**Morphology, ontogenesis and molecular characterization of *Atractos contortus*
Vörösváry, 1950 and *Stichotricha aculeata* Wrzesniowskiego, 1866
(Ciliophora, Stichotrichida) with consideration of their systematic positions.**

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Abstract

Atractos contortus, a rare highly spiralized hypotrich ciliate, has not been studied by modern methods. *Atractos contortus* and *Stichotricha aculeata* are currently assigned to the Spirofilidae. A Canadian population of *A. contortus* and an Idaho population of *Stichotricha aculeata*, were characterized using live observation, silver impregnation, scanning electron microscopy, and 18S rDNA sequencing. *Atractos contortus* has two frontal and one buccal cirrus, two marginal and two ventral rows arranged in left-handed helices, and four dorsal kineties. Left and right marginal rows make two and a half and three turns; left and right ventral rows are shortened. Frontoterminal, postoral, transverse and caudal cirri are absent. During morphogenesis the parental adoral zone is completely retained. Cirral rows replicate by intrakinetal and de novo processes. New right marginal and right ventral rows arise from the parental right ventral row. The parental right marginal row is morphogenetically inactive. Dorsal kineties replicate apokinetally. The parental dorsomarginal kinety and some parental marginal cirri persist for some time after cytokinesis. Phylogenetically, *A. contortus* groups with *Uroleptus* species and not spirofilids. The Idaho *S. aculeata* matches populations from previous reports. Monophyly of the Spirofilidae is rejected. Placement of genus *Atractos* in the family, Atractosidae nov. nom. is proposed.

Keywords: Canada; Dorsomarginalia; Morphogenesis; Phylogeny; *Uroleptus*; Spirofilidae

Introduction

The “hypotrichs” sensu Berger (2006) and Adl et al. (2012) represent a huge group of morphologically diverse and ubiquitously distributed ciliates immediately recognizable, even to the amateur microscopist, by their usually flattened bodies, distinctive adoral zone of membranelles, and compound ventral ciliary organelles (cirri). Despite being studied for centuries, the natural relationships within this group remain poorly resolved (Berger 2001; Foissner et al. 2004; Hu et al. 2011; Lynn 2008; Paiva et al. 2009).

Gelei (1929) erected the family Spirofilidae to accommodate hypotrich ciliates with distinctly spiraling or obliquely curved cirral rows, with *Spirofilum* (junior synonym of *Hypotrichidium* Ilowaisky, 1921) as type genus. Members of this group occur in marine, brackish, limnetic, and terrestrial habitats (Chen L. et al. 2013; Chen X. 2013; Chen et al. 2014; Froud 1949; Paiva and Silva-Neto 2007). Borror’s (1972) revision included seven genera in the family. Lynn (2008) assigned 13 genera, including *Kahliella* Tucolesco, 1962 as incertae sedis, to the Spirofilidae. A new marine genus, *Metastrongylidium*, added by Xu and Lei (2007) is considered a junior synonym of *Spiroamphisiella* by Berger (2008). The genus *Pseudouroleptus* Hemberger, 1985, originally included in the Amphisiellidae, was also assigned to the Spirofilidae by Chen et al. (2014). Although the genus *Urospinula* Corliss, 1960 was placed in the Spirofilidae by Corliss (1979), Lynn (2008) included it in the Ampisiellidae and, more recently, Heber et al. (2014) include it in the Psilotrichidae Bütschli, 1889. To date, sequences of 18S rDNA are known for only three of the spirofilid genera. Data on morphology and morphogenesis based on modern methods is similarly sparse. The monophyly of the Spirofilidae has been questioned, but undersampling has left this issue unresolved (Chen L. et al. 2013; Lynn and Small 2002; Paiva and Silva-Neto 2007; Xu and Lei 2007). Thus there is a pressing need for

increased taxon sampling in this group, including detailed morphologic, morphogenetic and molecular data (Chen L. et al. 2013; Paiva and Silva-Neto 2007; Paiva et al. 2009; Xu and Lei 2007).

Vörösváry (1950) described *Atractos contortus*, a distinctive hypotrich with a highly spiralized ciliature and placed the monotypic genus in the Spirofilidae. Tuffrau and Fleury (1994) designated the genus incertae sedis in the Oxytrichidae. Jankowski (1975) transferred the genus to a new monotypic family, Atractidae, but then abandoned this family status, assigning it again to Spirofilidae (Jankowski 2007). Corliss (1979) and Lynn (2008) considered Atractidae as a synonym of the Spirofilidae. *Atractos contortus* has, perhaps, the most highly spiralized ciliature of the hypotrichs in general and the spirofilids in particular. This curious species, depicted in only a single line drawing until now, has not been characterized by modern methods, and, with the exception of one listing without illustration, it has not been reported since its discovery in eastern Europe (Babko and Kovalchuk 1992). A population of *A. contortus*, recently found in Alberta, Canada, provided sufficient material for a detailed morphologic, morphometric, morphogenetic, and molecular characterization of this unusual hypotrich. *Stichotricha aculeata* Wrzesniowskiego, 1866 is also currently assigned to the Spirofilidae (Lynn 2008). A brief description, morphometry, and an 18S rDNA sequence for this species from Idaho populations are also included in this report.

Material and Methods

Collection data. *Atractos contortus* was found in a freshwater puddle in Calgary, Alberta, Canada in September of 2014 (see details in Occurrence and ecology section). Subcultures in site water and Eau de Volvic mineral water with wheat or rice grains to stimulate growth of bacteria for food were unsuccessful. Subcultures with green algae (*Golenkiniopsis* sp.) as a food source

supported a light growth of ciliates for about one week. The raw culture was maintained for about six weeks at which point the population collapsed.

Stichotricha aculeata was found in a shallow ephemeral puddle (Population 1) and a soil percolate from the bank of a rainwater catchment pond (Population 2) near Boise, Idaho in December 2014 and June 2013 respectively. Raw cultures were maintained for several weeks. The morphometry (Table 2), protargol preparations (Fig. 8B–D), and gene sequencing were based on Population 1 and the scanning electron microscope image (Fig. 8E) is from Population 2. Identification was based on Foissner (1980), Foissner et al. (1991), Gelei (1954) and Kahl (1932).

Morphologic methods. Living cells were studied at magnifications of 100–1000× with brightfield, phase- and differential interference contrast illumination using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA), an Olympus BX53 microscope (Olympus America, Center Valley, PA, USA), and a Flex digital camera with calibrated Spot imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Swimming motion, flexibility, contractility and cell shape were observed under the stereomicroscope (Amscope, Irvine, CA, USA). Encystation of *A. contortus* was induced by starvation in filtered (0.22 µm pore size) site water. Cysts were observed at intervals for 14 d. Cysts of *S. aculeata* were not studied. Protargol and methyl green-rhodamine (Waldeck, Münster, DE) staining was done according to Foissner (2014). Specimens for scanning electron microscopy were fixed with a 1:1 solution of 2.5% glutaraldehyde and 2% osmium tetroxide, processed according to Foissner (2014) and examined at 8–10 kV in a Hitachi S-3400N scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan). In vivo measurements were made from photomicrographs of freely swimming cells using calibrated

software. Counts and measurements were made at magnifications of 630–1000×. Measurements of protargol-impregnated specimens were made directly with an ocular micrometer and from microphotographs using calibrated software. Statistical analyses were performed using MedCalc for Windows, version 11.2 (MedCalc Software, Mariakerke, Belgium). Drawings of live cells were done freehand and drawings of silver-impregnated cells were done with the aid of a drawing attachment.

Assessment of phototaxis in *A. contortus*. A simple assessment of phototaxis was carried out by placing a 30 mm culture dish containing filtered site water and approximately 20 live cells under the transmitted-light stereomicroscope with half the illuminated field covered by aluminum foil. Cell behavior was observed for about 20 min to determine any preference of the cells for the light or dark half of the dish. This process was repeated with fresh cells three times.

DNA extraction, amplification and sequencing. Single cells were selected from raw samples, washed three times in sterile mineral water, placed individually in PCR tubes containing 50 µl 10 mM Tris–Cl buffer (pH 8.5), and stored at –20 °C prior to extraction. DNA extraction, amplification and sequencing were done as previously described (Bourland et al. 2012). Chromatograms were manually edited using 4-Peaks (Griekspoor and Groothuis 2006) and assembled into contiguous sequences using CAP3 (Huang and Madan 1999).

Phylogenetic analyses. An alignment, comprising 18S rDNA sequences of 50 hypotrich taxa with two oligotrichid and two choreotrichid taxa as outgroup, was constructed using MUSCLE (Edgar 2004) with default settings and further refined by eye. Taxon names and accession numbers are given in Figs 9 and 10 and Table 1. Two new 18S rDNA sequences are included (*Atractos contortus* KP233896 and *Stichotricha aculeata* KR701611). Neither resolution of nodes nor support values were improved by sequence alignment in R-Coffee (data not shown)

taking secondary structure into account (Wilm et al. 2008). Maximum likelihood analysis was implemented using ML + rapid bootstrap analysis, auto MRE option and the GTRGAMMA substitution model in raxmlGUI with 600 bootstrap pseudoreplicates (Pattengale et al. 2010; Silvestro and Michalak 2012; Stamatakis 2006). Posterior probabilities were determined using MrBayes 3.2.3 (Ronquist et al. 2012) on the CIPRES Portal V 1.15 (Miller et al. 2010), with support from four simultaneous MCMC chains run for 1,035,000 generations and sampling every 1000 generations. Convergence was assessed using Are We There Yet (Nylander et al. 2008). The first 25% of sampled trees were discarded as burn-in prior to tree reconstruction and a 50% majority-rule consensus tree was produced. Neighbor joining and maximum parsimony analyses were done using MEGA version 6 (Tamura et al. 2013). For neighbor joining, the TIM3+I+G substitution model, 1000 bootstrap pseudoreplicates and partial deletion with site coverage cutoff of 10% were used. For MP analysis the subtree-pruning-regrafting search method was used with 1000 bootstrap pseudoreplicates, 10 randomly added initial trees and sites with < 10% coverage treated as missing data. Trees are presented as cladograms with support values from the best maximum likelihood tree mapped onto the Bayesian inference tree and the maximum parsimony support values mapped onto the neighbour joining tree using TreeGraph 2 (Stöver and Müller 2010). According to Hillis and Bull (1993) bootstrap values <70 were considered as low, 70–94 as moderate, and ≥95 as high support. For posterior probabilities, values <0.70 were considered as low, 0.70–0.94 as moderate, and ≥0.95 as high support.

Topology hypothesis testing. Constrained trees were generated and compared with the unconstrained (i.e. best-scoring) maximum likelihood tree topology generating a file of per-site log-likelihoods using RaxML (Silvestro and Michalak 2012). The file of per-site likelihoods was fed to the appropriate executables in CONSEL ver. 0.1k to complete the approximately unbiased

(AU) test for various constrained topologies (Shimodaira 2002, 2008; Shimodaira and Hasegawa 2001). A p -value of < 0.05 was chosen for rejection of the null hypothesis that the negative log likelihoods of the constrained trees and best unconstrained maximum likelihood tree did not significantly differ.

Terminology. Terminology is according to Foissner and Al-Rasheid (2006) and Lynn (2008). To avoid confusion, the usual terms of morphologic orientation are used, that is, the “ventral” surface is that bearing the adoral zone in its left anterior part; the opposite surface is considered “dorsal”; when seen from the ventral aspect, “right” and “left” cell margins are on the viewer’s left and right respectively. Classification generally follows Lynn (2008) except as noted. The informal term “hypotrich” sensu Berger (2006) refers to Spirotricha excluding oligotrichs and euplotids and “spirofilds” refers to taxa currently assigned to family Spirofilidae by Lynn (2008) and others (Chen et al. 2014).

Results

Class Spirotrichea Bütschli, 1889

Subclass Stichotrichea Small and Lynn, 1985

Order Stichotrichida Fauré-Fremiet, 1961

Family Atractosidae nom. nov.

1975 Atractidae fam. nov.– Jankowski, Akad. Nauk. SSSR, Zool. Inst. Leningrad year 1975, p. 27.

1979 Atractidae fam. nov. – Jankowski, Trudy zool. Inst., Leningr. p. 75 (junior synonym of Atractidae Jankowski, 1975).

2001 Atractidae Jankowski, 1975 – Berger, Catalogue of Ciliate Names. 1. Hypotrichs, p. 105 (Atractidae Jankowski, 1979 designated a junior synonym).

Remarks. Atractidae Jankowski, 1975 is a junior homonym of the nematode family Atractidae Railliet, 1917, thus a replacement family name is required in accordance with the ICZN (International Code of Zoological Nomenclature 1999, Article 29.6). The entire genus name, *Atractos*, is combined with the required suffix, -idae in accordance with recommendation 29A of the ICZN (1999).

Diagnosis. Medium to large (80– >200 µm) Stichotrichida with some helical cirral and dorsal bristle rows making two or more complete turns around the cell axis. Midventral complex absent. At least one dorsomarginal kinety. Parental right marginal row morphogenetically inactive, right marginal cirral row develops from right ventral row anlage. Dorsal kinety fragmentation during morphogenesis absent. All dorsal kineties replicate de novo in proter and opisthe.

Type genus. *Atractos* Vörösváry, 1950

Genus *Atractos* Vörösváry, 1950

- 1950 *Atractos* g. n. – Vörösváry, Ann. Biol. Univ. Szeged. 1, pp. 372, 384.
- 1972 *Atractos* Vörösváry, 1950 – Borror, J. Protozool. pp. 19, 12. (revision).
- 1979 *Atractos* Vörösváry, 1950 – Corliss, The Ciliated Protozoa, p. 216.
- 1979 *Atractos* Vörösváry, 1950 – Jankowski, Trudy zool. Inst., Leningr. 86, p. 50 (revision of order Hypotrichida Stein, 1859).
- 1983 *Atractos* Vörösváry, 1950 – Curds et al., In: Part II, Synopses of the British Fauna, p. 372 (taxonomic key).
- 1985 *Atractos* – Small and Lynn, In: Illustrated Guide to the Protozoa, p. 453.
- 1994 *Atractos* Vörösváry, 1950 – Tuffrau and Fleury, In: Traité de Zoologie. Tome II, Fasc. 2, p. 144 (brief familial review).

- 2001 *Atractos* Vörösváry, 1950 – Aescht, Denisia 1, p. 27 (catalogue of ciliate generic names).
- 2001 *Atractos* Vörösváry, 1950 – Berger, Catalogue of Ciliate Names. 1. Hypotrichs, p. 11.
- 2002 *Atractos* Vörösváry, 1950 – Lynn and Small, In: The Illustrated Guide to the Protozoa, p. 448.
- 2007 *Atractos* – Jankowski, Phylum Ciliophora Doflein, 1901, In: Alimov, (Ed.), Protista. Part 2, p. 471.
- 2008 *Atractos* Vörösváry, 1950 – Lynn, The Ciliated Protozoa, p. 358.

Improved diagnosis including data from the Canadian population. Medium to large-sized (80– >200 μm) highly flexible, contractile Atractosidae; shape elongate, unflattened, distinct tail. Two marginal cirral rows; two ventral rows. Some cirral rows make at least three leftward helical turns around long axis. Frontal and buccal cirri present, frontoterminal, postperistomial, transverse and caudal cirri lacking. Dorsal kineties in strip bordered by right and left marginal rows.

Type species. *Atractos contortus* Vörösváry, 1950 by monotypy.

Species included. *Atractos contortus* Vörösváry, 1950.

Etymology. Not provided in the original description but certainly from the Greek noun “ἀτρακτος” = spindle or arrow, in reference to the spindle shape of the cell. Masculine gender.

***Atractos contortus* Vörösváry, 1950**

- 1950 *Atractos contortus* sp. nov. – Vörösváry, Ann. Biol. Univ. Szeged. 1, p. 372 (Fig. 11).
- 1972 *Atractos contortus* Vörösváry, 1950 – Borror, J. Protozool. pp. 19, 12 (Fig. 28, modified from Vörösváry [1950]).
- 1985 *Atractos contortus* – Small and Lynn, In: The illustrated guide to the Protozoa. p. 454 (Fig. 14, from Borror [1972]).

- 1992 *Atractos contortus* – Babko and Kovalchuk, Free-living infusoria of the Sumsoi region, In: Sumsoi, M. of E. and P.I.M. of the town (Ed.), Problems of Protection and Conservation of the Natural Resources of the Sumsoi Region. Sumsoi. pp. 113–125. (record from ?Ukraine without morphologic data or illustration).
- 2001 *Atractos contortus* Vörösváry, 1950 – Berger, Catalogue of Ciliate Names. 1. Hypotrichs, p. 11.
- 2002 *Atractos contortus* – Lynn and Small, In: The illustrated guide to the Protozoa, p. 448 (Fig. 21, from Borror [1972]).
- 2007 *Atractos contortus* – Jankowski, In: Alimov, (Ed.), Protista. Part 2, Handbook on Zoology, p. 471 (no illustration).

Improved diagnosis based on the Canadian population and original description. Size about $170 \times 40 \mu\text{m}$ in vivo; elongate fusiform to narrowly clavate; elliptical to circular in cross-section; right dorsolateral margin convex, left margin more or less straight; slender rounded tail. Somatic cirri in shallow spiraling cortical furrows, cortical granules following dorsal bristle rows, inconspicuous in vivo but stain pink with methyl green-rhodamine. Two macronuclear nodules, one micronucleus adjacent to each. Adoral zone straight, 37 adoral membranelles on average, adoral zone not extending to right margin, membranelles composed of two long and one short file of basal bodies. Two frontal cirri and one buccal cirrus aligned longitudinally. Right marginal row begins on anterior dorsal side spiraling around long axis three turns, left marginal row begins at buccal vertex, makes two and one half turns; marginal rows confluent posteriorly. Two ventral rows shortened posteriorly, left more so than right. Three ordinary dorsal bristle rows, one dorsomarginal kinety. Semi-terrestrial.

Type locality. Vörösváry (1950) established the genus and species for a population from a shallow muddy puddle on the bank of Kalános stream (? eastern Hungary).

Etymology. Not specified in the original description, probably from the Latin adjective *contortus*, -a, -um (twisted together), referring to the highly spiralized cirral and dorsal bristle rows.

Voucher material. No record of type material could be found. Six voucher slides with many protargol-impregnated specimens, including several dividers, are deposited in the Biology Center of the Museum of Upper Austria, Linz (LI, accession numbers 2015/28-33). Pertinent specimens are circled in black ink on the cover glass. The 18S rDNA sequence from the Canadian population has been deposited in GenBank (accession no. KP233896 as *Atractos contortus*).

Description of the Canadian population (Figs 1-7; Table 2). Body size about 155–185 × 34–43 µm in vivo (n=10); 107–183 × 23–60 µm, length:width ratio about 4:1 (range 3–6:1) in protargol-impregnated specimens, up to 6.8:1 in scanning electron microscope specimens (Fig. 4A–D). Shape quite variable depending partly on amount and type of ingesta and aspect viewed, symmetric in ventral view, very slenderly to broadly fusiform or narrowly clavate, asymmetric when viewed laterally, right dorsolateral margin convex, left dorsolateral side more or less straight, sometimes forming short rounded left anterior “beak” at junction of right and left sides (Figs 1F–K, 2A–C, 4A–D). Not dorsoventrally flattened (i.e. more or less circular in cross section). Anterior end bluntly to narrowly rounded when viewed ventrally. Posterior end tail-like, varying from about 10–25% of body length (Figs 1F–K, 2A, B, 4A–D). Entire cell highly flexible and contractile, most obvious when seen rummaging through detritus (**video link here please**). Two macronuclear nodules in middle third of cell, anterior nodule 15 × 10 µm, usually

rounded cuneate, posterior nodule $18 \times 9 \mu\text{m}$, ellipsoidal, sometimes globular, especially in more slender specimens (Fig. 1I), macronuclear nodules connected by inconspicuous thread in some protargol preparations; nucleoli numerous, diffusely distributed, approximately $1.5 \mu\text{m}$. Two approximately $4 \mu\text{m}$ globular micronuclei, one adjacent to each macronuclear nodule. Single contractile vacuole at left margin slightly posterior to level of buccal vertex, collecting canals not observed. Cortical granules sparse, globular, approximately $1 \mu\text{m}$, colorless and inconspicuous in vivo, stain pink with methyl green-rhodamine, not seen in protargol preparations, arranged in loose bands along dorsal bristle rows and between cirral rows (Figs 2E, 3J). Cytoplasm colorless but cells appear dark grey under low power due to numerous approximately $3.5 \mu\text{m}$ refractive globules, $1\text{--}2 \mu\text{m}$ irregular crystals, $10\text{--}30 \mu\text{m}$ food vacuoles containing diatom frustules, green algae, fungal spores and mineral particles (Figs 1A, 3D, I). Swims at moderate pace rotating around long axis, more often seen darting frantically to and fro through dense detritus and silt particles. No phototaxis observed.

Cirral pattern constant, number of cirri of low variability (Table 2). Cirri of marginal and ventral rows about $16 \mu\text{m}$ long, cirral bases in distinct rectangular cortical pits arranged in shallow spiral furrows, comprised of three files of approximately seven basal bodies in anterior half, more posterior cirri composed of two files; right marginal row commences at anterior end, composed of about 97 cirri, left marginal row commences at buccal vertex, composed of about 82 cirri, marginal rows extend to posterior pole, right marginal making three full turns, the left two and a half. Left ventral row shortest, commences near distal end of adoral zone, spirals leftward less than one turn, ends on dorsal surface near right margin, composed of about 38 cirri, left ventral row cirri slightly larger, more widely spaced between anterior end and buccal vertex. Right ventral row commences at anterior end, spirals two turns around cell to end on dorsal

surface in posterior fifth of cell, composed of about 84 cirri (Figs 1A–M, 3A–F, H, 4A–D). Fibrillar associates of marginal and ventral cirral bases, and dorsal bristle dikinetids long, sinuous, overlap in intercortical strip in some protargol preparations (Fig. 1N). Absence of postperistomial, transverse, and caudal cirri checked in morphostatic, dividing protargol specimens, scanning electron microscope preparations. Invariably two frontal cirri one behind the other between anterior end of left ventral row and adoral zone, about same size as anterior cirri of left ventral row; invariably one buccal cirrus at anterior end of paroral membrane, slightly smaller than frontal cirri (Figs 1A, B, 2E, 3D, H, 4A, B).

Dorsal bristles about 4 μm long, invariably comprising four regularly spaced kineties in helical intermarginal cortical strip. Dorsal kineties 1–3 “ordinary”, commence very near left margin of distal end of adoral zone; kinety 4 a dorsomarginal kinety, commences just posterior to anterior end of right marginal row; kineties 2 and 3 extend to posterior pole, each composed of about 70 bristles, kineties 1 and 4 end subterminally, kinety 4 shortest (Figs 1A–H, 3A–F, 4A–D, H).

Adoral zone occupies 20–36% of body length, more or less straight, distal end not extending to right body margin, composed of 37 membranelles on average, membranelle structure notable for only three files of basal bodies, proximal two long, nearly equal in length, distal one composed of only two basal bodies (Fig. 4E–G), no gap in adoral zone, base of widest membranelle about 11 μm . Intermembranellar bolsters triangular in scanning electron microscope preparations (Fig. 4G). Buccal cavity narrow, deep, teardrop-shaped when viewed laterally, nearly obscured by frontal scutum and buccal lip in ventral view (Figs 1A, B, E–L, 2A–D, 3A, B, D, F, G, 4A–D). Undulating membranes in stylonychid pattern, paroral slightly curved leftward distally, endoral membrane only slightly shorter, paroral stichodyad, endoral

stichomonad; paroral situated in conspicuous approximately 3.5 μm high curved buccal lip in scanning electron microscope preparations, endoral deep in buccal cavity, obscured by cilia of adoral membranelles and paroral in scanning electron microscope preparations; paroral cilia about 8 μm long in protargol preparations (Figs 1A, B, E, G, 2E, 3H, 4A–C, E–G). Pharyngeal fibers inconspicuous in vivo, arise from proximal half of adoral zone, converge in approximately 14 μm long rightward-directed funnel in protargol preparations. Second group of fibers arise from inner ends of adoral membranelles and converge left of adoral zone in distinct parallel bundle (Figs 1E, 3G).

Spherical kinetosome-resorbing resting cysts form about 72 h after isolation of cells in food-free medium. Cells first settled and rounded up while slowly rotating. Kinetosome resorption began before ectocyst formed (Fig. 2F). At 72 h the cyst was about 50 μm across, cytoplasm was packed with 2 μm globules and smaller granular inclusions, the finely wrinkled ectocyst was approximately 1.5 μm thick (Fig. 2G, H). The globular nuclear mass was centrally positioned. A peripheral contractile vacuole continued to pulse (Fig. 1O, 2G). At 5 d, the contractile vacuole had disappeared (Fig. 2I). By one week, the diameter of the outer layer was unchanged but the cell contents contracted into an approximately 40 μm smooth sphere presumably enclosed by a thin endocyst. Clumps of bacteria adhered to the ectocyst layer. The cytoplasm became more homogeneous. The nuclear mass contracted into a thick tortuous ribbon (Fig. 1P, 2J). By 11 d, the cyst diameter decreased to about 44 μm and cell contents contracted further to a 34 μm slightly eccentric globular mass with irregular margins. The nuclear material became inconspicuous (Fig. 1Q, 2K). At two weeks, the outer cyst wall had a crumpled appearance (Fig. 2L). Excystment was not observed.

Morphogenesis of *Atractos contortus* (Figs 5A–F, 6A–G, 7A–H). Observations on morphogenesis must be considered preliminary since important middle stages were absent in protargol preparations and found only once in scanning electron microscope preparations (Fig. 7F, G). Thus, the details of macronuclear division (i.e. whether the two macronuclei fuse and divide twice, as usual, or divide only once as separate nodules), the origins of frontal cirrus 2 and the buccal cirrus, whether any left ventral row cirri participate in oral primordium formation, and whether the oral primordium contributes to the right ventral row remain speculative.

Oral apparatus. Mid-early division was the earliest morphogenetic stage observed. The oral primordium develops just to the left of the midportion of the left ventral row. Contribution of proximal left ventral row cirri to the primordium was not observed but cannot be excluded. At this stage, opisthe adoral membranelles begin to differentiate in an anterior to posterior direction. A smaller field of disorganized basal bodies develops just anterior to the oral primordium and its subsequent fate was not determined. A file of dikinetids, possibly the future paroral of the opisthe, develops between this field and the parental undulating membranes. Together, these also appear to be the primordium of the proter left ventral row (Figs 5A, C, 7A, B). At this stage the parental paroral and endoral membranes dedifferentiate. The developing opisthe oral apparatus rotates leftward to the side opposite that of the proter oral apparatus (Figs 5D, 6A, B, 7C, D, F). The proter and opisthe paroral and endoral membranes begin to differentiate, the latter from the oral primordium. Basal bodies of both new undulating membranes continue to resorb in proter and opisthe to achieve their morphostatic length and structure. The parental adoral membranelles are completely retained. The proter and opisthe continue their axial rotation until the oral apparatus of both occupies the same (i.e. ventral) aspect (Fig. 6D).

Frontal and buccal cirri: In early division, the parental frontal cirri and buccal cirrus are resorbed. The anteriormost proter frontal cirrus (probably homologous to frontal cirrus I/1, [Berger 1999]) forms at the anterior end of the reintegrating endoral membrane (Figs 5A, C). The origin of the proter buccal cirrus as well as the origin of the second (posterior) frontal cirrus could not be determined. The origin of the frontal cirri and buccal cirrus in the opisthe could not be determined.

Ventral and marginal cirral rows. The left marginal row in proter and opisthe develops intrakinetally and extends bidirectionally (Figs 7F, G). The parental left marginal row is resorbed. In the proter, the new left marginal row extends anteriorly between the new dorsal kinety 1 and the retained parental adoral zone. In the opisthe, the new left marginal row develops between the degenerating parental left marginal row and the distal portion of the new adoral zone (Figs 5D, F, 7D–G). The anterior end of the left marginal row in both proter and opisthe is at the level of the distal half of the respective adoral zones (Figs 5D, 6A, C, D, F, 7F, G). As morphogenesis proceeds, the anterior end of left marginal row in both proter and opisthe migrates posteriad to assume its morphostatic position abutting the buccal vertex. The proter left ventral row appears to develop apokinetally at the anterior end of the oral primordium extending bidirectionally (Figs 5C, D, F, 6A, C, D, F, G, 7A–G).

Right ventral and marginal row development is more complex. The parental right ventral row splits in the midportion of the cell (Fig. 5A, C). Primordia develop at the posterior ends of the anterior and posterior fragments (Fig. 5C). The anterior fragment extends bidirectionally to create the new right ventral row for both proter and the anterior portion of the opisthe right ventral row while the posterior fragment forms the new right marginal row (Figs 5C, 7F). The opisthe right ventral row may result from fusion of the intrakinetel part already described and an

apokinetally derived posterior segment (Fig. 7F, G). A contribution to the right ventral row by the group of basal bodies at the anterior oral primordium was not observed but cannot be excluded due to the limited stages of division available for study. However, the parental right marginal row is morphogenetically inactive (i.e. it does not contribute to the new ciliature of either proter or opisthe). In the proter, the new right ventral and right marginal row extend anteriorly to the left of the parental rows which are not resorbed at this point (Fig. 7F).

Resorption of parental right marginal cirri is not complete until after cytokinesis (Figs 6G, 7H).

Dorsal ciliature. All dorsal kineties develop de novo in proter and opisthe (i.e. parental rows do not contribute to formation of new rows) at two levels. New “ordinary” proter and opisthe dorsal kineties 1–3 develop between the new left marginal row and the resorbing parental dorsal kineties (Figs 5D, F, 6F, 7D, E). The new dorsomarginal kinety develops separately in proter and opisthe from a de novo primordium between the parental dorsomarginal row and the new proter right marginal row and extends posteriad (Fig. 5F). The parental dorsomarginal kinety remains largely intact, undergoing resorption only after cytokinesis (Figs 6G, 7H). The opisthe dorsomarginal kinety develops to the left of the new opisthe right marginal row (Fig. 6F).

Nuclear apparatus. Formation of replication bands in each macronuclear nodule is the first indication of division. As these approach the proximal poles of the two macronuclear nodules, and prior to micronuclear mitosis, nucleoli began to disappear (Figs 5B, E, 6B, E, 7A–E).

Fusion of the macronuclear nodules was not observed but may have been missed. An atypical mode of macronuclear division (i.e. division of individual macronuclear nodules without fusion as in *Pseudokeronopsis*) cannot be excluded (Hu and Song 2001). Micronuclear mitosis occurs in conjunction with amitotic division of the macronuclear nodules (Figs 5B, E, 6B, E).

Proter postdividers are distinguishable by the broadly obconical posterior end, the tail developing later after cytokinesis (Fig. 7H). Opisthe dividers retain the parental “tail” but have a broadly truncate anterior end that gradually becomes narrowly rounded to assume its normal morphostatic shape.

Molecular characterization and phylogenetic analyses (Figs 9, 10). Three separate but identical 18S rDNA sequences were obtained from single cells. The sequence consists of 1727 bp with a G/C content of 45%. The edited alignment comprised 55 taxa and 1732 positions including gaps. The topology of the Bayesian (BI) 50% majority-rule consensus and maximum likelihood trees showed several poorly-supported conflicts between methods in *Atractos-Uroleptus* clade (Fig. 9). *Atractos contortus* clusters with five uroleptids (*Uroleptus gallina*, *U. piscis*, *U. sp. MD-2012*, *U. sp. WJC-2013* and *Uroleptus lepisma*) in a clade strongly supported by Bayesian inference (0.99) and weakly by maximum likelihood (61%). Neighbor joining and maximum parsimony trees were less well resolved but also grouped *A. contortus* with uroleptids (neighbor joining 58%, maximum parsimony 53%).

The placement of *Hypotrichidium paraconicum* was weakly supported in all methods. The species branches between the large oxytrichid clade and the stylonychines in Bayesian and maximum likelihood trees but is grouped within the oxytrichid clade in neighbor joining and maximum parsimony trees (Figs 9, 10). Despite the incongruence between methods, *Hypotrichidium paraconicum* branches distantly from other spirofilid taxa in all trees. It should be noted that an 18S rDNA sequence for *Hypotrichidium conicum*, the type species of the Spirofilidae, is lacking. An 18S rDNA sequence with accession no. JQ918371 was originally submitted to GenBank with the source organism name *Hypotrichidium conicum*. The source organism was later identified as a new species, *H. paraconicum*, however the source organism

name has not been corrected and is still listed as *H. conicum* (JQ918371) in GenBank (Chen L. et al. 2013).

Spirofilid taxa were scattered throughout the tree. The monophyly of the Spirofilidae, as currently constituted, is rejected by the AU test ($p < 0.05$). Monophyly of the clade with *A. contortus* and *Uroleptus* or with *A. contortus* and the strongylidiids was not rejected (Table 2). The relationships between non-stylonychine oxytrichids, *Uroleptus*, the *Strongylidium*-*Pseudouroleptus* clade and *Atractos* are poorly resolved, forming a polytomy in 50% majority consensus trees (Figs 9, 10).

Occurrence and ecology. Vörösváry (1950) found *A. contortus* in the company of *Bursaria* in a depression containing water and mud with abundant diatoms and detritus beside a frozen freshwater stream in Hungary during winter (water temperature 7 °C, pH 6.5–7.2). The population described in the current report was discovered in a muddy ephemeral puddle in an alleyway in Alberta, Canada (51° 03' 00" N, 114° 04' 00" W) in September 2014 (mean ambient temperature approximately 19.5 °C). Covering water was non-saline (electrical conductivity 950 $\mu\text{S}/\text{cm}$) and slightly alkaline (pH 8.31). The sediment consisted of silty loam (mainly silt with smaller amounts of clay and little sand) mixed with fine detritus. *Atractos contortus* fed primarily on diatoms, green algae, chrysophytes and fungal spores. Cells often contained unusually numerous clay and silt particles, some large enough (up to $20 \times 40 \mu\text{m}$) to distort the cortex (Fig. 3E, I).

***Stichotricha aculeata* Wrzesniowskiego, 1866**

Improved diagnosis based on Idaho populations and previous descriptions (Foissner 1980; Foissner et al. 1991; Gelei 1954; Kahl 1932). Medium-sized (80–200 μm) Stichotrichida. Slender, elongate, anterior half forms a narrow, curved neck bearing adoral zone. Flexible and

contractile, body widest just posterior to cytostome; posterior end tapered or truncate. Inhabits cylindrical detritus-covered lorica; sometimes gregarious but not truly colonial. Zoochlorellae absent. Two macronuclear nodules, each with adjacent micronucleus. Four cirral rows in cortical furrows spiral about one half turn around long axis; two dorsal kineties, left shorter than right, additional scattered dorsal dikinetids in posterior half. Two to four frontal and one buccal cirrus. Adoral zone straight, about half body length, comprising about 40 adoral membranelles, distalmost 2 or 3 membranelles elongated. Paroral stichomonad, on cell surface without buccal lip, about half length of adoral zone, endoral slightly sigmoid.

Voucher material. No record of type or voucher material could be found. Two voucher slides with many protargol-impregnated specimens from Idaho Population 1 are deposited in the Biology Center of the Museum of Upper Austria, Linz (LI, accession numbers 2015/34-35). Pertinent specimens are circled in black ink on the cover glass. The 18S rDNA sequence from this population has been deposited in GenBank (accession no. KR701611 as *Stichotricha aculeata*).

Description of the Idaho population (Fig. 8A–E, Table 2). Body size about $90\text{--}125 \times 18\text{--}25 \mu\text{m}$, length:width ratio about 5:1 in vivo ($n=20$); $64\text{--}98 \times 16\text{--}25 \mu\text{m}$, length:width ratio about 4.3:1 (range 3.4–5.2:1) in protargol-impregnated specimens. Shape slender, elongate fusiform, mid-body elliptical in cross section. Anterior half of cell forms narrow dorsally curving neck bearing adoral zone of membranelles; posterior end tapered to rounded point or narrowly truncate. Cell flexible and contractile. Two widely separated macronuclear nodules, ellipsoid to globular, each with numerous $1.5 \mu\text{m}$ diameter nucleoli, in protargol preparations; usually one lenticular, approximately $4 \mu\text{m}$ -long micronucleus adjacent to each macronuclear nodule (2 of 19 cells had three micronuclei).

Single contractile vacuole at left margin slightly posterior to level of buccal vertex. Cortical granules absent; cytoplasm colorless, scattered small refractive globules; food vacuoles contain bacteria, green algae. Inhabits brown, granular, translucent lorica up to 550 μm long, about 45 μm diameter. Loricae may agglomerate (up to 15) but do not intercommunicate. Cells often flee loricae and swim at moderate pace rotating on long axis.

Cirral pattern constant, single left and right marginal row and two ventral rows, cirri about 10 μm long, posteriormost cirri elongated (about 15 μm). Cirri in left-spiralling cortical furrows, more loosely-spaced posteriorly. Left marginal row extends from buccal vertex to posterior pole; left ventral row begins just below anterior end, extends to posterior pole; right ventral row about same length as left; right marginal row extends from base of neck region to posterior pole, rows make up to one half turn around long axis. Usually four inconspicuous frontal cirri, difficult to distinguish from distal ends of ventral rows; invariably one buccal cirrus at level of distal endoral membrane, easily overlooked in protargol preparations since often superimposed on endoral membrane (Fig. 8C, E). Possibly two caudal cirri, not confirmed beyond doubt since dividers were not observed. Postperistomial and transverse cirri absent. Two dorsal kineties, left about length of adoral zone, right bipolar; sparse, scattered dorsal dikinetids posteriorly, dorsal bristles up to 15 μm .

Adoral zone straight, distal end not extending to right body margin, occupies about 50% of body length, composed of 39 membranelles on average, membranelles of usual structure, about 3.5 μm wide at widest point, distalmost two or three membranelles distinctly elongated up to 14 μm . Buccal cavity, short, tear-drop shaped at base of neck region. Paroral membrane composed of single file of basal bodies more widely-spaced distally, extends from buccal vertex about 75% length of adoral zone, situated on cell surface without buccal lip in scanning electron microscope

preparations (Fig. 8E); endoral membrane shorter (about 14 μm), slightly sigmoid, stichomonad. Pharyngeal fibers not seen. Dividers and cysts not observed.

Molecular characterization and phylogenetic analyses (Figs 9, 10). The 18S rDNA sequence, obtained from a single cell, consisted of 1724 base pairs with a G/C content of 45%. In all analyses *S. aculeata* branches between the amphisiellid clade and the gonostomatids and not with any other spirofilids, however support values are only low to moderate. Monophyly of the Spirofilidae including *S. aculeata* is rejected by the AU test with or without *A. contortus* (Table 3).

Occurrence and ecology. *Stichotricha aculeata* is widely distributed in terrestrial and aquatic habitats but has not been reported from Antarctica, Central or South America (Foissner 1998). *Stichotricha aculeata* is a common inhabitant of freshwater and terrestrial ecosystems in Idaho. It was found in a shallow muddy ephemeral puddle with decaying grass (Population 1) and soil percolates from the margin of a rainwater catchment pond (Population 2) near Boise, Idaho (43°38'35.82" N, 114°09'23.33" W, el. 1057 m; 43°39'06.60" N, 116°17'43.84" W, el. 813 m respectively). Ambient temperature at the time of collection was approximately 2 °C for (Population 1) and 19 °C (Population 2). Food items include bacteria and green algae. Loricae are covered with fine brown detritus.

Discussion

Is the Canadian population of *A. contortus* conspecific with the type population?

Vörösváry's (1950) description of *A. contortus* was based on live observations and the Bresslau opal blue method. The Canadian population matches the original description well in terms of size, shape, nuclear morphology, flexibility and contractility, movement, habitat, food preference

(diatoms), general pattern of the ciliature, and extent of the adoral zone. Despite its relative simplicity, the highly spiralized ciliary pattern can be difficult to interpret.

It is likely that the original description contains several misobservations: 1) structure of the adoral membranelles (two files vs. three in Canadian population). The distal file of only two basal bodies was almost certainly overlooked. Several other, apparently distantly related, taxa have adoral membranelles composed of only three files including *Etoschothrix*, *Erniella*, and *Hypotrichidium tisiae* and *H. faurei* sensu Tuffrau (1972; the latter two taxa with three long files) making it likely that such oligemerized membranelles evolved convergently (Foissner and Al-Rasheid 2006; Tuffrau 1972); 2) pattern of the left marginal row (commencing at the anterior end vs. commencing at the buccal vertex in Canadian population). Vörösváry thought frontal cirri were absent and likely misinterpreted the frontal and buccal cirri as the anterior part of the left marginal row (depicted in Fig. 11, Vörösváry 1950); 3) spiralization of ciliature (four cirral rows extending from anterior to posterior end in four helical turns vs. posteriorly shortened ventral rows and marginal rows making only two and one half to three turns in Canadian population). Cirral row length and the precise number of cirral helical turns is almost impossible to determine in vivo due to rapid motion and pronounced cortical flexibility. Interpretation of these features can be difficult even in protargol preparations thus, correlation of in vivo, protargol and scanning electron microscope observations was required for their accurate determination in the present study. It is likely, therefore, that the single figure in the original report reflects some misinterpretations of cirral row length and extent of spiralization; 4) negative phototaxis (present vs. absent in Canadian population). Vörösváry interpreted the preference of *A. contortus* for rummaging in detritus as an indication of light aversion. This was not confirmed by the admittedly crude assessment of phototaxis used in the present study. The

tendency of *A. contortus* to root among silt and detritus is most likely a feeding behavior rather than negative phototaxis.

Comparison of *A. contortus* with similar species. *Atractos contortus* can be distinguished from other spiralized hypotrichs (e.g. *Chaetospira*, *Hypotrichidium*, *Spiroamphisiella*, *Stichotricha*, and *Strongylidium* species) by its lack of dorsoventral flattening, absence of transverse and caudal cirri, and by the more than two complete helical turns of the marginal cirral rows and the bipolar helical intermarginal cortical strip bearing the dorsal bristle rows (Foissner et al. 1991; Froud 1949; Hu and Song 2001; Kahl 1932; Li et al. 2007; Paiva and Silva-Neto 2007; Xu and Lei 2007). *Atractos contortus* can be distinguished from *Microspiretta plancticola* by its shape (slender vs. broad), morphology of the adoral zone (straight, comprised of about 37 membranelles vs. distinctly spiral, comprised of about 100 membranelles), the morphology of the macronuclei (two nodules, separate or connected by inconspicuous thread vs. dumbbell-shape with distinct isthmus) and dorsal bristles (inconspicuous vs. stiff, distinctly elongated) (Gelei 1954).

Comparison of *A. contortus* with *Uroleptus*. Despite the above considerations, the recovery of the *A. contortus*-*Uroleptus* clade in phylogenetic trees created with the four different statistical methods employed was a surprise since morphologic and morphogenetic characters such as dorsoventral flattening, a zigzag midventral complex (Berger 2006), frontoterminal cirri, caudal cirri, transverse cirri, and formation of intrakinetal dorsal kinety primordia, all features of uroleptids, are absent in *A. contortus*. At low magnifications, *A. contortus* can easily be confused with *Uroleptus* species which also have a distinct “tail”. Closer study will reveal the absence of the uroleptid “zigzag” midventral cirral complex in *A. contortus*.

However *A. contortus* does share some morphologic characters with *Uroleptus* species including: 1) a pisciform (i.e. “tailed”) shape; 2) a highly flexible cortex; 3) presence of at least one dorsomarginal kinety. Morphogenetic similarities include: 1) the location, de novo origin, and morphology of the early oral primordium; 2) retention of an intact parental adoral zone during morphogenesis; 3) absence of dorsal kinety fragmentation during morphogenesis; 4) persistence of some parental dorsomarginal kineties and marginal cirri after cytokinesis (Eigner 2001; Foissner and Stoeck 2008; He et al. 2011; Olmo 2000).

The *Atractos-Uroleptus* clade was recovered in both Bayesian and maximum likelihood trees with strong and weak support respectively (Fig. 9). Bayesian inference probably overestimates nodal support while bootstrap support from maximum likelihood methods probably underestimates it (Simmons et al. 2004). This clade is also recovered (albeit with weak support and differing internal structure) in maximum parsimony and neighborhood-joining trees (Fig. 10). Monophyly of the *Atractos-Uroleptus* clade is not rejected in the AU test (Table 3). As noted by others, single gene trees, particularly those based on the highly conserved 18S rRNA gene of hypotrichs, may lack the phylogenetic signal to allow confident resolution of many relationships in this group (Paiva et al. 2009; Yi and Song 2011). Caution is also warranted in interpreting the clustering of *Atractos* and *Uroleptus* since only four of approximately 30 nominal *Uroleptus* species have been characterized by modern methods (He et al. 2011). Berger (2008) considered *Uroleptus* (at least preliminarily) to be one of the “non-oxytrichid Dorsomarginalia” (i.e. flexible hypotrichs with at least one dorsomarginal kinety but lacking dorsal kinety fragmentation during morphogenesis). The recent report of a flexible hypotrich with dorsomarginal kineties, lacking dorsal kinety fragmentation and yet having 18S rDNA sequences placing it well within the “oxytrichid Dorsomarginalia” clade, challenges dorsal

kinety fragmentation as an apomorphy of Oxytrichidae sensu Berger (2008) (Singh and Kamra 2013). However misidentification of some populations has likely caused confusion in hypotrich taxonomy (Shao et al. 2011).

A possible relationship of *A. contortus* to uroleptids will require reevaluation as more multi-gene trees are published and the morphology and morphogenesis of more *Uroleptus* species and spirofilids are characterized in detail.

Family classification. While acknowledging the thorny taxonomic and nomenclatural problems of the type genus *Uroleptus* Ehrenberg, 1831, Foissner and Stoeck (2008) erected the family Uroleptidae with the diagnosis “Very flexible midventral hypotrichs forming a distinct clade within the oxytrichids in molecular trees”. Despite the suggested molecular phylogenetic relationship, its morphology (i.e. absence of a mid-ventral complex) indicates *Atractos* does not belong to the Uroleptidae. Molecular data and morphology (i.e. the presence of a dorsomarginal kinety) exclude a close relationship between *Atractos* and the few spirofilids described to date (Chen L. et al. 2013; Chen X. et al. 2013; Foissner et al. 1991; Gelei 1954; Paiva and Silva-Neto 2007). Furthermore, Spirofilidae sensu Lynn (2008) is very likely nonmonophyletic. The unusual de novo mode of dorsal kinety replication in *A. contortus* has been documented in only two other hypotrichs viz. *Cotterillia bromelicola* and *Engelmanniella mobilis* (Berger 2011; Foissner and Stoeck 2011; Wirnsberger-Aeschl et al. 1989). However, in the latter two species at least some dorsal kineties reproduce as usual (i.e. intrakinetally) whereas all proter and opisthe dorsal kineties form de novo in *A. contortus*. The phylogenetic analyses indicate that this feature probably evolved independently at least three times in the hypotrichs (Figs 9, 10). Because the combination of distinctive morphologic and morphogenetic features, together with the phylogenetic analyses, preclude placement of *Atractos* in one of the existing stichotrichid

families, creation of Atractosidae nom. nov. pro Atractidae Jankowski, 1975 in the order Stichotrichida Fauré-Fremiet, 1961 is proposed.

Systematic position of *Stichotricha aculeata*. The morphology of the Idaho populations of *S. aculeata* closely matches previous descriptions from Austria, Hungary and Germany (Foissner 1980; Foissner et al. 1991; Froud 1949; Gelei 1954; Kahl 1932). Jankowski (1979) created the subfamily Stichotrichinae with *Stichotricha* as the type genus but abandoned it in a more recent revision (2007) in which *Stichotricha* was omitted from the Spirofilidae, possibly as an oversight. Borror and Evans (1979) speculated on the establishment of separate families for the “top-shaped” spirofilids (*Hypotrichidium* and *Microspiretta*) and the slender “tubicolous” taxa (*Stichotricha* and *Chaetospira*) but didn’t formalize this as a nomenclatural act. It should be noted that the authors misidentify a marine *Chaetospira* species (apparently lacking endosymbiotic zoochlorellae) as *Stichotricha secunda* Kahl, 1932 (Borror and Evans, 1979, Fig. 15). The current study clearly separates *Stichotricha aculeata* from other spirofilids, but does not provide sufficient information to confidently place it in an established family in Stichotrichida or justify creation of a new family. The 18S rDNA sequence of the type species, *S. secunda* has not been reported. Systematic placement awaits detailed ontogenetic data and evaluation of additional molecular markers for the *Hypotrichidium*-like and slender, “tubicolous” *Stichotricha*-like taxa.

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Legends

Fig. 1A–Q. *Atractos contortus*, Canadian population from life (1A, O–Q) and after protargol impregnation (1B–N). **A.** Ventral view of representative specimen. **B–E.** Ventral (B), dorsal (C), right lateral (D), and left lateral (E) views of separate specimens. **F–K.** Body shape variations after protargol impregnation. **L.** Schematic of ventral (black) and dorsal (gray) courses of cirral rows (solid bands) and dorsal kineties (dashed lines) showing the shortened left (arrowhead) and right (arrow) ventral rows. **M.** Ventral view of cirral rows at posterior pole (ventral in black, dorsal in gray). **N.** Dorsal view of fibrillar associates of cirral row and dorsal kineties. **O–Q.** Resting cysts at three (O), seven (P), and eleven (Q) days showing contractile vacuole (black arrow) and nuclear material white arrows). AZM, adoral zone of membranelles; BC, buccal cirrus; DK1–4, dorsal kineties 1–4; EM, endoral membrane; FC1, 2, frontal cirrus 1 and 2; HS, intermarginal helicoid cortical strip; LMR, left marginal row; LVR, left ventral row; Ma, macronucleus, Mi, micronucleus; PF, pharyngeal fibers; PM, paroral membrane; RMR, right marginal row; RVR, right ventral row. Scale bars: 50 μm (F–K), 25 μm (A–E, L, M, O–Q), 10 μm (N).

Fig. 2A–L. *Atractos contortus*, Canadian population from life (A, B, I brightfield; C–E, G, J–L differential interference contrast) and in scanning electron microscope (F, H). G, I–L show same

specimen. **A, B.** Left lateral views. **C.** Right lateral view showing contractile vacuole (arrow) and macronuclear nodules (arrowheads). **D.** Left lateral view showing left and right marginal (black and white arrows respectively) and left and right ventral rows (black and white arrowheads respectively). **E.** Ventral view of compressed specimen showing margin of frontal scutum (white arrowhead), frontal cirri (black arrowheads), buccal lip (white arrowhead) and cortical granules (black arrows). **F.** Preencystment stage showing rounded cell with resorbing left marginal and ventral rows (black and white arrows respectively) and right marginal and ventral cirri (black and white arrowheads respectively). **G, I–L.** Same resting cyst at 3 d (G), contractile vacuole functioning (arrow), 5 d (I), contractile vacuole has disappeared, 7 d (J), cell contents have contracted and nuclear material forms a thick ribbon (arrow), 11 d (K), outer cyst wall has contracted slightly and cell contents continue to shrink. Nuclear material has become inconspicuous. Clumps of bacteria adhere to the ectocyst (arrowheads), 14 d (L), ectocyst appears crumpled (arrowheads). **H.** Outer wall of resting cyst. Ma, macronucleus; LVR, left ventral row; RMR, right marginal row; RVR, right ventral row. Scale bars: 50 μm (A–D), 25 μm (E–L).

Fig. 3A–J. *Atractos contortus*, Canadian population after protargol impregnation (A–I) and after staining with methyl green-rhodamine (J). **A.** Left lateral view showing the dorsomarginal kinety (black arrows) and dorsal kinety 1 (white arrows) and the left (black arrowhead) and right (white arrowhead) marginal row. **B.** Right lateral view of same cell (A) showing the posterior end of the shortened left ventral row (white arrowhead). **C.** Dorsal view. **D.** Ventral view showing distortion of left marginal row by ingested silt particle (white arrowhead) and an ingested diatom frustule (black arrowhead). **E.** Dorsal view of same (D) specimen showing left (white arrow) and

right (black arrow) marginal rows. **F.** Left lateral view. **G.** Left lateral view showing bundle of fibers (white arrow) arising from bases of the adoral membranelles (black arrow) and the inconspicuous pharyngeal fibers (arrowhead). **H.** Ventral view of anterior ciliature. **I.** Ventral view showing large ingested silt particle (asterisk). **J.** Sparse pink-staining cortical granules. BC, buccal cirrus; DK1–4, dorsal kineties 1–4; EM, endoral membrane; FC1, 2, frontal cirrus 1, 2; G, cortical granules; LMR, left marginal row; LVR, left ventral row; Ma, macronucleus; PM, paroral membrane; RMR, right marginal row; RVR, right ventral row. Scale bars: 25 μ m (A–J).

Fig. 4A–H. *Atractos contortus*, Canadian population in scanning electron microscope. **A.** Left ventrolateral view of a very slender specimen showing the two frontal cirri (black arrowheads), the buccal cirrus (white arrowhead), the dorsomarginal (black arrow) and first dorsal kinety (white arrow). Note the nearly cylindrical body shape. **B.** Left ventrolateral view showing frontal scutum (asterisk) and right wall of the buccal lip (arrowhead). **C.** Left lateral view showing adoral membranelles (black arrowhead), paroral membrane (white arrowhead), anterior cirrus of left marginal row (black arrow) and dorsal kineties 2 and 3 in the tail portion (white arrow). **D.** Dorsal view showing the posterior end of the left ventral row (white arrowhead), dorsal kinety 1 (white arrow) and the dorsomarginal kinety (black arrow). Note the posterior shortening of the dorsomarginal kinety (black arrowhead). **E.** Left ventrolateral view showing the two cilia comprising the distal file of the adoral membranelles (white arrowheads), the paroral membrane cilia (black arrowhead), the frontal scutum (white asterisk), and the right wall of the buccal lip (black asterisk). **F.** Left lateral view showing the two long files of the adoral membranelles (white arrowheads), the buccal cirrus (black arrow), the paroral membrane cilia (white arrow), and bristles of dorsal kinety 1 (black arrowheads). **G.** Left ventrolateral view showing triangular

intermembranellar bolsters (white arrowheads). **H.** Posterior view showing dorsal kinety 1 (white arrow) the posterior shortening of the dorsomarginal kinety (black arrow), the confluent posterior ends of the left marginal (white arrowhead) and right marginal row (black arrowhead). LMR, left marginal row; LVR, left ventral row; RMR, right marginal row; RVR, right ventral row. Scale bars: 25 μm (A–F), 10 μm (G, H).

Fig. 5 A–F. *Atractos contortus*, Canadian population after protargol impregnation. **A.** Left dorsolateral view of late early divider showing probable left ventral row primordium (black arrows) and the primordium for right ventral and marginal rows (black arrowhead) arising from the posterior part of the split parental right ventral row (white arrow). The anterior part of the right ventral row lies between the primordium and the parental left ventral row (white arrowhead). The right marginal row is morphogenetically inactive. The undulating membrane primordia form from the parental structures. **B.** Nuclear changes in same specimen as (A). Macronuclear replication bands near the proximal end of each nodule and nucleoli disappear (black arrowheads). The micronuclei become ellipsoid and begin to enlarge (white arrowheads). **C.** Left dorsolateral view of slightly later early divider than (A). The primordium for the right ventral (white arrowhead) and right marginal (black arrowhead) rows separates. The left ventral row primordium (black arrow) extends between the paroral membrane primordium and the parental left ventral row. Frontal cirrus 1 forms at the distal end of the endoral membrane primordium (white arrow). **D.** Left lateral view of mid late divider. The parental and opisthe adoral zones are rotated 180° with respect to one another (cf. Fig. F, same specimen), the frontal and buccal cirri have been replaced, the proter undulating membranes are reforming and the parental adoral zone is fully retained. The developing right ventral row is split (black arrowhead)

and the posterior part of the developing right marginal row extends between the old parental row and the old dorsal kinety 4. Opisthe dorsal kineties 1–3 develop de novo (white arrows) and parental dorsal kineties 1–3 are resorbing (small black arrow). The parental dorsomarginal kinety persists (small white arrow). **E.** Nuclear events in same specimen as (D). Macronuclear nodules begin amitotic division (black arrowheads. Whether the nodules previously fuse into a single mass and subsequently divide is unknown. Micronuclei are in telophase of mitosis (white arrowheads). **F.** Dorsal view of proter. The new dorsomarginal kinety of the proter (white arrow) develops de novo between the new and the old parental right marginal row. The parental dorsomarginal kinety persists (black arrow) and new dorsal kineties 1–3 form de novo (white arrowheads). The opisthe dorsomarginal kinety develops adjacent to the new right marginal row (black arrowhead). Opisthe frontal and buccal cirri (small white arrow) have formed but their origin is unclear. The opisthe left marginal row is seen (small black arrow). BC, buccal cirrus; DK4, dorsal kinety 4 (dorsomarginal kinety); EM, endoral membrane; FC, frontal cirri; LMR, left marginal row; LVR, left ventral row; PM, paroral membrane; RMR, right marginal row; RVR, right ventral row. Scale bars: 25 μ m (A, C, D, F).

Fig. 6A–G. *Atractos contortus*, Canadian population after protargol impregnation. **A.** Left ventrolateral view of proter in late divider. Proter left and right ventral rows extend bidirectionally (small black and white arrows respectively). New dorsal kineties have formed de novo in proter and opisthe (black and white arrowheads respectively). The opisthe dorsomarginal kinety forms adjacent to the new right marginal row. Opisthe cirral rows develop bidirectionally. **B.** Nuclear events in same specimen as (A). Nucleoli reappear in the dividing macronuclear nodules now connected by a narrow bridge (black arrowheads). Micronuclei continue to migrate,

now connected by spindle remnants (white arrowheads). **C.** Dorsal view of proter of same specimen as (A). The opisthe adoral is nearly fully differentiated but still rotated 180° with respect to the proter. **D.** Ventral view of late divider. The opisthe has nearly completed its 360° leftward rotation so that proter and opisthe adoral zones present on the same (ventral) aspect. The proter left marginal row extends from the adoral zone to the division furrow (white arrowheads). **E.** Nuclear events in same specimen as (D). **F.** Dorsal view of same specimen as (D). Some proter parental right marginal cirri (black arrowheads) and the parental dorsomarginal kinety (small black arrow) persist. **G.** Right dorsolateral view of proter daughter cell after cytokinesis. Note the bluntly conical posterior end. The parental dorsomarginal kinety (black arrowheads) persists between the new dorsomarginal kinety (white arrowheads) and the new dorsal kineties 1–3 (black arrows) but will ultimately be completely resorbed. A few scattered parental right marginal cirri have not yet been resorbed. DK4, dorsal kinety 4 (dorsomarginal kinety); EM, endoral membrane, LMR, left marginal row; LVR, left ventral row; PM, paroral membrane; RMR, right marginal row; RVR, right ventral row. Scale bars: 25µm (A, C, D, F, G).

Fig. 7A–H. *Atractos contortus*, Canadian population after protargol impregnation (A–E, H) and in scanning electron microscope (F, G). **A.** Left dorsolateral view of late early divider showing the oral primordium (white arrowhead) and the primordia of the right ventral (white arrow) and right marginal rows (black arrow). **B.** Left dorsolateral view of slightly late early divider than (A) showing the oral primordium (white arrow), primordia of left ventral (black arrow) and right marginal (white arrowhead) row extending from the poster part of the split right ventral row, and the anterior part of the split right ventral row (black arrowhead). **C.** Dorsal view of proter in late divider showing the proter and opisthe dorsomarginal kineties (white arrow and black arrow

respectively), the opisthe dorsal kinety 1 (small black arrow), the opisthe frontal cirri (white arrowheads) and buccal cirrus (black arrowhead). **D.** Left lateral view of mid-late divider showing new proter left marginal (white arrow), right marginal (black arrowhead), left ventral (white arrowhead) row, and new opisthe dorsal kineties 1–3 (black arrow). **E.** Dorsal view of proter in late divider showing unresorbed parental right marginal cirri (white arrowhead) and new opisthe dorsal kinety 1 (black arrow) and dorsomarginal kinety (white arrow). **F.** Dorsal view of proter in late mid divider showing overlapping segments in the new opisthe right ventral row (black arrowheads). **G.** Right dorsolateral view of proter daughter cell after cytokinesis showing the persisting parental right marginal cirri (black arrow) and parental dorsomarginal kinety (white arrowheads) that will soon be completely resorbed. DK1, 4, dorsal kineties 1 and 4; LMR, left marginal row; RMR, right marginal row; UM, undulating membranes. Scale bars: 25 μm (A–H).

Fig. 8A–E. *Stichotricha aculeata*, Idaho population from ephemeral puddle (A, from life; B–D, after protargol impregnation) and Idaho freshwater pond (F, in scanning electron microscope).

A. Specimen protruding from lorica (white arrows). Several of the anteriormost adoral membranelles are enlarged (arrowhead). Anterior dorsal bristles are elongated (black arrow). **B.** Right ventrolateral view. Arrows indicate the paroral membrane. **C.** Ventral view. **D.** Left dorsolateral view. **E.** Ventral view showing elongated adoral membranelle (small arrow) and left marginal (white arrowhead), left ventral (black arrow), right ventral (white arrow), and right marginal (black arrowhead) rows. BC, buccal cirrus; DK1, 2, dorsal kineties 1 and 2; EM, endoral membrane; FC, frontal cirri; LMR, left marginal row; LVR, left ventral row; Ma,

macronucleus; PM, paroral membrane; RMR, right marginal row; RVR, right ventral row. Scale bars: 25 μm (A, E), 10 μm (B–D).

Fig. 9. Cladogram based on 18S rDNA; Bayesian inference and maximum likelihood. *Atractos contortus* is separated from the other spirofilids (black arrows). First number of nodal support is the posterior probability from Bayesian inference followed by bootstrap support from maximum likelihood. Black dots indicate full support in both methods, – indicates < 50% bootstrap support and bracketed values indicate topologic incongruence between the two methods. ^a*Uroleptus lepisma* is a synonym of *Paruroleptus lepisma* (Foissner 1998). Taxon names and GenBank accession numbers for stylonychines in collapsed node are given in Table 1. Newly sequenced taxa are in bold type. BI, Bayesian inference; ML, Maximum likelihood.

Fig. 10. Cladogram based on 18S rDNA; neighbor joining and maximum parsimony. First number of nodal support is the bootstrap value from neighbor joining followed by the bootstrap value from maximum parsimony. Black dots indicate full support in both methods, – indicates <50% bootstrap support and bracketed values indicate topologic incongruence between the two methods. Black arrows indicate spirofilids. ^a*Uroleptus lepisma* is a synonym of *Paruroleptus lepisma* (Foissner 1998). Taxon names and GenBank accession numbers for stylonychines in collapsed node are given in Table 1. Newly sequenced taxa are in bold type. MP, maximum parsimony; NJ, neighborjoining.

Table 1. Stylonychine taxa included in Figs 9, 10

Taxon name	GenBank accession no.
<i>Gastrostyla steinii</i>	AF508758
<i>Histriculus histrio</i>	FM209294
<i>Laurentiella strenua</i>	AJ310487
<i>Onychodromus grandis</i>	AJ310486
<i>Pattersoniella vitiphila</i>	AJ310495
<i>Pleurotricha lanceolata</i>	AF508768
<i>Steinia sphagnicola</i>	AJ310494
<i>Sterkiella cavicola</i>	GU942565
<i>Sterkiella histriomuscorum</i>	FJ545743
<i>Sterkiella nova</i>	AF508771
<i>Stylonychia ammermanni</i>	JX885703
<i>Stylonychia lemnae</i>	AM233908
<i>Stylonychia lemnae</i>	AM086648
<i>Stylonychia mytilus</i>	AM086655
<i>Stylonychia notophora</i>	FM209297
<i>Styxophrya quadricornuta</i>	X53485
<i>Tetmemena bifaria</i>	AJ310495
<i>Tetmemena pustulata</i>	AF396973

Table 2. Morphometric data on Canadian population of *Atractos contortus* (upper line) and Idaho population of *Stichotricha aculeata* ^a

Characteristic	Mean	M	SD	CV	Min	Max	n
Body, length	141.7	140.0	21.06	14.9	107.0	183.0	31
	80.8	78.5	9.60	11.9	64.0	98.0	22
Body, width	34.8	34.0	7.49	21.5	23.0	60.0	31
	19.0	19.0	2.42	12.8	16.0	25.0	22
Body, length : width	4.2	4.1	0.73	17.5	3.0	5.8	31
	4.3	4.3	0.44	10.4	3.4	5.2	22
Adoral zone of membranelles, length	38.8	40.0	4.29	11.1	29.0	46.0	31
	39.9	40.5	4.78	12.0	30.0	50.0	22
Adoral zone of membranelles, % body length	27.8	27.0	4.08	14.7	20.0	36.0	31
	50.0	48.5	5.71	11.4	38	58	22
Adoral membranelles, no.	37.5	37.0	2.66	7.1	31.0	43.0	31
	38.4	39.0	3.22	8.4	33.0	45.0	19
Adoral membranelles, width	7.4	7.0	1.22	16.6	5.0	11.0	25
	3.5	3.5	0.46	13.1	3.0	4.0	21
Paroral membrane, length	—	—	—	—	—	—	—
	25.8	25.0	2.61	10.1	20.0	30.0	17
Endoral membrane, length	—	—	—	—	—	—	—
	14.4	15.0	1.70	11.8	12.0	17.0	17
Frontal cirri, no.	2.0	2.0	0.00	0.0	2.0	2.0	31
	3.9	4.0	0.46	11.8	2.0	4.0	19
Buccal cirri, no.	1.0	1.0	0.00	0.0	1.0	1.0	31
	1.0	1.0	0.00	0.0	1.0	1.0	21
Cirral rows, no.	4.0	4.0	0.00	0.0	4.0	4.0	31
	4.0	4.0	0.00	0.0	4.0	4.0	20

Left marginal row cirri, no.	81.6	81.5	9.91	12.1	68.0	113.0	26
	24.7	24	4.06	16.4	18.0	32.0	17
Rigth marginal row cirri, no.	95.7	97.0	7.70	8.0	82.0	114.0	27
	31.4	31.0	3.58	11.4	26.0	412.0	16
Left ventral row cirri, no.	38.3	38.0	3.45	9.0	32.0	46.0	26
	38.9	40.0	4.09	10.5	30.0	44.0	14
Right ventral row cirri, no.	84.8	84.0	7.58	8.9	71.0	105.0	27
	36.5	36.0	3.93	10.8	29.0	43.0	15
Dorsal kinety, no.	4.0	4.0	0.00	0.0	4.0	4.0	31
	2.1	2.0	0.30	14.4	2.0	3.0	21
Dikinetids in dorsal kinety 1 (per 20 μ m)	10.3	10.0	2.36	22.8	7.0	15.0	28
	—	—	—	—	—	—	—
Macronucleus, no.	2.0	2.0	0.00	0.0	2.0	2.0	31
	2.0	2.0	0.00	0.0	2.0	2.0	22
Anterior macronucleus, length	15.5	15.0	2.91	18.8	10.0	22.0	30
	12.4	12.0	3.20	14.5	10.0	16.0	22
Anterior macronucleus, width	10.0	10.0	1.53	15.3	7.0	13.0	30
	7.1	7.0	0.98	13.9	5.0	9.0	22
Posterior macronucleus, length	18.0	18.5	4.07	22.7	11.0	25.0	30
	11.2	11.0	2.00	18.0	8.0	16.0	22
Posterior macronucleus, width	8.9	9.0	1.29	12.8	6.0	11.0	30
	7.0	7.0	1.40	20.2	5.0	10.0	22
Micronucleus, no.	2.0	2.0	0.00	0.0	2.0	2.0	31
	2.1	2.0	0.32	15.0	2.0	3.0	19
Anterior micronucleus, diameter	4.3	4.3	0.43	10.0	3.6	5.0	31
	2.5	2.2	0.61	24.0	2.0	4.0	18
Posterior micronucleus, diameter	4.2	4.1	0.42	10.0	3.5	5.0	31
	2.2	2.0	0.28	23.6	2.0	4.0	20

^aBased on protargol-impregnated specimens (Procedure A, Foissner 2014), values for *S. aculeata* from Idaho Population 1. CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Min, minimum value; *n*, no of specimens studied; SD, standard deviation of the mean.

Table 3. Analyses of topological constraints.

Constraint ^a	-Log-likelihood	AU test (<i>p</i>)	Conclusion
None	-9530.72	0.620	–
<i>Atractos contortus</i> + <i>Uroleptus</i> spp.	- 9530.72	0.444	Not rejected
<i>Atractos contortus</i> + <i>Strongylidium</i> spp. + <i>Pseudouroleptus caudatus</i>	- 9553.14	0.122	Not rejected
<i>Atractos contortus</i> + <i>Hypotrichidium paraconicum</i> + <i>Uroleptus</i> spp.	- 9577.45	0.005	Rejected
<i>Strongylidium</i> spp. + <i>Pseudouroleptus</i> <i>caudatus</i>	- 9530.72	0.620	Not rejected
<i>Atractos contortus</i> + <i>Hypotrichidium paraconicum</i> + <i>Stichotricha aculeata</i> + <i>Strongylidium</i> spp. + <i>Pseudouroleptus caudatus</i>	-9615.12	2e-06	Rejected
<i>Hypotrichidium paraconicum</i> + <i>Stichotricha</i> <i>aculeata</i> + <i>Strongylidium</i> spp. + <i>Pseudouroleptus</i> <i>caudatus</i>	-9610.28	3e-04	Rejected

^a Taxa constrained to be monophyletic.

AU – approximately unbiased test. *p* = probability.

Figure3A-J

Figure4A-H

Figure6A-G