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Morphology of three *Litonotus* species (Ciliophora: Pleurostomatida) from China seas, with brief notes on their SSU rDNA-based phylogeny

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Abstract

The morphology and ciliary pattern of three brackish pleurostomatid ciliates, *Litonotus gracilis* spec. nov., *L. tropicus* spec. nov., and *L. duplostriatus*, were investigated. *Litonotus gracilis* differs from its congeners by body size (200–400 × 15–40 μm in vivo), body shape (slenderly spindle-shaped, long neck), the number of somatic kineties (6–7 left and 11–17 right somatic kineties), long bar-shaped extrusomes arranged along oral slit, tiny cortical granules arranged like honeycomb, one subterminally located contractile vacuole and, usually, four macronuclear nodules. *Litonotus tropicus* is characterized by four contractile vacuoles dorsally located, 8–11 right and four or five left somatic kineties. *Litonotus duplostriatus* is lanceolate-shaped, with 11–14 right and five or six left somatic kineties, one subterminally located contractile vacuole, fusiform-shaped extrusomes distributed along oral slit. *Litonotus dragescoi* Pan et al., 2013 is not a valid name, it still be named as *Litonotus fasciolatus* (basionym *Loxophyllum fasciolatus* Dragesco, 1966). Molecular phylogenetic analyses based on SSU rDNA sequence data indicate that neither the family Litonotidae nor the genus *Litonotus* is monophyletic, and *L. gracilis* has a closer relationship with the genus *Kentrophyllum* than with other *Litonotus* species.

Keywords: *Litonotus*; Morphology; New species; Pleurostomatida; SSU rDNA

Introduction

The ciliate genus *Litonotus* Wrzesniowski, 1870 belongs to the order Pleurostomatida Schewiakoff, 1896, a ubiquitous and diverse group of periphytic ciliates that sometimes play an important role in sewage plants and can be used to monitor the water quality (Foissner et al. 1995; Gong et al. 2005; Vd'ačný and Rajter 2014). It is diagnosed by right somatic kineties terminating anteriorly along perioral kineties and the absence of dorsally positioned extrusomes (Foissner 1984; Lynn 2008).

Among over 75 nominal species in this genus, fewer than half have been adequately studied with standard taxonomic

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methods (Blatterer and Foissner 1988; Chen et al. 2011; Foissner 1978, 1984; Foissner et al. 1995; Kahl 1931, 1933; Lin et al. 2008, 2009; Song and Wilbert 1989). Recent research, however, is shedding new light on this genus, both through descriptions of several new species and by identifying some critical features for species identification. These include: (i) the shape and distribution pattern of extrusomes; (ii) the number of somatic kineties, particularly left somatic kineties; (iii) the number and position of contractile vacuoles; (iv) the cortical granules; (v) the number of macronuclear nodules; (vi) the cell shape; and (vii) the furrows on the left side (Foissner 1984; Foissner et al. 1995; Lin et al. 2008, 2009; Petz et al. 1995; Song and Wilbert 1989).

Ever since Gao et al. (2008) provided the first SSU rRNA gene sequence from this genus, *Litonotus* has appeared to be non-monophyletic in nearly all phylogenetic studies, although the node supports were not high and its monophyly has not been conclusively rejected by AU tests (Pan et al. 2010, 2013, 2014; Vd'ačný et al. 2011, 2014; Wu et al. 2013, 2014; Zhang et al. 2012).

Recently, we isolated three *Litonotus* species from Chinese coastal waters. The SSU rRNA gene sequence of *Litonotus gracilis* spec. nov. and *L. duplostriatus* are also sequenced so as to reveal their phylogenetic relationship and the phylogeny of *Litonotus*.

Material and Methods

Sample collection (Fig. 1)

The Zhanjiang population of *Litonotus gracilis* spec. nov. was isolated from a mangrove wetland (Fig. 1B) in Gaoqiao Town (21°34′08″ N, 109°45′20″ E), Zhanjiang, China on March 26, 2010, with the water temperature ca. 19.7 °C, salinity ca. 23.9‰, and pH 7.8. The Daya Bay population of *L. gracilis* was sampled from a mangrove wetland in Daya Bay (22°44′17.70″ N, 114°31′59.05″ E) on November 28, 2011, with the water temperature 23.5 °C, salinity 11‰, and pH 7.4.

Litonotus tropicus spec. nov. was collected from coastal water (Fig. 1A) off Donghai Island (20°56′36″ N, 110°31′49″ E), Zhanjiang, on April 7, 2010. The water temperature was 24.2 °C and the salinity was 13.9‰.

Litonotus duplostriatus was sampled from a sand beach in the estuary of Weihe River, Changyi (37°07′01″ N, 119°30′07″ E), China on May 16, 2009. The water temperature was ca. 23 °C and the salinity was 27‰.

Samples were collected with water and surface sediments (<5 cm in depth) during the ebb tide. After transferred to the laboratory, samples were isolated into Petri dishes and raw cultures were maintained at room temperature (ca. 25 °C). Rice grains were used to enrich the growth of bacteria as a food source for ciliates.

Morphology and terminology

Living cells were isolated from raw cultures with a micropipette under stereomicroscopy, and observed using bright field and differential interference contrast microscopy (Foissner 2014). Protargol staining was used to reveal the ciliary pattern, following the method of Wilbert (1975). Living individuals were examined at 100–1250× magnification; measurements were carried out with an ocular micrometre; drawings of stained specimens were performed at 1250× with the aid of a camera lucida. Terminology and systematics mainly follow Lynn (2008) and Foissner (1984).

DNA extraction, sequencing and comparison

The genomic DNA extraction was performed using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) according to Zhang et al. (2014). The universal oligonucleotide primers (forward 5′-AACCTGGTTGA TCCTGCCAGT-3′ or 5′-GAAACTGCGAATGGCTC-3′; reverse 5′-TGATCCTTCTG CAGGTTCACCTAC-3′) designed by Medlin et al. (1988) and Elwood et al. (1985) were used for PCR amplifications of the SSU rDNA. The PCR amplification, cloning and sequencing were performed as described in Fan et al. (2014).

Phylogenetic analyses

Other nucleotide sequences used in the present analyses were obtained from the GenBank/EMBL databases and the accession numbers of the sequences are as presented in Fig. 6. Firstly, alignment of the SSU rDNA sequences was done using the GUIDANCE algorithm (Penn et al. 2010b) following the default parameters in the GUIDANCE web server (Penn et al. 2010a) and further manually modified with BioEdit 7.0 (Hall 1999). The final alignment of 1563 characters for 36 taxa was used to construct the phylogenetic trees. Then, Maximum Likelihood (ML) analyses were performed with RAxML-HPC2 v8.1.11 with the GTRCAT model (Stamatakis 2006; Stamatakis et al. 2008) on the CIPRES Science Gateway V. 3.3 (http://www.phylo.org; Miller et al., 2010). Support came from 1000 bootstrap replicates. The Bayesian inference (BI) analyses were performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) under the GTR + I + G evolutionary model, which was selected as the most appropriate model in MrModeltest v.2.2 (Nylander 2004). Four simultaneous Markov Chain Monte Carlo algorithms (MCMC) were run for 2,000,000 generations, sampling every 100th generation, and discarding the first 5000 trees as burn-in. The remaining trees were used to calculate the posterior probabilities of the majority rule consensus tree (Zhao et al. 2014).