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Current view on phylogeny within the genus *Flabellula* Schaeffer, 1926 (Amoebozoa: Leptomyxida)

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Abstract

The molecular phylogeny of *Flabellula* Schaeffer, 1926 has been updated by analysing 18S rRNA and actin gene sequences of 19 new strains collected and characterised by the authors over the past ten years. The genus *Flabellula* Schaeffer, 1926 (Amoebozoa: Leptomyxida) is a taxon in which species delineation based on morphological data by themselves is insufficient or even misleading. The description of two novel species, *F. schaefferi* n. sp. and *F. sawyeri* n. sp., is justified by the congruence of morphological data with 18S rRNA and actin gene sequence phylogenies, in-silico secondary structure prediction of the V2 region in the 18S rRNA, and by recognition of species-specific sequential motifs within this region.

Keywords: Amoebozoa; Tubulinea; Phylogeny; Taxonomy; 18S rRNA; Actin

Introduction

In the modern classification system of eukaryotes (Adl et al. 2012), *Flabellula* Schaeffer, 1926 has its place in Leptomyxida Pussard and Pons, 1976 belonging to the supergroup Amoebozoa. The most basic points of the taxonomic history of the genus, including the establishment of morphology-based generic criteria (Schaeffer 1926), their amendment (Bovee 1965), the addition of ultrastructural features (Page 1980), and the verification of morphological generic criteria in the descriptions of new species, have been discussed in a series of studies (Michel and Smirnov 1999; Page 1968, 1971, 1980, 1983, 1991; Smirnov 1999; Smirnov and Goodkov 1999). The most detailed light microscopic data are available for *Flabellula citata* Schaeffer, 1926 (the type species) and *F. baltica* Smirnov, 1999. A polymorphic life history has been described and partly documented for both these species (Fenchel 2010; Schaeffer 1926). Recently, Smirnov et al. (2017) listed 10 nominal *Flabellula* species, *F. citata*, *F. baltica*, *F. calkinsi* Bovee, 1965, *F. demetica* Page, 1980, *F. trinovantica* Page, 1980, *F. kudoi* (Singh et Hanumaiah, 1979), *F. reniformis* (Schmoller, 1964), *F. hoguae* Sawyer, 1975, *F. pomeranica* Kudryavtsev, 2017 in Smirnov et al. (2017), and *F. pellucida* Schaeffer, 1926 whose generic assignment, however, has been questioned.

Molecular characterization of flabellulid amoebae started with representatives of *Paraflabellula*, the genus that was morphologically separated from *Flabellula* by Page and Willumsen (in Page 1983). The first 18S rRNA gene sequences used in a molecular study were those of *Paraflabellula reniformis* (Schmoller, 1964) and *P. hoguae* (Sawyer, 1975); they appeared in a molecular re-assessment of the leptomyxid amoebae by Amaral-Zettler et al. (2000). Later, these sequences appeared in several other phylogenetic reconstruc-

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tions (e.g., Nikolaev et al. 2005). The first *Flabellula* species to be subjected to a phylogenetic analysis was *F. citata* (strain CCAP 1529/2 along with another six strains of similar light microscopic and ultrastructural features) (Dyková et al. 2008). Recently, a phylogenetic analysis based on 18S rRNA gene sequences of five of the ten nominal species of *Flabellula* (those mentioned above) has been published by Smirnov et al. (2017) as a part of their review of leptomyxid amoebae. The morphological and molecular analysis of *Flabellula* and *Paraflabellula* spp. published in the same paper resulted in the formal invalidation of *Paraflabellula* due to the lack of substantial differences between these two genera, *Flabellula* remaining a valid genus based on the rule of priority. The assemblage of six strains analysed along with *F. citata* was not considered adequate to be completed with a description of new species (Dyková et al. 2008). One clonal strain of this assemblage (SMA17/I) was later identified with *F. baltica* (Smirnov et al. 2017). Over the past ten years we have collected and characterised a set of 19 new strains (candidates for comparison with *Flabellula* spp.) and thus more than tripled the number of strains included in our former study (Dyková et al. 2008). This fact, along with new data available in the DDBJ/EMBL/NCBI databases, and the improvement of analytic methods, motivated us to attempt a species determination of seven strains and description of two new *Flabellula* spp. in this paper.

Material and Methods

The set of amoeba strains involved in this study (Table 1) consisted of (i) 22 strains from our collection that were isolated by ourselves from marine materials of different nature and diverse geographical origin and (ii) 2 strain representatives of nominal *Flabellula* species obtained from the Culture Collection of Algae and Protozoa (UKNCC). The strains from our collection were selected by light microscopic features of trophozoites that either fully or partially matched the *Flabellula* generic diagnosis. Final inclusion in the study of partially matching strains was preceded by preliminary sequencing of their 18S rRNA genes and Blast searches. The methods of isolation, subculturing, and observation of amoebae were those detailed in Dyková et al. (2008).

DNA extraction, amplification, and cloning

In this study, we used archived DNA samples from the strains included in Dyková et al. (2008) and Dyková and Kostka (2013) plus samples from strains isolated more recently. DNA samples of the latter strains were extracted using the Exgene^{TM} Tissue kit (GeneAll Biotechnology, Seoul, Republic of Korea). Nearly complete 18S rRNA genes were amplified using the 18e (Hillis and Dixon 1991) and Erib10 primers (Barta et al. 1997). Actin genes were amplified using the primers designed by Tekle et al. (2007). The PCR conditions, same for both genes, were described in Tyml and Dyková (2017) whereas the purification of PCR products, molecular cloning and sequencing were conducted as in Tyml et al. (2017).

Phylogenetic reconstruction

Newly obtained sequences were checked and assembled using Geneious R10 (Biomatters, Auckland, New Zealand) and then deposited into the DDBJ/EMBL/NCBI databases under Acc. Nos LC340972–LC341039. In total, four sequence datasets of Tubulinea were prepared for this study: Dataset A consisted of 3 newly generated and 50 publicly available 18S rRNA gene sequences. Dataset B, consisting of 29 sequences, was created from dataset A by including only those strains represented by actin gene sequences as well. Dataset C was composed of 71 newly produced and 151 publicly available sequences of actin gene (as of August 2017) omitting duplicates, which in this case were mostly represented by molecular clones. Dataset D was derived from the dataset C associated 29 actin gene sequences from DNA samples of the strains corresponding to those included in dataset B. As recommended by Lahr et al. (2011), the shortest-branching sequences of molecular clones were selected. All datasets (A–D) were aligned by MAFFT v. 7 (Katoh and Standley 2013) using the G-INS-i strategy in datasets A and B and the translation-align algorithm for datasets C and D. Accession Nos of sequences involved in the study are listed in Table 2. The alignments were trimmed using the -gt 0.3 -st 0.001 option of trimAl v. 1.2 (Capella-Gutiérrez et al. 2009). For the alignment of A and the concatenated alignments of B + D, ModelFinder (Kalyaanamoorthy et al. 2017) chose GTR + R4 and GTR + R4/SYM + R4, respectively, as the best-fit models using the Bayesian information criterion (BIC). For the alignment of C, Smart Model Search (Lefort et al. 2017) chose GTR + G + I as the best-fit model using the BIC. Maximum likelihood analyses were executed in PhyML v. 3 (Guindon et al. 2010) for the alignment of C whereas the alignment of A and the concatenated alignments of B + D were executed in IQ-TREE v. 1.5.5 (Nguyen et al. 2015) with the standard bootstrap analysis (Felsenstein 1985), Approximate Bayes test (Anisimova et al. 2011), and SH-aLRT branch test (Guindon et al. 2010). The final trees were formatted using iTol v. 4 (Letunic and Bork 2006) and Illustrator v. 22 (Adobe Systems, San Jose, CA). The secondary structure of the V2 genus-specific region of 18S rRNA was predicted in silico using mfold web server (Zuker 2003) with default parameters.

Results

Light microscopy

Regular maintenance and observation of agar-plate cultures through Petri dishes facilitated the preliminary selection