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Morphology and molecular phylogeny of *Holostichides terrae* nov. spec. (Ciliophora: Spirotrichea) with discussion on the possible non-monophyly of *Holostichides*

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Abstract

In a study to investigate ciliate diversity, we discovered a new soil ciliate. *Holostichides terrae* nov. spec. was examined and identified based on observations of living cells and stained specimens. In addition, the nuclear SSU rRNA gene along with morphology was analyzed to infer its phylogenetic position. The new species closely resembles *H. dumonti*, but can be distinguished by the morphology of the pharynx (with rod-shaped structure vs. lacking) and the number of frontoterminal cirri (invariably two vs. usually more than two). Molecular analyses indicate that the genus *Holostichides* is not monophyletic, and *H. terrae* is closely related with the genera *Birojimia* and *Hemicycliostyla*, both of which have a pharynx with rod-shaped structures, as also seen in *H. terrae*.

Keywords: Korea; New species; SSU rRNA gene; Terrestrial ciliate

Introduction

The genus *Holostichides* Foissner, 1987 occurs in terrestrial or semi-terrestrial habitats and comprises four species, including a recent one (Berger 2006; Kim et al. 2017): *H. chardezi* Foissner, 1987 (type species); *H. dumonti* Foissner, 2000; *H. heterotypicus* Kim et al., 2017; and *H. typicus* (Song and Wilbert, 1988) Eigner, 1994. Based on the midventral complex comprising midventral pairs and midventral row(s) as an autapomorphy, Berger (2006) assigned the genus to the Bakuellidae Jankowski, 1979.

Recently, Kim et al. (2017) described *H. heterotypicus* and provided its SSU rDNA sequence, which is the only genetic data available from GenBank for this genus. Their gene tree indicated *H. heterotypicus* is not a member of Bakuellidae, but rather clusters with *Extraholosticha sylvatica*. In addition, they emphasized that presence of caudal cirri in combination with absence of transverse cirri might be distinct features of bakuellids.

During the examination of ciliate diversity in South Korea, we isolated a *Holostichides* morphotype from a moss sample on a mountain, which was identified as a new species based on morphological and molecular attributes. Herein, we report its morphology along with SSU rRNA gene tree and discuss its phylogenetic position with focus on the non-monophyly of this genus.

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Material and Methods

Sample collection and identification

Holostichides terrae was discovered in a moss sample taken from Sogeumgang (valley) of Odaesan National Park, S. Korea, in April 2017. The sample was air-dried for two months and rewetted with mineral water to induce excystment using the non-flooded Petri dish method (Foissner et al. 2002). A raw culture was maintained for morphometry and DNA analyses. We have checked all hypotrichs in the culture and did not find any species that could be misidentified as *H. terrae*. We analyzed SSU rDNA sequences of four individuals and they were completely identical.

Living cells were observed under a stereomicroscope (SZ11, Olympus, Japan) and light microscope (BX53, Olympus; inverted microscope Eclipse Ti-U, Nikon, Japan) using bright field and differential interference contrast (DIC) at magnifications of 50–1000×. The cells were then impregnated with acetone developer and protargol ('Procedure A' of Foissner 2014). The protargol powder was manually synthesized, following the method prescribed by Pan et al. (2013) with slight modification (Kim and Jung 2017). General terminology follows Lynn (2008), specific terms for urostylids follow Berger (2006), and the type of buccal lips follows Foissner and Al-Rasheid (2006).

DNA extraction, PCR amplification, sequencing

Four cells were isolated from the raw culture and each cell was transferred to a 1.5 mL tube with distilled water using a micro-capillary. Genomic DNA of the cells was extracted using a RED-Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. The conditions for PCR were as follows: denaturation at 94 °C for 1 min 30 s, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 58.5 °C for 30 s, and extension at 72 °C for 3 min, and a final extension step at 72 °C for 7 min. The PCR product that nearly covered the entire SSU rRNA gene was amplified using slightly modified versions of two primers (New Euk A and LSU rev4) that were described by Sonnenberg et al. (2007). After amplification, the PCR products were purified using a MEGAquick-spin Total Fragment DNA Purification Kit (iNtRON, Korea), and DNA sequencing was performed using two internal primers (18SF790v2: 5′-AAA TTA KAG TGT TYM ARG CAG-3′ and 18SR300: 5′-CAT GGT AGT CCA ATA CAC TAC-3′) and an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). We compared the four partial sequences (approximately 800 bp) of the 3′ end of the SSU rRNA gene obtained from the New Euk A primer, and they showed completely identical sequences. Next, one of the four cells was fully sequenced to acquire the entire SSU rRNA gene. Therefore, only one sequence was deposited in GenBank.

Phylogenetic analysis

To infer the phylogenetic position of the new species, SSU rRNA gene sequences of 132 ciliates were retrieved from the NCBI database, including 130 hypotrichs and two oligotrichs, *Novistrombidium orientale* (FJ422988) and *Strombidium stylifer* (DQ631805), as outgroups. The sequences were aligned using Muscle alignment (Edgar 2004) in Geneious 9.1.6 (Kearse et al. 2012), and both ends of the alignment were manually trimmed using Geneious. The best-fit model of substitution for phylogenetic analysis was selected using jModelTest 2.1.10 (Darriba et al. 2012). We selected the model TIM2 + I (0.5080) + G (0.4630) based on the Akaike information criterion (AIC). IQ-TREE 1.5.3 was used to render maximum likelihood (ML) trees, with 1000 bootstrap replicates (Nguyen et al. 2015). A consensus ML tree was annotated using ggtree in R (Yu et al. 2017). Pairwise distances were calculated using Mega 5.2.2 (Tamura et al. 2011).

Tree topology test

We performed the approximately unbiased (AU) test on the trees (i.e. topologically constrained or unconstrained) to assess statistical significance of the topological constraints (see below) using CONSEL ver. 0.20 (Shimodaira and Hasegawa 2001). The trees were inferred in PAUP* v4.0b10 (Swofford 2003) using the ML criterion and a heuristic search with TBR branch swapping and 10 random sequence addition replicates. PAUP* was used to calculate per-site log likelihoods of the best and constrained ML trees under the best-fit model. The following constraints were statistically tested: (1) monophyly of the genus *Holostichides* (*H. heterotypicus* and *H. terrae*); and (2) monophyly of the species with rod-shaped structures in the pharynx (*Birojimia soyaensis*, *Hemicycliostyla franzi*, *H. sphagni*, and *Holostichides terrae*).

Results and Discussion

Holostichides terrae nov. spec. (Figs. 1A–J, 2A–F, 3A–F, Fig. 4, Table 1)

Diagnosis

Body size 150–200 × 30–50 μm in vivo, slender to elongate elliptical shape, posterior body end narrower than anterior end; cortex flexible but acontractile. Nuclear apparatus composed of 37–75 macronuclear nodules and 3–10 micronuclei. Contractile vacuole with distinct collecting canals. Cortical granules distributed along cirri and dorsal kineties, colorless, 2.0–2.5 × 1.0 μm in vivo, circular to elliptical in top view, elongated elliptical to rod-shaped in lateral view; 33–43 adoral membranelles, undulating membranes in *Oxytricha* pattern; pharynx with conspicuous rod-shaped