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Morphology, ontogenesis, and molecular phylogeny of an Indian population of *Cyrtohymena* (*Cyrtohymenides*) *shii*, including remarks on the subgenus

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Abstract

The freshwater ciliate *Cyrtohymena* (*Cyrtohymenides*) *shii* (Shi et al., 1997) Shao et al., 2012 (Hypotricha, Oxytrichidae), isolated from Barsey Rhododendron Sanctuary of The Eastern Himalayas, is slightly flexible, measures about 150 μm × 50 μm in life and possesses citrine cortical granules randomly distributed singly and in small clusters. Cells of our Indian population have five or six dorsal kineties arising from multiple fragmentation of the third dorsal anlage. The subgenus *Cyrtohymenides* includes species with multiple dorsal kinety fragmentation namely *C.* (*C.*) *aspoecki* (type species), *C.* (*C.*) *australis*, and the present species. Ventral morphogenesis of the genus *Cyrtohymena* has been reported only for the type species *C. muscorum*. Notable features of the Indian population include formation of frontal anlagen from four parental cirri, two more parental cirri possibly contribute to these anlagen later, and the formation of primary primordia which later split transversely to form two sets, one for each daughter cell. 18S rDNA sequence of the Indian population matches with those of two populations of *C. citrina*; it also clusters with *Afrokeronopsis aurea*, a neokeronopsid, with which it interestingly shares some morphological features, supporting the CEUU hypothesis.

Keywords: *Cyrtohymena* (*Cyrtohymenides*) *shii*; Hypotricha; India; Morphogenesis; Phylogeny; 18S rDNA

Introduction

The oxytrichid genus *Cyrtohymena* Foissner, 1989 is well characterized by its sole autapomorphy, namely, the distinctive hook-shaped arrangement of the paroral and deep, transparent buccal cavity in an 18 frontal–ventral–transverse oxytrichid body architecture. So far 17 species belonging to this genus have been reported (Berger 1999; Foissner 2004; Shao et al. 2012; Shi et al. 1997) with *C. muscorum* as the type species. Foissner (2004) and Shao et al. (2012) reassigned species with multiple dorsal kinety fragmentation, namely *C. australis* Foissner, 1995, *C. aspoecki* Foissner, 2004, and *C. shii* Shi et al., 1997 to the subgenus *Cyrtohymena* (*Cyrtohymenides*) Foissner, 2004.

In the first-ever survey of the ciliate fauna of The Eastern Himalayas, a population matching *C.* (*C.*) *shii* was found from a water catchment area at the entry point of the Barsey Rhododendron Sanctuary. The Indian population has been investigated because of its distinct morphological and morphogenetic traits erstwhile not known or only partially known for the genus. Additionally, 18S rDNA sequence of the population has been used for studying phylogenetic relationships.

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Material and Methods

Collection and cell cultures

Samples were collected in January 2010 from a water catchment area close to the entry point of the Barsey Rhododendron Sanctuary (27°15′ to 27°27′N; 88°01′ to 88°23′E), Sikkim, India and were examined to record the presence of ciliate fauna. *Cyrtohymena* (*Cyrtohymenides*) *shii* was found in the water soil interface. Encysted cells, present in the peripheral soil of the water body, could be easily excysted by the non-flooded petridish method (Foissner 1987). Other ciliates observed in the samples from the same water body were *Oxytricha longigranulosa*, *Pattersoniella vitiphila*, *Stylonychia ammermanni*, *Sterkiella histriomuscorum*, *S. cavicola*, *Frontonia* sp., *Colpoda* sp., *Paramecium caudatum* and *Tetrahymena* sp. Soil from the soil water interface was acidic (pH 5.7) with high organic carbon (2.6%). Clonal cultures of *C.* (*C.*) *shii* were grown in Pringsheim medium with the green alga *Chlorogonium elongatum* as the food organism (Ammermann et al. 1974) at optimum growth temperature of 18 ± 2 °C (s).

Morphological and morphogenetic analyses

Observations of live cells were made using differential interference contrast microscopy. Protargol staining was used for visualizing surface ciliature (Kamra and Sapra 1990). Observations on nuclei were made from Feulgen stained cells (Chieco and Derenzini 1999; Feulgen 1914). Biometric characterization was done at a magnification of 1000× directly from the Leica software IM50 image manager. Leica camera DFC320 was used for photography. Line diagrams were prepared using CorelDRAW(R) Graphics Suite-Version 12.0 software. To illustrate the changes during division morphogenesis, parental cirri are depicted by contour whereas new ones are filled in.

Classification is according to Foissner (1989), Berger (1999), and Lynn (2008). Terminology is according to Borror (1972), Martin (1982), and Wallengren (1900).

Genomic DNA isolation, amplification of 18S rDNA, and sequencing

Genomic DNA was extracted as per the protocol for cultured animal cells using DNeasy Blood and Tissue Kit (QIAGEN GmbH, Hilden, Germany). 18S rDNA was amplified by the universal eukaryotic primers EukA and EukB (Medlin et al. 1988). The protocol for amplification reaction was according to Foissner and Stoeck (2011). The resulting PCR product was purified with the QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and sequenced directly through ABI 3730 automated sequencer using the two universal forward (5′-AACCTGGTTGATCCTGCCAGT-3′) and reverse (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) primers and additionally three internal primers: two forward (5′-ATTGGAGGGCAAGTCTGGTG-3′ and 5′-ACACTGACGCATACAGCGAG-3′) and one reverse (5′-GCCCATGCGATTCGATCAGT-3′).

18S rDNA sequence analysis and phylogenetic tree construction

18S rDNA sequence was submitted to a BLAST search (http://www.ncbi.nlm.nih.gov) to find closely related sequences; 18S rDNA sequences of a total of 26 taxa were retrieved. 18S rDNA sequence of *Urostyla grandis* of family Urostylidae was retrieved to be used as outgroup. Sequences were aligned using CLUSTAL X2 sequence analysis software (Larkin et al. 2007). The resulting alignments were checked and corrected manually to remove ambiguous nucleotide positions at the beginning and end of the fragments. Phylogenetic analysis was performed with the MEGA (Molecular Evolutionary Genetics Analysis) software package version 5.0 (Tamura et al. 2011) available at http://www.megasoftware.net. The evolutionary distances were computed using the GTR + G + I nucleotide substitution model (Saitou and Nei 1987) in Maximum-likelihood tree. A Neighbor-joining tree using Maximum Composite Likelihood method (Tamura et al. 2004) and a Maximum parsimony tree were also constructed to check the relative stability of tree topologies. In each case tree construction was done using 1,000 bootstrap replicates. The analysis involved 27 nucleotide sequences. There were a total of 1721 positions in the final dataset. Distance matrix was constructed by Maximum Composite Likelihood method for nine taxa that clustered with *C.* (*C.*) *shii* Indian population in Maximum-likelihood, Maximum parsimony, and Neighbor-joining trees.

The nucleotide sequence determined in this study has been deposited in GenBank database under accession number JQ513386.

Results

Cyrtohymena (*Cyrtohymenides*) *shii* Indian population

Description (Figs 1A–C, 2A, B, 3A–C and Table 1): Body oblong, right margin usually slightly concave, left margin slightly convex, widest in midline, gradually narrowing posteriorly, dorso-ventrally flattened, slightly flexible, about 150 μm × 50 μm in vivo (Figs 1A, 3A) and 138 μm × 51 μm on average when impregnated with protargol (Figs 2A, B, 3B, C). Length:width ratio 2.5:1. Movement rapid; prey upon smaller encysting or excysting cells (Fig. 4B). Abundant brilliant citrine cortical granules make cells appear slightly yellowish green at low magnification; individual granules about 1 μm in cross section,