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Morphology, morphogenesis and molecular phylogeny of a new brackish water subspecies, *Neourostylopsis flava paraflava* nov. subsp. (Ciliophora, Hypotrichia, Urostylidae), with redefinition of the genus *Neourostylopsis*

Tengyue Zhang[a,b,1], Hongli Qi[c,1], Tengteng Zhang[a], Yalan Sheng[a], Alan Warren[d], Chen Shao[b,*]

[a] *Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China*
[b] *The Key Laboratory of Biomedical Information Engineering, Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an 710049, China*
[c] *Tianjin Key Laboratory of Aqua-Ecology and Aquaculture, College of Fisheries, Tianjin Agricultural University, Tianjin 300384, China*
[d] *Department of Life Sciences, Natural History Museum, London SW7 5BD, United Kingdom*

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Abstract

The morphology, morphogenesis and small subunit (SSU) rRNA gene-based phylogeny of *Neourostylopsis flava paraflava* nov. subsp. were investigated. *Neourostylopsis flava paraflava* nov. subsp. was separated from *N. flava flava* nov. stat. by habitat (brackish water vs. fresh water), pretransverse cirri (absent in all specimens vs. present in 15% of specimens), the numbers of frontal cirri (8–15 vs. 6–8) and left marginal cirral rows (6–9 vs. 4–5). The main morphogenetic features of *N. flava paraflava* nov. subsp. are as follows: (1) streaks I–VI (or I–VIII, deduced from morphological data) produce the bicorona; (2) the oral primordium and frontoventral-transverse cirral anlagen in the opisthe are formed de novo on the cell surface; (3) the numerous macronuclear nodules fuse into a branch-like mass; (4) two pretransverse ventral cirri are formed initially but disappear in the later stages; and (5) some of the frontoventral-transverse cirral anlagen develop in a primary mode. Based on SSU rDNA sequence data, phylogenetic analyses show a close relationship between *N. flava paraflava* nov. subsp., *N. flava flava* nov. stat. and other *Neourostylopsis* species. An improved diagnosis for *Neourostylopsis* is provided: Urostylidae with five or more frontal cirri which form an indistinct or distinct bicorona; pretransverse cirri present or absent; transverse cirri present; buccal cirri present; two frontoterminal cirri; midventral complex composed of midventral pairs only; more than one row of marginal cirri on each side which derive from individual anlagen within each parental row; caudal cirri lacking; three dorsal kineties; endoral and paroral rather long, endoral straight, paroral distinctly curved anteriorly.

Keywords: Brackish water; Ontogenesis; SSU rDNA sequence; Systematics; Taxonomy; Urostylida

Introduction

A comprehensive guide to urostylid hypotrichs was provided by Berger (2006). According to this detailed revision, urostylid hypotrichs are characterized by the midventral complex which is usually composed of ventral cirral pairs forming

*Corresponding author.
E-mail address: shaochen@mail.xjtu.edu.cn (C. Shao).
[1] Both authors contributed equally.

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a more or less distinct 'zig-zag' pattern. They are one of the most diverse and complex groups of hypotrichs. Consequently their biodiversity and systematics have been the focus of much recent research (Chen et al. 2016, 2017b; Foissner 2016; Gao et al. 2016, 2017; Huang et al. 2014; Kim et al. 2017; Li et al. 2018; Liu et al. 2017; Luo et al. 2017a,b; Yan et al. 2016a,b; Yi and Song 2011; Zhao et al. 2015).

The genus *Neourostylopsis* was erected by Chen et al. (2013) with *N. flavicana* (Wang et al., 2011) Chen et al., 2013 as the type species. This genus was originally defined as follows: marine or brackish Urostylidae with frontal and transverse cirri clearly differentiated; buccal cirri present; two frontoterminal cirri; midventral complex composed of midventral pairs only and not extending posteriad beyond the mid-region of the cell; more than one row of marginal cirri on each side which derive from individual anlagen within each parental row; caudal cirri lacking (Chen et al. 2013). Since its establishment, four species have been assigned to the genus *Neourostylopsis*: *N. flavicana, N. songi* (Lei et al., 2005) Chen et al., 2013, *N. orientalis* Chen et al., 2013 and *N. flava* Pan et al., 2016. Knowledge of morphogenetic processes in *Neourostylopsis*, however, is restricted to one middle stage for *N. flavicana* and a few stages of cell regeneration in *N. songi* (Lei et al., 2005; Wang et al., 2011). Thus, several key features of the morphogenesis, which are considered significant in determining the phylogenetic relationships, are unknown for this genus.

In November 2016, an unknown hypotrichous ciliate was isolated and collected from a brackish water stream near Qingdao, northern China. Observations of its morphology, both in vivo and following protargol staining, demonstrated that it represents a novel subspecies of *N. flava*. This gave us an opportunity to reveal new information on both the morphology and morphogenesis of *Neourostylopsis*. Furthermore, the SSU rDNA of the new isolate was sequenced and analyzed in order to assess its phylogenetic position.

It is noteworthy that the frontal cirri in *N. flava flava* and the present subspecies are not clearly differentiated but rather they are arranged in an indistinct bicorona, which is not consistent with the genus diagnosis. Nevertheless, all *Neourostylopsis* species cluster together in phylogenetic trees based on SSU rDNA sequence data (Lyu et al. 2018; Pan et al. 2016). Hence, the diagnosis of the genus is questionable and the pattern of frontal cirri in the other three congeners should be checked. An improved diagnosis of *Neourostylopsis* is provided based on a combination of previously published information and the new morphological and morphogenetic data revealed in the present study. In addition, we re-examined the protargol-stained slides of *N. flava flava* and found that pretransverse ventral cirri are absent in about 85% individuals and that dorsal bristles ahead of several outermost right marginal rows are present, but were overlooked by Pan et al. (2016).

Material and Methods

Sampling and cultivation

Samples were collected on 5 November 2016 from a brackish water stream (35°56′18″N; 120°12′44″E) that connects a lake in Tangdao Bay Park with Tangdao Bay, Qingdao, China (Fig. 1). The water temperature was about 13 °C and the salinity was 6‰. Submerged plant material, i.e. bark and rotten leaves, was collected together with water from the stream. Individual *Neourostylopsis* cells were isolated in the laboratory using micropipettes and clonal cultures were established at room temperature (23 °C) using a mixture of boiled mineral water and seawater giving a final salinity of 6‰. Clonal cultures were established for DNA extraction. Other cultures were established for morphogenetic investigation. The diatom *Diploneis ovalis* was isolated from the samples and cultivated separately as a food source for the ciliates.

Morphology and morphogenesis

Ciliates were observed in vivo using bright field and differential interference contrast microscopy (Olympus BX53, Tokyo, Japan). The protargol staining method of Wilbert (1975) was used to reveal the infraciliature. The protargol reagent was synthesized following the protocol of Pan et al. (2013). Counts and measurements of stained specimens were performed at a magnification of 1000×. Line diagrams of stained cells were made with the aid of a drawing device. To illustrate the changes occurring during morphogenesis, old (parental) ciliary structures are depicted by contour whereas new structures are shaded black (Chen et al. 2017a). For general and specific terms, see Berger (2006, 2008). For the designation of the frontoventral-transverse (FVT) cirri and FVT cirral anlagen, the numbering system of Wallengren (1900) is used. The protargol-stained slides of *N. flava flava* were re-examined (Slides numbers: FYB-2011032701-01, 02, 03 in Laboratory of Protozoology, Ocean University of China).

DNA extraction, PCR amplification, and gene sequencing

Genomic DNA extraction, polymerase chain reaction (PCR), and sequencing of the SSU rDNA were carried out according to Huang et al. (2016) and Zhao et al. (2017). One or more cells were isolated from the clonal cultures and washed four times with culture water (0.22 μm filtered) to remove potential contaminants. Extraction of genomic DNA was performed using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, but we modified the volume by using 25% of suggested volume for each solution. Primers 82F (5′-GAAACTGCGAATGGCTC-3′) and 18s-R (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) were used