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Considerations on the Systematic Position of *Uronychia* and Related Euplotids Based on the Data of Ontogeny and 18S rRNA Gene Sequence Analyses, with Morphogenetic Redescription of *Uronychia setigera* Calkins, 1902 (Ciliophora: Euplotida)

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Summary. Characteristics found in morphogenetic process of the *Uronychia setigera* Calkins, 1902 during binary division and the molecular data of several euplotid genera inferred from 18S rRNA gene sequence analyses are compared and discussed. The results indicate that the traditional order Euplotida might comprise 3 paraphyletic groups: the eueuplotids, i.e. euplotids with a 5-cirral anlagen mode and a subcortical origin of the oral primordium during morphogenesis (we call them the "typical" euplotids in the text), gastrocirrhids (the pseudoeuplotids) and discocephalids, the last of which is possibly a convergent lineage. The results suggest also that *Uronychia* represents a primitive type within typical euplotids in phylogeny. Based on these analyses, a conclusion is suggested which consists of essentially the following points: (1) The traditional euplotids are likely a paraphyletic assemblage with differently originated groups. As a suggestion based on the new definition, the euplotids *s. l.* contains at least 2 subgroups: the eueuplotids and the sister group, gastrocirrhids (pseudoeuplotids) with multi-anlagen mode which is a divergent or apomorphic feature. (2) The systematic position of discocephaline remains unclear, but possibly an intermediate group between the euplotids *s. str.* and stichotrichs. We agree, however, that it is presently placed within the order Euplotida, as an outer group of other two assemblages. (3) The morphogenetic feature of 5-cirral-anlagen-mode is highly conservative and should be hence weighted with more phylogenetic value(s) in systematic analysis. (4) We suppose that some features like the origin mode of the leftmost frontal (formed from the UM-anlage), the situation of undulating membranes (e.g. two in number, curved in appearance) represent apomorphic features, and should not be over-evaluated, which caused often the diverse structures of the phylogenetic trees. (5) Euplotids *s. str.* might be derived from the 5-anlagen ancestor-type of hypotrichs *s. l.*, which is possibly also the ancestor of the current stichotrichs.

Key works: 18S rDNA gene sequences, Ciliophora, euplotids, morphogenesis and phylogeny, *Uronychia setigera.*

INTRODUCTION

Typical euplotid ciliates, as widely accepted for a long time, are regarded as a monophyletic lineage and a sister group with other traditional hypotrichs *s. l.* (or stichotrichs), which contains several well-known taxa, e.g. *Euplotes, Diophrys, Uronychia, Aspidisca, Gastrocirrhus, Discocephalus* (Deroux and Tuffrau 1965, Hartwig 1973, Curds and Wu 1983, Small and Lynn 1985, Song and Wilbert 2000). However, evidence (accumulated recently from molecular investigations) suggests that these ciliates might be a paraphyletic assemblage (Chen and Song 2001, 2002). This result is also partly supported by the interpretation derived from

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non-molecular phylogenetic analyses, which indicate that the highly specialized *Uronychia* branches very early from the main cluster, and represents possibly a marginalized lineage against other euplotids (Song 1995).

The small *Uronychia* species, *U. setigera* Calkins, 1902, is commonly found in littoral biotopes (Calkins 1902, Kahl 1932, Bullington 1940, Song and Wilbert 1997). Its morphology and infraciliature were recently redefined by Song and Wilbert (1997), albeit it is often confused with its congener, *Uronychia binucleata* (under various names, details see Song and Wilbert 1997). To unravel the phylogenetic position and the systematic relationships of *Uronychia* among related taxa, the 18S rRNA gene data based on several euplotid genera and morphogenetic characteristics revealed in *U. setigera* have been re-examined/re-analyzed and, as a new contribution, more detailed descriptions for this species are presented as well in the current paper.

Thus, our paper has two main aims: (1) we will check again the morphogenetical data obtained in those well-known genera of euplotids to find/question hiatuses which are seemingly ignored in last time; (2) Based on both ontogenetical and DNA sequence data, to address the systematic relationships between/among the taxa which are traditionally arranged in euplotids.

MATERIALS AND METHODS

Morphological and morphogenetical studies

Population of *Uronychia setigera* used for morphogenetic studies was collected from coastal waters near Qingdao (36°08' N; 120°43' E), China. After isolation, pure cultures were kept in the Laboratory of Protozoology, OUC. For molecular work, clonal cultures were established and maintained in sterilized seawater at room temperature with rice grains as food source to enrich bacteria. Cells in division were impregnated using 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.

Genomic DNA extraction, PCR amplification, sequencing and phylogenetic analyses

Nine species with original sequence data are concerned in the present studies, which were identified based on both *in vivo* observation and silvered (protargol and silver nitrate methods) specimens. DNA extraction, isolation and PCR reaction were performed as previously described (Chen *et al*. 2002).

Sequences analyzed are accessible at GenBank/EMBL with the following codes: *Aspidisca steini* AF305625 (Chen and Song 2002), *Diophrys appendiculata* AY004773 (Chen and Song 2001), *Euplotidium arenarium* Y19166 (Petroni *et al*. 2000), *Euplotes charon* AF492705 (Chen and Song, unpubl), *E. crassus* AJ310492 (Bernhard *et al*. 2001), *E. eurystomus* AF452707 (Chen and Song, unpubl), *E. magnicirratus* AJ549210 (Petroni *et al*. 2002), *E. minuta* AJ310490 (Bernhard *et al*. 2001), *E. parawoodruffi* AF452708 (Chen and Song, unpubl), *E. rariseta* AF492706 (Chen and Song, unpubl), *E. vannus* AY004772 (Chen and Song 2002), *E. woodruffi* AF492707 (Chen and Song, unpubl), *E. raikovi* AJ305251 (Petroni *et al*. 2002), *Holosticha multistylata* AJ277876 (Shin *et al*. 2000), *Laboea strobila* AF399151 (Snoeyenbos-West *et al*. 2002), *Oxytricha ferruginea* X53486 (Chen and Song, unpubl), *Phacodinium metchnikoffi* AJ277877 (Shin *et al*. 2000), *Protocruzia* sp. AF194409 (Shin *et al*. 2000), *Sterkiella nova* (= *Oxytricha nova*) X03948 (Elwood *et al*. 1985), *Strombidium purpureum* U97112 (Hirt *et al*. unpubl), *Stylonychia lemnae* AJ310497 (Bernhard *et al*. 2001), *Uronychia transfuga* AF260120 (Chen and Song 2001).

The 18S rRNA gene sequences were aligned with 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). PHYLIP (Phylogeny Inference Package, Version 3.57) (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). PAUP v4.0b1 was used for the maximum parsimony (MP) analysis (Swofford 1998). Of the 2037 aligned sites, 540 phylogenetically informative characters were used to find the most parsimonious tree using the heuristic search method. Both parsimony and distance data were bootstrap resampled 1,000 times (Felsenstein 1985).

Terminology and systematics

Basically, the terminology and systematics used in the present paper are according to Corliss (1979) and Lynn and Small (2002). However, the higher systematic classification or contents have changed greatly since Corliss' review and are still in argument. Thus we use mainly a vernacular terminology in the text, e.g. stichotrichs, euplotids, hypotrichs or add *sensu lato* (*s. l.*) *stricto* (*s. str.*) to the taxa. Some terms are recalled to avoid possible confusion:

Cirral anlagen. This word is used basically for stichotrichs and euplotids to describe the primordia which will generate late frontoventral transverse cirri, including the undulating membrane anlage (UM-anlage) and streaks which appear ventrally to the right of buccal field. In these primordia, the UM-anlage usually gives rise to one leftmost frontal cirrus. However, this is not the case at least in several euplotids, e.g. *Uronychia*, *Aspidisca* and *Euplotes*, in which the left most cirrus is formed *de novo* either near the UM-anlage (in the opisthe), or completely isolated (in the proter); hence, this term is defined in the present work, only for these 5 streak-like primordia (the UM-anlage is excluded). The cirral anlagen I-V here are equivalent to the anlagen II-VI by some other researchers (i.e., UM-anlage is the No. 1) (Foissner 1996, Eigner 1997, Berger 1999).

Epicortical (opposite to **subcortical**). This term refers to the most common mode of primordia formation in morphogenesis, of which the basal bodies of primordia occur on the cell surface rather than subsurface (beneath the cortex).

Eueuplotids (opposite to **pseudoeuplotids**). This term indicates euplotids with a 5-cirral anlagen mode and subcortical origin of the oral primordium during morphogenesis; it excludes the pseudoeuplotids which do not have such features.

RESULTS

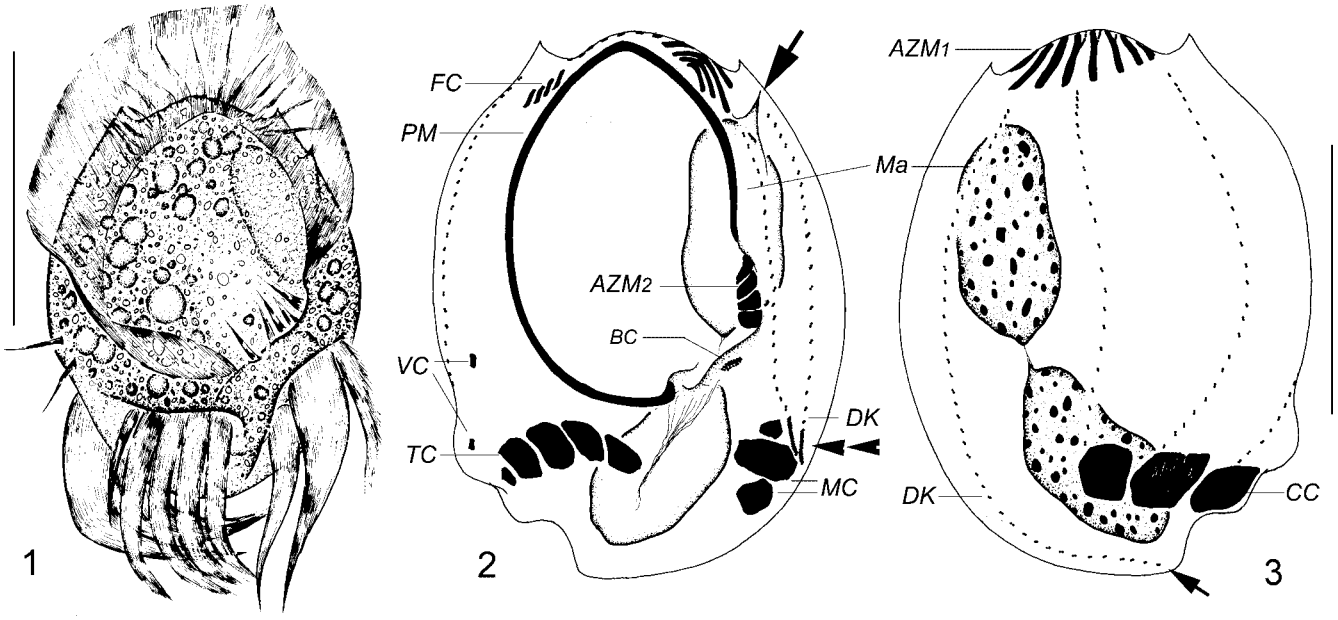
Morphology and infraciliature of *Uronychia setigera* (Figs 1-7, 9-10)

The morphology of living cells is slightly different from that previously described (Song and Wilbert 1997, Chen *et al*. 2003); hence it is only briefly redescribed here.

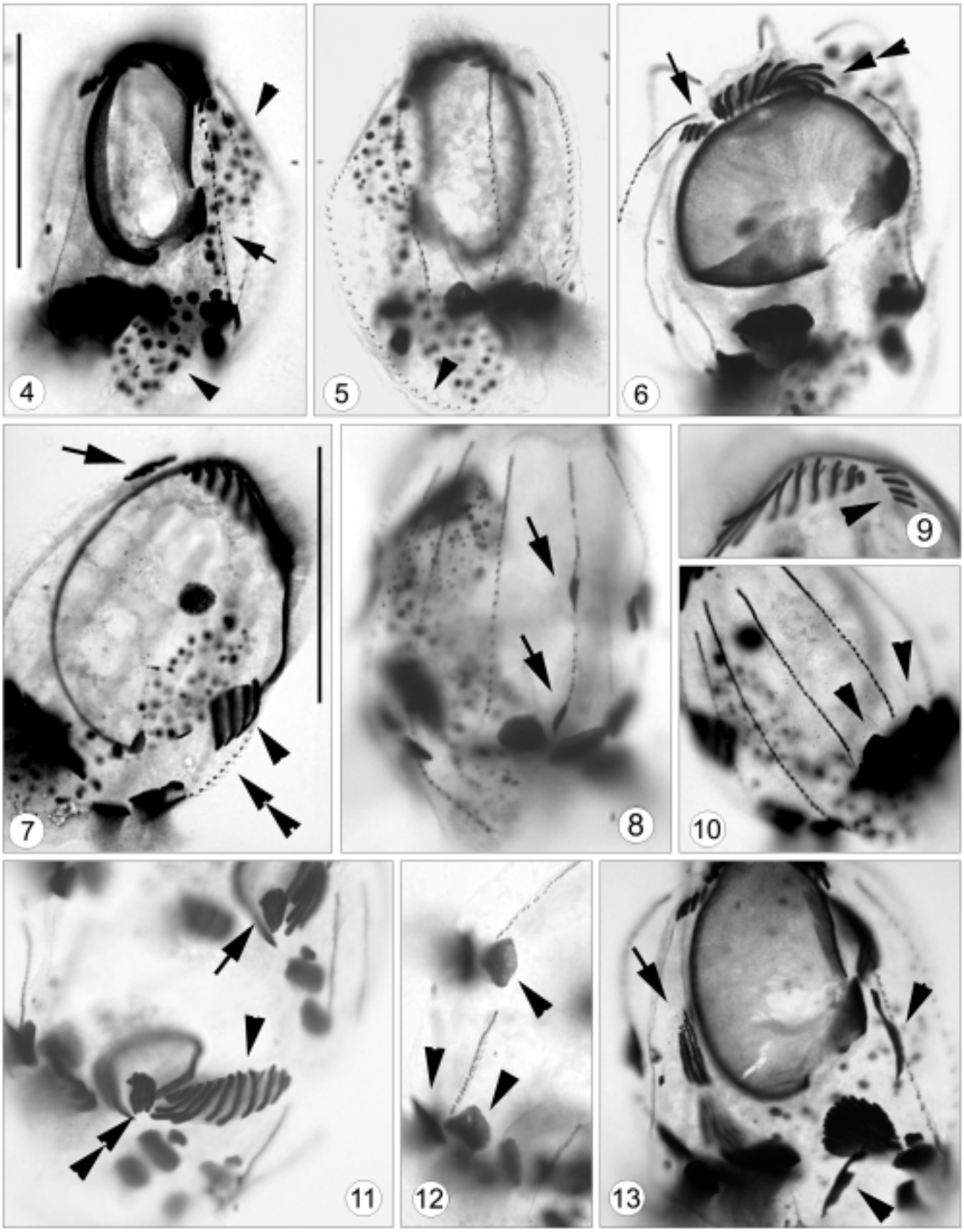
Newly sampled cells show about the same size, *ca* 50-60 × 40-50 µm *in vivo*, while after culture for weeks their size becomes rather variable; from 50 to maximum 90 µm in length with body shape generally oval (as shown in Fig. 1). Buccal field enormous, extending over 60% of body length. Lateral spur-like protrusions on left margin often less conspicuous in this population than the forms described by Song and Wilbert (1997) and Chen *et al*. (2003) (Fig. 2, arrow). Ventrally 2 concaves in posterior portion of cell, where transverse (TC) and left marginal (MC) cirri insert, whereas on dorsal side, a third depression corresponding position of caudal cirri (Fig. 3, CC). Cytoplasm colourless to greyish, usually comprising many to numerous granules (2-8 µm across). No food vacuoles detected (Fig. 1). Two macronuclear nodules (Ma), connected by funiculus (Figs 2-4). Micronucleus not observed. Movement typical of *Uronychia*: rapidly jumping sideways or backwards, swimming very fast while rotating around its longitudinal axis. During pause, all cirri stiffly spread as shown in Fig. 1.

Structure of adoral zone of membranelles (AZM) genus typical (Figs 2, 3, 6, 7). Invariably 11 adoral membranelles in anterior part (AZM_1); cilia of membranelles about 15 µm long. Posterior part (AZM_2) composed always of 4 membranelles (bases about 10 µm in length; Fig. 7, arrowhead), in which basal bodies are irregularly arranged (not in rows) like those in primordia. One small cirrus-like membranelle (named buccal cirrus here, BC) apart from AZM_2 and inconspicuous, with the base about 3 µm long (Figs 2, 7). Paroral membrane (PM) mighty and surrounding buccal field, with right end near buccal cirrus, often slightly crook-like (Fig. 2); cilia of PM about 30 µm long (Fig. 1).

Somatic ciliature as described before (Song and Wilbert 1997) except the number of basal body pairs in



Figs 1-3. *Uronychia setigera in vivo* (**1**) and after protargol impregnation (**2, 3**). **1** - ventral view of a typical individual; **2** - ventral view, to show the general infraciliature; arrow marks the spine which is often lower positioned, whereas double-arrowheads indicate the fragment-like part at the posterior end of the leftmost two dorsal kineties; **3** - dorsal view of the same specimen as in Fig. **2**, to show the dorsal kineties and the nuclear apparatus; arrow marks the 3rd kinety which curves and extends to the posterior margin. $AZM_{1,2}$ - anterior and posterior parts of the adoral zone of membranelles, BC - buccal cirrus, CC - caudal cirri, DK - dorsal kinety, FC - frontal cirri, MC - marginal cirri, Ma - macronuclei, PM - paroral membrane, TC - transverse cirri, VC - ventral cirri. Scale bars - 40 µm.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the leftmost kinety (Fig. 4, arrow), which is slightly higher than that previously reported (17-25 *vs. ca* 15). Invariably 4 membranelle-like frontal (FC), 2 inconspicuous ventral (VC), 5 strong transverse (TC) and 3 left marginal cirri (MC), while on dorsal side 3 mighty caudal cirri on right margin (Figs 3, 5-7, 9, 10). Totally 6 dorsal kineties, of them 2 to 3 on margin or ventrally positioned, in which basal bodies at the post ends of the leftmost 2 kineties are always densely packed (Fig. 2, double-arrowhead).

Morphogenesis in binary fission (Figs 8, 11-36)

Morphogenesis of *Uronychia* species in binary division was studied by many authors, which repeatedly confirmed that the developmental pattern of cortical structure as well as nuclear apparatus within congeners exhibit extremely similar mode (Dembowska 1926, Taylor 1928, Fauré-Fremiet 1964, Hill 1990, Wilbert 1995, Song 1996). Hence only the important events and some details which were insufficiently described in previous investigations are documented here.

Stomatogenesis. Morphogenesis starts with the appearance of a small patch of kinetosomes, the opisthe's oral primordium (POP), within a subsurface or subcortical pouch positioned between cytostome and left marginal cirri (Fig. 23). Slightly later, the proter's oral primordium (AOP) appears, also subcortically, anterior to the parental AZM_2 (Fig. 24), in this stage, membranelles in the POP begin to align (Fig. 24, double-arrowhead). Both the POP and AOP develop then by rapid proliferation of kinetosomes (Fig. 26), and soon an additional anlage, the primordium for paroral membrane (UM-anlage), appears within the subcortical pouch, which is located opposite to the posterior end of the AOP and POP, respectively (Fig. 28, arrowheads; 30). Subsequently, two groups in the POP, each with 11 and 5 membranelles, and two groups both with 5 membranelles in the AOP are differentiated and finally migrate onto the surface of cell (Figs 11, 14, 15, 17, 32, 34). Meanwhile, the UM-anlage begins to lengthen and then develops along the right edge of each opening pouch, at mid portion of which one short anlage (for the leftmost frontal cirrus) occurs *de novo* in both proter and opisthe (Figs 15; 32, arrowheads). In late stages, two groups of membranelles in both oral primordia are separated, and among them the first one migrates anteriorly. As in its congeners, the first part in the proter will replace the posterior 5 membranelles of AZM_1, while the anterior (parental) 6 are retained (Fig. 35, arrow). In the second group of AZM in both dividers, the most posterior membranelle (the smallest one) moves apart from the other 4 and then to the final position as the "buccal cirrus" (Figs 34, 35).

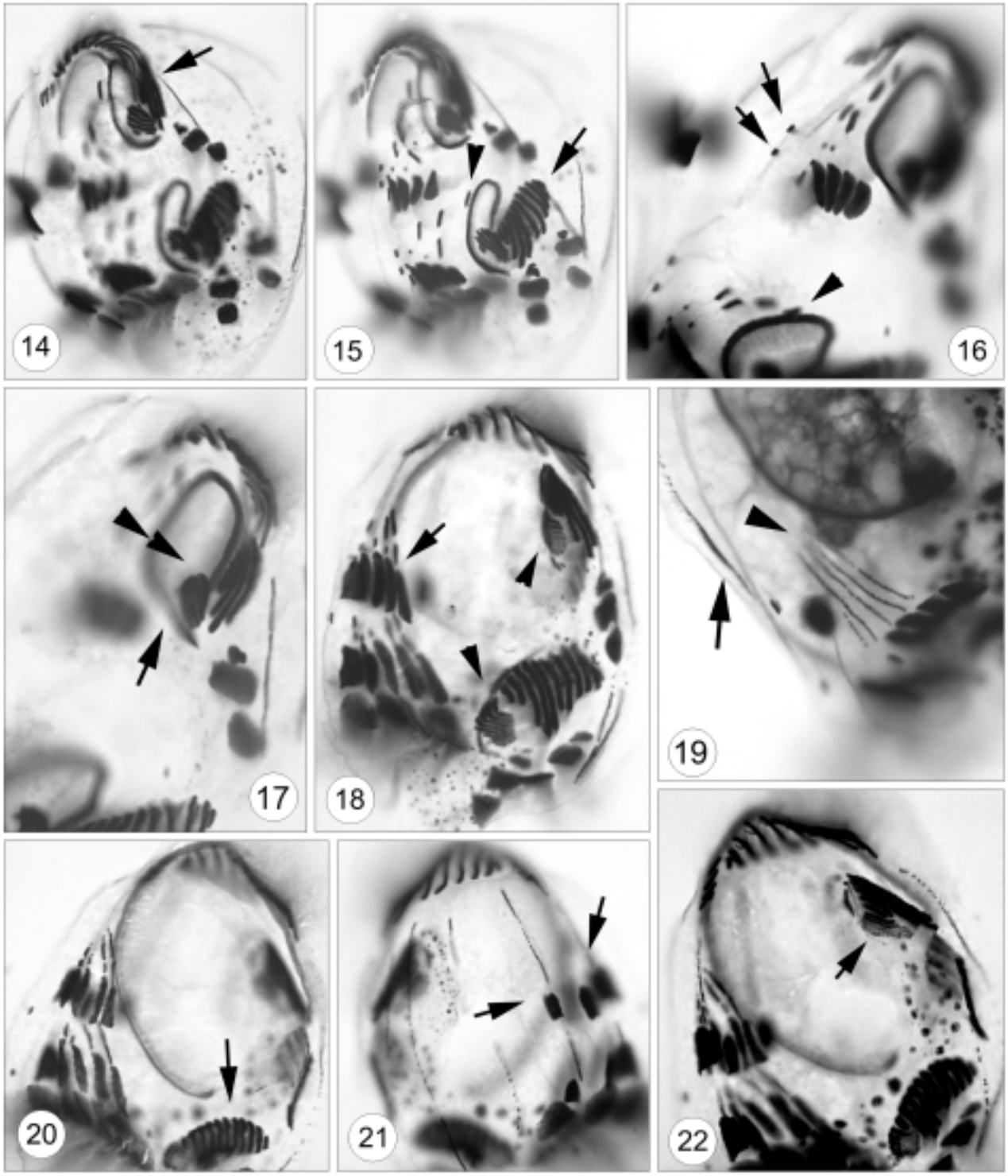
Development of the somatic ciliature. At about the same time as the oral primordia are formed, basal bodies for FVT-cirral primordia (CA) develop on the cell surface as first 4, and then 5 streaks (anlagen I-V) anterior to the transverse cirri (Figs 19, 23, 24). Clearly no parental ciliary organelles are involved in the formation of these new primordia. Then each of 5 anlagen extends to its maximum length before they divide into two sets (Figs 19, arrow; 26, double-arrowheads). Subsequently, the later events within the cirral anlagen in both dividers undergo in the same mode as in previous descriptions (Song 1996, Shi and Song 1999): streaks broaden, break apart and then migrate developing as distinct cirri (Figs 18, 20, 22, 30, 32, 34, 35).

According to the segmentation of cirri, the cirral anlagen I-V give rise to the pattern of 3:3:2:2:3, respectively, while the leftmost cirrus is formed *de novo* near the UM-anlage as described above. Nevertheless, only 11 cirri in total are formed in non-divisional stage following the pattern of 3:2:1:1:3, that is, one segment from each of anlagen II-IV will be resorbed before/after cell division is finished.

The anlagen of left marginal cirri for proter and opisthe are formed also *de novo* and separately on the cell surface near the old AZM_2 and the marginal cirri (Figs 13, arrowheads; 24, arrows), which are then enlarge and segmented to form the cirri for daughter cells (Fig. 28, double-arrowheads).

On dorsal side, the proliferation of new basal bodies occurs at two levels within each of the six old kineties. The development of these anlagen seems to follow a

Figs 4-13. Photomicrographs of *Uronychia setigera*, to show the non-divisional (**4-7, 9, 10**) and divisional stages (**8, 11-13**). **4** - ventral view, arrow points the leftmost kinety, arrowheads mark the macronuclei; **5** - dorsal view of the same specimen as in Fig. 4, arrowhead indicates the long 3rd dorsal kinety; **6** - anterior view, to show the first part of AZM (AZM_1, double-arrowheads) and the frontal cirri (arrow); **7** - ventral view, arrowhead marks the 2nd part of AZM (AZM_2), double-arrowheads indicate the leftmost kinety, arrow indicates the frontal cirri; **8** - dorsal view, arrows mark the newly formed caudal cirri; **9** - dorsal view of anterior portion, arrowhead marks the frontal cirri; **10** - dorsal view, arrowheads indicate the dominant caudal cirri; **11** - ventral view, arrow and double-arrowheads mark the newly formed AZM_2 in both proter and opisthe, respectively, while arrowhead indicates the AZM_1 in the opisthe; **12** - dorsal view, arrowheads mark the caudal cirri in both dividers; **13** - ventral view, arrowheads mark the new marginal cirri, arrow indicates the FVT-cirral anlagen (only 4 are recognized in this specimen). Scale bars - 50 μm.

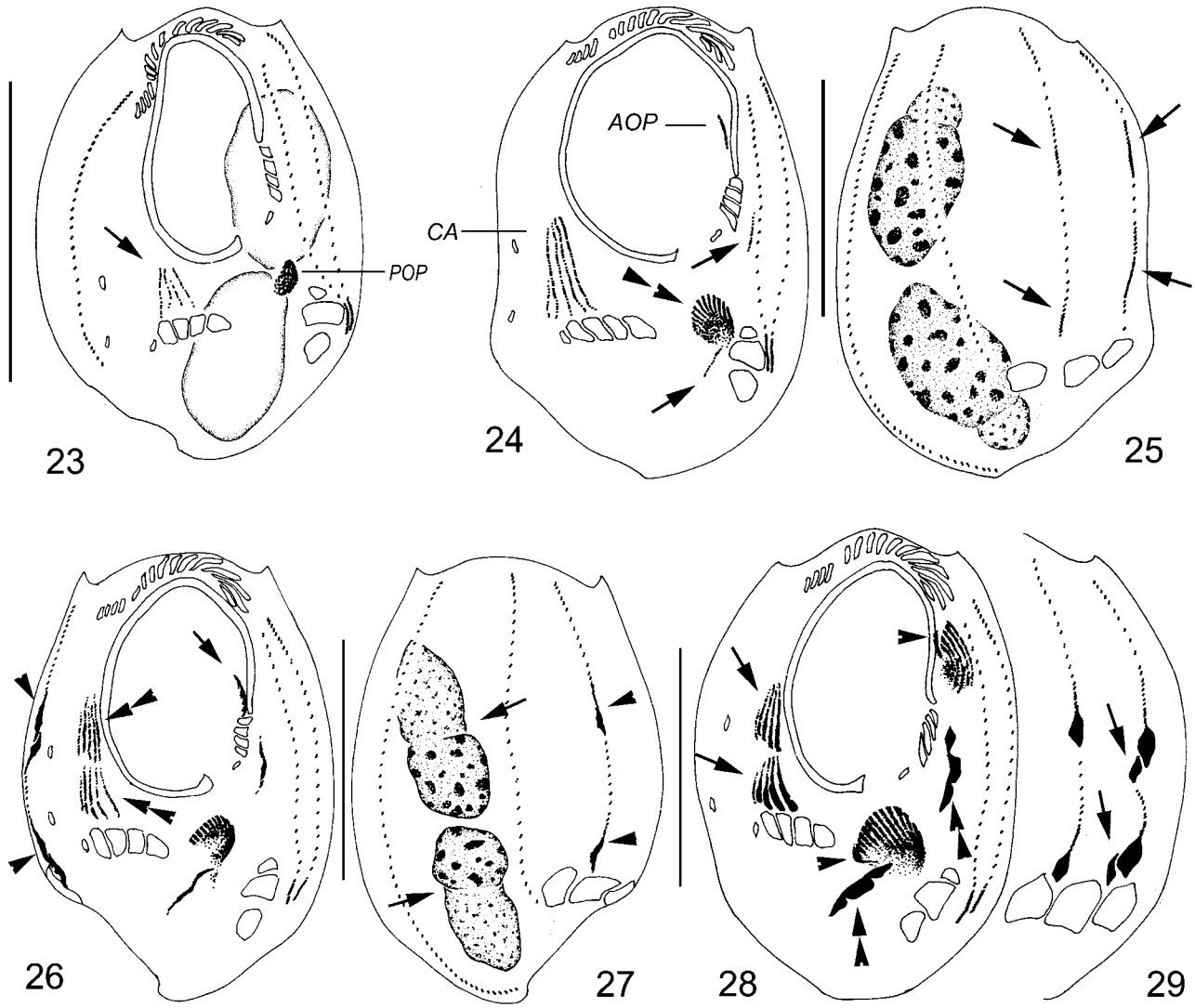


Figs 14-22. Photomicrographs of *Uronychia setigera* in morphogenesis. **14, 15** - ventral views of the same specimen focusing on different levels; arrow in Fig. 14 marks the newly build AZM_1 which just replaces the old structure, arrow in Fig. 15 marks the AZM_1 of the opisthe, while arrowhead indicates the leftmost frontal cirrus derived from the paroral membrane anlage; **16, 17** - ventral views of the same specimen at different focus levels; arrows in Fig. 16 indicate the two inconspicuous ventral cirri, double-arrowheads in Fig. 17 mark theAZM_2 of the proter (note that the paroral membrane, arrow, is positioned at different level); **18** - ventral view, arrowheads mark the AZM_2 in both dividers, whereas arrow indicates the segmented cirral anlagen; **19** - ventral view, arrow marks the newly formed caudal cirrus in the rightmost dorsal kinety, which is just as a slender patch of kinetosomes, while arrowhead indicates the 5 "primary" cirral anlagen; **20** - ventral view, arrow marks the AZM_1 in the opisthe; **21** - dorsal view, arrows mark the caudal cirri of the proter; **22** - ventral view, arrow marks the posterior portion of the oral primordium in the proter, where the last membranelles of the AZM_2 are still in formation.

Table 1. Summary of data on morphogenesis in five well-known typical euplotid genera, with emphasis on the features excluding other non-euplotidous spirotrichs. (AZM - adoral zone of membranelles, FVT - frontoventral transverse, UM - undulating membrane).

Characters	*Uronychia*	*Aspidisca*	*Euplotes*	*Diophrys*	*Certesia*
FVT-cirral anlagen	5 primary streaks	5 primary streaks	5 primary streaks	5 primary streaks	5 primary streaks
Oral primordium in the proter	present	absent	absent	absent	absent
Origin of the oral primordium in the opisthe	subcortically	subcortically	subcortically	subcortically	subcortically
Fate of the parental adoral zone of membranelles during division	invariably 5 proximal membranelles will be replaced by the OP in the proter	completely retained will be renewed by de-differentiating and rebuilding mode	completely retained	proximal portion	completely retained
Formation of the 2nd part of AZM	present	present	absent	absent	absent
Undulating membrane in the proter	rebuilt by the *de novo* newly-formed UM-anlage	old one retained no UM-anlage	old one retained, no UM-anlage	completely renewed the new UM-anlage*	completely renewed by the new UM-anlage*
Formation of the par- and endoral membranes from the UM-anlage	no	no	no	yes	no
Origin of the leftmost frontal cirrus	*de novo*, near the UM-anlage	*de novo*	*de novo*	derived from the UM-anlage	derived from the UM-anlage
Formation of the left marginal cirri	*de novo*, from MC-anlage	absent	*de novo*, from MC-anlage	*de novo*, from MC-anlage	from MC-anlage, *de novo*
No. of membranelles formed by oral primordia	invariable	variable	variable	variable	variable
Origin of dorsal kinety anlagen	intrakinetally, secondary**	as left	as left	as left	as left
Formation of caudal cirri	from rightmost two anlagen, with a complex mode***	no caudal cirri formed	from rightmost one anlage, with a complex mode	from rightmost two anlagen, with a complex mode	no caudal cirri formed
Arrangement of basal bodies in cirri/membrane/ membranelles in non-division stage	irregular and anarchic, in “anlage” state	well-developed, in rows	well-developed, in rows	well-developed, in rows	well-developed, in rows
Data sources	Hill 1990, Song 1996, Shi and Song 1999, Present work	Diller 1975, Hill 1979, Song 2003, Deroux and Tuffrau1965	Bonner 1954, Tuffrau *et al*. 1976, Washburn and Borror 1972	Hill 1981, Song and Packroff 1993, Song and Wilbert 1994	Wicklow 1983

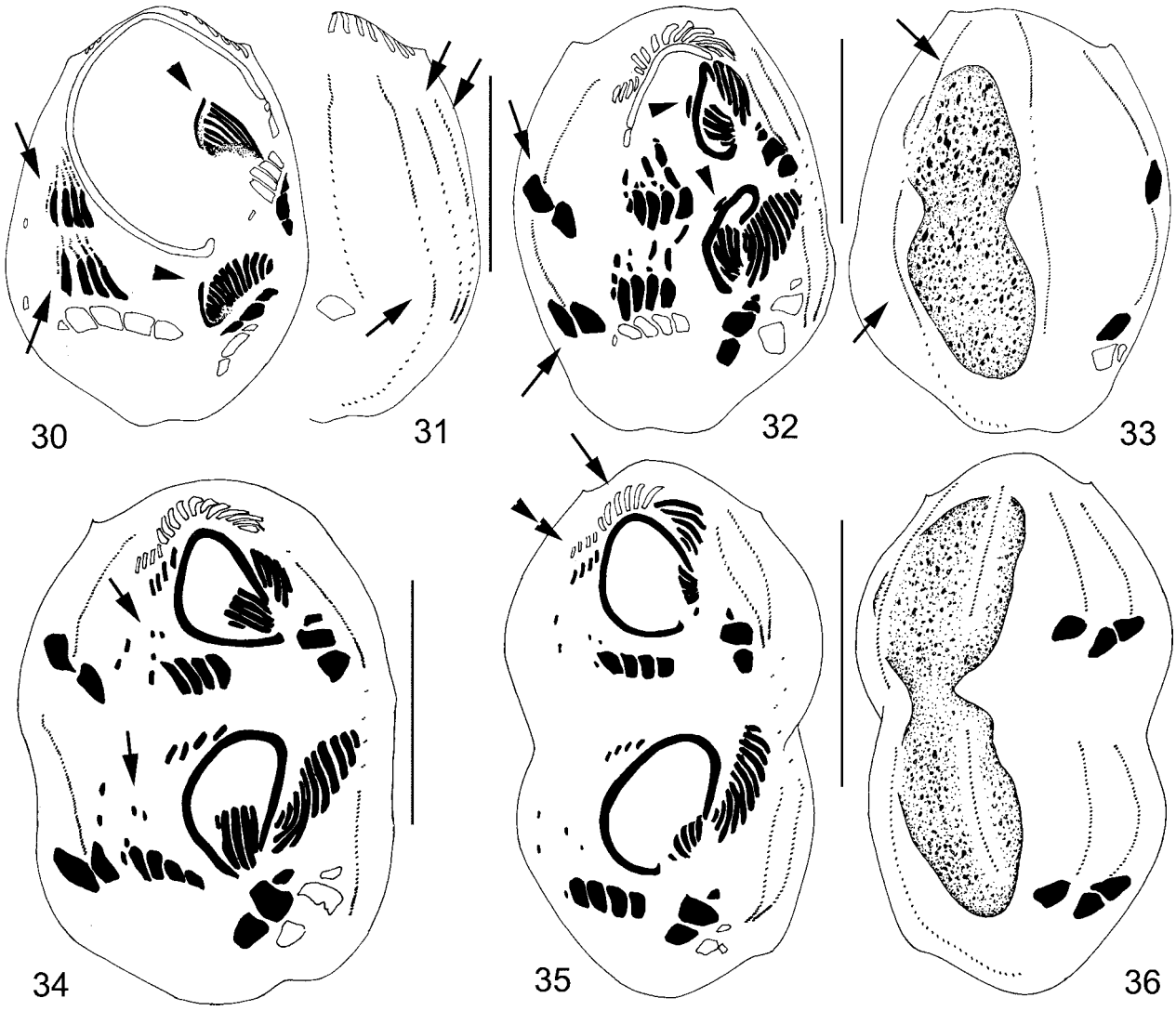
* This process is undertaken yet not *de novo*, but by a “de-differentiating and then rebuilding *in situ*” mode, basal bodies of old structures join the formation of the new anlage;
** Sometimes inconspicuously or difficult to outline the new anlagen because newly proliferated basal bodies appear within the parental strucutures; *** In this mode, two or more caudal cirri are developed from the posterior end of the rightmost anlage.



Figs 23-29. *Uronychia setigera* in morphogenesis. **23** - ventral view of an early divider, arrow marks the cirral anlagen (note that there are only four streaks at this stage); **24** - ventral view (note that the membranelles begin to generate, double-arrowheads), arrows mark the anlagen of the marginal cirri; **25** - dorsal view of the same specimen as Fig. 24, arrows mark the new caudal cirri formed within the 2 rightmost dorsal kineties; **26, 27** - ventral and dorsal views, arrowheads in mark the newly formed caudal cirri, double-arrowheads indicate the cirral anlagen (note they are in 2 groups); arrows in Fig. 26 marks the oral primordium of the proter, while in Fig. 27 mark the macronuclear replication bands; **28, 29** - ventral and dorsal views of the same specimen, to show the newly formed paroral membrane anlage (arrowheads), anlagen of marginal cirri (double-arrowheads) and 2 groups of cirral anlagen (arrows in Fig. 28); arrows in Fig. 29 mark the 2nd caudal cirrus generated from the rightmost dorsal kinety anlage in both dividers, respectively. AOP - anterior oral primordium, CA - cirral anlagen, POP - posterior oral primordium. Scale bars 50 μm.

gradient from right to left (Figs 25, 31, 33, arrows). Caudal cirri are generated as in other congeners (and even as in *Diophrys*)(Song and Packroff 1993): two caudal cirri are formed at the posterior end of the rightmost anlage in both proter an opisthe, while the second primordium (from right) gives rise to the third one (Figs 8, 12, 21, 27, 29, 36).

As a conclusion, the morphogenesis in *Uronychia setigera*, as in its congeners, can be summarized as follows: (1) The oral primordium (OP) in both proter and opisthe develops *de novo* in a subcortical pouch, respectively, in which the new membranelles formed in the proter's OP will replace the leftmost 5 parental ones; 6 old membranelles will be retained for the proter. (2)



Figs 30-36. *Uronychia setigera* in morphogenesis. **30** - ventral view, arrows mark the thread-like cirral anlage, arrowheads indicate the short paroral membrane anlage in both dividers; **31** - dorsal-to-ventral view of the same specimen, to show the leftmost 3 dorsal kineties, note kinetosomes in some portions are arranged more densely due to the proliferation of basal bodies in order to form the "anlagen" for the dorsal kineties; **32** - ventral view, arrows mark the new caudal cirri; **33** - dorsal view of the same specimen as in Fig. 32, arrows refer the 3rd kinety anlage in both proter and opisthe; **34** - ventral view, arrows mark ventral cirri; **35** - ventral view, arrow marks the old membranelles which will retain for the proter, while double-arrowheads indicate the old frontal cirri; **36** - dorsal view of the same specimen as in Fig. 35, to show that the fused macronuclear mass is in division. Scale bars 50 μm.

Highly specialized undulating membrane generates from the isolated UM-anlage which is formed and develops independently from the OP within the same subcortical pouch. (3) Five primary FVT-cirral anlagen appear *de novo* on the cell surface, which divide and give rise to 2 sets of cirral anlagen for the proter and opisthe. (4) Three frontal, 2 ventral and 5 transverse cirri derive from the 5 FVT-cirral anlagen in both daughter cells, usually 2 to 3 extra ventral cirri generated from these anlagen will be resorbed; the left marginal cirri develop from the marginal-anlage; all these primordia are formed *de novo*. (5) The leftmost frontal cirrus develops *de novo* on the cell surface in both dividers, and has no connection with the UM-anlage. (6) Origination of 3 caudal cirri is involved in the 2 rightmost dorsal kineties with a multi-segmentation mode.

Molecular phylogenetic trees constructed from complete 18S rRNA gene sequences

The bootstrap trees constructed from 18S rRNA sequences are demonstrated in Figs 37, 38. The maximum-parsimony analyses in our work provide strong

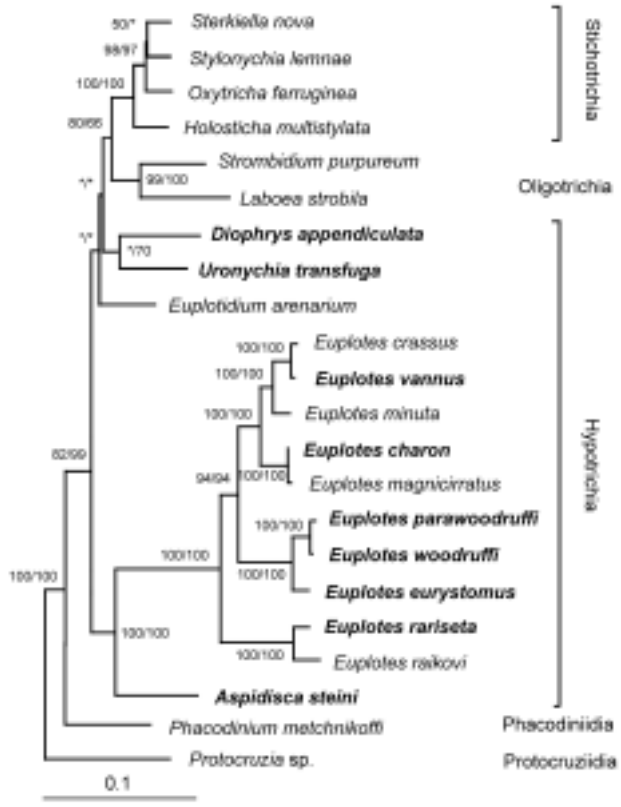


Fig. 37. Distance tree of the spirotrichous ciliates inferred from complete 18S-like, small subunit ribosomal RNA gene sequences. Evolutionary distances were calculated by the Kimura (1980) two-parameter correction model and constructed by the Fitch and Margoliash (1967) least-squares [LS] method. The numbers at the nodes represented the bootstrap percentages of 1,000 for the LS method followed by the bootstrap values for the Saitou and Nei (1987) neighbor-joining [NJ] method. Asterisks indicate bootstrap values less than 50%. Evolutionary distance is represented by the branch length to separate the species in the figure. The scale bar corresponds to 10 substitutions per 100 nucleotide positions. The euplotid species sequenced by the present authors are represented in **boldface**.

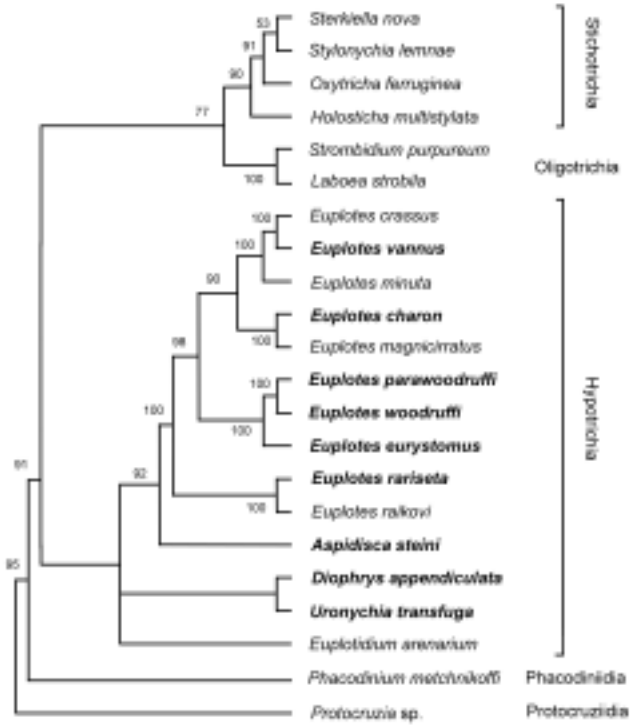


Fig. 38. A maximum-parsimony tree of the spirotrichous ciliates constructed from complete 18S-like small subunit ribosomal RNA gene sequences. The numbers at the forks exhibit the percentage of times the group occurred out of the 1,000 trees. No significance is placed on branch lengths connecting the species. The euplotid species sequenced by the present authors are represented in **boldface**.

support for the monophyly of typical taxa in Euplotina while the genus *Euplotidium*, the only Gastrocirrhidae, of which the sequences data are available, branches from them at low level (Fig. 38). The distance-matrix tree gives slightly different result (Fig. 37), i.e. the 18S rRNA data locate *Uronychia* and *Diophrys* within the Stichotrichia clade (the hypotrichs *s. l.*) though with very lower (lower than 50%) bootstrap support. *Euplotidium* is also grouped with them. All *Euplotes* species and *Aspidisca* are clustered together in a separate clade. In agreement with Borror and Hill (1995) who concluded that *Diophrys* and *Uronychia* are more closely related to each other than to *Euplotes* (hence as a different family, Uronychiidae), both trees support this arrangement and indicate also that the genus *Aspidisca* might separate from them at family level as well. All results obtained reflect consistently that *Euplotidium* (or Gastrocirrhidae) is a sister group to all of them.

DISCUSSION

Identification of *Uronychia setigera*

According to the morphological redescription by Song and Wilbert (1997), the well-known genus *Uronychia* contains now 3 valid morphospecies, *U. transfuga*, *U. binucleata*, and *U. setigera* with numerous synonyms, hence considerably fewer in number than previously believed (Taylor 1928, Kahl 1932, Bullington 1940, Fenchel 1965, Agamaliev 1971, Dragesco and Dragesco-Kernéis 1986, Valbonesi and Luporini 1990, Petz *et al.* 1995). Shortly later, one more species with beaded macronuclei and a long row of ventral cirri on the right

Table 2. Comparison of groups among traditional euplotids *s. l.* and oxytrichids (outer group).

Characters	eueuplotids	gastrocirrhids	discocephaline*	oxytrichids**
Cirral anlagen, number	invariably 5 in number	non-5, usually more than 6	non-5, usually more than 6	invariably 5 in number
Type of development of FVT-cirral anlagen	primary, *de novo*	primary, *de novo*	primary, possibly *de novo* ?	secondary, non-*de novo*
Origin of the oral primordium	within a subsurface pouch	on cell surface, *de novo*	on cell surface, *de novo*	on cell surface, *de novo*
Body shape	generally flattened and oval	subconical, non-oval	cephalized, elongated	typically elongated
Marginal cirri	absent, or only on left side	absent, or only on left side	on both left and right sides	on both sides or only on left side, grouped
Distribution of the frontoventral cirri	usually non-grouped***, reduced in number	non-grouped, in rows	grouped, like that in oxytrichids	characteristically grouped
Caudal cirri	present (few) or absent	absent	present and many, usually in several longitudinal rows	usually present, 3 in number
Origin of caudal cirri	(if present) generated from leftmost 2 dorsal kinety anlagen when present	-	formed as that mode in eueuplotids (from leftmost kinety anlagen)	one formed at posterior end of each kinety
Cilia in dorsal kineties	very short, stub-like	very short, stub-like	often very long, bristle-like	long or short
Adoral zone of membranelles	dominant, *ca* 1/2 or more of cell length	dominant, as in left	less than 1/3, like in other oxytrichids	generally less than 1/3 of cell length
Undulating membranes	mostly single, two in *Diophrys*	single	well-developed two	well-developed two
Origin of the leftmost frontal cirrus	*de novo* except in *Diophrys*****	from anterior end of UM-anlage	from anterior end of UM-anlage	from anterior end of UM-anlage
Macronuclear apparatus	beaded or in 1-2 segments, (band- or sausage-like in the latter)	as in eueuplotids number, sparsely distributed within the cell	numerous in	mostly two, oval in shape
Data sources	original	Hu and Song 2003	Wicklow 1982	Berger 1999

* This type is represented by *Discocephalus ehrenbergi*; ** Based largely on *Oxytricha*-complex; *** Frontal and ventral cirri in some genera (e.g. *Diophrys, Uronychia*) are in two groups; **** This cirrus in *Diophrys* derives from the UM-anlage.

cell margin, *Uronychia multicirrus*, was added (Song 1997).

The small form, *Uronychia setigera*, as defined by Song and Wilbert (1997), differs from the closely-related *U. binucleata* in lower number of basal bodies in the leftmost dorsal kineties (*ca* 20 *vs*. *ca* 30), conspicuously smaller size in natural water (*ca* 60 μm *vs*. about 100 μm in length) and presence of the lateral spur or spine at

about anterior 2/3 of cell on left margin (*vs*. absent or inconspicuous in *U. binucleata*) (Calkins 1902, Kahl 1932, Curds and Wu 1983, Song and Wilbert 1997). This conclusion was recently confirmed by researches using DNA-fingerprinting and ARDAR riboprinting methods (Chen *et al*. 2003).

Some noticeable morphogenetic features in *Uronychia*

The morphogenesis in all three well-known morphospecies of *Uronychia, U. transfuga, U. binucleata* and *U. setigera* has been repeatedly investigated even in last decades using silver methods. Among them, the largest form with beaded macronuclei, *U. transfuga* was studied by Wilbert and Kahan (1981) and Wilbert (1995), although only the main stages were documented. The species described by Hill (1990) under the name *Uronychia transfuga* and by Song (1995) under the name of *U. uncinata* would be populations of *U. binucleata* (see Song and Wilbert 1997). The small one, *U. setigera* was likely only once described (Shi and Song 1999), which matches completely the results of the present work.

All the hitherto data demonstrate that fissional events in this genus are highly stable and conservative, hence, the general morphogenetic pattern is shared by all known congeners. These data also suggest that *Uronychia*, compared with other related taxa, e.g. *Euplotes, Aspidisca, Diophrys*, possesses more primitive features: (1) UM-primordium forms only a single anlage-like membrane; (2) all ciliary organelles are present, and (3) most ciliary organelles appear to be at early developing stage considering the arrangement of basal bodies which are uniquely distributed in an anarchic mode. In addition, 2 caudal cirri developing from the posterior end of a single kinety anlage (the leftmost one) is possibly also a plesiomorphic characteristic, for the anterior one might represent the non-loosened kinety corresponding to some oxytrichids, in which the rightmost DK-anlage (of the dorsal group) is usually segmented to form the 4th kinety and the newly formed kinety bears the caudal cirrus (Eigner 1997, Foissner 1996, Berger 1999). This mode is seen also in *Euplotes* and *Diophrys* (Tuffrau *et al*. 1976, Hill 1981, Song and Packroff 1993, Song and Wilbert 1994), and implying that it is a conservative feature having a long evolutionary history.

Considering the general process of the development of the oral apparatus, *Uronychia* exhibits the combination of 3 unusual stomatogenetic characteristics: (1) oral primordium in the proter develops *de novo* and subcortically as well; (2) AZM divided into 2 parts, and (3) the piece-together-mode of the AZM_1 in the proter. Among these, the most noticeable event is the point 3, namely the formation of AZM_1 in the proter: it is (invariably !) composed of 6 parental (retained) and 5 newly formed membranelles. This phenomenon is unique even in all spirotrichids, though a "similar" process is seen also in several other non-euplotids, e.g. *Hemigastrostyla* and some urosylids. In these taxa, nevertheless, the proter's oral primordium develops unexceptionally on the cell surface (epicortically) and forms a variable number of membranelles (Hemberger 1982, Song and Hu 1999), i.e. very likely a convergent similarity.

As given in the morphogenetic descriptions (see Results), some newly formed ventral cirri will be resorbed before/after cell division is completed, i.e. one cirrus from the each of cirral anlagen II-IV will be dissolved. Clearly, this process might be individual-dependent: in some cases, those to-be-dissolved cirri could be retained for a short time after division as we observed in some individuals.

Another event needs to be clarified: the leftmost frontal cirrus in both proter and opisthe is formed *de novo* rather than generated from the UM-primordium like that in most hypotrichs *s. l.* This situation is seen also in at least two other morphologically rather specialized genera, *Aspidisca* (Diller 1975, Hill 1979, Song 2003) and *Euplotes* (Washburn and Borror 1972, Tuffrau *et al*. 1976), this implys that this might be a plesiomorphic character.

Hill (1990) described that the paroral membrane in *Uronychia binucleata* (called *U. transfuga*) divides and overlaps at the anterior end before completion of division, thus forming the right and left oral membranes. This description is, however, neither supported by the morphological (Song and Wilbert 1997) nor by the morphogenetic observations (Song 1996, called *U. uncinata*). In *U. setigera,* the present work and a previous report (Shi and Song 1999) substantiate the same conclusion: UM-primordium gives rise to only a single structure. Similar results are also obtained in *U. transfuga* (Wilbert and Kahan 1981, Wilbert 1995).

Systematic relationships and position of *Uronychia* and related euplotids

Euplotids traditionally comprise of at least 12 traditional genera assigned by Corliss (1979) to 3 families, Aspidiscidae, Euplotidae and Gastrocirrhidae. They are characterized by reduced somatic ciliature, rigid body and mostly oval/flattened shape, absence of right mar-

ginal cirri, dominant AZM, and subcortical mode of stomatogenesis (Borror 1972, Tuffrau *et al*. 1976, Corliss 1979, Curds and Wu 1983, Dragesco and Dragesco-Kernéis 1986). This classification is generally accepted by most taxonomists, though some related or "sister groups" were subsequently added into this increasingly comprehensive complex, and its systematic arrangement was often slightly redefined (Small and Lynn 1985, Hill and Borror 1992, Borror and Hill 1995). The updated system is suggested by Lynn and Small (2002), in which the order Euplotida consists of 2 suborders: the Euplotina with "typical" euplotids (5-anlagen-mode) plus gastrocirrhids, while another suborder, Discocephalina, is composed of some cephalized marine groups (Wicklow 1982).

In euplotids *s. l.*, most genera in Aspidiscidae and Euplotidae belong to the type of 5-cirral anlagen (5 transverse cirri in non-dividing stage) or "typical" euplotids (eueuplotids, see terminology) though only 5 of them, *Aspidisca, Euplotes, Certesia, Diophrys* and *Uronychia,* have been both morphologically and morphogenetically studied (Washburn and Borror 1972, Wicklow 1983, Hill 1990, Song and Packroff 1993, Song and Wilbert 1994). Different from these eueuplotids, the discocephalins with the representative genus *Discocephalus,* demonstrates distinctly diverse morphology and a highly different mode with multi-cirral anlagen and epicortical stomatogenesis (Wicklow 1982) (Table 2). In Gastrocirrhidae, the genus *Gastrocirrhus* is likely the only one, in which the morphogenesis is partly known (Hu and Song 2003). Based on the description by Hu and Song, 5 features can be recognized: (1) the oral primordium develops on cell surface; (2) FVT-cirri generate from many (more than 5) cirral anlagen; (3) no marginal and caudal cirri are formed; (4) paroral membrane is a single structure, (5) one streak-like UM-anlage develops *de novo* in both proter and opisthe, from which the leftmost frontal cirrus is generated. This reflects that *Gastrocirrhus* is an intermediate type between *Discocephalus* and other typical euplotids mentioned above (Table 2).

The main morphogenetic features found in 5 "typical" euplotid genera are tabulated (Table 1). Among these, the most significant characters are: (1) oral primordium develops subcortically; (2) five *de novo* formed primary FVT-cirral anlagen; (3) single undulating membrane generates from the isolated UM-anlage (yet presence of two membranes in *Diophrys* is clearly an apomorphic character); (4) the leftmost frontal cirrus develops completely separated from the UM-anlage (again, it is exceptional in *Diophrys* which exhibits a stichotrichous mode); (5) multi-segmentation of caudal cirri (when present); (6) no right marginal cirri generated. Considering so many morphological and morphogenetical features shared by diverse taxa, it is unlikely that these euplotids develop from different ancestors convergently, so that combination of these characters should be, in our opinion, regarded to be critical criteria for a monophyletic complex. The multi-anlagen-mode (non-5-cirral anlagen) is seemingly an apomorphic character, which derives divergently from (?) the 5-anlagen type as revealed by investigations on *Amphisiella annulata* by Berger (2004). The morphogenesis of this stichotrich ciliate supports that, apart from 5 normal cirral streaks, there is always an inconspicuous, extra anlage being formed which develops to the 6th transverse cirrus. The epicortical origin of the oral primordium is definitely a primary character (vs. subcortical mode) for it occurs in numerous highly diverse stichotrichs/ hypotrichs, which indicates it is impossible due to convergent evolution. Hence a reasonable surmise is that, within the order Euplotida, the gastrocirrhids are indeed a closely related group to the eueuplotids, which are as pseudoeuplotids belonging to the same suborder as suggested by Lynn and Small (2002). This view is basically supported also by molecular data (Chen and Song 2001, 2002; Chen *et al*. 2002), based on which gastrocirrhids are almost always clustered with other eueuplotid clade (Figs 37, 38). As to the discocephalids/discocephaline, they could be, considering their morphological and morphogenetic features (no molecular data in this group available yet), possibly a marginalized or even a paraphyletic group to the euplotine, which are even more closely related to oxytrichids, e.g. grouped frontoventral cirri, 2 UM-membranes, epicortical origin of oral primordium, elongated body shape and generally presence of both marginal rows (Table 2). Nevertheless, further evidences are needed to indicate in which position this group should be placed.

With reference to the AZM in the proter, at least 4 basic patterns can be recognized in all spirotrichs (Hemberger 1982, Foissner 1996, Eigner 1997, Berger 1999, Hu *et al*. 2003): (1) The parental structure is intact and retained for the proter, e.g. in many euplotids, oxytrichids, many amphisiellids, discocephalids and kahliellids. (2) Posterior portion of AZM is partly renewed after de-differentiation of old membranelles and then a re-building process, in which no new OP is involved, e.g. *Diophrys* and some urostylids. (3) The old AZM will be completely replaced by a new one which

is formed by a *de novo* appeared OP, like in most urostylids. (4) (Only in the posterior portion) partly renewed by the newly formed structure, hence piecing together with the retained old part (the distal part), e.g. *Uronychia, Hemigastrostyla*. Thus, euplotids (both eueuplotids and pseudoeuplotids) exhibit 3 different patterns of the formation/rebuild of the oral structure. This suggests that this diversity is likely due to divergent evolution (from an ancestor type) though evidence could not be traced in the morphogenesis.

Finally, an interesting point of discussion is the relationship between euplotids and hypotrichs *s. l.* (stichotrichs) or the origin of the euplotids if we consider that two assemblages have close connection as most hitherto information has revealed. Our opinion is that the euplotids are not the most primitive forms, though there is still no firm evidence showing that stichotrichs is the forerunner of euplotids. The most "critical" reasons are from morphogenetic data, e.g. 5-anlagen pattern and the subcortical origin of the oral primordium. The former feature is shared by both euplotids and many highly dedifferentiated stichotrichs (e.g. oxytrichids, kahliellids *vs*. the primitive *Kiitricha*), hence not the most primitive taxa. This indicates clearly that this similarity does not derive from parallel evolution. Whereas the latter feature, the oral primordium originating subcortically, should be also an apomorphic characteristic because in most other lower ciliates (non-spirotrichs) oral primordium develops on the cell surface (Foissner 1996). Other evidence comes from the morphological features: (1) many euplotids have only left marginal cirri or simply absent (divergent from predecessors with both left and right ones); (2) presence of bipartite AZMs is surely an apomorphic feature. However, considering some primitive characteristics in euplotids, e.g. most genera have only a single undulating membrane, 2 caudal cirri develop from a single dorsal kinety anlage, the ancestor of euplotids is also less likely any current type of stichotrichs. We presume that, especially because of the fact that 5-anlagen pattern is widely seen in many taxa in oxytrichids as well, euplotids (at least the eueuplotids) derive from a forerunner possessing the 5-anlagen mode, which might be also the predecessor of many present hypotrichs *s. l.*

In conclusion, our new points of views on taxonomy of euplotids are: (1) The traditional euplotids could be a paraphyletic assemblage consisting of "true" and outer groups. As a suggestion based on the new definition, the order Euplotida *s. l.* contains at least 2 subgroups: the eueuplotids or "typical" euplotids, a monophyly consisting of the taxa with the pattern of 5-cirral-anlagen as well as the subcortical origin of the oral primordium, and the sister group, gastrocirrhids (or pseudoeuplotids), with multi-anlagen feature which is a divergent, or apomorphic feature. (2) The systematic position of discocephaline remains unclear, but possibly an intermediate group between the euplotids *s. str.* and stichotrichs; However, we agree that it is presently placed within the order Euplotida, as an outer group of other two assemblages. (3) The morphogenetic feature of 5-cirral-anlagen-mode is highly conservative and should be hence weighted with more phylogenetic value(s) in systematic analysis. (4) We suppose that some features like the origin mode of the leftmost frontal (formed from the UM-anlage), and the situation of undulating membranes (e.g., two in number and curved in appearance) represent apomorphic features, and should not be over-evaluated. (5) Euplotids *s. str.* might be derived from the 5-anlagen ancestor-type of hypotrichs *s. l.*, which is possibly also the ancestor of the current stichotrichs.

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