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Morphogenesis and Molecular Phylogeny of a Soil Ciliate *Uroleptoides longiseries* (Foissner, Agatha and Berger, 2002) Berger, 2008 (Ciliophora, Hypotrichia)

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ABSTRACT

Morphogenesis of the hypotrich ciliate *Uroleptoides longiseries*, isolated from sandy soil beside the Yellow River, Suide, Yulin, Shaanxi Province, China, was investigated using protargol staining. The main events during binary fission are as follows: (1) the long frontoventral row is formed by a single anlage; (2) five frontoventral-transverse cirral anlagen are formed in primary mode; (3) only the posterior part of the parental adoral zone of the membranelles is renewed; and (4) the oral primordium of the opisthe is formed intrakinetally. This is the first detailed record of all stages of morphogenesis for *Uroleptoides*. We also provide the first record of the small subunit ribosomal DNA (SSU rDNA) sequences for *U. longiseries*. Phylogenetic analyses based on the SSU rDNA sequences data show that *Uroleptoides longiseries* clusters with *U. magnigranulosa* with moderate to high support which together form a clade with *Orthoamphisiella breviseries*. These three species share the morphogenetic feature of the long frontoventral row being formed by a single anlage.

Keywords: Hypotrich; Ontogeny; Phylogenetic; Sandy; SSU rDNA.

THE hypotrichous family Amphisiellidae is characterized by the presence of the amphisiellid median cirral row, which arises from two or three anlagen (Berger 2008; Eigner and Foissner 1994; Jankowski 1979). Wenzel (1953) established the genus *Uroleptoides*, which was characterized by the presence of a buccal cirrus, two or more cirri left of the anterior portion of the amphisiellid median cirral row which usually terminates behind the mid-body, postperistomial cirrus lacking, transverse cirri present, one right and one left marginal row, usually three dorsal kineties, dorsomarginal row, kinety fragmentation, and caudal cirri lacking. Hemberger's (1982) decision to assign *Uroleptoides* to Amphisiellidae was based on its possession of a long frontoventral row which he interpreted as the amphisiellid median cirral row. However, it was not known whether the frontoventral row of *Uroleptoides kihni* (the type species) originates from two or three anlagen (= supposed apomorphy of the amphisiellids) or from only one anlage, as, for example, in *Orthoamphisiella*.

To date, details of morphogenesis of the genus *Uroleptoides* remain unclear, the only previous record being a partial description based on two individuals of *U. terricola* (Berger 2008; Hemberger 1982). The existing information does not clarify whether the long frontoventral row originates from two or three anlagen as in amphisiellids or forms from only a single anlage as in other hypotrichs.

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In this work, *Uroleptooides longiseris* was isolated from sandy soil beside the Yellow River, Yulin, Shaanxi Province, China. This provided the opportunity to perform a detailed study of its morphogenesis and phylogenetic position for the first time. The main aim of the study is to gain a better understanding of the morphogenetic diversity of *Uroleptooides*.

MATERIALS AND METHODS

Sampling and cultivation

Samples of sandy soil from beside the Yellow River, Suide, Yulin, Shaanxi Province, China (37°24'29"N; 110°37'46"E) were collected on 9 May 2015. Culture was maintained at ca. 20–24 °C in mineral water for several days with rice grains as food resource for bacteria. Specimens undergoing morphogenetic events were examined in protargol impregnated slides (Wilbert 1975). Drawings of the impregnated specimens were made with the help of a camera lucida. To illustrate the changes occurring during morphogenetical processes, old (parental) cirri and adoral membranelles are depicted by contour, whereas new ones are shaded black. Terminology is according to Berger (2008) and Lynn (2008).

Voucher material

Three slides with protargol stained specimens have been deposited in the collection of the Laboratory of Protozoology, Xi'an Jiaotong University, China (accession numbers wjy2015050901B–D).

DNA extraction, PCR amplification, and sequencing

Three cells of *Uroleptooides longiseris* were isolated and repeatedly washed using sterile distilled water. Then they were transferred to a 1.5-ml microfuge tube with a minimum volume of water. Genomic DNA was extracted from cells using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The SSU rDNA was amplified using the eukaryotic universal primers 18S-F (5'-AAC CTG GTT GAT CCT GCC AGT-3') and 18S-R (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') (Medlin et al. 1988).

Phylogenetic analyses

Our phylogenetic analyses included SSU rDNA sequences of *Uroleptooides longiseries* and 74 other hypotrichs from the GenBank database (for accession numbers, see Fig. 5). *Apodiophrys ovalis*, *Diophrys scutum*, *Paradiophrys zhangii* and *Uronychia multicirrus*, were used as outgroup taxa. All sequences were aligned using the GUIDANCE web server (<http://guidance.tau.ac.il/>, Penn et al. 2010). Maximum likelihood (ML) analyses were performed using RAxML-HPC2 on XSEDE v8.2.9 (Stamatakis 2014; Stamatakis et al. 2008) on the online server CIPRES Science Gateway (Miller et al. 2010). The reliability of internal branches was assessed using a nonparametric bootstrap method with 1000 replicates. Bayesian inference (BI) analysis was carried out with MrBayes on XSEDE v3.2.6 (Ronquist et al. 2012) on CIPRES Science Gateway using the GTR + I + G model selected by Akaike Information Criterion (AIC) in MrModeltest v2 (Nylander 2004). Markov chain Monte Carlo simulations were run with two sets of four chains for 2,000,000 generations with a sample frequency of 100 generations. The first 5,000 trees were discarded as burn-in (25 %). All remaining trees were used to calculate posterior probabilities using a 50 % majority rule consensus. MEGA v5 (Tamura et al. 2011) was used to visualize the tree topologies.

RESULTS

Morphology of Chinese population

Cell in vivo ca. $190\text{--}350 \times 25\text{--}35 \mu\text{m}$ in size ($n = 8$). Ratio of length to width after protargol staining about 4.6:1 (Fig. 1E, F). Body usually slender, highly flexible but not distinctly contractile, often slightly to distinctly twisted about main axis (Fig. 1C), dorsoventrally flattened about 2:1 (Fig. 1A, B). Contractile vacuole about $12 \mu\text{m}$ across when fully extended, located at about $2/5$ of body length near left margin; collecting channels not observed (Fig. 1B). Four ellipsoidal macronuclear nodules, about $20 \times 10 \mu\text{m}$ in size (in vivo), longitudinally orientated along cell's main axis or slightly left of it (Fig. 1F). Cortical granules colorless, about $0.5\text{--}1.5 \mu\text{m}$ across; there are two kinds of arrangement patterns, the ones grouped in rosettes around dorsal cilia on dorsal side, and the other arranged with usually about two granules or in pairs near marginal cirri on ventral side, some larger granules usually impregnate with protargol (Fig. 1D, F). Cytoplasm colorless, with many food vacuoles ca. $5\text{--}15 \mu\text{m}$ in diameter.

Adoral zone occupies ca. 17% of body length and composed of 25–31 membranelles. Paroral and endoral in *Australocirrus*-pattern. Three distinctly enlarged frontal cirri form concave row; one buccal cirrus; usually three cirri left of anterior portion of frontoventral row; frontoventral row consists of 45–55 cirri and extends to about 70% of body length; usually four transverse cirri located near posterior end of body. One right and one left marginal row comprising 68–80 and 63–82 cirri respectively, not confluent posteriorly (Fig. 1E). Dorsal ciliature composed of three bipolar dorsal kineties. Caudal cirri absent (Fig. 1F).

Divisional morphogenesis

Stomatogenesis

The first evidence of stomatogenesis during cell division is the appearance of a small patch of basal bodies, that is, the opisthe's oral primordium, which is located in the frontoventral row, indicating that parental basal bodies are incorporated in the primordium (Fig. 2A, 4A). Then the oral primordium becomes a long, narrow anarchic field posterior to the parental adoral zone of membranelles (AZM) (Fig. 2B, 4B). Later, the basal bodies of the oral primordium proliferate (Fig. 2C, 4C) and the new adoral membranelles begin to develop at its anterior end (Fig. 2D, 4D). Subsequently, the primordium for the undulating membranes (UM-anlage) is formed to the right of the oral primordium as a long streak of basal bodies (Fig. 2D, E, 4D).

During the later stages, the anterior end of the newly built AZM bends to the right and the differentiation of membranelles is completed forming the new oral structure for the opisthe. At this stage the leftmost frontal cirrus is generated from the anterior end of the UM-anlage (Fig. 2F, 3A, 4H). Meanwhile, the undulating membranes anlage splits longitudinally to form two streaks from which the endoral and paroral derive (Fig. 3C, 4K). Initially, these streaks lie close together in parallel but later they separate, arch, and intersect optically at around their mid-point (Fig. 3C, E, G, 4K).

In the proter, some membranelles (or part of some membranelles) at proximal end of AZM dedifferentiates and the basal bodies differentiate to form the new membranelles to complete the proter's AZM (Fig. 2E, F, 3A, C, 4E, G, J). During the mid- to late stages the differentiation of membranelles is almost completed (Fig. 3C, E, G). The first sign of the formation of the UM-anlage is the dedifferentiation of the posterior portion of the endoral (Fig. 2B, 4B). Slightly later, dedifferentiation proceeds in the anterior portions of both paroral and endoral, and the posterior portion of endoral may be absorbed at this stage (Fig. 2C, D, 4C, D). In subsequent stages, the development of the UM-anlage follows a similar pattern to

that in the opisthe, i.e. the leftmost frontal cirrus is produced from the anterior end and during late phases, the paroral and endoral are regenerated (Fig. 2F, 3A, C, E, G, 4G, J).

Development of the frontoventral-transverse cirri

The development of the somatic ciliature begins with the formation of the frontoventral-transverse cirral anlagen (FVT-anlagen). The buccal cirrus (II/2), cirrus III/2 and the rearmost IV/2 cirrus contribute to the formation of FVT-anlagen II, III, IV, respectively (Fig. 2B, 4B). Then, with the proliferation of basal bodies, these primordia are formed to the right of the proximal end of the AZM as several fine streaks (Fig. 2C, 4C). Later, all the FVT-anlagen fragment in the middle to form two sets of secondary anlagen, one for each dividing part (Fig. 2D, 4D). Subsequently, anlage V is formed in the long frontoventral cirral row. In total, five FVT-anlagen (including the UM-anlage) occur both in proter and opisthe (Fig. 2E).

Subsequently, five cirral anlagen develop independently in both dividing parts. The streaks then broaden, break apart and migrate to their final positions as distinct cirri, the parental structures have been almost completely resorbed (Fig. 2F, 3A, C, E, G, 4E, G, H, J, K, M).

Development of marginal rows and dorsal kineties

As is usual for most hypotrichs, the marginal rows and dorsal kineties form an anlage each in the proter and opisthe by intrakinetal proliferation of basal bodies. No caudal cirri are formed. (Fig. 2G, 3A–H, 4G, H, J, K, M).

Division of nuclear apparatus

The division of the nuclear apparatus proceeds in the usual way for hypotrichs (Berger 2008). During the middle stages the macronuclear nodules fuse into a single mass which subsequently divides into two, then each nodule divides again. Micronuclei were observed to divide mitotically (Fig. 2G, 3B, D, F, H, 4F, I, L).

Reorganisation

Only two reorganisational stages were observed. These indicated that the main processes of cortical development in reorganisers are similar to those in the proter. These include: (1) only the posterior part of the parental AZM is renewed and the oral primordium develops in situ; (2) the undulating membranes anlage generates the first frontal cirrus; (3) four frontoventral-transverse anlagen are formed; (4) the marginal rows and dorsal kineties originate and develop intrakinetally.

Phylogenetic analyses based on SSU rDNA sequences

The SSU rDNA sequence of *Uroleptooides longiseries* has been deposited in GenBank with the accession number MH143251. The length and GC content of the new sequence are 1,753 bp and 45.52%, respectively.

The topologies of the ML and BI trees are similar, therefore we only present the ML tree (Fig. 5). These analyses reveal that *Uroleptooides longiseries* clusters with *Uroleptooides magnigranulosa* with moderate to high support (ML/BI, 70/1.00), and together these group with *Orthoamphisiella breviseries* with moderate to strong support (ML/BI, 59/1.00). This assemblage then forms a weakly supported polytomy (node values below 50 in ML) with *Bistichella cystiformans* and *Bistichella variabilis*.

The SSU rDNA sequence similarity between *Uroleptooides longiseries* and *U. magnigranulosa* is 98.5%.

DISCUSSION

Morphogenesis

Uroleptooides was established by Wenzel (1953) who classified it as a genus of Amphisiellidae. Eight valid species of *Uroleptooides* are recognized, namely, *U. binucleatus*, *U. kihni*, *U. longiseries*, *U. magnigranulosa*, *U. multinucleatus*, *U. polycirratatus*, *U. raptans* and *U. terricola* (Berger, 2008). So far, the morphogenesis of *U. longiseries* is only detail studied among the genus *Uroleptooides*. The main characteristic events during morphogenesis in *U. longiseries* can be summarized as follows: (1) the long frontoventral row formed by only a single anlage; (2) five FVT-anlagen are formed in primary mode; (3) in the proter, the posterior part of the parental AZM is renewed; in the opisthe, the oral primordium is formed

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intrakinetally; (4) cirral streaks II–V generate one transverse cirrus each at their posterior ends; (5) the left and right marginal anlagen develop intrakinetally; (6) dorsal morphogenesis follows a typical *Gonostomum*-pattern; and (7) the macronuclear nodules fuse to form a single mass.

Hitherto, the only record of morphogenesis in *Uroleptoidea* was *Uroleptoidea terricola* for which a partial description was made based on two dividers (Berger 2008; Hemberger 1982). This species differs from *U. longiseries* in the formation of oral primordium (apokinetally vs. intrakinetally) and the number of FVT-anlagen (seven vs. five) (Berger 2008).

One of the most remarkable morphogenetic features in *Uroleptoidea longiseries* is that the long frontoventral row formed by only a single anlage, which, according to current knowledge, is different from amphisiellids. In most amphisiellid genera, the long frontoventral row (= amphisiellid median cirral row) is formed by anlage VI (anterior portion) and anlage V (posterior portion); while in some amphisiellid species, the row is tripartite and the middle portion is formed by anlage IV. If the amphisiellid median cirral row is formed from a single anlage only, the species has to be removed from the amphisiellids. Furthermore, in the present SSU rDNA tree, *Uroleptoidea* forms a clade with *Bistichella cystiformans*, *B. variabilis* and *Orthoamphisiella breviseries*, all of which are non-amphisiellids, and are widely separated from the Amphisiellidae. These findings could justify establishment of a new genus for *U. longiseries*. However, morphogenesis and molecular data are not available for *U. kihni*, the type species of *Uroleptoidea*, therefore we refrain from the establishment of a new genus.

Hemberger (1982) assigned *Uroleptoidea* to the Amphisiellidae, basically because of the frontoventral row. Shi et al. (1999), Shi (1999), and Lynn & Small (2002) followed this proposal. Berger (2008) tentatively accepted this classification, although with the caveat that the mode of formation of the frontoventral row of *U. kihni*, i.e., whether it is formed by two or three anlagen (= supposed apomorphy of the amphisiellids) or by only one anlage as in *Orthoamphisiella*, remains unknown. If it is formed from a single anlage only, *Uroleptoidea* has to be removed from the amphisiellids.

Phylogenetic analyses

Among these *Uroleptooides* species, SSU rDNA sequence data are available only for *U. longiseriis* and *U. magnigranulosa*, which as expected, cluster together in the present phylogenetic analyses (Fig. 5). The morphological similarity of these two species that support this close relationship include the possession of three distinctly enlarged frontal cirri, one buccal cirrus and cortical granules present. They can, however, be clearly separated by the number of macronuclear nodules (four in *U. longiseriis* vs. two in *U. magnigranulosa*), and the termination of posterior end of the long frontoventral row (70% of body length in *U. longiseriis* vs. 47% of body length in *U. magnigranulosa*). *Orthoamphisiella breviseriis* is sister to the *U. longiseriis* and *U. magnigranulosa* clade. The close relationship between these three species is supported by the long frontoventral row being derived formed by a single anlage (Berger 2008, 2011). Data on the morphogenesis and gene sequence are currently lacking for most congeners including the type species, *U. kihni*. Further studies are therefore needed in order to enhance our understanding of the phylogeny and systematics of *Uroleptooides*.

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Figure 1 Photomicrographs of *Uroleptoides longiseris* from life (A–D) and after protargol staining (E, F). (A, B) Ventral views of representative individuals, arrow shows the contractile vacuole. (C) Ventral view to show high flexibility of cell. (D) Distribution of cortical granules on dorsal side (arrows). (E) Ventral view of a representative individual to show ventral ciliature, arrow shows the long frontoventral row and arrowhead marks the buccal cirrus. (F) Dorsal view to show dorsal kineties (arrows) and macronuclear nodules (arrowheads). CG, cortical granules; FC, frontal cirri; LMR, left marginal row; RMR, right marginal row; TC, transverse cirri. Scale bars = 50 μm (A, B); 50 μm (E, F).

Figure 2 *Uroleptoides longiseris* morphogenesis (A–G) and reorganisation (H, I) after protargol staining. (A) Ventral view of a very early divider, showing the newly formed oral primordium in the opisthe. (B) Ventral view of an early divider, to show that basal bodies in oral primordium form an elongated field, the dedifferentiation of the posterior portion of endoral (arrow) and the cirri II/2, III/2 and the rearmost cirrus IV/2 join in the formation of the frontoventral-transverse cirral anlagen (arrowheads). (C) Ventral view, arrows mark the frontoventral-transverse cirral anlagen, arrowhead shows the undulating membranes anlage. (D) Ventral view, to show the frontoventral-transverse cirral anlagen (arrows) and the newly formed membranelles (arrowhead). (E) Ventral view, arrows mark the frontoventral-transverse cirral anlagen III, arrowheads show the frontoventral-transverse cirral anlagen formed in the frontoventral row and double-arrowhead depicts reorganisation of parental adoral zone of membranelles. (F, G) Ventral and dorsal view of middle divider, arrows show the frontoventral-transverse cirral anlagen formed in the frontoventral row, arrowheads mark the first frontal cirrus separated from the undulating membranes anlage in

each filial product and double-arrowhead depicts the dorsal kineties anlage. **(H, I)** Ventral views of early and middle reorganisers, double-arrowheads show the dedifferentiation of parental adoral zone of membranelles, arrowheads depict the first frontal cirri separated from the undulating membranes anlagen and arrows show the frontoventral-transverse cirral anlagen. DKA, dorsal kineties anlagen; LMA, left marginal anlagen; Ma, macronuclear nodules; OP, oral primordium; RMA, right marginal anlagen. Scale bars = 50 μ m.

Figure 3 Middle and late morphogenetic stages in *Uroleptooides longiseris* after protargol staining. **(A–D)** Ventral and dorsal views of middle dividers, arrows mark the frontoventral-transverse cirral anlagen, which are formed in the frontoventral rows, arrowheads mark the first frontal cirrus separated from the undulating membranes anlage in each daughter cell. **(E–H)** Ventral and dorsal views of late dividers, arrows in E and G show the frontoventral-transverse cirral anlagen n, while arrows in F and H depict newly formed transverse cirri. DK, dorsal kineties; DKA, dorsal kineties anlagen; LMA, left marginal anlagen; LMR, left marginal row; Ma, macronuclear nodules; Mi, micronuclei; RMA, right marginal anlagen; RMR, right marginal row. Scale bars = 50 μ m.

Figure 4 Photomicrographs of *Uroleptooides longiseris* during morphogenesis after protargol staining. **(A)** Ventral view of an early divider, to show the oral primordium in the opisthe. **(B)** Ventral view, arrow depicts the dedifferentiation of the parental undulating membranes, arrowheads show that cirri II/2, III/2 and the rearmost cirrus IV/2 join in the formation of the frontoventral-transverse cirral anlagen. **(C)** Ventral view, arrows mark the frontoventral-transverse cirral anlagen, and arrowhead shows the undulating membranes anlage. **(D, E)** Ventral views to show the frontoventral-transverse cirral anlagen (arrows) and the dedifferentiation of parental adoral zone of membranelles (arrowhead). **(F)** Dorsal view, to show the macronuclear nodules. **(G, H, J)** Ventral views, arrows show the frontoventral-transverse cirral anlagen n, arrowheads show the first frontal cirrus originates from the undulating membranes anlage in each dividing part, double-arrowhead marks the reorganisation of membranelles in the proter. **(I, L)** Dorsal views, to show the fusion and division of the macronuclear nodules. **(K, M)** Ventral views of late dividers, arrows show the frontoventral-transverse cirral anlage n and circle marks the newly formed transverse cirri. LMA, left marginal anlagen; LMR, left marginal row; Ma, macronuclear nodules; OP, oral primordium; RMA, right marginal anlagen; RMR, right marginal row. Scale bars = 35 μ m.

Figure 5 Maximum likelihood (ML) tree based on the SSU rDNA sequence data. The newly sequenced *Uroleptooides longiseris* is indicated in bold. Support values for nodes are for ML and BI, respectively (ML/BI). Disagreements in ML and BI tree topologies that could not be represented on the reference ML tree are indicated by “-”. Fully supported branches are marked with solid circles at the nodes. All branches are drawn to scale. The scale bar corresponds to 0.01 expected substitutions per site.

Table 1. Morphometric characterization of the Chinese population of *Uroleptooides longiseris*.

Character ^a	Min	Max	Mean	M	SD	CV	n
Body length	170	260	203.5	200	27.4	13.5	10
Body width	32	66	45.8	42	12.0	26.2	10
Body length: width, ratio	3.50	5.47	4.59	4.74	0.70	15.7	10
Adoral zone, length	30	43	35.5	35	4.3	12.1	10
Adoral zone length: body length, ratio	0.16	0.19	0.17	0.18	0.01	5.4	10
Adoral membranelles, no.	25	31	26.7	26	1.9	7.1	10
Buccal cirri, no.	1	1	1.0	1	0	0	20
Frontal cirri, no.	3	3	3.0	3	0	0	20
Cirri left of anterior end of frontoventral row, no.	3	3	3.0	3	0	0	10
Transverse cirri, no.	4	5	4.4	4	0.5	11.7	10
Cirri in left marginal row, no.	63	82	71.9	72	5.0	7.0	10
Cirri in right marginal row, no.	68	80	74.6	75	3.8	5.1	10
Cirri in frontoventral row, no.	45	55	50.2	50	2.6	5.1	10
Dorsal kineties, no.	3	3	3.0	3	0	0	10
Dorsal kinety 1, bristles, no.	25	34	30.0	29	3.0	9.9	10
Dorsal kinety 2, bristles, no.	28	37	32.7	34	3.5	10.6	10
Dorsal kinety 3, bristles, no.	26	33	29.5	29	2.4	8.2	10
Macronuclear nodules, no.	4	4	4.0	4	0	0	10
Micronuclei, no.	1	3	2.2	2	0.6	28.7	10

Macronuclear nodule, length	14	25	18.3	18	3.4	18.8	10
Macronuclear nodule, width	5	10	7.2	7	1.4	19.4	10
Micronucleus, length	3	8	5.2	5	1.4	26.9	10
Micronucleus, width	2	3	2.6	3	0.5	19.5	10
Anterior body end to right marginal row, distance	4	15	9.5	10	3.8	39.8	10
Anterior body end to left marginal row, distance	21	38	28.3	29	4.9	17.2	10
Anterior body end to buccal cirrus, distance	14	23	18.2	18	3.2	17.3	10
Anterior body end to frontoventral row, distance	4	10	6.5	6	2.1	31.8	10
Anterior body end to posterior end of frontoventral row, distance	130	168	153.6	159	13.2	8.6	10

^a All data are based on protargol-stained specimens, measurements in μm . Abbreviations: BC, buccal cirrus; CV, coefficient of variation in %; Max, maximum; Mean, arithmetic mean; Min, minimum; n, sample size; no., number; SD, standard deviation.









