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European Journal of Protistology 61 (2017) 20–28

European Journal of PROTISTOLOGY

www.elsevier.com/locate/ejop

Redescription of the halophile ciliate, *Blepharisma halophilum* Ruinen, 1938 (Ciliophora, Heterotrichea, Heterotrichida) shows that the genus *Blepharisma* is non-monophyletic

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Received 18 April 2017; received in revised form 17 July 2017; accepted 19 July 2017
Available online 24 July 2017

Abstract

We collected a heterotrich ciliate from hypersaline waters on the Cape Verde island Sal. After living observation and protargol-staining, it closely matched *Blepharisma halophilum* Ruinen, 1938. However, because (i) Ruinen most likely described several different species as *B. halophilum*, and (ii) he provided only very scarce diagnostic features, we redescribe this species with state-of-the-art methods according to modern standards. This will facilitate an accurate identification of this ciliate species, globally distributed in hypersaline water bodies: halophile *Blepharisma*, about 100–250 μm *in vivo*; 32–42 adoral membranelles; 13–17 somatic kineties, including 2–4 anterior kinety fragments; single elongated macronucleus; cortical granules colourless. Its SSU rRNA gene sequence is very similar (99.7%) to an uncultured organism (environmental sequence) obtained from the hypersaline Lake Tyrrell in Australia. In phylogenetic analyses, these two sequences form a cluster, which with nearly full support (ML/BI, 99/1.00) branches as sister to the Fabreidae, showing for the first time that *Blepharisma* is yet another non-monophyletic ciliate genus.

Keywords: *Blepharisma*; Halophile; Morphology; SSU rRNA gene; Taxonomy

Introduction

Species of the ciliate genus *Blepharisma* Perty, 1849 (class Heterotrichea, order Heterotrichida) are cosmopolitan organisms and have been reported to occur in various types of habitat. Most records are from freshwater (Aescht and Foissner 1998; Fernandes et al. 2013; Foissner 1980, 1989; Isquith et al. 1965; Kahl 1932) and marine habitats (Al-Rasheid 1999; Borror 1963; Kahl 1932), but a few studies have also reported the occurrence of *Blepharisma* in extreme environments such as hypersaline water (Al-Rasheid et al. 2001; Post et al. 1983). Species may vary considerably in size (50 μm–1 mm) and shape (ellipsoidal, pyriform with round posterior to long and thin while tapering at the posterior into a tail-like point; Kahl 1932, 1933). However, there are common diagnostic features of the genus *Blepharisma* which include a non-contractile and laterally compressed body and a paroral membrane composed of dikinetids and differentiated into two parts, one of which is with closely-spaced dikinetids in a

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http://dx.doi.org/10.1016/j.ejop.2017.07.004

zig-zag pattern, the other one less closely arranged in one line (Giese 1973; Hirshfield et al. 1965; Lynn and Small 2002). Most *Blepharisma* species have a reddish or pink colour, originating from granules of the photosensitive pigment blepharismin which is located just under the plasma membrane of most, but not all, *Blepharisma* species (Miyake et al. 1990). This colouration usually makes identification relatively straightforward.

There are nearly fifty *Blepharisma* species reported (Giese 1973). Surprisingly, only few of these species have been studied using state-of-the-art methods (Warren et al. 2017). Consequently, the taxonomic history of this genus is a story of confusion, re-naming and re-organisation (Giese 1973; Hirshfield et al. 1965). Thus, it is not surprising that the status of the Blepharismidae and the subgeneric classification of *Blepharisma* based on some morphological criteria, such as the macronuclear structures, are not supported by ontogenetic data (Aescht and Foissner 1998; Lynn 2008). Consequently, more recent studies have a stronger focus on the molecular phylogeny of *Blepharisma* rather than their taxonomy and biodiversity (Fernandes et al. 2016; Shazib et al. 2014; Yan et al. 2016). These analyses all show *Blepharisma* to be a monophyletic clade.

Recently we isolated a *Blepharisma* species from hypersaline waters of the Cape Verde island Sal. This population matches the superficial description of a *Blepharisma* species (*Blepharisma halophila*, now *B. halophilum*) by Ruinen (1938), which the author observed in hypersaline samples from Australia, India and Europe (for revision, see Giese 1973). Unfortunately, Ruinen (1938) only provides a few morphological criteria, making it impossible to precisely identify the species and at the same time impeding the assignment of the currently described morphotype to a new taxon. Therefore, we describe here the *Blepharisma* detected in Sal as *Blepharisma halophilum* and provide a state-of-the art species description, which will make future identification straightforward. Interestingly, despite its unbiased *Blepharisma*-morphology, the SSU rDNA sequence of this species does not cluster within the presumably monophyletic *Blepharisma* clade, which challenges our current understanding about the evolutionary history of the Blepharismidae.

Material and Methods

Sample collection and identification

Blepharisma halophilum was collected from an ancient solar saltern (Salina de Pedra de Lume, lat: 16°46′22.88″N, long: 22°54′3.53″W) on the island of Sal, Cape Verde, in September 2015. The salterns are of natural origin, occurring below sea-level in the centre of a volcano crater, where a natural salt lake formed through infiltration of the crater with sea water. Residual heat from volcanic activities and intense solar radiation resulted in evaporation, leaving behind a nearly salt-saturated thalassohaline environment. Salinity of the pond water from which *B. halophilum* was isolated was 280‰. *Blepharisma halophilum* was maintained in sterilised water (salinity 250‰) from solar salterns Ses Salines, Es Trenc, Spain. The addition of two or three autoclaved wheat grains per 25 ml of culture medium served the enrichment of indigenous bacteria from the habitat water as food. All cells for both the morphological studies and the DNA extraction were from the non-clonal culture which was based on the collection of several individuals of this species.

Live cells were observed under a bright-field and differential interference contrast microscope. The protargol staining method was used to reveal the ciliature and the nuclear apparatus (Wilbert 1975). The protargol reagent used in this staining procedure was made according to the protocol of Pan et al. (2013). Illustrations of both live cells and protargol-stained specimens were freehand sketches based on photomicrographs. Measurements were performed at 200–1000× magnification. Terminology and systematics are according to Lynn (2008) and Shazib et al. (2014).

DNA amplification and sequencing

DNA extraction and PCR amplification of the small subunit rDNA (SSU rDNA) and phylogenetic analyses were conducted as described previously (Foissner et al. 2014). In short, genomic DNA was extracted from ten specimens of *Blepharisma halophilum* using the DNEasy Tissue Kit (Qiagen). The partial SSU rDNA was amplified using the universal eukaryotic primers Euk82 (5′-GAA[AGT]CTG[CT]GAA[CT]GGCTC-3′; Lopez-Garcia et al. 2001) and U1517R (5′-ACGGCTACCTTG TTACGACTT-3′; Shopsin et al. 1999). Amplification with Phusion Taq (NEB, MA, USA) was as follows: 30 s at 98 °C, followed by 30 identical amplification cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 1 min, extension at 72 °C for 45 s and a final extension at 72 °C for 2 min. The purified PCR product (MiniElute kit, Qiagen) was cloned into a vector using the NEB PCR cloning kit with the pMiniT vector. Sequences were obtained with the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) on an ABI 3730 automated sequencer. We sequenced four different clones which were subsequently analysed in CodonCode Aligner (CodonCode Corporation, MA, USA).

Phylogenetic analyses

The SSU rDNA sequence of *Blepharisma halophilum* was aligned with sequences of 45 other heterotrich ciliates obtained from NCBI GenBank database. Two *Gruberia* spp. were chosen as the outgroup according to Yan et al. (2016). The accession numbers of all sequences used in this study are provided in Fig. 3. The alignment was conducted with Muscle 3.7 (Edgar 2004) and refined using BioEdit v. 7.0.5 (Hall 1999) to remove highly variable sites. The final alignment comprised 1611 nucleotide positions and 46 taxa, which was