9% of reads were retained and assembled into 129,114 contigs (maximum length: 14,811 bp, N50: 1,159 bp) using Trinity. After removing the redundancy and contamination of bacteria, rRNA and mitochondria, 117,978 contigs were retained (Table 1).

Two datasets from single cell (single-cell 1 and single-cell 2) were analysed both separately and after combination. Compared with the data from the mass-culture, the single-cell transcriptome recovered much less contigs, and even the combined data did not exhibit much improvement (117,978 vs. 71,498 contigs). The N50 and maximum length of the mass-culture transcriptome are higher than those of single-cell transcriptomes. The number of predicted genes longer than 50 amino acids in the mass-culture is about two-fold higher than that in the single-cell-combined transcriptome (92,114 vs. 44,219). After CDS prediction, 21,782 and 12,007 peptide sequences were annotated in the mass-culture and single-cell transcriptomes, respectively (Figure 2d). In the annotation results, single-cell transcriptome has higher percentage of genes in cellular component and biological process, while mass-culture transcriptome data have higher proportion of genes in molecular function.

To determine whether the number of reads affects the assembly quality and size, we used the same number of reads (7,433,129 pair-end reads, which is all of the combined two single-cell RNA-seq reads and part of the mass-culture RNA-seq reads) with the same length (125 bp), and assembled them via Trinity (Table S2). Similarly, the N50 and maximum length of the mass-culture transcriptome are still higher than those of single-cell transcriptomes (1,234 vs. 833 bp). The number of predicted genes longer than 50 amino acids in the mass-culture is much higher than that in the single-cell-combined transcriptome (55,798 vs. 32,805).

The predicted protein sequences of the mass-culture transcriptome were binned into 47,158 protein clusters, more than that of the combined single-cell transcriptome (14,313). The largest protein cluster of the mass-culture transcriptome contained 1,790 proteins with a protein kinase function. For the peptides from both the mass-culture and single-cell-combined transcriptomes, 54,752 protein clusters were produced.

3.2 | Analysis of the mass-culture transcriptome

The culture-based dataset was analysed further to assess the content of the *H. grandinella* transcriptome. The Web Gene Ontology Annotation (WEGO) analyses of the annotated proteins mainly focused on cell and intracellular in cellular component, binding in molecular function and metabolic process in biological process.

The predicted peptides were first blasted with the protein sequences of eight other ciliate genomes to investigate

TABLE 1 The assembly comparison of *Halteria grandinella* between mass-culture and single-cell transcriptome data

	Mass-culture	Single-cell-1	Single-cell-2	Single-cell-combined
Assembly size (M)	97.3	33.4	24.5	41.8
Contigs (*n*)	129,114	53,874	50,021	75,270
N50 (bp)	1,159	746	558	658
Maximum length (bp)	14,811	5,524	3,349	5,710
Remove redundancy	119,845	51,631	48,664	72,325
Remove bacteria	118,041	51,149	48,120	71,532
Remove rDNA	117,993	51,136	48,105	71,512
Remove mitochondria	117,978	51,123	48,090	71,498
Predicted gene number (aa ≥ 50)	92,114	33,768	29,926	44,219
Predicted gene size (M)	61.0	19.8	14.3	23.6
GC (%)	48.4	44.5	45.6	44.7

Note: Mass-culture, the transcriptome data from mass cells obtained in the present work; Single-cell-1 and Single-cell-2, the transcriptome data from two cells using the single cell sequencing technology from Lynn and Kolisko, (2017). Single-cell-combined, merge the data of Single-cell-1 and Single-cell-2.

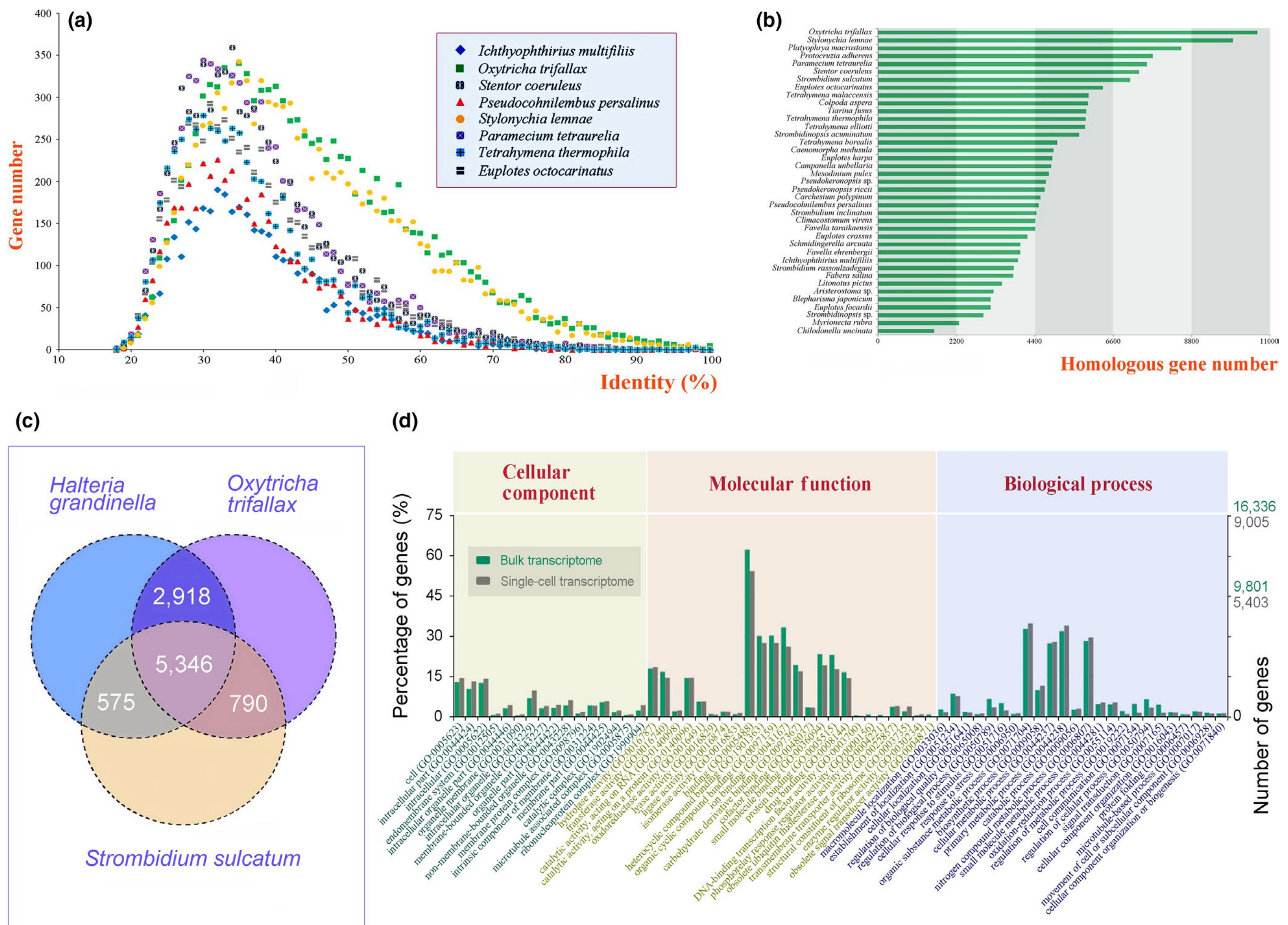


FIGURE 2 The analyses of *Halteria grandinella* transcriptome. (a), the distribution of the protein sequence identity between *H. grandinella* and eight ciliate genomes; (b), the homologous gene numbers between *H. grandinella* and other ciliates; (c), Venn diagram of orthologous gene groups among *H. grandinella*, *Strombidium sulcatum* and *Oxytricha trifallax*; (d), GO term enrichment analysis of the predicted genes in mass-culture and single-cell transcriptomes of *H. grandinella* [Colour figure can be viewed at wileyonlinelibrary.com]

sequence similarity (Figure 2a). The sequence identities between *H. grandinella* and the two hypotrichs *O. trifallax* and *S. lemnae* were higher than that with other genomes. The predicted peptides of *H. grandinella* were then blasted with the protein sequences of ciliates that are available for both genomes and transcriptomes downloaded from NCBI and MMETSP (Figure 2b). *O. trifallax* and *S. lemnae* are the foremost two species sharing homologous genes with *H. grandinella* in the amount of 10,645 and 9,964, respectively.

To gain further insights into the relationships among *H. grandinella*, oligotrichs and hypotrichs, the proteins predicted from the *H. grandinella* transcriptome were grouped with the transcriptome or genome of the oligotrich *S. sulcatum* and hypotrich *O. trifallax* using OrthoFinder (Figure 2c). *H. grandinella*, *O. trifallax* and *S. sulcatum* shared 5,346 ortholog groups. Except the orthologs shared by these three species, *H. grandinella* and *O. trifallax* shared 2,918 specific ortholog groups, far more than that shared specifically by *H. grandinella* and *S. sulcatum* (575).

3.3 | Codon usage in *Halteria grandinella*

To evaluate the amino acid codon usage of *H. grandinella*, the mass-culture transcriptome data of *H. grandinella* were blasted against all the ciliate protein sequences in NCBI and MMETSP, and the 6,141 contigs with sequence identity ≥ 50% and alignment length ≥ 300 amino acids were retained for analyses (Figure 3a, Table S3). Among the 3,105,110 amino acid codons of the 6,141 contigs, AAG and GAG were the two most common amino acid codons at a percentage of 5.55% and 4.66%, respectively. Leucine (L) had the highest proportion at 9.41% (292,248) among the 20 amino acids, and the least frequent amino acid was Tryptophan (W, 0.99%, 30,681). Compared with A/U-ending codons, G/C-ending codons were more frequently used for the 6,141 contigs (G/C-ending: 1,866,739 vs. A/U-ending: 1,238,251).

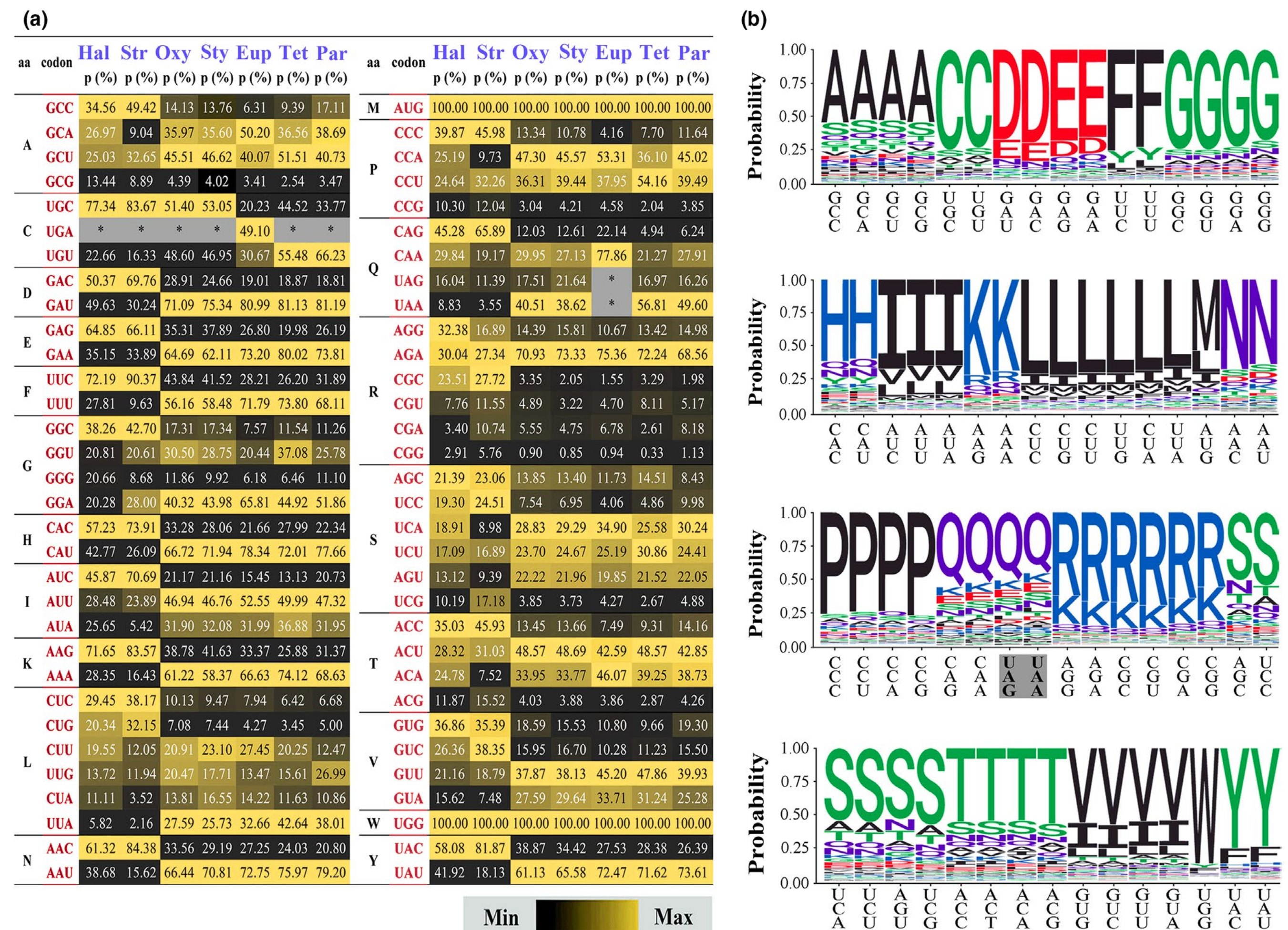
Among the amino acid codons in the coding regions, 14,447 UAA, 26,228 UAG and 120 UGA were detected

(Figure 3a). For both UAA and UAG, the highest frequency amino acid is Glutamine (Q) with at proportions of 45.55% and 50.93%, respectively (Figure 3b). The 120 UGA and the very low frequency (<5%) amino acids (except for Glutamine) encoded by UAA and UAG may be subject to noise. Besides, we sequenced the macronuclear sequences of ATP-dependent 26S proteasome regulatory subunit gene and actin II gene. In these two genes, there are UAA and UAG positioned within the coding regions (mostly corresponding to Glutamine), while UGA is used as a stop codon (Figure S1).

There is an obvious usage bias among the synonymous codons in our results (Figure 3a). For example, for the four amino acid codons encoding for Glutamine, UAA and UAG have lower percentage compared with CAG and CAA, especially UAA that encodes only 8.83% with Glutamine. The codon usage of single-cell-combined transcriptome has similar tendency with that of mass-culture data, although has a few differences (Table S3). Amino acid codon usage of *S. sulcatum*, *O. trifallax*, *S. lemnae*, *E. octocarinatus*, *T. thermophila* and *P. tetraurelia* was also analysed and compared. Interestingly, for the 18 amino acids that have synonymous codons, the codon usage bias of *H. grandinella* is much more similar to that of *S. sulcatum*, while the bias of the other five species are more akin to each other, despite not being phylogenetically related to each other.

3.4 | Phylogenomic analyses

We used the RBH approach to identify orthologs between *H. grandinella* and the other 42 species (40 ciliates and two *Plasmodium* species as outgroups). A final dataset containing 132 proteins with 47,263 amino acids from 43 taxa was used to conduct phylogenomic analyses with both algorithms of maximum likelihood (ML) and Bayesian inference (BI). The concatenated trees of ML and BI algorithms shared a similar topology; therefore, only the ML tree was shown in Figure 4a with the support values from both ML and BI methods.



(a)

aa	codon	Hal p (%)	Str p (%)	Oxy p (%)	Sty p (%)	Eup p (%)	Tet p (%)	Par p (%)
A	GCC	34.56	49.42	14.13	13.76	6.31	9.39	17.11
	GCA	26.97	9.04	35.97	35.60	50.20	36.56	38.69
	GCU	25.03	32.65	45.51	46.62	40.07	51.51	40.73
	GCG	13.44	8.89	4.39	4.02	3.41	2.54	3.47
C	UGC	77.34	83.67	51.40	53.05	20.23	44.52	33.77
	UGA	*	*	*	*	49.10	*	*
	UGU	22.66	16.33	48.60	46.95	30.67	55.48	66.23
D	GAC	50.37	69.76	28.91	24.66	19.01	18.87	18.81
	GAU	49.63	30.24	71.09	75.34	80.99	81.13	81.19
E	GAG	64.85	66.11	35.31	37.89	26.80	19.98	26.19
	GAA	35.15	33.89	64.69	62.11	73.20	80.02	73.81
F	UUC	72.19	90.37	43.84	41.52	28.21	26.20	31.89
	UUU	27.81	9.63	56.16	58.48	71.79	73.80	68.11
G	GGC	38.26	42.70	17.31	17.34	7.57	11.54	11.26
	GGU	20.81	20.61	30.50	28.75	20.44	37.08	25.78
	GGG	20.66	8.68	11.86	9.92	6.18	6.46	11.10
	GGA	20.28	28.00	40.32	43.98	65.81	44.92	51.86
H	CAC	57.23	73.91	33.28	28.06	21.66	27.99	22.34
	CAU	42.77	26.09	66.72	71.94	78.34	72.01	77.66
I	AUC	45.87	70.69	21.17	21.16	15.45	13.13	20.73
	AUU	28.48	23.89	46.94	46.76	52.55	49.99	47.32
	AUA	25.65	5.42	31.90	32.08	31.99	36.88	31.95
K	AAG	71.65	83.57	38.78	41.63	33.37	25.88	31.37
	AAA	28.35	16.43	61.22	58.37	66.63	74.12	68.63
L	CUC	29.45	38.17	10.13	9.47	7.94	6.42	6.68
	CUG	20.34	32.15	7.08	7.44	4.27	3.45	5.00
	CUU	19.55	12.05	20.91	23.10	27.45	20.25	12.47
	UUG	13.72	11.94	20.47	17.71	13.47	15.61	26.99
	CUA	11.11	3.52	13.81	16.55	14.22	11.63	10.86
	UUA	5.82	2.16	27.59	25.73	32.66	42.64	38.01
N	AAC	61.32	84.38	33.56	29.19	27.25	24.03	20.80
	AAU	38.68	15.62	66.44	70.81	72.75	75.97	79.20

aa	codon	Hal p (%)	Str p (%)	Oxy p (%)	Sty p (%)	Eup p (%)	Tet p (%)	Par p (%)
M	AUG	100.00	100.00	100.00	100.00	100.00	100.00	100.00
P	CCC	39.87	45.98	13.34	10.78	4.16	7.70	11.64
	CCA	25.19	9.73	47.30	45.57	53.31	36.10	45.02
	CCU	24.64	32.26	36.31	39.44	37.95	54.16	39.49
	CCG	10.30	12.04	3.04	4.21	4.58	2.04	3.85
Q	CAG	45.28	65.89	12.03	12.61	22.14	4.94	6.24
	CAA	29.84	19.17	29.95	27.13	77.86	21.27	27.91
	UAG	16.04	11.39	17.51	21.64	*	16.97	16.26
	UAA	8.83	3.55	40.51	38.62	*	56.81	49.60
R	AGG	32.38	16.89	14.39	15.81	10.67	13.42	14.98
	AGA	30.04	27.34	70.93	73.33	75.36	72.24	68.56
	CGC	23.51	27.72	3.35	2.05	1.55	3.29	1.98
	CGU	7.76	11.55	4.89	3.22	4.70	8.11	5.17
	CGA	3.40	10.74	5.55	4.75	6.78	2.61	8.18
	CGG	2.91	5.76	0.90	0.85	0.94	0.33	1.13
S	AGC	21.39	23.06	13.85	13.40	11.73	14.51	8.43
	UCC	19.30	24.51	7.54	6.95	4.06	4.86	9.98
	UCA	18.91	8.98	28.83	29.29	34.90	25.58	30.24
	UCU	17.09	16.89	23.70	24.67	25.19	30.86	24.41
	AGU	13.12	9.39	22.22	21.96	19.85	21.52	22.05
	UCG	10.19	17.18	3.85	3.73	4.27	2.67	4.88
T	ACC	35.03	45.93	13.45	13.66	7.49	9.31	14.16
	ACU	28.32	31.03	48.57	48.69	42.59	48.57	42.85
	ACA	24.78	7.52	33.95	33.77	46.07	39.25	38.73
	ACG	11.87	15.52	4.03	3.88	3.86	2.87	4.26
V	GUG	36.86	35.39	18.59	15.53	10.80	9.66	19.30
	GUC	26.36	38.35	15.95	16.70	10.28	11.23	15.50
	GUU	21.16	18.79	37.87	38.13	45.20	47.86	39.93
	GUA	15.62	7.48	27.59	29.64	33.71	31.24	25.28
W	UGG	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Y	UAC	58.08	81.87	38.87	34.42	27.53	28.38	26.39
	UAU	41.92	18.13	61.13	65.58	72.47	71.62	73.61

FIGURE 3 The amino acid and codon usage of *Halteria grandinella*. (a), the codon usage bias of *H. grandinella*. The percentage of synonymous codons is indicated by gradient colours filled from black (minimum) to yellow (maximum). (b), the percentage of the predicted amino acids encoded by UAA and UAG. Hal, *Halteria grandinella*; Str. *Strombidium sulcatum*; Oxy, *Oxytricha trifallax*; Sty, *Stylonychia lemnae*; Eup, *Euplotes octocarinatus;* Tet, *Tetrahymena thermophila*; Par, *Paramecium tetraurelia* [Colour figure can be viewed at wileyonlinelibrary.com]

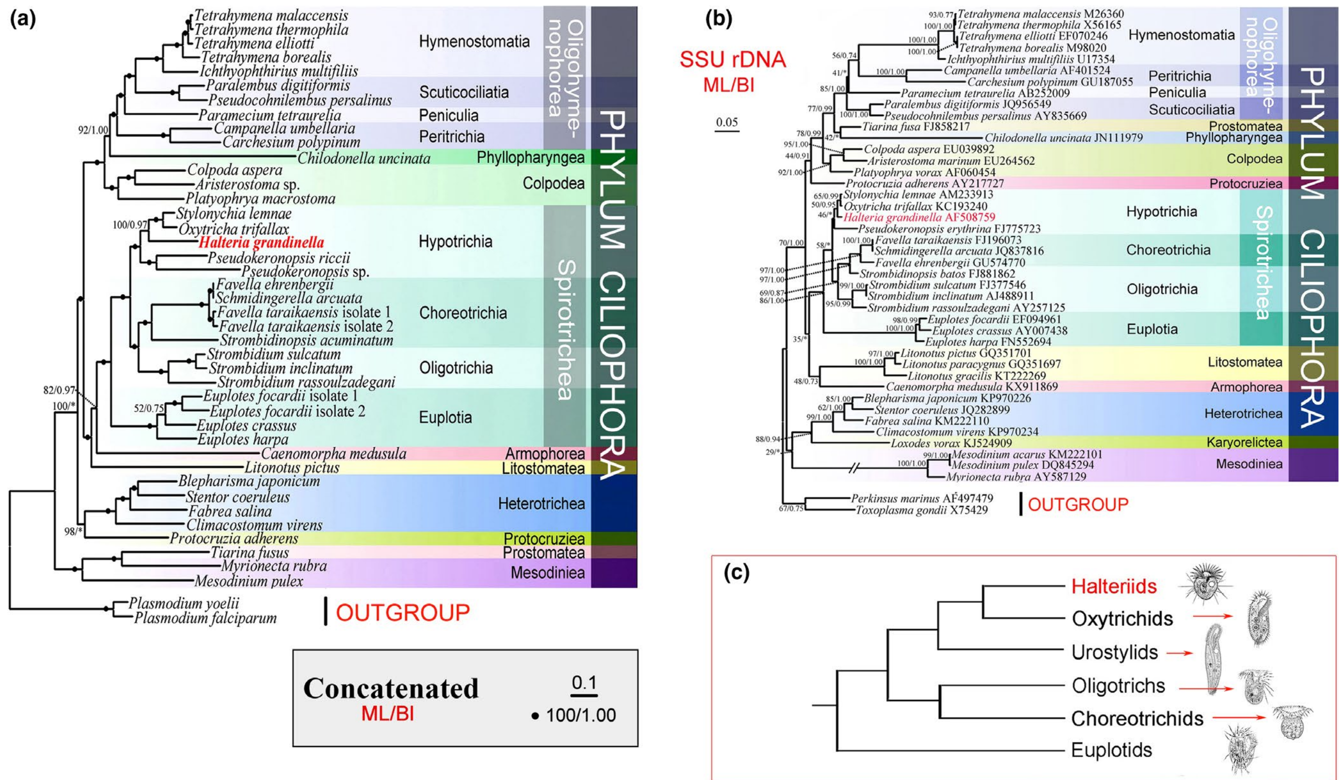


FIGURE 4 Maximum-likelihood trees based on a concatenated alignment of 132 proteins (a) and SSU rDNA sequences (b). Numbers at each node are ML bootstrap support and Bayesian posterior probability, respectively. The black dots indicate the full bootstrap support in both ML and BI trees. The scale bar corresponds to ten and five substitutions per one hundred nucleotide positions in concatenated and SSU rDNA trees, respectively. (c), the hypothesized evolution relationships of the core groups in the class Spirotrichea based on the current phylogenetic reconstruction [Colour figure can be viewed at wileyonlinelibrary.com]

In the concatenated trees, the class Spirotrichea is monophyletic and clusters with the classes Armophorea and Litostomatea. Within Spirotrichea, the subclass Euplotia is placed as an early branching lineage. The subclasses Choreotrichia and Oligotrichia form a fully supported clade, which then groups with Hypotrichia with full support. *H. grandinella* is positioned within Hypotrichia by clustering with *O. trifallax* and *S. lemnae* with strong support (100% ML, 0.97 BI), which is consistent with the SSU rDNA trees (Figure 4b). Among the 132 single-gene trees, *H. grandinella* clusters within Hypotrichia in 74 trees while it groups with oligotrichs in only six trees (data not shown).

4 | DISCUSSION

4.1 | Comparison between mass-culture and single-cell transcriptomes

The single-cell transcriptome recovered much less genes compared with the data from the mass-culture. The previously reported transcriptomes of *H. grandinella* are sequenced from two cells using single-cell sequencing techniques (Lynn & Kolisko, 2017). The assembly size, contig numbers, N50 and predicted genes are much less than those of the mass-culture transcriptome. The first explanation is that the genes expressed in single cell at one time could be less than those expressed in mass-culture. The second explanation might be the PCR bias during cDNA synthesis and amplification (Gawad, Koh, & Quake, 2016; Zhang et al., 2006; Zong, Lu, Chapman, & Xie, 2012). Amplification bias can result in gene loss with the coverage from about 30% to 90% (Gawad et al., 2016; Hou et al., 2015; Huang, Ma, Chapman, Lu, & Xie, 2015; Kolisko, Boscaro, Burki, Lynn, & Keeling, 2014; Lao, Xu, & Straus, 2008; Zhang et al., 2006, 1992; Zong et al., 2012). Besides, the PCR amplification can cause fragmentation of the assembled contigs (X. Chen, Wang, et al., 2018; Zong et al., 2012). However, despite the fact that gene recovery of single-cell sequencing techniques is less efficient than those of the mass-culture method, it is obviously much more efficient than traditional cloning and sequencing to recover transcripts in isolated cells (De Bourcy et al., 2014; Tang et al., 2009; Wu et al., 2014). Moreover, the transcriptome assembled by combining the reads of single-cell-1 and single-cell-2 can recover more genes and has longer N50, which indicates that we can increase the number of parallel samples to obtain more data when using single-cell sequencing techniques.

For GO annotation, mass-cultured and single-cell transcriptomes have some differences, for example single-cell transcriptome recovered higher percentage of genes in cellular component and biological process while mass-culture transcriptome data recovered higher proportion of genes in molecular function (Figure 2d). This might be because that transcriptomes from mass-culture represent the average stage of the whole population, while single-cell datasets reflect living conditions of individual cells, which might be in different living conditions or cell process. Besides, it is possible that only the majority of highly expressed genes are amplified by single-cell transcriptomes, while some lowly expressed genes are not represented in the data due to the limitation of single-cell sequencing techniques.

4.2 | The codon bias and stop codon reassignment of *Halteria grandinella*

Synonymous codons are used with different frequencies in many organisms, known as codon bias, which can reflect a balance between selection, mutation and genetic drift (Bulmer, 1991; Shah & Gilchrist, 2011). The codon bias in *H. grandinella* is more similar with *S. sulcatum* compared with *O. trifallax*, *S. lemnae*, *E. octocarinatus*, *P. tetraurelia* and *T. thermophila* (Figure 3a). Previous studies reported that amino acid codon usage was driven by GC content (Knight, Freeland, & Landweber, 2001). The GC content of the coding region in *H. grandinella*, *S. sulcatum*, *O. trifallax*, *S. lemnae*, *E. octocarinatus*, *P. tetraurelia* and *T. thermophila* is 48%, 52%, 31%, 33%, 32%, 28% and 22%, respectively. The similarity of GC content between *H. grandinella* and *S. sulcatum* might explain why their codon bias resembles each other more faithfully.

The standard stop codons in *H. grandinella* are reassigned (UGA as stop codon while UAA and UAG encoding Glutamine), which is consistent with other hypotrichs and oligotrichs (Swart, Serra, Petroni, & Nowacki, 2016). In most eukaryotes, UAA, UAG and UGA are standard stop codons and do not encode amino acid (Knight et al., 2001; Ling, Patrick, & Dieter, 2015). However, in ciliates, the reassignments of standard stop codons UAA, UAG and UGA to amino acids are detected frequently, which show high divergence and evolve independently in various ciliate lineages (Eliseev, Kryuchkova, Alkalaeva, & Frolova, 2010; Lozupone, Knight, & Landweber, 2001; Swart et al., 2016). For example, in *O. trifallax*, *Strombidium inclinatum* (Cl: Spirotrichea) and *T. thermophila* (Cl: Oligohymenophorea), UAA and UAG encode Glutamine whereas UGA is stop codon (Horowitz & Gorovsky, 1985; Swart et al., 2016); in *Euplotes* (Cl: Spirotrichea), UAA and UAG are stop codons whereas UGA encode Cysteine (Meyer & Heckmann, 1991); in *Blepharisma japonicum* (Cl: Heterotrichea), UAA and UAG are stop codon and UGA encodes Tryptophan (Lozupone et al., 2001; Swart et al., 2016); in *Condylostoma magnum*, UAA, UAG and UGA can either encode amino acid (UAA and UAG encode Glutamine, UGA encode Tryptophan) or be used as stop codons in a context-dependent manner (Swart et al., 2016). The stop codons for UAA, UAG and UGA are decoded by the N-terminal domain of eukaryote class-1 polypeptide release factor (eRF1) (Eliseev et al., 2010; H. Song, Mugnier, et al., 2000; Swart et al., 2016). In some ciliates, the features of the eRF1 protein molecule are changed and can recognize only one or two stop codons (Eliseev et al., 2010; Lekomtsev et al., 2007; Seit-Nebi et al., 2002).

4.3 | The systematic position of *Halteria grandinella*

In the concatenated phylogenomic trees, *H. grandinella* falls within the subclass Hypotrichia by clustering with oxytrichids *O. trifallax* and *S. lemnae* (Figure 4a), which is consistent with the topologies inferred from SSU rDNA, internal transcribed spacers (ITS), 5.8S rDNA, LSU rDNA, α-tubulin and actin I genes (Gao et al., 2016; Hu et al., 2011; Lynn & Sogin, 1988; Paiva et al., 2009). The multigene tree in Lynn and Kolisko (2017) based on the single-cell transcriptome of *H. grandinella* revealed similar topology to our concatenated trees. Moreover, *H. grandinella* has more orthologous genes with the oxytrichid *O. trifallax* than the oligotrich *S. sulcatum* (Figure 2c). All these analyses support that *H. grandinella* has a closer relationship with hypotrichs than oligotrichs.

As discussed in Lynn and Kolisko (2017), it is reasonable to hypothesize that the similar morphology (e.g. globular body shape, apical adoral membranelles and reduced somatic ciliature) of halteriids and oligotrichs might be evolution-adaptation to the planktonic lifestyle, and their similar enantiotropic cell division is evolved to provide enough locomotive force to separate the dividing cells using the developing oral ciliature instead of reduced somatic ciliature (Foissner et al., 2007; Lynn & Kolisko, 2017). Besides, we concur that the long cilia of halteriids, also called jumping bristles, might be specialized cirri or homologous to the cirri in hypotrichs. Notably, all the species in the hypotrich genus *Hypotrichidium* Ilowaisky, 1921 have several spiral cirral rows, which can be considered as an intermediate stage between halteriids and traditional hypotrichs (Chen, Liu, Liu, Al-Rasheid, & Shao, 2013). Moreover, some characteristics (e.g. both paroral and endoral membranes present, stomatogenesis epiapokinetal, somatic ciliature originates de novo) can also indicate a closer relationship between halteriids and hypotrichs (Chen, Wu, El-Serehy, Hu, & Clamp, 2018; Lu et al., 2018; Luo et al., 2017; Petz & Foissner, 1992; Song, 1992).

Hence, our results support the opinion that *H. grandinella* might be an "oligotrich-like" hypotrich. Based on the morphological and phylogenetic data, the evolutionary relationship of the core spirotrichs is hypothesized as follows: the

ancestor of euplotids was firstly isolated from the ancestor of other spirotrichs, which then divided into two groups: one was the ancestor of oligotrichs and choreotrichs, and the other was the ancestor of hypotrichs; within hypotrichs, the ancestor of urostylids and/or stichotrichids separated firstly, while halteriids and oxytrichids shared the most common ancestor (Figure 4c). Nonetheless, the hypothesis here needs to be tested in the future, especially that many taxa are still missing. We believe that with the genomic/transcriptomic data of more and more taxa being available, incorporated analyses of phylogenomic and morphological/morphogenetic results will depict a clearer evolutionary story.

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