Morphogenesis of the marine spirotrichous ciliate, *Trachelostyla pediculiformis* (Cohn, 1866) Borror, 1972 (Ciliophora, Stichotrichia), with consideration of its phylogenetic position

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Received 27 October 2006; received in revised form 27 December 2006; accepted 24 March 2007

**Abstract**

The cortical development during binary fission of the relatively poorly known stichotrich ciliate, *Trachelostyla pediculiformis* (Cohn, 1866) Borror, 1972, found in coastal waters near Qingdao, China, was investigated using the protargol impregnation method. The morphogenetic process reveals some pretty unusual characteristics, which do not follow the *Oxytricha*-pattern: (1) the parental oral apparatus is entirely renewed from an oral primordium formed de novo in the proter; (2) in the proter, the parental undulating membranes are not involved in the formation of the newly formed oral primordium; both undulating membrane-anlagen (UM-anlage) and frontoventral-transverse cirral anlagen (FVT-anlagen) develop from the oral primordium in the proter; (3) the dorsal kineties (DK) are generated in a unique way, that is, in both dividers, two separate groups of DK-anlagen develop in the right- and left-most DK, generate all the DK and evolve to replace the old structures; (4) three caudal cirri are formed at the posterior ends of three right-most dorsal kinety anlagen; (5) eight frontal, five ventral and five transverse cirri are derived from six streaks, namely, the UM-anlage and 5 FVT-anlagen; the cirri are segregated from these anlagen in the pattern 1:3:3:3:4:4 (from left to right) in the *Oxytricha* mode. Based on both SSrRNA gene sequencing and morphogenetic data, the systematic positions of the genus *Trachelostyla* Borror, 1972 as well as the family Trachelostylidae Small and Lynn, 1985 are briefly analyzed. The results indicate that this genus/family could be a highly isolated lineage and might be ancestral to other well-known oxytrichids.

**Keywords:** Marine ciliate; Ontogenesis; Phylogeny; Stichotrichia; SSrRNA gene sequences; *Trachelostyla pediculiformis*

**Introduction**

The marine stichotrich, *Trachelostyla pediculiformis*, was recently redescribed and neotypified by Gong et al. (2006). Its ciliature shows generally similar features to that of oxytrichids, e.g. 18 frontoventral-transverse (FVT) cirri, constantly 11 cirri in the frontal area and two ventral ones together with five enlarged transverse cirri, three inconspicuous caudal cirri and six dorsal kineties (DK) with prominent cilia. To the authors’ knowledge, the morphogenesis of no species of this genus (or even of any member of the whole family) has been fully investigated (Berger 1999; Foissner 1996), hence, morphogenetic characteristics important for
assessing its phylogenetic position are still unknown (Gong et al. 2006; Kool 1970).

Recently, we were fortunate to find a population of *T. pediculiformis* in a sandy beach near Qingdao, north China, and successfully kept a pure culture in the laboratory, which allowed a detailed observation of its morphogenesis. In this paper, the details of its morphogenesis and a comparison with some closely related taxa are presented. In addition, we present data about the systematic position of these poorly known ciliates.

**Materials and methods**

**Morphogenetic studies**

The population of *T. pediculiformis* used for morphogenetic studies was collected from the top 10 cm of sandy littoral sediments of Jiaozhou Bay near Qingdao (36°10′0″N; 120°14′30″E), China. Specimens were maintained for several weeks in the laboratory at room temperature. Squeezed wheat grains were added to the medium as a food source to enrich bacteria. Cells in division were selected and then impregnated using the protargol method (Wilbert 1975). Drawings were made with the help of a camera lucida at 1250× magnification. For clarity, parental cirri are shown in diagrams of morphogenetic stages only by outline, whereas new ones are shaded black.

Terminology and systematics are mainly according to Gong et al. (2006), Berger (1999) and Lynn and Small (2002).

**Phylogenetic analyses**

The SSrRNA gene sequences of species used in tree construction in the present work are available in GenBank/EMBL. The alignment was edited by a computer-assisted procedure, Clustal W (Version 1.80) (Thompson et al. 1994), and refined by considering the conservation of both primary and secondary structures (Elwood et al. 1985). The computer program, MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001) was used for Bayesian tree construction with 100,000 cycles for the Markov chain Monte Carlo algorithm under the GTR model of substitution (Lanave et al. 1984; Rodriguez et al. 1990; Tavare 1986) and considering a gamma-shaped distribution of the rates of substitution among sites. The PHYLIP package, version 3.57c (Felsenstein 1995) was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using the Kimura (1980) two-parameter model. Distance-matrix trees were then constructed using the Fitch and Margoliash (1967) least-squares (LS) method and the neighbor-joining (NJ) method (Saitou and Nei 1987).

**Results**

**Divisional morphogenesis of *T. pediculiformis* (Figs 1–5)**

**Oral primordia and cirral streaks**

Morphogenesis commences apokinetically with the appearance of two fields of sparsely distributed basal bodies (oral primordia) about 1/3 and 2/3 along the anterior/posterior axis (Figs 1C and 4A). Evidently, no old structures (cirri, UM) are involved in the formation of these two anlagen and thus all the old cirri and fibres nearby remain intact.

These two oral fields (primordia) develop with further proliferation of basal bodies. Development in both dividers is very similar and takes place at about the same pace. Cirral streaks develop slightly later at the right of the primordia (Figs 1D and 4B).

In the following stage, the new membranelles begin to organize at the anterior ends of primordia in both proter and opisthe. Two groups of undulating membrane-anlagen (UM-anlagen, Figs 1F and 4E) and frontoventral-transverse cirral anlagen (FVT-anlagen) consisting of five streaks develop to the right of the oral primordia (Figs 1F and 4E). A middle divider is shown in Figs 1H and 4F. The old structures, e.g., UM and proximal end of adoral zone begin to be resorbed. The new membranelles and UM-anlage differentiate posteriad in each divider. A single cirrus develops from the anterior end of the UM-anlage, later becoming the leftmost frontal cirrus. Commencing at the anterior ends of FVT streaks and proceeding posteriad, cirri form at the ends of the FVT-anlagen in both dividing parts (Fig. 1H). Commonly, more than 18 cirral segments were identified in this stage, but the “surplus” ones were later resorbed (Figs 1H and 4F).

Subsequently, the anterior end of the newly built adoral zone of membranelles bends to the right and the differentiation of membranelles is almost completed forming the new oral structures for both the opisthe and proter. The segregation of cirri from FVT-anlagen is complete (Figs 1J and 4H).

Thus, the UM-anlage and the FVT-anlagen I–V generate 1:3:3:3:4:4 cirri, respectively, in both in the proter and the opisthe, as most oxytrichids. That is, in each daughter cell: 3 anterior-most frontal cirri originate (one each) from the UM-anlage and cirral anlagen I and II; 4 frontoventral cirri develop from anlagen II (1), III (1), V (2); one buccal cirrus comes from anlage I; 3 “ventral cirri” are differentiated from anlagen III (1) and IV (2); while 2 pre-transverse ventral
Fig. 1. Early and middle stages of morphogenesis in *Trachelostyla pediculiformis* (after protargol impregnation; (C, F) after Gong et al. 2006). A, B. Ventral and dorsal views of the same specimen, to demonstrate the general pattern of the infraciliature. C. Ventral view at an early stage to show the oral primordia in the proter (POP) and opisthe (OP). D, E. Ventral and dorsal views of the same specimen at an early stage to show that three streaks are formed on the right of the oral primordia in both proter and opisthe. Note the macronuclear replication bands. F, G. Slightly later stage, ventral and dorsal views of the same specimen, indicating the UM-anlage and 5 FVT-cirral streaks that are formed in each divider with further proliferation of basal bodies. Arrows mark the dorsal kinety anlagen. H, I. Ventral and dorsal views of the same specimen of a mid-divider to demonstrate the segmentation of the FVT-anlagen; the first frontal cirrus is generated from the UM-anlage in each divider, and anlagen of the left and right marginal cirri develop in the old structures (arrowheads). Note the macronuclear nodules fuse into a single mass. Arrows indicate the dorsal kinety anlagen. J. Slightly later stage; new FVT-cirri now distinct. Arrows mark three dorsal kinety anlagen which stretch in both directions with proliferation of basal bodies. AZM, adoral zone of membranelles; BC, buccal cirrus; CC, caudal cirri; DK, dorsal kinety; DKA, dorsal kinety anlagen; EM, endoral membrane; FC, frontal cirri; FVC, frontoventral cirri; LMR, left marginal row; Ma, macronucleus; Mi, micronucleus; OP, opisthe's oral primordium; PM, paroral membrane; POP, proter's oral primordium; PVC, postoral ventral cirri; PTVC, pretransverse ventral cirri; RMR, right marginal row; TC, transverse cirri. Scale bar = 50 μm.
Fig. 2. Middle and late stages of morphogenesis in *Trachelostyla pediculiformis* (after protargol impregnation). **A, B.** Ventral and dorsal views of the same specimen at a middle stage to show the fragmentation of the left-most dorsal kinety anlagen (arrows) and the migration of the cirri. **C, D.** Ventral and dorsal views of a later middle stage divider (same specimen), to demonstrate the further fragmentation of the left-most dorsal kinety anlagen (arrows). **E, F.** Ventral and dorsal views of a slightly later divider (same specimen) to show the stage in which the caudal cirri are differentiated (arrowheads). Note the 1st (left-most) dorsal kinety anlage is in fragmentation (arrows in E). Arrows in F demonstrate the division of the micronuclei. **G, H.** Ventral and dorsal views of a late stage divider (same specimen); arrowheads mark the caudal cirri. Scale bars = 40 μm.
cirri are generated from anlagen IV (1) and V (1). Each of these five cirral anlagen contributes one transverse cirrus. In the final stages (Figs 2A, C, E, G, 4J, K and 5A, B, D, E), the formation of the adoral zone in both dividers is completed and each has almost acquired its definitive shape. The endoral and paroral membranes are derived from the UM-anlage by splitting longitudinally. The new cirri migrate to form the mature cortical pattern while absorption of the parental structures continues.

Anlagen of marginal cirri
The new marginal rows in this species are formed in a typical Oxytricha manner (Berger 1999), both marginal row anlagen develop within the parental rows, i.e., the new structures are built by basal bodies derived from the disaggregated old marginal cirri (Figs 1H and J). These new marginal cirri subsequently develop and replace the old ones (Figs 2A, C, E and G).

Dorsal anlagen and caudal cirri
The new DK are generated in a unique manner: at the beginning, one dorsal kinety anlage (DKA) develops intrakinetally within each of the parental left- and right-most kineties of both daughter cells (Figs 1F and G). And then, two DKA are formed in the right-most dorsal kinety of each daughter cell while only one develops in the left-most dorsal kinety (Figs 1H, I and 4G). Subsequently, three DK develop almost synchronously with proliferation of basal bodies (Fig. 1J).

Subsequently, in middle and late dividers, the left-most DKA fragments at the middle region in each daughter cell, thereby forming four anlagen from this DKA in each cell (Figs 2A, C, 4J, K and 5A, B, F). Thus, six DK are formed, which then stretch continuously in both directions (Figs 2E, 3G and 5H).

During the morphogenetic process, three caudal cirri are formed at the posterior ends of the right-most three (i.e. the 4th, 5th and 6th) DK (Figs 2E, G, H and 5A, B, F, H). No dorsomarginal kinetics are formed.

Nuclear division
The nuclear apparatus divides in a usual way as in most other stichotrichs. All macronuclear nodules fuse to form a big mass during the mid-divisional stage (Fig. 1I) and then, before the cell division is finished, the mass divides successively to form usually 16 macronuclear nodules in each daughter cell. Micronuclei divide in later morphogenetic stages, leaving two in each daughter cell (Fig. 2F). A less-common feature is that replication bands were observed in macronuclear nodules of almost all the interphase cells or very early morphogenetic stages (Figs 1E, G and 4C, D, I, L).

Physiological regeneration (Figs 3A and B)
The main process in reorganizers is similar to that in dividers though a complete sequence was not found. From the data available from our observations, the following conclusions can be given: (a) the parental adoral zone of membranelles and undulating membranes are entirely replaced by new ones; (b) all the cirri, including the frontoventral-transverse cirri, marginal rows and the ciliature on dorsal side, are formed and develop in the same way as in cell division.

Molecular phylogenetic tree constructed from complete SSrRNA gene sequences (Fig. 6)
The Bayesian tree constructed from partial SSrRNA gene sequences shown in Fig. 6 has a similar topology to that given by Gong et al. (2006) who used fewer taxa in their alignment. Our analyses also support with high bootstrap values the morphological and ontogenetical conclusions on the monophyly of the urostylid and oxytrichid clades which were reached in the phylogenetic studies on stichotrichines by Foissner et al. (2004). As shown in Fig. 6, T. pediculiformis forms a cluster with
Fig. 4. Photomicrographs of *Trachelostyla pediculiformis* in morphogenesis (after protargol impregnation). **A.** Ventral view of an early divider, to indicate the oral primordia in both the proter (arrowhead) and opisthe (arrow). **B.** Slightly later stage, to demonstrate the oral primordia develop with further proliferation of basal bodies in the proter (arrowhead) and the opisthe (arrow). **C.** Macronuclear nodules of a divider of the stage shown in B. Arrows mark the replication bands. **D.** Macronuclear nodules of a divider of the stage shown in E. **E.** Ventral view of a later early divider, to demonstrate the FVT-anlagen and oral primordia in both dividers. Arrow marks the UM-anlage in the opisthe. **F, G.** Ventral and dorsal views of a middle stage divider (same specimen); arrow in F marks the DKA before fragmentation, while arrow in G marks the DKA on the right side of the cell. **H.** Ventral view of a divider in mid-division stage, showing the fragmentation of the FVT-anlagen in the opisthe. **I.** A cell in interphase, arrows indicate the replication bands. **J, K.** Ventral views of a middle divider (same specimen), to show the fragmentation of the leftmost DKA in the proter (arrow in J) and opisthe (arrow in K). **L.** Ma of a cell just after division, to show the macronuclear nodules in the process of division. Scale bars = 25 µm.
two *Gonostomum* species as well as *Orthoamphisiella breviseries* and *Hemiurosoma terricola*, but with only poor bootstrap support by NJ (40%) and LS (59%) methods. This isolated clade clusters as a sister group to typical oxytrichids, which indicates that *Trachelostyla* might be an intermediate type between Amphisiellidae and Oxytrichidae, as supported by the morphogenetic results obtained in the present work. Otherwise, it

![Photomicrographs of Trachelostyla pediculiformis (after protargol impregnation). A–C. Ventral views of a middle stage divider (same specimen), to show the fragmentation of the left-most DKA in the proter (arrow in B) and opisthe (arrow in A). Note the three caudal cirri are formed at the posterior ends of dorsal kineties 4–6 in both dividers (arrowheads in A, B). Arrow in C indicates the micronucleus. D–G. Ventral D–F and dorsal G views of a slightly later divider (same specimen), to demonstrate the migration of the FVT-cirri. Arrow in F marking the caudal cirrus on dorsal kinety 4, and arrows in G indicate the caudal cirri on new dorsal kineties 5 and 6. H. Ventral view of a very late divider, to show four dorsal kineties on the left side of the cell and the caudal cirrus (arrowhead). I. A very late divider showing the division of the Ma. J. Ma of a divider in late divisional stage. Scale bar = 25μm.](image-url)
Fig. 6. Bayesian tree inferred from partial nucleotide sequences of small subunit rRNA (SSrRNA) genes of spirotrichous ciliated protozoa. The first number on branch points indicates the Bayesian posterior probability percentage derived using the MrBayes program, while the second and third numbers indicate percentage bootstrap support indices from 1000 bootstrap estimations using distance-matrix based neighbor joining (NJ) and least-squares (LS) methods, respectively, using the Phylip package. Some values less than 50% are indicated by asterisks. Evolutionary distance is represented by the horizontal branch length to separate the species in the figure. The scale bar corresponds to five substitutions per 100 nucleotide positions.
might represent a form ancestral to other well-known oxytrichids.

**Discussion and conclusions**

**Main morphogenetic features of *T. pediciformis***

Based on the Qingdao population, the main morphogenetic characteristics can be summarized as follows:

(a) The parental oral apparatus is entirely renewed by the proter's oral primordium which appears de novo in the frontal region; the parental undulating membranes are clearly not involved in the formation of this newly formed oral primordium in the proter.

(b) The segmentation of the 5 FVT-cirral anlagen follows the typical *Oxytricha* mode, that is, in the pattern of 3:3:3:4:4.

(c) Both UM-anlage and FVT-anlagen develop (uniquely) from the same oral primordium, formed de novo in the proter.

(d) The mode of generation of DK is of a “one group type”, but is rather unusual in that two anlagen are formed in the right-most dorsal kinety while only one develops within the left-most dorsal kinety in each divider. Then, in later stages, the DKA in the left-most dorsal kinety fragments three times in the mid-part to generate four kineties. Thus, six dorsal rows are formed during the division process.

(e) Three caudal cirri are formed at the posterior ends of the 4th, 5th and 6th DKA, which is clearly different from the process in oxytrichids.

(f) The beginnings of macronuclear replication are observed before any other morphogenetic processes are evident.

**Systematic position of the genus *Trachelostyla***

Both morphological and molecular data on *T. pediciformis* have justified the establishment of the family Trachelostylidae (Gong et al. 2006; Small and Lynn 1985). The present work offers a complete morphogenetic description and adds another molecular phylogenetic analysis based on Bayesian and distance-matrix methods, which permits re-assessment of the family Trachelostylidae.

In general, the developmental pattern of *T. pediciformis* conforms to that of oxytrichids, i.e., (1) 5 FVT-anlagen mode and (2) streaks segregating in the pattern of 1:3:3:3:4:4. However, it differs conspicuously in some other morphogenetic features from typical oxytrichids such as *Oxytricha, Urosomoida, Tachysoma, Coniculostomum* or *Stylonychia* (Berger, 1999; Song 2004a) in the following points: (1) the old AZM is renewed completely in the proter, which is the pattern found in most lower stichotrichs (vs. parental AZM is retained); (2) the oral primordium, UM-anlage and FVT-anlagen in the proter develop from the same anergic field of basal bodies (vs. UM-anlage and FVT-cirral streaks develop from different primordia); (3) the old UM does not join in the formation of the newly formed oral primordium in the proter (vs. UM-anlage always formed by the old structure via a process of dedifferentiation), and (4) the generation mode of DK is of “one group type” (primary, vs. two-groups in most oxytrichids) (see Song 2004b), and all three caudal cirri derive from the right-most 3 DK-anlagen (or kinetics) (vs. generally two from left-most kinetics and one from the right-most one, when caudal cirri are present).

Considering the origin and processing mode of the ciliature on the dorsal side, *Trachelostyla* must be extremely unusual: in all known oxytrichids (*s. l.* including *Gonostomum*), invariably three old DK are concerned in the formation of the new anlagen, while in the present form, only two old ones are involved though two kinetics will be formed from the right-most anlage. In addition, the fragmentation of the DK-anlagen in *Trachelostyla* is also unique: the DK-anlage fragmentation occurs only in the single, left-most primordium, while in almost all known typical oxytrichids, when this process exists, fragmentation takes place in the 3rd one from left (Berger 1999; Gupta et al. 2006; Naqvi et al. 2006; Song 1990, 2004a).

When Small and Lynn (1985) established the family Trachelostylidae, they assigned both *Trachelostyla* and *Gonostomum* to this taxon, which is clearly different from Berger’s system (1999), in which the latter belongs to Oxytrichidae. The analyses based on SSrRNA gene sequencing generally support Small and Lynn’s assignment, these two genera branching, along with amphisiellids, separately from other oxytrichids. However, this topological structure is unambiguously challenged by the morphogenetic data, in which *Gonostomum* differs in many ways from *Trachelostyla*, and, at the same time, exhibits more similarities to oxytrichids (Berger 1999; Song 1990, 2004a). The probable explanation is that this tree, based upon sequencing of a single gene, does not properly reflect the real relationship between these two genera, and that they could belong to two sister clades at the family level.

We tentatively conclude from the available morphogenetic and molecular evidence that: (1) Trachelostylidae, represented by the genus *Trachelostyla*, might be a lower but sister group to the closely related family Oxytrichidae; (2) *Gonostomum* should be assigned to Oxytrichidae (*s. l.*); (3) some other poorly known genera which were previously placed in Trachelostylidae (e.g. *Hemisincirra, Lamtostyla, Terricirra* and *Spirotrachelostyla*) should be regarded systematically as *incertae sedis* until further studies have been performed.
Acknowledgments

This work was supported by “the Natural Science Foundation of China” (Projects nos. 30430090; 30570236; 40506033; 40676076). Our thanks are also due to Mr. Yangang Wang, Laboratory of Protozoology, KLM, Ocean University of China, for collecting the sample.

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