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Molecular Phylogeny and Species Delimitation of Heterotrich Ciliates (Alevolata, Ciliophora, Heterotrichea)

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Ciliates (Ciliophora) are single-celled heterotrophic eukaryotic group of protozoans that emerged more than one billion years ago, and characterized by the hair-like organelles called cilia, and sexual reproduction of conjugation, and having dimorphic nuclei called macronuclei and micronuclei presenting unique genetic phenomena. They are widely distributed in diverse aquatic and terrestrial habitats, regarded as important trophic linkers in food webs of many ecosystems. Ciliates are currently divided into two subphyla and 11 classes, among these, heterotrich ciliates, i.e., class Heterotrichea, are characterized by somatic dikinetids bearing postciliodesmata, oral apparatus consisting of a paroral membrane and an adoral zone of membranelles, as well as features of nuclear division involving extramacronuclear microtubules. Although phylogenetic relationships among heterotrichs have been analysed previously multiple times, but deeper nodes of the tree remain poorly resolved. To resolve the phylogenetic relationship of heterotrichs, we have accumulated morphological data since Lee & Shin (2009), and acquired the nucleotide sequences of small-sized genetic markers (18S, ITS1-5.8S-ITS2 and 28S rRNAs genes) based on Sanger sequencing. By utilizing these data, we performed a family-level phylogenetic study with establishing two new families and reorganizing the widely used scheme of Lynn (2009). This study has been received attention and accepted by the community (Adl et al., 2019). After this study, several investigators repeated phylogenetic study of heterotrichs. However, the instability of some high-level lineages was not resolved. To resolve it, we have adopted a transcriptome (next-generation sequencing)-based study. Consequently, most nodes in the phylogenetic tree were relatively stable. However, family-level relationships using small-sized genetic markers and transcriptome sequence data are inconsistent and are still resolved partially. Therefore, a more comprehensive study for the phylogeny and evolution of heterotrichs will be needed in future. Moreover, among the heterotrichs, we are interested in the species of the genus *Spirostomum* because they occur at high frequencies in natural environments and are commonly used as biological indicators for assessing water quality and serve as model organisms for applied ecology and symbiosis research. However, some *Spirostomum* species may still be difficult to be identified due to their morphological plasticity and the existence of cryptic species. The delimitation of the species in *Spirostomum* congeners has been started by us (Shazib et al., 2016) by using both primary and secondary gene sequence-structure. In this study, we have focused on the secondary structure of the ITS2 molecule. Our analyses of the ITS region revealed a complex genealogical structure within the genus *Spirostomum*. However, boundaries among *Spirostomum* species could not be unambiguously determined. Therefore, we continued further research on this topic, by adding two more marker genes of CO1 and α-tubulin and applying Bayseian coalescent method. As a result (Shazib et al., 2019), the species complex of *Spirostomum minus* might have a different origin with *S. teres*, and tentatively concluded that *S. teres* had ancestral traits, suggesting that *S. yagiui* and *S. dharwarensis* were derived from *S. teres*. However, *Spirostomum minus* and *S. teres* have different origins, and it is suggested that more comprehensive data should be supplemented to solve the problem. On that account, we have performed transcriptome analysis of 24 populations of six morphospecies of *Spirostomum*. Phylogenomic trees have been reconstructed by using the curated gene families, and the transcripts diversity was estimated by using their paralogs present in the gene trees. We have assessed the genetic structure of species by analyzing ~1,000 gene trees for the species boundaries and evolutionary relationships among *Spirostomum* species using transcriptome data. By applying this state-of-art approach may unambiguously resolve existing taxonomic problems and suggests a new horizon of phylogenetics to understanding biological species.

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