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The Dancing Star: Reinvestigation of *Artodiscus saltans* (Variosea, Amoebozoa) Penard 1890

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***Artodiscus saltans,* first described by Penard (1890), has a unique morphology. Without genetic data it could not yet been reliably placed into a wider taxonomical context. We present morphological data for *A. saltans* from different aquatic habitats of four European countries. We subjected three cells of one strain from Germany to molecular analyses and, interestingly, obtained six different rDNA sequences. Phylogenetic analyses of these SSU rDNA sequences revealed that *A. saltans* branches close to the amoebozoan *Multicilia marina* (Variosea, Amoebozoa).**

Key words: Ribosomal genes; amoebae; Conosa; *Multicilia*; flagellate; *Paramphitrema*.

Introduction

Artodiscus saltans was discovered and described by Penard in 1890 after he collected some specimens from a flooded pasture near Wiesbaden, Germany. He found similar specimens in subsequent years in Lake Geneva; along its shore and in up to 30 m water depth (Penard 1905). However, since Penard's description, records have been very scarce. In the last century it was found by Rainer (1968), Siemensma (1973, unpubl.) and De Groot (1979). Only recently have reports of *A. saltans* increased (e.g. Frouz 2013, Czech Republic, several photomicrographs and videos available online by Revello 2012, Uruguay; Opitz 2013, Austria; Voelcker and Clauß 2015, Germany, and Capilla 2016, Spain, as well as the records that are the subject of this publication (Table 1)).

Penard (1890) described *A. saltans* as a reddish, spherical cell, being very plastic (hence its generic name; the prefix *arto* is derived from the Greek word αρτοϱ for a piece of dough) with fast and continuous changes of its shape during locomotion. He noticed that, although difficult to see, the cells were surrounded by a layer of very fine 'mucus' in which very small plates were embedded. It was, in his words, one of the most interesting species he ever had observed, because of its 'extraordinary mobile' flagella-like 'pseudopodia' by which the 'animals' were 'dancing vividly' and sometimes 'swimming as fast as a flagellate'. In 1903 he emended his description, writing that the cell body usually was spherical, but with a tendency to become uneven and angular, being surrounded by a distinct membranous envelope that followed every deformation

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Table 1. Sampling spots of *A. saltans*, morphological characters and reference to figures.

Sampling spots	Coordinates	Observation date	n	Diameter cell body in μm*	Length of flagella in μm**	position of nucleus	theca	Fig.	Habitat
Wipperfürth, Germany	51.127368, 7.380871	May, 2018	1	13 × 10	34 ± 1 (n = 4)	centric	smooth	1 F	small stream, running water, approx. depth 15–20 cm
Spiegelplas, Netherlands	52.274259, 5.071945	1973	1	NA	NA	eccentric	xenosomes		lake shore, sandy bottom, CSSW
Gaasterland, Netherlands	52.885363, 5.561656	May, 2012	3	20–24	41	eccentric	xenosomes	1D,E	ditch, thick layer of organic sediment, CSSW
Spiegelplas, Netherlands	52.261307, 5.067952	Jan., 2013	20	19–21	45	ecc./centric	smooth/xeno.	1H	lake shore, sandy bottom, CSSW
Sankt Ulrich, Austria	47.534666, 12.573009	June, 2013	1	9	32	centric	smooth		river, backwater, thin layer of organic sediment, CSSW
River Saar, Germany	49.621619, 6.564232	Oct., 2014	2	9–13	34–37	centric	smooth	1A	river, turbid running water, between green algae on rock
Laegieskamp, Netherlands	52.277634, 5.136724	April, 2014	3	5–10	27–31	centric	smooth	1C	ditch, with *Chara* sp. and *Sphagnum,* CSSW
Naardermeer, Netherlands	52.282330, 5.119572	Jan., 2015	5	13–15	39–47	centric	smooth		ditch, thick layer of organic sediment, CSSW
Castricum, Netherlands	52.548240, 4.617177	Sept. 2015	2	10–15	41–52	centric	smooth	1 G	artificial dune pond, sandy bottom, CSSW
Île-Grande, France	48.795937, 3.585810	June, 2015	2	10–11	27–30	centric	smooth		old washing basin, brackish water, CSSW
IJsselmeer, Netherlands	53.074981, 5.344705	July, 2015	1	12	26–30	centric	smooth	1B	lake shore, sandy bottom, CSSW

CSSW = optically clear stagnant and shallow water.
NA = No data available.
*measured from photomicrographs; as the cells have a constantly changeable shape, we tried to estimate the diameter of a spherical cell.
**measured from photomicrographs; we only measured projections that were clearly visible. Most projections were partly out of focus.

of the cell and was covered with granules and very small plates (Penard 1903, 1904, 1905).

Based on the unique morphological characters of *A. saltans* it could not be reliably placed within a wider taxonomic context. Since the Thecofilosea (Cercozoa, Rhizaria) and the Variosea (Amoebozoa) exhibit morphological characters common to *A. saltans*, both represent taxa that could possibly accommodate this species. Both taxa accommodate eukaryvorous and bacterivorous flagellates, amoebae and amoeboid flagellates. Thecofilosean species characteristically possess a more or less conspicuous organic theca, sometimes with embedded or attached xenosomes (Dumack et al. 2017), while variosean flagellates exhibit a protuberate base from which the flagella emerge (Berney et al. 2015). To ascertain the phylogenetic affiliation of *A. saltans*, we obtained part of its SSU rDNA sequence and conducted phylogenetic analyses.

Results

Ecological and Morphological Observations

The observed and isolated specimens of *A. saltans* were collected from different aquatic habitats located in Austria, France, Germany and the Netherlands (Table 1). All specimens were found in freshwater except one that occurred in slightly brackish water in an old fisherman's washing pool on the French Atlantic coast, where *Enteromorpha intestinalis* was a dominant species. All but one location had optically very clear water. Only the river Saar had turbid water; specimens there were collected from sessile algae on a stone just below the surface. All aquatic habitats were shallow with a layer of organic sediment of variable thickness. The water bodies were stagnant or running.

A. saltans was never abundant in any sample. It was never found at first observation, but when wet mounts were left over for one or more days in a wet chamber, specimens emerged from the debris and could be observed moving along the cover glass. We never found evidence that this species multiplied in the wet mounts. Usually the cells disappeared again after a few days.

The cell body contained one nucleus with a single small centric nucleolus and the cytoplasm appeared slightly greenish under phase contrast. Specimens collected from Spiegelplas and Gaasterland had a relatively large, eccentrically placed nucleus (diameter ~9 μm) with one or three small nucleoli (~2 μm) (Fig. 1D, E, H), though some specimens from Spiegelplas had a centric and smaller nucleus with a relatively large central nucleolus (diameters ~4 μm and ~2 μm respectively). Specimens from all other locations had a relatively small and more centric nucleus (diam. ~3–5 μm) with a relatively large central nucleolus (Fig. 1A, B). Specimens isolated from the river Wupper in Wipperfürth had a length of ~13 μm, a width of ~10 μm, flagella-length of 34 ± 1 μm and a span width of ~80 μm (Fig. 1F). Measurements of all observed specimens are summarized in Table 1.

All observed specimens had a flexible membranous theca that followed the constantly changing outline of the cell. Some cells bore a theca with embedded small granules and/or rod-shaped elements (Fig. 1D, E, H), while most cells had a smooth theca (Fig. 1A, B, G). Only large cells sometimes showed a small space between theca and cytoplasm (Fig. 1E). The small elements were embedded in the membranous theca; we never observed these elements laying on the test.

Individuals from all sampling locations had a variable number of flagella. Small specimens had about 5–6 flagella and larger specimens up to 12, respectively. However, the exact number was very difficult to count in living specimens. Although all flagella were mobile, the cell usually had one or two flagella that moved more actively, making slow rowing movements. In fast locomotion the cell had one leading flagellum with two trailing flagella behind in the same plane, all three together shaped like a Y (Supplementary Material Videos S1, S2). These three flagella hardly moved, while the other flagella made rowing movements in the direction of the leading one. Despite the slow movements of the flagella, they propelled the organism relatively quickly in the direction of the leading flagellum (see Fig. 1C). Some specimens of *A. saltans* showed one short and stiff flagellum and at one occasion even two (Fig. 1G). When mechanically disturbed, the cells tended to swim actively to the water surface, making isolation a difficult task.

In larger specimens all flagella had a thickened and slightly tapering base. Using light microscopy it was not possible to detect if this base was part of the flagellum or a tubular bulge of the theca (Fig. 1 D, E, Supplementary Material Videos S1, S2). In one specimen this base was covered by the same kind of material as embedded in the theca.

In almost all specimens the flagella were more or less evenly distributed over the cell surface, but in material from Spiegelplas we observed some specimens that occasionally became spindle-shaped within some minutes, with 1 to 4 flagella at each pole. We observed that these spindle-shaped cells

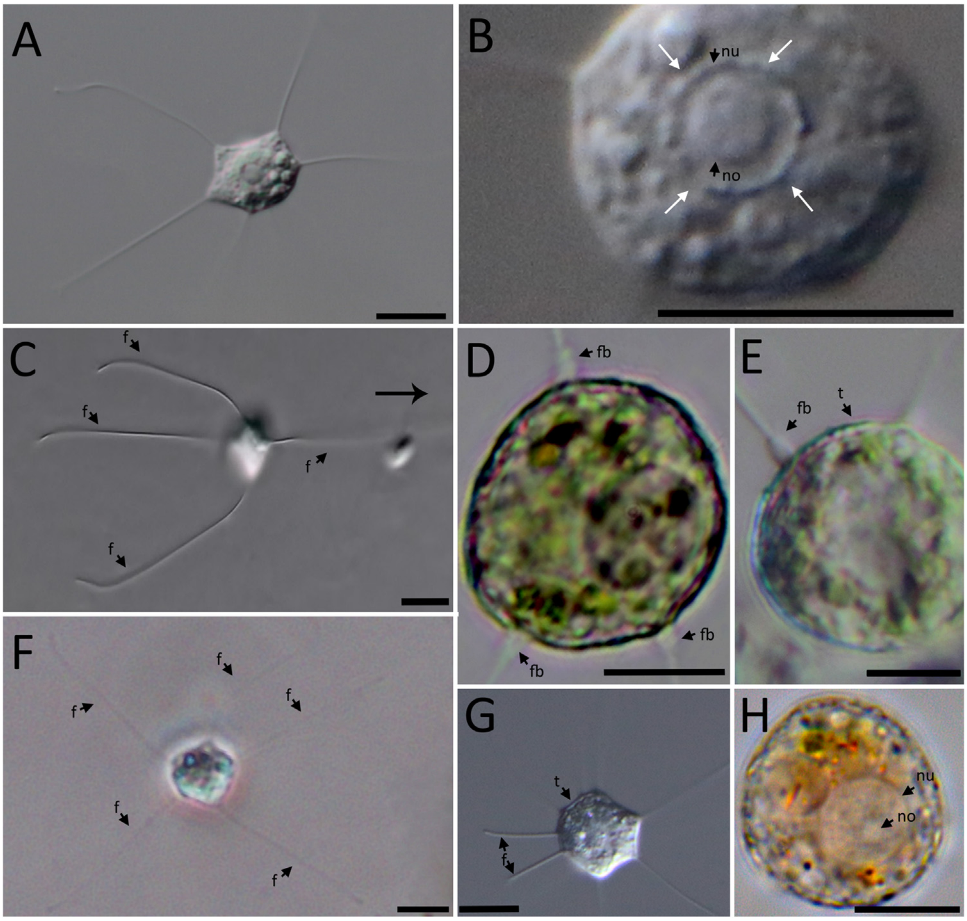

Figure 1. Cellular features of *Artodiscus saltans* presented by 7 different individuals (A, B, C, D + E, F, G, H) by light micrographs. Consult Table 1 for an assignation of the pictures to the reported strains. **(A)** Overview of the cell body. **(B)** Close up of the spherical nucleus (highlighted by white arrows) with one spherical nucleolus. **(C)** Close up of a swimming cell with three trailing flagella, one leading flagellum and the direction of movement of the cell (indicated by black arrow). **(D)** View of the thickened and tapering base of the flagella. **(E)** Close up of the thin covering of the cell's surface with some scale-like elements. **(F)** Overview of one of the individuals used for phylogenetic analyses. (**G**) Specimen with two short flagella. (**H**) Specimens with relatively large nucleus and small nucleolus. Abbreviations: f-flagellum; fb-base of flagellum; no-nucleolus; nu-nucleus; t-theca. Scale bars: 10 μm.

transformed into spherical cells with their flagella spreading all over the cell surface within minutes (Supplementary Material Data S1).

Phylogenetic Analysis

Partial SSU rDNA sequences with a length of 1450 bp were obtained and subjected to phylogenetic analyses (Fig. 2). PCRs with single or a few cells as template repeatedly resulted in partially ambiguous sequences. Subsequently two different PCR products, one obtained from a single cell and another obtained from two cells, were subjected to cloning. We obtained two SSU rDNA genotypes from the single cell PCR and the PCR performed with two cells as template resulted into four obtained SSU rDNA genotypes. The six obtained sequences had reoccurring patterns that were seen in different combinations (Fig. 3). These variable sites were distributed throughout the whole sequenced stretch of the SSU rDNA and occurred thus in variable regions and more conserved regions of the sequence. All sequences were to 98–99% identical to each other (Fig. 3).

We were able to recover all the clades as Berney et al. (2015) in their phylogenetic analyses, although we deliberately excluded some long branches (like myxomycetes, see discussion of

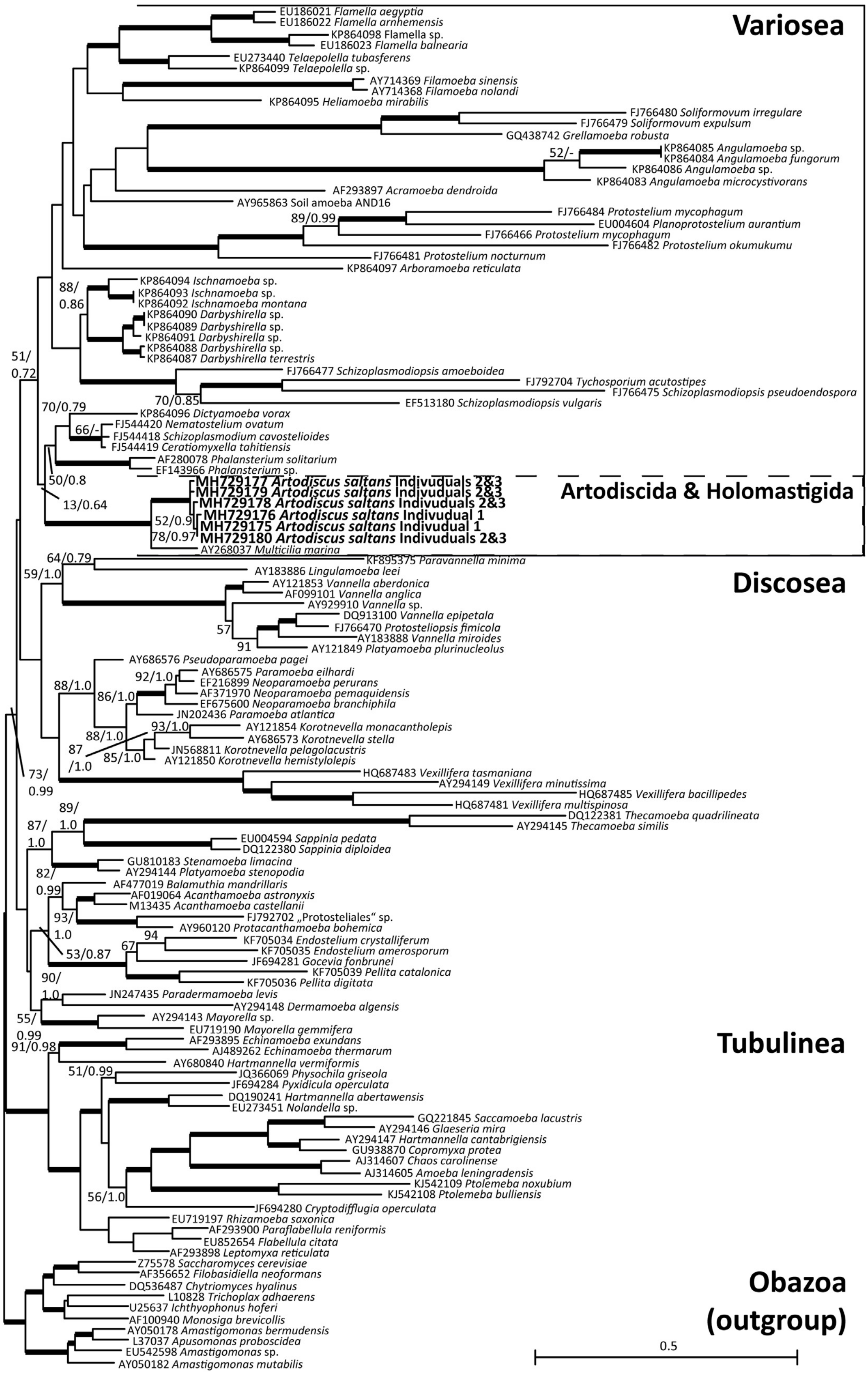

Figure 2. SSU rDNA phylogeny of the Variosea and other Amoebozoa with selected Obazoa as outgroup.

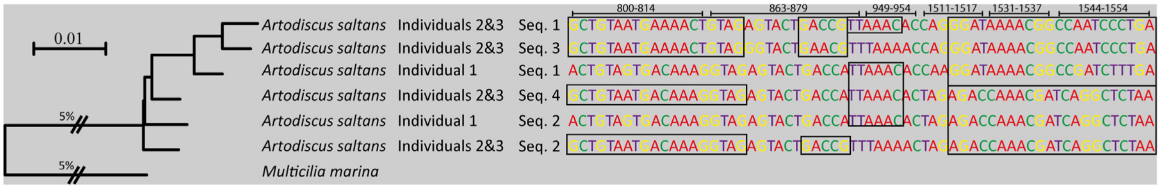

Figure 3. Comparison of the multiple sequences obtained from *Artodiscus saltans* consisting of a phylogenetic tree of uncut SSU rDNA sequences on the left and on the right selected sites are chosen and represented to illustrate similarities of the sequences. Positions of the chosen sites in respect to the full length of the uncut alignment are given at the top. The drawn boxes show selected similar sites in the different individuals.

SSU rDNA sequence characteristics in Berney et al. 2015). In our analyses, all six newly generated SSU rDNA sequences form a maximally-supported clade in both Maximum Likelihood and Bayesian analyses and branch as the sister group to *Multicilia marina*, again with maximal support. The partial SSU rDNA sequences from *A. saltans* and *M. marina* were only about 66% identical, due to the long insertions in the *M. marina* sequence.

Discussion

Morphological Analysis

In his original diagnosis Penard (1890) described the cell surface of *A. saltans* as a layer of very fine mucus embedded with very small endogenous plates, but in 1903 he emended this diagnosis, noticing that the cell was enveloped with a flexible theca (he referred to it as 'membrane'), more or less clearly separated from the cytoplasm and reinforced with exogenous elements. In 1904 he wrote that his description of the mucus sheath with small endogenous plates had been an assumption under the impression that *A. saltans* was a heliozoan and that similar heliozoans had a mucus sheath embedded with endogenous plates. We did not observe a thin mucus sheath as it was described in Penard's early diagnosis (Penard 1890), but could clearly detect a flexible theca in all observed specimens as later described by Penard (1903, 1904, 1905).

Penard (1903) noticed that some rare individuals were almost colourless. In contrast to that, most of our specimens and especially the smaller ones were colourless and without granules on the surface of the theca. Smaller specimens always had concave lateral sides. Only some specimens from Gaasterland and Spiegelplas had clear granules and were conspicuously coloured (Fig. 1D, E, H). Isolates of the Wipperfürth strain were slightly greenish in phase contrast (Fig. 1F). However, we assume the cell colour depends on the consumed food organisms.

Penard is ambiguous in the description of the size and shape of the nucleus. He described the nucleus as round and compact (Penard 1903, 1904) and as relatively large with a relatively small nucleolus (Penard 1905). However, his drawings (Penard 1903, 1904, 1905) show a small compact nucleus with a large nucleolus. In all his reports, Penard (1890, 1903, 1904, 1905) noted that the position of the nucleus was eccentric to very eccentric. We observed a large eccentric nucleus with a small nucleolus only in specimens from Spiegelplas and Gaasterland (Fig. 1H). All other specimens had a smaller, more central nucleus with a relatively large central nucleolus (Fig. 1A, B). Only specimens from Spiegelplas contained both types of nuclei. In contrast Rainer (1968) noticed that the nucleus of *A. saltans* was eccentric with a large nucleolus.

In general, specimens of *A. saltans* with a smooth membranous theca had a small centric nucleus with a relatively large nucleolus, while specimens with xenosomes embedded in their theca had a large eccentric nucleus with a relatively small nucleolus (Table 1). We cannot explain these differences in nuclear position, shape and size. It may indicate either phenotypic plasticity, potentially due to different life history stages, or the existence of more than one species of *Artodiscus*.

Penard (1890) reported that this flagellate (he referred to it as an amoeba) was supposed to have 7-12 slowly moving flagella, with only 4 or 5 visible at a time, which extend with a thickened, somewhat tapering base through pores of the envelope. This

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Shown is the maximum likelihood tree. The support levels of the tree are shown on the respective branches (ML/BI) if support was over 50% BP/0.8 PP. Bold lines: BP support of ≥95%.

agrees very well with our observations: about 5–6 flagella were present in small specimens and up to 12 flagella in larger ones. These flagella were evenly distributed over the spherical cell body.

According to Penard (1890) the cell body of specimens he observed measured between 18–23 μm excluding the length of the flagella. Our specimens measured 5–30 μm (n = 27). De Groot (1979) reported cell sizes of 27–40 μm (n = 4). Revello (2012) measured 40 μm (n = 1) and Opitz (2013) mentioned 27 μm (n = 1). Again, this large difference in cell size could be an indication of different life history stages or more than one *Artodiscus* species. The latter would best explain the morphological similarities of specimens from Spiegelplas and Gaasterland (Fig. 1D, E, H) which were larger and more spherical, had a theca embedded with minute plate-like elements and a large eccentric nucleus with small nucleolus. Their morphology differed from the specimens with more concave sides, a relatively smooth theca and a smaller, more central nucleus and large nucleolus (Fig. 1A, B) that we collected from all other locations. However, in Spiegelplas we also found both types besides each other.

Penard (1890, 1903, 1904, 1905) is not clear about the shape and size of the nucleus, but otherwise our observations exactly correspond to his description. Therefore, we consider our observed protists to be *A. saltans.* Further investigations are needed to clarify whether the genus is monotypic or not.

Interestingly, Penard (1903, 1904, 1905) remarked that *A. saltans* closely resembles *Paramphitrema lemanense* (Penard, 1903) in several aspects. This thecate amoeba was described by Penard (1903) as more or less spindle-shaped, sometimes almost spherical, with a flexible theca and with two flagella-like projections, one extending from each pole of the cell. He wrote "at rest, these projections arise on a small mass of clear plasma, spread out at each of the poles, which then divides immediately into two, three, four or more filaments, very fine, delicate, generally straight, sometimes bifurcated." (Penard 1903). Our observations on the transformation of specimens from Spiegelplas (Supplementary Material Data S1) that were able to elongate and show a bipolar flagellar position indicate that both species might be closely related or even identical. If so, this would give a first indication of the taxonomic position of *Paramphitrema*, a testate amoeba genus currently still considered as *incertae sedis* (Kosakyan et al. 2016).

Ecological Analysis

Rainer (1968), who found *A. saltans* in Germany, gave some data concerning its ecology. He found this species in the organic sediment of stagnant backwaters, being parts of a river not reached by the current, and in *Sphagnum*-dominated bogs. The pH of the waters where he collected *A. saltans* was always between 5.4 and 7.6. De Groot (1979) found this species in small, clear, running streams (pH 7–8) with sandy bottoms near Arnhem, the Netherlands. He compared this habitat with spring-fed streams and their connected lakes in the Alps, where Penard had found similar species. He concluded that the environmental factors in the oligotrophic mountain streams and lakes were comparable with those of the Dutch lowland running waters, although he did no comparable measurements. We only conclude that *A. saltans* prefers shallow, clear, stagnant water with a layer of organic sediment.

Phylogenetic Placement of *Artodiscus saltans*

Cavalier-Smith (2013) established the Order Artodiscida Cavalier-Smith, 2013 together with Family Artodiscidae Cavalier-Smith, 2013 (*Artodiscus*, *Tetracilia*) on the basis of morphology. Our phylogenetic analyses showed that *A. saltans* belongs to the phylum Conosa, which either bear flagella or are expected to have secondarily lost them. Flagellate species of Conosa typically possess a cone of microtubules (Cavalier-Smith et al. 2015, 2016). The bases of the flagella of *A. saltans* bear cones as we have shown in Figure 1, consistent with an affiliation of *A. saltans* with the Conosa. Conosa are morphologically highly diverse and comprise the Variosea, Archamoebae and Mycetozoa (Cavalier-Smith 2009, Cavalier-Smith et al. 2015, 2016; Kang et al. 2017). Single-gene phylogenies challenged the validity of Conosa due to long branches of many taxa, however multi-gene trees gave increasing support for its monophyly (Cavalier-Smith et al. 2015; Kang et al. 2017).

Our phylogenetic analyses supported that *A. saltans* belongs to the class Variosea, branching as a sister clade of *M. marina*. *Multicilia* is placed in the order Holomastigida Lauterborn, 1895 and family Multiciliidae Poche, 1913 (see Cavalier-Smith 2013). *M. marina* was described as an intermediate form between flagellates and heliozoans (Cienkowski 1881). A close relationship between *A. saltans* and *M. marina* is supported by their similarity in a considerable number of morphological traits. *M. marina* cells are also spherical with flagella that move slowly (Mikrjukov and Mylnikov 1998). *Multicilia marina* moves by flagella with irregular, apparently uncoordinated agitations, except one anterior and one posterior flagellum that seem responsible for the direction of movement (Mikrjukov and Mylnikov 1996, 1998). However, *M. marina* and *A. saltans* differ in their inhabited ecosystems. *M. marina* is reported to be a marine flagellate (Mikrjukov and Mylnikov 1998).

The six different SSU rDNA sequences from three individuals of *A. saltans* indicate a genetic variability within *A. saltans* cells. This may hint at multiple sequences variants amongst the copies of the ribosomal operon in *A. saltans* cells or a complex life history, possibly including fusion and genetic exchange, i.e. sex. Although a complex life history was reported for *M. marina* (Mikrjukov and Mylnikov 1998), no fusion of cells or sexual reproduction was yet confirmed.

Methods

Sampling and observations: Specimens of *A. saltans* were collected at several locations in Austria, France, Germany and the Netherlands (Table 1). All specimens except from the Wipperfürth strain were collected with a large wide mouth pipette with an internal opening of 5 mm. It was used to collect the uppermost layer of the sediment. Each time, a wide-mouthed bottle was filled with about 1 cm of sediment, transported to the lab and kept at room temperature on a windowsill on the north side. Small amounts of sediment were placed with a pipette on microscope slides and covered with a 24 × 32 mm cover slip. These wet mounts were kept in moisture chambers for at least one day before observations started. Living specimens of *A. saltans* were observed with an Olympus BX51 microscope with following objectives: 10XAPLN, 20 × 0.75 APO, 60 × 0.90 APO with correction collar and 100 × 1.30 oil, all with DIC. They were filmed and photographed with a Canon D70 camera. Adobe Photoshop was used for processing and measuring.

The Wipperfürth strain was sampled by scraping near-surface sediments with a plastic tube. The sampled material was transferred into a 24-well-plate. Pictures and videos were taken with a Nikon digital sight DS-U2 camera (program: NIS-Elements V4.13.04) with a Nikon TE2000-E inverted microscope (up to 400x magnification, phase contrast). All attempts to culture *A.saltans* isolates were not successful.

DNA extraction, amplification, cloning and sequencing: Specimens of the Wipperfürth strain were isolated and subjected to SSU rDNA sequencing. Two PCRs were setup, one with a single cell and another with two cells. The individuals were placed with approx. 1 μl of culture medium into sterile 200 μl PCR tubes which contained 4 μl ddH$_2$O. Directly after isolation 4.6 μl PCR mixture was added, including 1.7 μl ddH2O, 1 μl Thermo Scientific Dream Taq Green Buffer, 1 μl of 10 μM forward and reverse primers each, 0.2 μl 10 μM dNTPs and 0.1 μl DreamTaq polymerase (Thermos Fisher Scientific, Dreieich, Germany). The whole SSU rDNA was amplified with the primers EukA and EukB (Medlin et al. 1988). In a second step, semi-nested re-amplifications were performed in 50 μl reaction mixtures by using the primer pairs EukA + 1300R (Quintela-Alonso et al. 2011) targeting the 5' part and 590 F (Quintela-Alonso et al. 2011) + EukB for the 3' part of the gene.

Eight μl of the PCR products were purified by adding 0.15 μl of exonuclease, 0.9 μl FastAP and 1.95 μl ddH$_2$O to 8 μl of the reamplified PCR product and then heated for 30 min at 37 °C, and subsequently for 20 min at 85 °C. The Big dye Terminator Cycle sequencing Kit (Thermo Fisher Scientific, Dreieich, Germany) and an ABI PRISM automatic sequencer were used for the sequencing. After several attempts of sequencing which resulted in partially ambiguous SSU rDNA sequences, PCR products were subjected to cloning.

For cloning, 40 μl of the PCR products were cleaned with GenJet PCR purification kit (ThermoFisher Scientific), cloned with the Promega pGEM-T EasyVector in combination with the JM109 competent bacteria and subjected to sequencing as described above, including necessary primer walking. The partial sequences (1450 bp length) were manually checked for sequencing errors before they were assembled into sequence contigs. A BLASTn (2.3.0, default parameters) screening revealed an affiliation to the variosean *Multicilia marina*.

To create a dataset for phylogenetic analyses, SSU rDNA sequences of various amoebozoan taxa were obtained from the NCBI GenBank database (last date of accession: January 21, 2019). The sequences were downloaded and aligned with MAFFT version 7 (Katoh and Standley 2013). 1777 sites were used for the alignment of which 25.15% were invariant. Phylogenetic trees were inferred with the GTR + I+G model in RAxML (Stamatakis 2014) with 200 random taxon additions for the maximum likelihood tree search and a 1000 replicate bootstrap analysis and MrBayes (Altekar et al. 2004; Ronquist and Huelsenbeck 2003). The Bayesian analysis was set up with a sampling of every 100 and a diagnosis of every 500 trees and 25% of burn-in. It was conducted on the online platform CIPRES (Miller et al. 2010) and reached average standard deviation of split frequencies <0.01 for the last 75% of the sample after 21,355,000 generations. The sequences were submitted to the NCBI database under the accession numbers: MH729175, MH729176, MH729177, MH729178, MH729179, MH729180.

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Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.protis.2019.06.002.

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