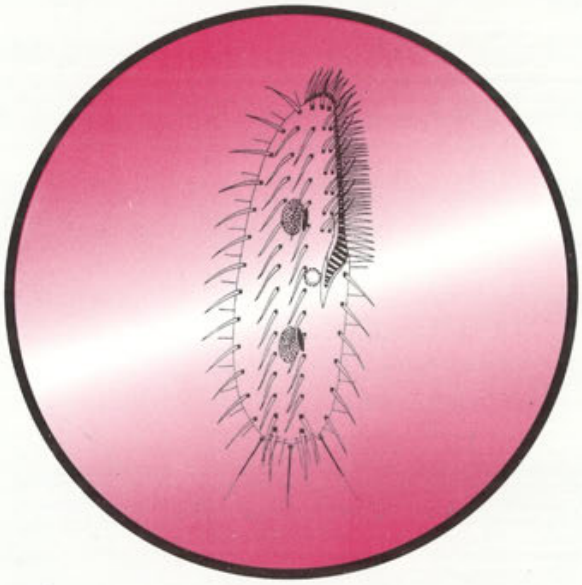
ACTA PROTOZOOLOGICA



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Could Foraminiferal Zooxanthellae be Derived from Environmental Pools Contributed to by Different Coelenterate Hosts?

John J. LEE[1,2], Charles G. WRAY[3,4] and Charles LAWRENCE[1]

[1]Department of Biology, City College of City University of New York; [2]Department of Invertebrates, American Museum of Natural History, New York City; [3]Department of Entomology, American Museum of Natural History, New York City; [4]Department of Geology and Geophysics, Yale University, New Haven, USA

Summary. We isolated the zooxanthellae from two closely related species of "living sands" (soritid foraminifera), *Amphisorus hemprichii*, from the Gulf of Eilat, Red Sea, and *Marginopora kudakajimensis*, from Kudaka Island, Japan. After extraction of their DNA, amplification by PCR, cloning and sequencing, we compared their nuclear small-subunit rDNA sequences to those already published. Phylogenetic reconstructions indicate that the two dinoflagellates isolated from the foraminifera do not represent sister taxa. The *Symbiodinium sp. isolated from Marginopora kudakajimensis* was a sister taxon to *Symbiodinium pulchrorum* isolated from an Hawaiian anemone, *Aiptasia pulchella*, while the isolate from *Amphisorus hemprichii* was a sister taxon to the type of the genus, *S. microadriaticum* from the upside down jelly fish, *Casssiopeia xamachana*. Since the two foraminiferal hosts evolved from a common ancestor, the topologies of their phylogenetic trees should be similar if the symbionts and hosts were mutually associated during their evolutionary diversification (Rowan 1991). We anticipated that the two *Symbiodinium* spp. isolated from soritacean hosts would be more closely related to each other than to those from invertebrates. Since they were not, we are left with the question how were they acquired? We have shown in diatom-bearing larger foraminifera that hosts can acquire new species of symbionts by feeding on them. By analogy to the diatom-bearing forms, we speculate that the dinoflagellate-bearing soritid foraminifera acquired their symbionts from an environmental pool contributed to by coelenterates in their habitat, rather than by common inheritance, and speculate they may still be doing so.

Key words. Soritid foraminifera, *Amphisorus hemprichii, Marginopora kudakajimensis, Symbiodinium*, zooxanthellae.

INTRODUCTION

Symbiotic associations, between marine invertebrates, or between protists, and unicellular algae, are common in oligotrophic seas and are broadly viewed as significant mechanisms for adaptation to those habitats which are well illuminated and shallow. Although the fact is not widely appreciated, larger foraminifera, popularly known as "living sands", are extremely abundant in these tropical and semitropical marine habitats. They have also left fossil remains (e.g. the limestone of the pyramids of Egypt) which attest to their abundance in similar ancient seas. The majority (four of seven) of modern families of larger foraminifera are hosts for diatom endosymbionts, but one family, Soritaceae, is the host for symbiotic dinoflagellates. The dominance of diatoms in

Address for correspondence: J.J. Lee, Department of Biology, City College of City University of New York, Convent Avenue at 138 St., New York, NY 10031, USA; Fax: 212 650 8585

foraminiferal endosymbioses is in contrast to the phenomenon in a large number of invertebrate hosts where symbiotic dinoflagellate partners are most common. Many of the dinoflagellates belong to the *Symbiodinium*-complex. It was once believed that one species, *Symbiodinium microadriaticum*, was a pandemic symbiotic partner in a wide range of marine invertebrate and foraminiferal hosts (Freudenthal 1962, Smith and Douglas 1987, Smith 1993). Both stages of *Symbiodinium* have very simple morphology with few diagnostic characters. Ideas changed as isoenzyme (Schoenberg and Trench 1980a, b, c) and chromosomal (Blank and Trench 1986, Blank 1987, Trench and Blank 1987, Blank et al. 1988) evidence showed that the diversity was much greater than was previously believed (reviews Trench 1987, Blank 1992). Subsequent research has completely dispelled the pandemic concept and a large number of endosymbiotic dinoflagellates have been recognized (Trench 1992, 1993; Banaszak et al. 1993; McNally et al. 1994). Seven dinoflagellate genera which have some endosymbiotic species, *Amphidinium, Aureodinium, Gloeodinium, Gymnodinium, Prorocentrum, Scrippsiella* and *Symbiodinium*, are now recognized (Trench 1993). Data comparing nuclear ss rRNA diversity in invertebrate isolates reinforced the conclusion that *Symbiodinium*-like zooxanthellae represent a collection of distinct species (Rowan and Powers 1991b, 1992; McNally et al. 1994). Six restriction fragment length polymorphisms (RFLPs) were detected. Each host contained only one algal RFLP type. Phylogenetic relationships were estimated by comparing the ss rRNA from isolates from 22 host species. Closely related algae were found in dissimilar hosts suggesting that there is no correlation between microalgal taxa and host taxa (Trench 1993). Since it was already suspected from partial morphological and fine structural studies that *Symbiodinium* sp. (spp.) or *Symbiodinium*-like species were the endosymbionts in the soritids, we were stimulated to characterize and relate them to the growing constellation of described endosymbiotic dinoflagellates (Banaszak et al. 1993, Trench 1993, McNally et al. 1994).

MATERIALS AND METHODS

Dinoflagellate isolation. The hosts of one isolate, *Amphisorus hemprichii*, were collected with the aid of SCUBA at approximately 25m depth in a sea grass bed of *Halophila stipacea* near Wadi Taba, at the boarder between Israel and Egypt on the Gulf of Eilat, Red Sea. The second hosts, *Marginopora kudakajimensis*, were collected at low tide in the backreef of Kudaka Island, Okinawa Prefecture, Japan. Both hosts were taken by air by the senior author to the City College of New York where the isolations of the symbionts were accomplished. We knew at the onset that the isolations would be difficult because we had previously studied the surfaces of the hosts in the SEM. Both are heavily colonized by diatoms and bacteria when the hosts are alive. Crevices between the joints in the plate-like chamberlet walls are common habitats for species of diatoms belonging to the genera *Amphora* and *Cocconeis*. We have also observed endolithic colonization. For these reasons, we took the unusual steps necessary to totally remove the colonized calcium carbonate from the shells. In our previous work with other types of symbiont-bearing larger foraminifera, which are not colonized, it was only necessary to crack open the tests to release the symbionts. Ten hosts of each species were transferred to sterile sea water in the wells of sterilized spot plates placed on the stage of a dissecting microscope equipped with both above stage fiber optic cold light and below stage darkfield illumination. There they were aseptically and carefully brushed with ethyl alcohol sterilized sable artist brushes (#0000). After 10 aseptic changes of sea water and brushes, the specimens were examined at relatively high magnification (x 60), using both types of illumination, to see if all surfaces of the test had been freed of as many of the colonizing contaminants as could be removed at this stage of the process. The hosts were then transferred to sea water containing 1 mM EDTA and observed under the dissecting microscope as they were being decalcified. When the process was completed, the decalcifying medium was withdrawn and replaced with sterile normal sea water. The hosts were then dissected with sterile glass needles which were used to tear open the organic layers of the chamberlet walls and release the symbionts. Individual,and small groups of symbionts were removed from the wells with the aid of 10 µl capillary pipettes and transferred to ISM medium (Lee et al. 1980) which contained GeO_2 (0.1 µM) to inhibit the growth of any contaminating diatoms which might have survived the washing process.

Morphology. The isolates from both hosts were examined by both phase contrast microscopy and electron microscopy. Specimens examined in the SEM were fixed in 1% glutaraldehyde in 0.2M sodium cacodylate buffer, washed in sea water, and post fixed in 1% OsO_4 in sea water. They were then dehydrated in acetone, transferred to amyl acetate, critically point dried, and coated with 10 nm of Au in a sputter coater. Specimens examined in the TEM were also fixed in 1% glutaraldehyde in 0.2M sodium cacodylate, washed in sea water. and post fixed in 1% OsO_4 in sea water. A culture of *Symbiodinium pilosum* was sent to us by Dr. Robert Trench, University of California, Santa Barbara, so that we could examine it comparatively. Measurements of 50 properly oriented specimens of each type were made with the aid of optical calipers, an accessory of the Zeiss DSM (Model 950) which we used in the study. Because of the rarity of the motile stage of the isolate from *M. kudadakajiminensis*, we were only able to find 7 properly oriented specimens to measure.

Molecular methodology. One culture of *Symbiodinium* from each host was harvested by centrifugation. DNA isolation followed the techniques of Rowan and Powers (1992). PCR reactions were carried out in a Perkin-Elmer 4000 thermal cycler programmed with a standard cycle of one min. at 94°C, 1.25 min. at 42°C, 2 min.

at 72°C for 35-45 cycles. The product was run at 130 V for 40 min. on a 1% agarose gel in 1X TBE buffer and stained with ethidium bromide. PCR and sequencing oligonucleotide primers were designed after Medlin et al. (1988). Positive amplifications of the complete nuclear small-subunit (18S) rDNA were cloned using the components of the TA cloning kit from Invitrogen. Cloned DNA was sequenced as described by Toneguzzo et al. (1988). The divergent regions of the 18S rDNA were sequenced for multiple clones to investigate sequence heterogeneity; no nucleotide differences were apparent in the regions examined. Complete clones were sequenced for both strands. DNA sequence was read directly into the computer using Macvector 4.11 (Eastman Kodak) and verified using the program's computer voice assisted read back. DNA sequences were aligned using MALIGN (version 3.1) (Wheeler and Gladstein 1992). Complete dinoflagellate 18S rDNA sequences taken from the NCBI data base (release 6.0) and incorporated into the alignments and phylogenetic reconstructions were: *Cryptothecodinium cohnii* (Rowan and Powers 1992) (M34847), *Gloeodinium viscum* (McNally et al. 1994) (L13716) *Prorocentrum micans* (Rowan and Powers 1992) (M14649), *Symbiodinium microadriaticum* (Rowan and Powers 1992), *S. pilosum* (Rowan and Powers 1992), *S. meandrinae* (McNally et al. 1994) (L13718), *S. corculorum* (McNally et al. 1994) (L13717), *S. pulchrorum* (Rowan and Powers 1992). Phylogenetic reconstructions were carried out using PAUP (Swofford 1991). A single most parsimonious tree was found after branch and bound search using PAUP. The sequence from the *Symbiodinium* sp. isolated from *Marginopora kudakajimensis* has been assigned - GENBANK number U10893; that from the *Amphisorus hemprichii* isolate was assigned U10892.

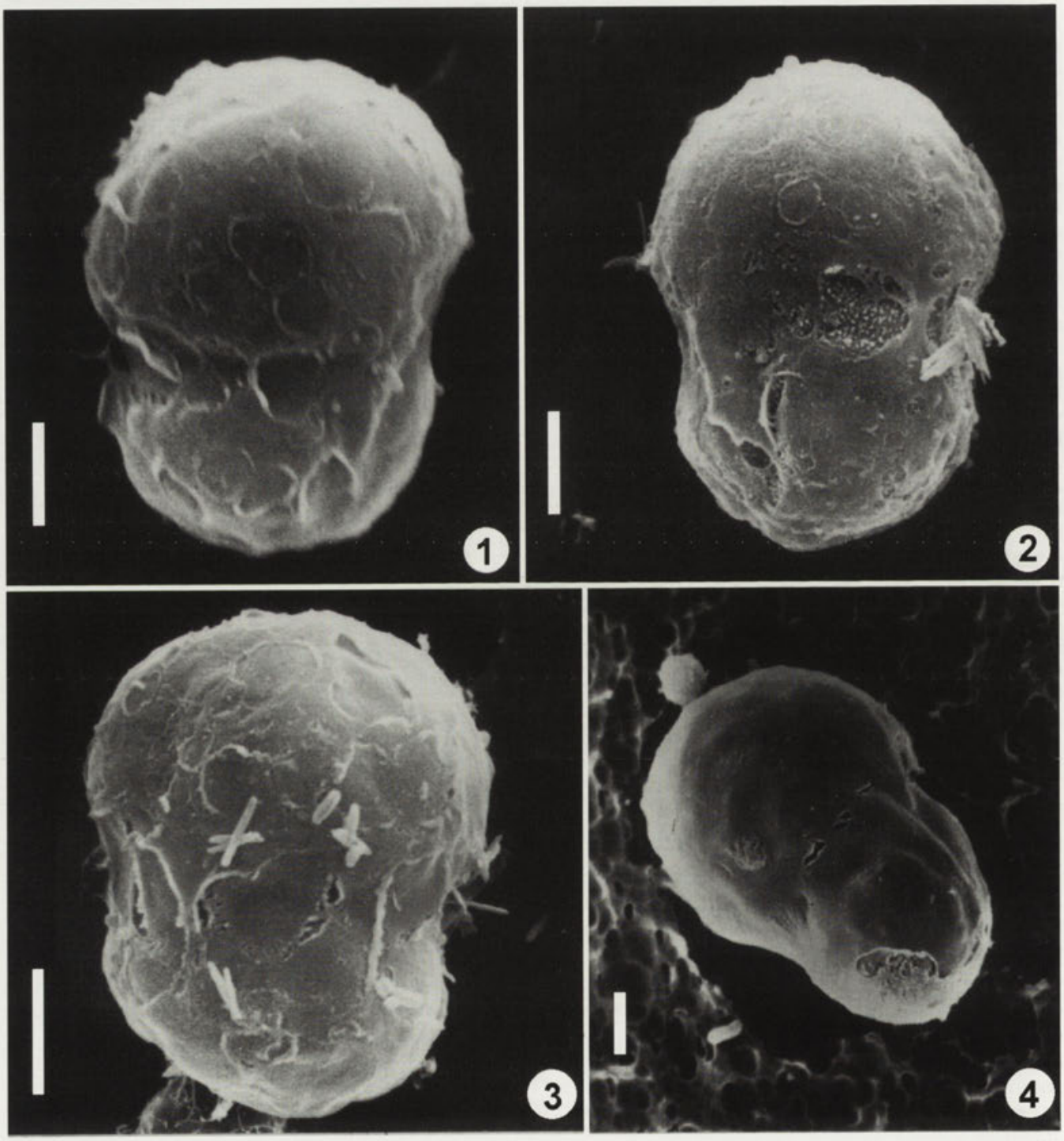
RESULTS

Phase contrast microscopic observations of the gross morphology of the dinoflagellate endosymbionts we isolated from both species of foraminifera suggested that they also belong to the *Symbiodinium*-complex. SEM observations showed that the zoospore of the *Amphisorus*-isolate is covered with fine subsurface alveoli which could be interpreted as protoscales (Fig.1), a feature not found on either *S. microadriaticum, Symbiodinium* sp. from *Marginopora kudakajimensis* (Fig.4), or *S. pilosum* (Figs. 13, 14). The surface of the zoospore of *Marginopora kudakajimensis* isolate, a stage rarely seen in cultures, is quite smooth. The cingulum of the isolates from both *Marginopora kudakajimensis* and *Amphisorus hemprichii* is quite shallow. The epicone of both isolates is very slightly larger than hypocone. The mean of the transapical axis of the isolate from *A. hemprichii* was 12.4 µm (± 0.53); that of the isolate from *M. kudakajimensis* was 11.1. The cingular axis of the former was 7.5µm (± 0.34) and of the latter was 7.2 µm. We found two different gymnodinoid flagellates in the culture of *S. pilosum* sent to us (Figs. 13, 14). The most common one has a deep cingulum with extended margins and a smooth surface (Fig. 13). The epicone of the other is larger than the hypocone. It also more elongate (transapical axis: cingular axis; ~ 2:1) and has a surface with raised knobs from which fine threads or filaments seem to be extruded (Fig. 14).

Vegetative stages of both foraminiferal isolates varied in shape from spherical, oblate spherical, to bean-shaped (Figs. 7, 9-12). Newly formed sibling cells, still within their parental wall, are bean-shaped (Figs. 11, 12). The individual vegetative cells of the *Amphisorus*-isolate were smaller (8.1 µm ± 1.2) than those of the *Marginopora kudakajimensis* isolate (12.4 µm ± 0.8). The surface of the vegetative stage of the latter is slightly rough (Fig. 7) when compared to that of the former (Fig. 9). At higher magnification small irregularly spaced pores are observable (Fig. 8). As expected from the original description of the species (Trench and Blank 1987) the surfaces of the vegetative cells of *S. pilosum* were covered with fine spike-like projections (Fig. 13).

Serial fine sections of vegetative stages of the *Amphisorus* isolate suggest that this species has approximately 70 condensed DNA nodes (chromosomes?) (Figs. 7, 10, 11). The vegetative stages of the *Marginopora kudakajimensis* isolate seem to have even more condensed DNA nodes, perhaps as many as 90 (Figs. 5, 12). One can observe a irregular fuzzy coating on the surface of the vegetative stage of *S. pilosum*; the surfaces of the same stage of *S. microadriaticum* and the two foraminiferal isolates were smooth.

Phylogenetic analysis of zooxanthellae 18S rDNA sequences (Fig. 15) indicates that the foraminiferal dinoflagellates are not sister taxa: the isolate from *A. hemprichii* is related to *S. microadriaticum* (isolated from the upside down jellyfish *Cassiopeia*) and the isolate from *M. kudakajimensis* is sister to an isolate from an Hawaiian anemone, *Aiptasia pulchella* (Fig. 16). In order to avoid the problematic assumptions associated with bootstrap resampling (Sanderson 1989, Hillis and Bull 1993), we investigated the robustness of our phylogenetic hypothesis (Fig. 16) through derivation of Bremer support (Bremer 1988, Källersjö et al. 1992) There is strong phylogenetic support placing both foraminiferal dinoflagellate endosymbiotic sequences within a *Symbiodinium* clade and *S. pulchrorum* and *Symbiodinium* sp. (Kudaka Jima) sister taxa. Strict consensus of the 245 phylogenetic hypotheses at least 16 steps longer than our most parsimonious tree (Fig. 16) still resolve the



Figs. 1-3. SEMs of the zoospore stage of *Symbiodinium* sp. isolated from the foraminiferan *Amphisorus hemprichii*. Each figure is from a different preparation of the symbiont. Fine envelope with irregular scale-like subpelicular amphaesmal vesicles visible on the surface. Bars - 2µm
Fig. 4. SEM of the zoospore stage of *Symbiodinium* sp. isolated from the foraminiferan *Marginopora kudakajimensis*. Bar - 2µm

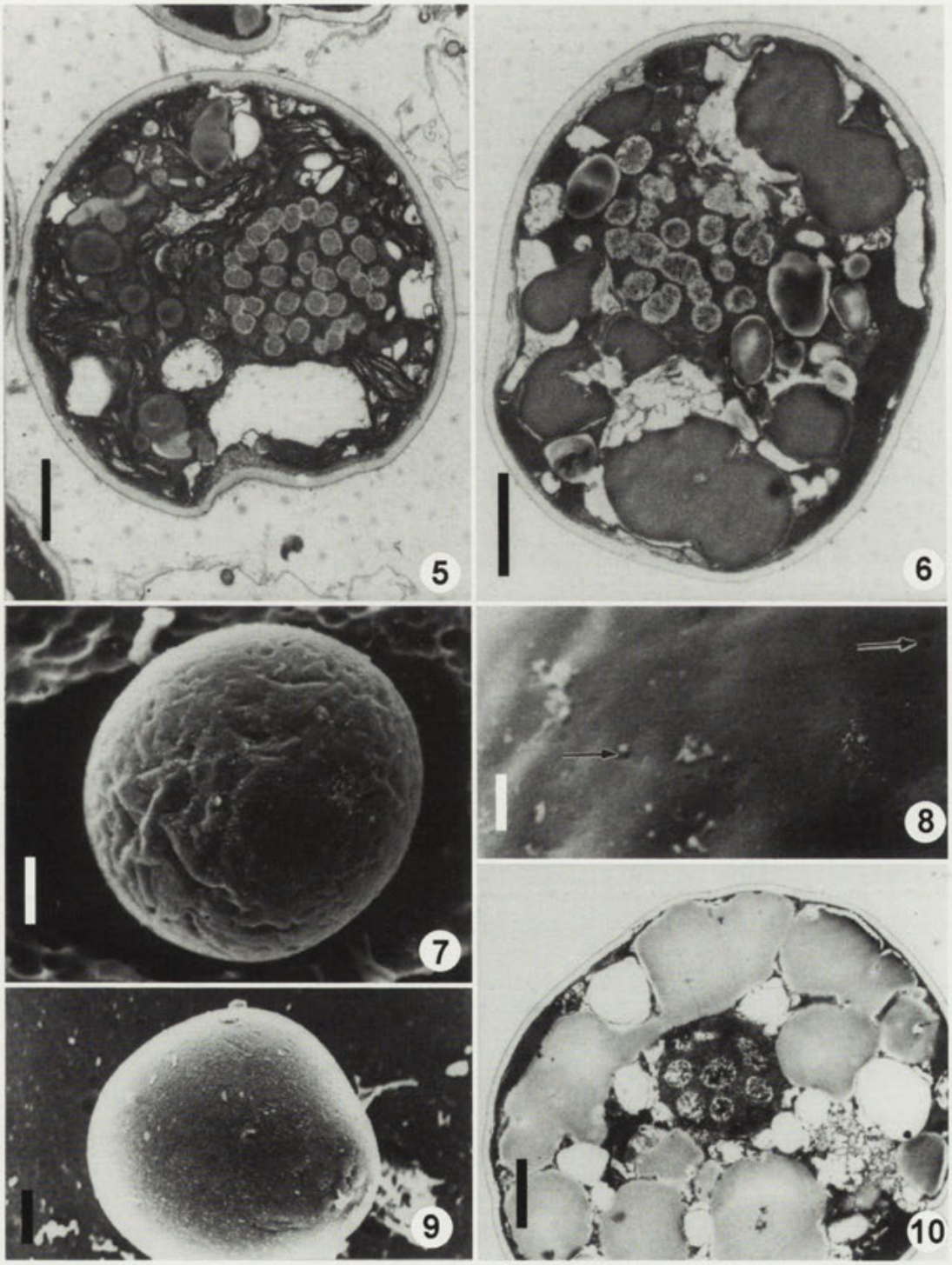


Fig. 5. TEM of the vegetative stage of *Symbiodinium* sp. from *A. hemprichii.* Section is through the middle of the nucleus and shows 28 condensed chromosome units in this view. Bar - 2µm
Fig. 6. TEM of the vegetative stage of *Symbiodinium* sp. from *M. kudakajimensis.* Section is through the middle of the nucleus and shows 17 condensed chromosome units in this view. Bar - 2µm
Fig. 7. SEM of the vegetative stage of *Symbiodinium* sp. from *M. kudakajimensis.* Figure shows the irregular rough surface of the *Symbiodinium sp. isolates from this host. Bar* - 2µm
Fig. 8. Higher magnification of the surface of the same specimen as in Fig. 7 showing fine irregular pores on the surface. Bar - 500 nm
Fig. 9. SEM of the vegetative stage of *Symbiodinium* sp. from *A. hemprichii.* Figure shows the fine dimpled surface of the *Symbiodinium* sp. isolates from this host. Bar - 5µm
Fig. 10. TEM of the vegetative stage of *Symbiodinium* sp. from *M. kudakajimensis.* Section is through the periphery of the nucleus and shows 8 condensed chromosome units in this view. Bar - 2µm

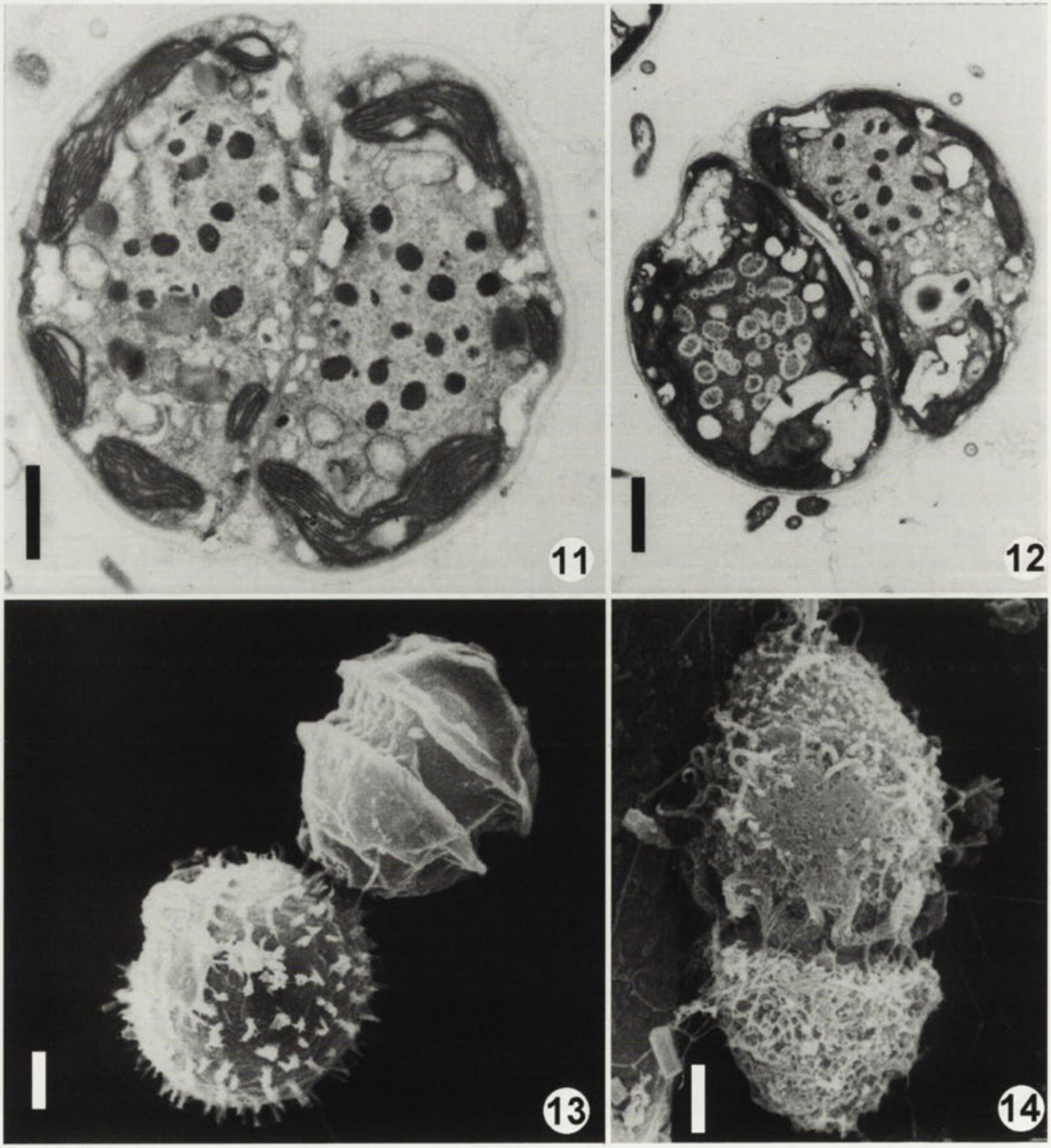


Fig. 11. TEM of a newly divided vegetative stage of *Symbiodinium* sp. from *M. kudakajimensis.* Newly divided vegetative cells tend to be oblate or bean-shaped spheroids. Section is through the middle of the nucleus and shows portions of 17 condensed chromosome units in this view. Bar - 2μm

Fig. 12. TEM of a newly divided vegetative stage of *Symbiodinium* sp. from *A. hemprichii.* Newly divided vegetative cells tend to be oblate or bean-shaped spheroids. Section is through the middle of the nucleus of cell on left and shows portions of 24 condensed chromosome units in this view. Bar - 2μm

Fig. 13. SEM of juxtaposed motile and vegetative stages of *S. pilosum* from a culture of the type provided by Dr. R. K. Trench to aid our ability to compare our new isolates. Bar - 2μm

Fig. 14. SEM of a different motile stage of *S. pilosum* from the same culture of the type provided by Dr. R. K. Trench to aid our ability to compare our new isolates. Bar - 2μm

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SYMB-Mk *    AACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCT
SYMB-Ah **   ............................-................................
             CAGTATAAG-CATTCAAATGGCGAAACTGCGAATGGCTCATTAAAGCAGTTATAGTTTAT
             ...........T..T.C.C.....................................A.....
             TTGATGGTCGCT-GCTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCATCCAA
             .........A.............................................-....C.A..
             GCCCGACTTCGCAGAAGGGTTGTGTTTATTAGATACAGAACCAA-CCAGGTTCTACCT-G
             A...A.........-........A.....................CG....C..CGT..G.
             TCATGTGGTGATTCATGATAA-CTTGACGAATCGTGTGGCCT-TGCCGACGATGCGTCAT
             .--.......................C..T............T..G...........A..T.
             TCAAGTTTCTGACCTATCAGGCTTCCGACGGTAGGGTATTGGCCTACCGTGGCAATGACG
             ...................-...........C.......G.....................
             GGTAACGGAGAATTAGGGTTTGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCT
             ..................................................G.G...
             AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAAGAAA
             ..........................................................
             TAACAATACAGGGCATCCATGTCTTGTAATTGGAATGAGTAGAACTTAAATCTCTTTATG
             ..........................................T.....C.C.......
             AGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGC-GTAATTCCAGCTCCAATAGCG
             .........CC..............................................
             TATATTAGAGTTGTTGCGGTTAAAAAGCACGTAGTTGGATTTCTGTTGAGGATGACCGGT
             .......A.....................T........-N.....................
             CCGCCCCCTGGGTGTGCATCTGGCTCAGCCTTGACATCTTCCTGAAGTACGTATCT-GCA
             .....TT.....................-..............A...A.............
             CTTGACTGTGTGGTGCGGTATTTGGGACATTTACCTTGAGGAAATTAGAGTGTTTCAAGC
             ...C.T.................A.....................................
             AAGCGCGCGCTTTGAATGCATTAGCATGGAATAATAAGATAGGACCTCAGTTCTATTTTG
             .....ATT..C......A.....................................--.....
             TTGGTTTCTAGAGCTGA-GGTAATGATTGATAGGGATAGTTGGGGGCATTCGTATTTAAC
             ..................G.......G.C.............-...................
             T-GTCAGAGGTGAAATTCTTGGATTTGTTAAAGACGGACTACTGCGAAAGCATTTGCCAA
             ..........................................................
             GGATGTTTTCATTGATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCCT
             ..........G...............................................
             AGTCTTAACCATAAACCATGCCAACTAGAGATTGGAGGTCGTTACTTGAATGACTCTTTC
             ................T.............................AT.C.....C...
             AGCACCTTATGAGAAATCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAA
             ..........................................................
             ACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTC
             ..................T.........................-...............
             AACACGGGGAAACTTACCAGGTCCAGACATAGTAAGGATTGACAGATTGATAGCTCTTTC
             ....-.....................................................
             TTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTA
             ..........................A.......................
             ATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAGTTACATGTAACCTCGGTTACATG
             ..........................................................
             GGCAA-CTTCTTAGAGGGAACACTTTGTGTGTCTAACGCAAGGAAGTTTGAGGCAATAAC
             ..................---.....................................
             AGGTCTGTGATGCCCTTAGATGTCCTGGGCTGCACGCGCGCTACACTGATGCGCTCAACG
             ..........................................................
             AGTTTATGATCTTGCCTGAAATGGTTGGGTAATCTTTTT-AAAATGCATCGTGATGGGGA
             .....GCA.................CC.............A.....................
             TAGAT-CATTGCAATTATTGATCTTCAACGAGAAATTCCTAGTAAGCGCGAGTCATCAGC
             .....C.........................G.....................
             TCGTGCTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAGTG
             ..........................................................
             ATCCGGTGAATAATTTGGACCGACGCAGTGCTCAGCTTCTGGACGTTGCGTTGGAAAGTT
             ...........T...C....T.....................................
             TCATGAACCTTATCACTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCT
             ..........................................................
             GCAGAAGGATCAA
             ............- 1812
```

Fig. 15. Complete nuclear small subunit rDNA from two foraminiferal endosymbiotic dinoflagellates. Sequence was generated using PCR amplification described by Rowan and Powers (1991b), cloned into the TA cloning vector (INVITROGEN) and sequenced using standard dideoxy termination methods. Dots represent identity to top line of sequence. * *Symbiodinium* sp. isolated from *Marginopora kudakajimensis*, ** *Symbiodinium* sp . isolated from *Amphisorus hemprichii*

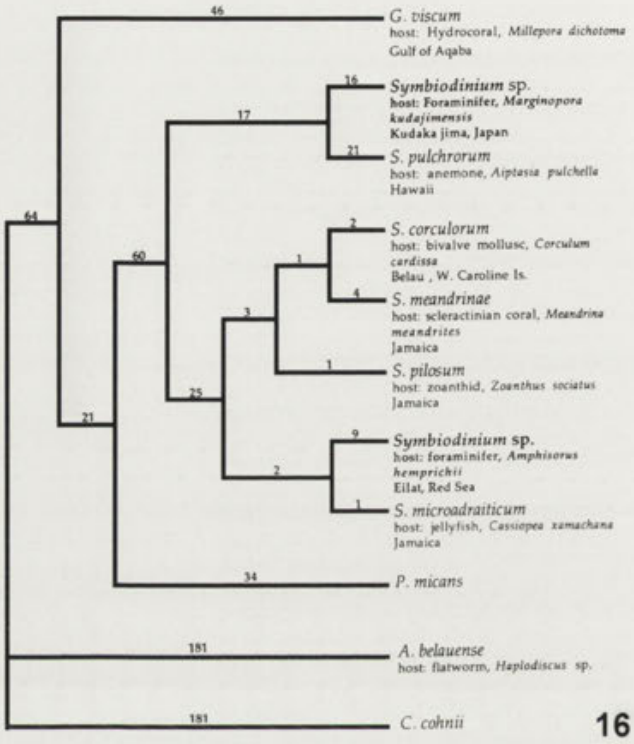

Fig. 16. Phylogenetic relationships within the genus *Symbiodinium*. Single most parsimonious tree for complete nuclear small subunit rDNA. Phylogeny was derived through branch and bound search using PAUP Consistency index 858, Retention index 716. Tree requires 689 steps. Taxa in bold sequenced in this study. Numbers above branches represent branch lengths. Phylogenetic reconstructions of partial 18S rDNA data, including *Gymnodinium* spp. indicate that the genus *Symbiodinium* is not monophyletic (McNally et. al. 1994)

Symiodinium clade and the early branching of *S. pulchrorum* and *Symbiodinium* sp. (Kudaka Jima).

DISCUSSION

The foraminiferan isolates are possibly divergent enough from each other, and the other members of the *Symbiodinium*-complex, to be characterized as new "species". But what is a species, subspecies, or variety in this complex? The criteria and the data are still in healthy dispute and reexamination (e.g. McNally et al. 1994). For example: McNally et al. (1994) suggest that the two ss rDNA sequences used by Rowan and Powers (1991b, 1992) in an attempt to produce "zooxanthellae-specific" primers (ss5Z, ss3Z) were an inadequate sample and were neither zooxanthella-specific or even dinoflagellate specific. They presented evidence (McNally et al. 1994, Fig. 1) that the primer in question would anneal to the 5' end of the ss rDNA from *S. pilosum, S. meandrinae, and S. pulchrorum* and the apicomplexans *Sarcocystis muris* and *Theileria annulata*, but not to the symbiotic dinoflagellates *S. corculorum, G. viscum,* or *A. belauense.* In addition the sequence that McNally and coworkers obtained from *Symbiodinium microadriaticum* was very different than the sequence obtained by Rowan and Powers (1992) from the same algal isolate. The simple morphological structure of *Symbiodinium* precludes easy separation on morphological grounds at the light microscopical level. In the SEM *S. pilosum* is quite distinctive. One important criterion used by Trench, Blank and coworkers (reviewed in Blank 1992 and Banaszak et al. 1993) to establish new taxa (species and subspecies) in the complex, (i.e. chromosome number, size and volume) has been brought under challenge by a new study (Udy et al. 1993). The results suggest that the regions of condensed nuclear DNA in dinoflagellates may not represent whole chromosomes, and so chromosome (condensed nuclear DNA nodes) counts are considered by them to be inappropriate for defining taxa of *Symbiodinium*. Blank (1987) did some very sophisticated image reconstruction to measure his condensed DNA nodes. Since Udy et al. (1993) studied natural populations, there is a possibility that the hosts they were examining may have had mixed symbiont populations. This question certainly begs reexamination. Being influenced by Udy et al. (1993), we did not attempt to reconstruct chromosomes in serial sections. The chromosome numbers we estimated were from mid-nuclear TEM sections and extrapolated to total nuclear volume. The numbers we estimated for our isolates fall well within the range (26, -98) found for other species of *Symbiodinium* (Blank 1992). The *Amphisorus* isolate, which in our molecular analysis was a sister group to *Symbiodinium microadriaticum,* has roughly the same number of condensed DNA nodes (97 vs. ~90) as the type. Corresponding data is not available for the sister taxon of the *Marginopora* isolate. The condensed DNA node number of the *Marginopora* isolate (~70) is close to the numbers reported for *S. goreauii* (74) and *S. pilosum* (78) (Blank 1992).

While asexual reproduction is the most common method of propagation in *Symbiodinium*, a sexual phase was observed and included in the original description (Freudenthal 1962) and in later studies (Taylor 1973, 1974). Unfortunately little is known of this phase of the life cycle (if it exists), or the genetics of populations, and potentiality for genetic exchange. The biological

species concept can not be employed to separate species in this genus. At least one investigator refutes the validity of the evidence for a sexual stage (Trench 1993). Rather than drawings of observations, more creditable new evidence would need to show meiotic processes and/or photographs of sexual recombination. Other differences (e.g. spectral forms of chlorophyll *a*, isoenzyme patterns, pyrenoid stalks, nuclear volume, motility patterns) in the species belonging to the *Symbiodinium*-complex have been observed (reviewed by Trench 1993). We are aware that one research group has established criteria for speciation of members of the *Symbiodinium*-complex. Evidence obtained by others to the contrary seems to be carefully refuted. Still, under the present conditions, we prefer to be conservative, and not name our two new isolates until a number of groups obtain data in agreement with each other, and a consensus is developed for criteria to separate species and subspecies of the genus *Symbiodinium*. It is our intention to isolate the endosymbionts from all the living soritids so that we can develop a comparative picture as to where these dinoflagellates fit in with those from invertebrates actively being studied by other groups. The endosymbiotic dinoflagellates from two additional soritid species, *Marginopora vertebralis,* and *Sorites marginalis,* have been already isolated in axenic culture and are in early stages of characterization.

An additional facet of the foraminiferal symbiont background comes from studies of diatom-bearing foraminifera. Individually, these hosts do not harbor particular species of diatoms. Overall, a very restricted group of approximately 20 species of small pennate diatoms were isolated and identified from several thousand foraminifera representing 10 host species (Lee et al. 1989, 1992; Lee and Anderson 1991). Six, taxonomically diverse, species were found in 75% of all the symbiont systems (Lee et al. 1992). Significant numbers of specimens harbored more than one species of diatom. As in the case of dinoflagellate-bearing foraminifera, the diatom-bearing hosts have an absolute dependence on the photosynthetic activity of their symbionts and actively feed on algae in their habitat (Lee and Anderson 1991). We were able to obtain nearly aposymbiotic hosts by incubating the hosts in DCMU in the light or by prolonged incubation of the hosts in the dark (reviewed in Lee and Anderson 1991). The nearly aposymbiotic hosts were then incubated in the sea (ambient light, photoperiod, temperature) in tissue culture flasks with mixtures of different symbiotic diatoms and chlorophytes and some species of free-living diatoms which were isolated in culture from the habitat where the hosts were collected and the flasks were incubated. The hosts were recolonized with endosymbionts during incubation. None of the free-living diatom species, or chlorophytes isolated as endosymbionts from a different group of symbiont-bearing foraminifera (Archaiadae) persisted (or could be isolated) from the hosts. There was a pecking order of host recolonizing species. Some species were retained rather than others. The recolonization process was followed with the aid of the TEM. Some endosymbiotic species were not digested by their hosts. We know that during asexual reproduction the parental cell passes its symbionts to their offspring. Many of the larger foraminifera are known to have sexual phases to their life cycles. Nothing is known about the acquisition of the endosymbiotic diatoms by zygotes. Symbionts were not found in TEM examination of the gametes of *Heterostegina depressa* the only larger foraminiferan where this stage has been studied. The plausible, but yet untested, hypothesis is that the zygotes acquire new symbionts from those released by their parents into the habitat. This could be a factor underlying the diversity we find in diatom species in diatom-bearing hosts from the same habitats. Whether gametes from parents with different symbionts can produce fertile offspring is an open question. Unlike the phenomenon found in diatom-bearing larger foraminifera, there is an example of diatom-host/symbiont specificity. *Convoluta convoluta* harbors the diatom *Lichmophora*, while a closely related flatworm *C. roscoffensis* harbors a very different symbiont, *Tetraselmis convolutae*. This is an example of two related hosts with very dissimilar symbionts.

Is it possible that there are parallels between the diatom-bearing and the dinoflagellate-bearing host/symbiont systems? Because of our very small sample size, we can not rule out the same diversity among dinoflagellate-bearing hosts as we found in the diatom-bearing ones. In fact, if we used the data from only our initial diatom isolations, and did no further work, the diversity of foraminiferal symbionts would never have been discovered. Other support comes from the fact that we also isolated an *Amphidinium* sp. from several specimens of *Amphisorous hemprichii*. Trench and Winsor (1987) found similar diversity in the flatworm, *Haplodiscus*.

Unlike the probable symbiont-bearing, highly successful, fusulinids, the Soriticea, which originated in the Upper Permian, survived late Paleozoic extinc-

tions. Approximately 155 MY later, during the Cenomanian (U. Cretaceous), their descendants evolved into families which flourished throughout the Cenozoic and are still abundant in modern tropical seas. Although some of the contemporary genera evolved as early as the Middle Eocene (50 MYBP, *Androsina*) and the Oligocene (40 MYBP, *Cyclorbiculina*) the majority of today's symbiont-bearing soritaceans (e.g. *Laevipeneroplis, Peneroplis, Archaias, Amphisorus, Sorites*, and *Marginopora*) arose in the Miocene (25 MYBP). Members of various soritacean families (Peneroplidae, Archaiasinidae, Soritidae, Alveolinidae) in contemporary tropical and semitropical seas are the hosts, respectively, for unicellular red, chlorophyte, dinoflagellate, and diatom endosymbionts. This symbiont diversity is in sharp contrast to many scleractinian corals which host only dinoflagellates (*Symbiodinium* spp. and others) in the same seas. Since most of today's scleractinian orders and families originated at various times in the Mesozoic (Wells 1956), and since many host dinoflagellates, it is reasonable to assume that the later evolving soritids acquire(d) their zooxanthellae from environmental pools contributed to by the corals in their habitat. The phenomenon of mass expulsion of zooxanthellae by corals is fairly common in nature. The question of acquisition is an intriguing one. Was it a one time event for each new foraminiferan species, or is it an ongoing process, as it seems to be in the diatom-bearing hosts?

We wonder if the gene, with which we worked, has the resolution needed to work at the specific level in the genus *Symbiodinium*. Other genetic domains (D1 and D8 of 24S rRNA) of dinoflagellates also seem to be useful in making inferences on the molecular phylogeny of these flagellates (Lenaers et al. 1991). No doubt, other genes, potentially useful for fine scale evolutionary studies, remain to be detected. For now we draw our conclusions from the only broad data base for the genus which is presently available.

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Comparative Studies of Experimental Giardiosis* in Mongolian Gerbils. I. Infections Induced with Different *Giardia* Isolates from Human

Anna C. MAJEWSKA

Department of Biology and Medical Parasitology, K. Marcinkowski University of Medical Sciences, Poznań, Poland

Summary. The aim of the studies was to compare and to correlate the pattern of experimental giardiosis (incubation and infection period, time of spontaneous elimination of infection, cyst output) induced in Mongolian gerbils with different *Giardia* of *duodenalis* morphological type (from patients with asymptomatic or symptomatic giardiosis) isolated in one geographic region, as well as the infectivity of isolates and the susceptibility of gerbils to reinfection with definite characteristics of *Giardia* isolates. Mongolian gerbils were susceptible to infection with all human *Giardia* isolates investigated. However, the infectivity of those isolates, the pattern of infections, as well as the susceptibility of gerbils to reinfection, differed markedly. The incubation period of primary infections was similar in all but one experiments but the time of spontaneous elimination of infection differed. In general, an intermittent cysts output was observed. The pattern of infection induced with trophozoites was similar to the infections induced with cysts of each *Giardia* isolate. The results of experimental infection of gerbils with *Giardia* isolates from patients with different clinical manifestations from one geographic region showed two patterns of giardiosis. The first one concerned *Giardia* isolates from patients with symptomatic giardiosis. This pattern was related to a low infectivity of the parasites and a short period of infection - shorter than one month. The second pattern, which involved *Giardia* isolated from patient with asymptomatic giardiosis, was connected with the high infectivity of the isolate and the long-term primary infection. The gerbils previously infected with *Giardia* from patients with symptomatic giardiosis were resistant to reinfection with either homologous or heterologous *Giardia* isolates. On the other hand, the animals previously infected with *Giardia* from man with asymptomatic giardiosis were susceptible, although not all of them, to reinfection. In that study atypical cysts in feces were observed sporadically in all experiments during the entire period of infections. It seems that the occurrence of atypical cysts in gerbils' feces depends rather on environmental conditions in experimental host than on the dying out of the parasite population. The general conclusion of this study is that the populations of *Giardia* (isolates) differ markedly in several properties which do not correlate, and the only feature which discriminates the populations is the ability to induce the symptoms and the pattern of infection.

Key words. *Giardia*, giardiosis, experimental infection, reinfection, Mongolian gerbil (*Meriones unguiculatus*).

INTRODUCTION

Human giardiosis caused by *Giardia intestinalis* (syn. *G. duodenalis, G. lamblia*) is one of the most common intestinal parasitoses in developed and developing countries. The pattern of this infection is variable. Clinical manifestations of giardiosis ranged fromasymptomatic to symptomatic cases with varying intensity and level of symptoms. The duration of human giardiosis extends from very short to long-term periods. Elimination of parasites may be spontaneous but chronic infections, despite a therapy, have also been observed, yet the reasons for such a range of clinical manifestations are unknown.

*According to Standardized Nomenclature of Parasitic Diseases (SNOPAD) made during ICOPA, Paris, 1990

Address for correspondence: A.C. Majewska, Department of Biology and Medical Parasitology, Karol Marcinkowski University of Medical Sciences, ul. Fredry 10, PL- 61-701 Poznań, Poland; Fax: (0-61) 52-71-92

The pattern of giardiosis depends on different factors but there is convincing evidence that the character of *Giardia* populations may influence the pattern of infection. Faubert et al. (1983), has reported that the pattern of cyst release in experimentally infected gerbils is characteristic of the *Giardia* species. Moreover, experiments in gerbils found different infectivity and virulence of human *Giardia* isolates (Aggarwal and Nash 1987, Visvesvara et al. 1988, Abaza et al. 1991). Also, studies that used molecular and immunological techniques demonstrated that *Giardia* human isolates were heterogeneous (De Jonckheere et al. 1989, Homan et al. 1992,Nash and Mowatt 1992 Isaac-Renton et al. 1993,).

The aim of the present studies was threefold: to compare the pattern of experimental giardiosis induced in Mongolian gerbils with different human *Giardia* isolates and to correlate the pattern of infection as well as the infectivity of isolates, and to determine the susceptibility of gerbils to reinfection with definite characteristics of *Giardia* isolates.

MATERIALS AND METHODS

Parasite isolates. For primary infection of gerbils, four axenic human *G. intestinalis* isolates, obtained from one geographic region were used: HP-63 from a patient with asymptomatic giardiosis, HP-10 and HP-34 from patients with clinical symptoms[1] (Kasprzak and Majewska 1985), and HP-109 from a patient with chronic giardiosis despite chemotherapy (Majewska, unpublished data). For reinfection studies, besides the above mentioned isolates, two animal *Giardia* isolates were used: LSLP-116 *Giardia* of *duodenalis* type from a lesser slow loris (Majewska and Kasprzak 1990) and *G. muris* from naturally infected gerbils.

Characteristics of *Giardia* isolates. The isolates were defined by the genomes (the isolates showed no significant differences although two strains, HP-10 and HP-63, showed a unique genetic pattern - De Jonckheere et al. 1989, Homan et al. 1992), dsRNA virus (found only in the trophozoites of HP-34 and HP-63 isolates - De Jonckheere and Gordts 1987), antigenicity (the isolates differed markedly - Kasprzak et al. 1987), the *in vitro* sensitivity to ornidazole and metronidazole (the *isolates* differed noticeably from sensitive to resistant - Kasprzak et al. 1984, Majewska et al. 1991), morphometry (analysis of axenic cultured trophozoites showed two different groups - Majewska et al. 1993) and the activity of surface membrane-associated lectin (the isolates could be divided into two groups and the lectin induced agglutination showed little evidence of representative carbohydrate moieties on the surface of trophozoites - Majewska and Kasprzak unpublished data).

Preparation and administration of inocula. Trophozoite inocula. The inocula were prepared from 3-day-old cultures maintained in BI-S-33 medium (Keister's medium, 1983 modified by Meloni and Thompson in 1987). The preparation of inocula was described elsewhere (Belosevic et al. 1983). The volume of the inocula was 0.1 ml, and the number of trophozoites was $2x10^5$.

Cyst inocula. The cysts were purified by a slightly modified procedure of Bingham et al. (1979) from fresh feces of gerbils experimentally infected with trophozoites. The number of viable cysts in the inoculum was $1x10^2$ or $1x10^5$.

The slightly anaesthesised gerbils were inoculated with trophozoites or cysts intragastrically.

Experimental animals. Six- to eight-week-old Mongolian gerbils (*Meriones unguiculatus*) from *Giardia*-free colonies were used in all experiments. The animals were maintained individually in cages with separate wire floors. Strict hygienic measures to prevent accidental infection were performed. Food, water, and bedding materials were autoclaved and clean cages were additionally disinfected prior to use. Ten days prior to the infection the animals were treated with Metronidazole (Polfa) - 10 mg per gerbil; nine daily stool examinations showed the animals to be free from intestinal parasites. Uninoculated and untreated gerbils maintained in the same room throughout the experiments as a control remained free from intestinal parasites during the entire period of the studies. The animals were weighed prior to and after the experiment.

Experimental design. Primary experimental infection. In each experiment one group of 7-8 animals was inoculated with trophozoites, and two groups of 5 gerbils were inoculated with two different inocula of cysts.

Reinfection. The gerbils primarily infected with trophozoites were reinoculated with $5x10^4$ cysts of homologous *Giardia* isolate on the 21st day after resolution of primary infection. The animals primarily infected with $1x10^5$ of cysts of HP-10, HP-34, HP-63 or HP-109 *Giardia* isolates were reinoculated on the 21st day after elimination of the primary infection with $5x10^4$ cysts of heterologous *Giardia* isolates (*G. muris*, HP-63, HP-34 and LSLP-116 respectively).

Quantitative examination of feces for cysts. The pattern of cyst release during primary infection and reinfection was monitored at 24h intervals until day 20th after spontaneous elimination of *Giardia*. Cysts were purified and concentrated by the sucrose gradient centrifugation method (Bingham et al. 1979). The total number of cysts released by individual gerbils in a 2h fecal collection period was counted daily in a hematocytometer (2 measures) and calculated for 1 g of feces. The average number and the standard error of excreted cysts were calculated for each group of animals in each experiment.

Autopsy examinations. Gerbils not used for reinfection, and those in which the reinfection ceased, as well as those which did not undergo reinfection were killed with ether overdose (after 20 consecutive days of *Giardia* cysts absence in feces). Intestinal smears were examined for *Giardia* trophozoites.

RESULTS

Mongolian gerbils were susceptible to infection with all four human *Giardia* isolates investigated. However, the infectivity of these isolates, the pattern of infections, as well as the susceptibility of gerbils to reinfection, differed markedly.

[1]The clinical picture in patients infected with *G. intestinalis* (symptomatic *vs* asymptomatic giardiosis) was determined in the course of observation conducted in the Clinic for Parasitic and Tropical Diseases, University of Medical Sciences, Poznań, Poland.

Infectivity of isolates. The HP-63 isolate from a patient with asymptomatic giardiosis was most infective; all animals inoculated with cysts and all but one inoculated with trophozoites became infected (Table 1). The isolates from patients with symptomatic giardiosis were less infective to gerbils (Table 1). The inoculation of gerbils with *Giardia* trophozoites resulted in the infection of small numbers of animals. However, the inoculation of a large number of cysts resulted in the infection of all gerbils. The HP-109 isolate from a patient with

Table 1

Results of experimental infections of Mongolian gerbils inoculated with different human *Giardia* isolates

Isolate	Inoculum of trophozoites (T) or cysts (C)	No. of animals inoculated/ infected	Incubation period (d.p.i.)	Elimination of infection (d.p.i.)	Maximal excretion rates of cysts ($\bar{x}10^3$) released in feces (x±SE)	
					during 2h	per 1g
HP-10	200000 T	7/3	6-7	23-28	117.2± 49.3	1167.4±443.9
	100000 C	5/5	6-8	27-32	178.8± 60.7	5344.1±1788.3
	100 C	5/0				
HP-34	200000 T	8/1	7	25	292.9 ±82.3	4184.9 ±1175.6
	100000 C	5/5	5-7	23-27	126.5±19.0	1733.6±364.6
	100 C	5/5	6-11	12-28	229.5±52.3	1403.6±503.3
HP-63	200000 T	8/7	6-13	9-85	100.0±38.2	910.5±335.7
	100000 C	5/5	4-7	21-43	232.8±114.4	4058.8±1788.8
	100 C	5/5	5-7	33-34	541.4±157.8	5360.2±1472.6
HP-109	200000 T	7/2	6-7	16-22	750.1±618.7	7501.0±6187.6
	100000 C	5/1	11	20	468.7±.0.0	2929.0±0.0
	100 C	5/0				

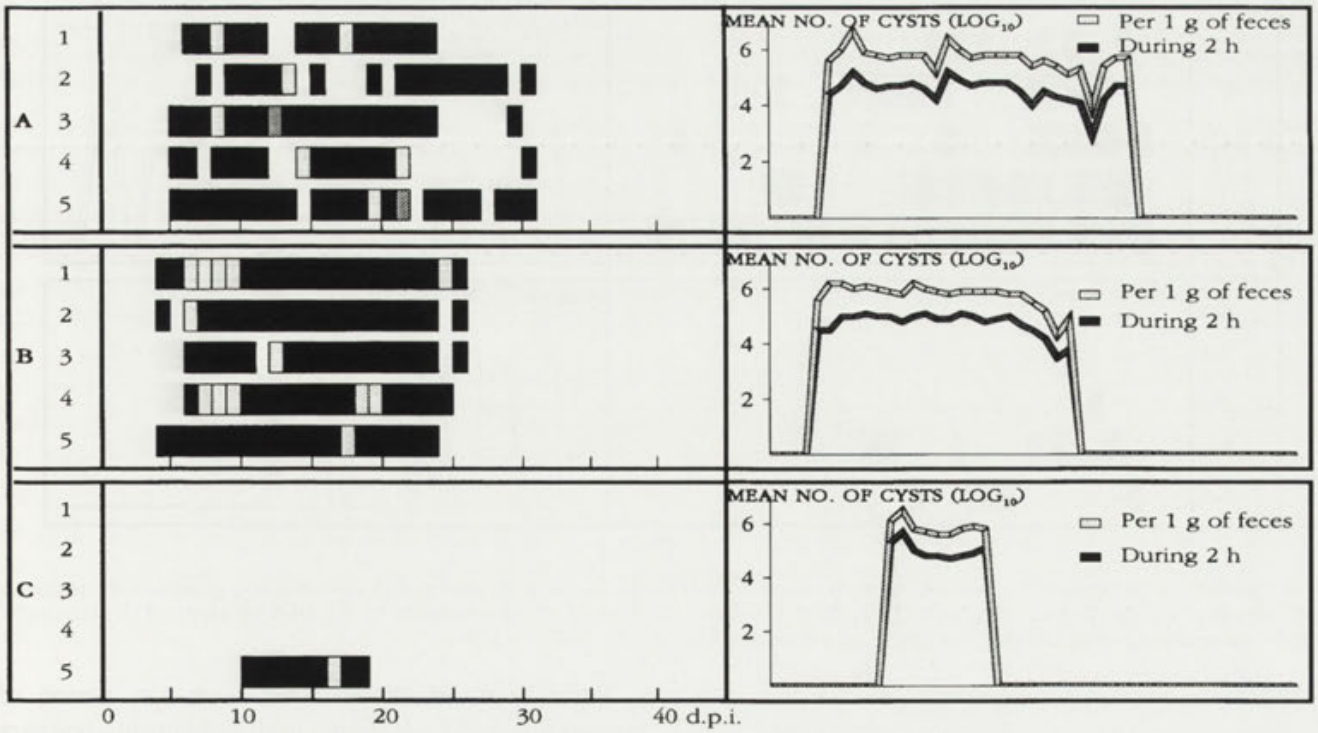

Fig. 1. Patterns of experimental infections in gerbils inoculated with *Giardia* isolates from patients with symptomatic giardiosis. A - pattern of cysts excretion by 5 gerbils inoculated with 10^5 cysts of HP-10 isolate; B - pattern of cysts excretion by 5 gerbils inoculated with 10^5 cysts of HP-34 isolate; C - pattern of cysts excretion by 5 gerbils inoculated with 10^5 cysts of HP-109 isolate; ■ - cysts in formed feces; ▒ -cysts and diahorrea; □ - atypical cysts in feces

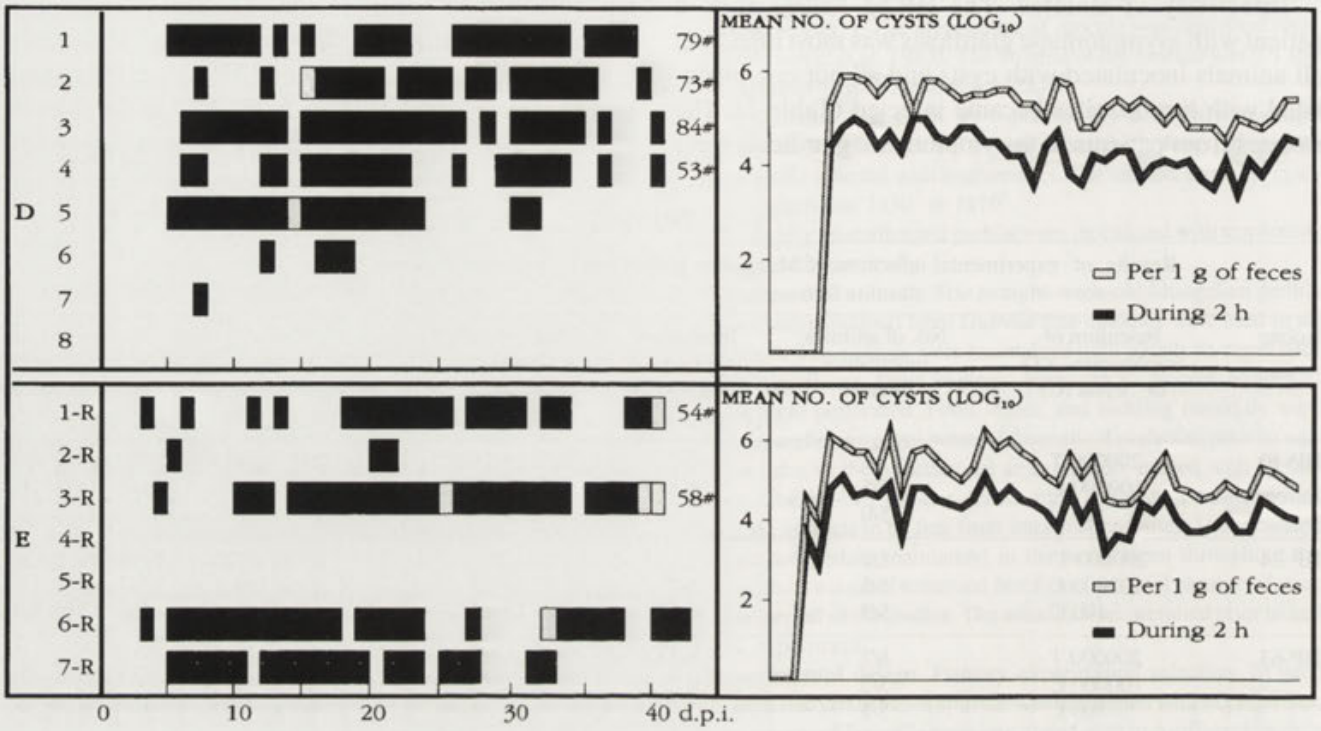

Fig 2. Patterns of experimental infections in gerbils inoculated with *Giardia* isolate from patient with asymptomatic giardiosis. D - pattern of cysts excretion by 8 gerbils inoculated with $2x10^5$ trophozoites HP-63; E - pattern of cysts excretion by 7 gerbils reinoculated (R) with $5x10^4$ cysts of homologous isolate; # - the last day of cysts release; for other explanations see Fig. 1

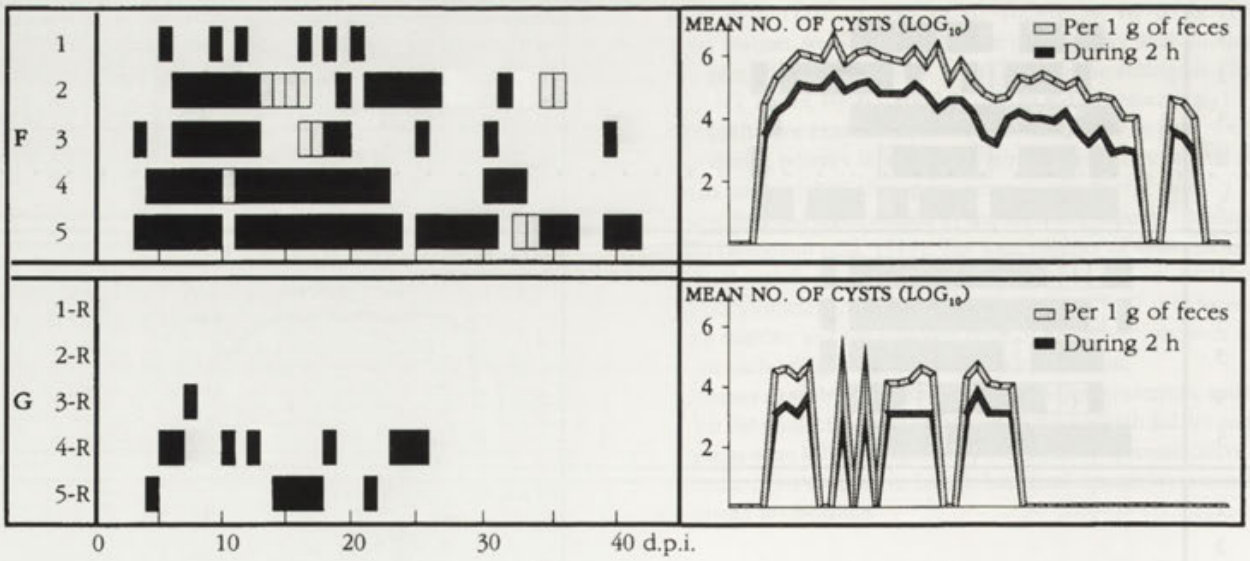

Fig 3. Patterns of experimental infections in gerbils inoculated with *Giardia* isolate from patient with asymptomatic giardiosis. F - pattern of cysts excretion by 5 gerbils inoculated with 10^5 cysts HP-63 isolate; G - pattern of cysts excretion by 5 gerbils reinoculated (R) with $5x10^4$ cysts of heterologous isolate (HP-34); for other explanations see Fig. 1

symptomatic chronic giardiosis was less infective; even the inoculation of a large number of cysts resulted in the infection of only one animal. The inoculation of low number of cysts of HP-10 and HP-109 isolates did not induce the infection.

Pattern of infections. The incubation period of primary infections was similar in all experiments, however in some cases, e. g. inoculation of trophozoites of HP-63 isolate or of a large number of HP-109 isolate cysts, the prepatent period was longer (Table 1; Figs. 1, 2).

The time of spontaneous elimination of infection - in case of *Giardia* isolates - differed markedly (Table 1, Figs. 1-3). In animals infected with isolates from humans with symptomatic giardiosis short periods of infection were observed. On the other hand inoculations with isolate from man with asymptomatic giardiosis resulted in long-term infections of the gerbils.

The pattern of infection induced with trophozoites was similar to the infections induced with cysts of each *Giardia* isolate. In general, an intermittent cysts output was observed, and the infection induced with HP-109 isolate was an exception. The maximum excretion rates of cysts in feces as well as the term of cyst output peak differed distinctly and also depended on an animal. In all experiments gerbils sporadically released atypical cysts in feces (Figs. 1, 3). The atypical cysts were sausage-shaped or triangle-shaped and stained blue in the iodine mount. In animals infected with HP-10 isolate diarrhoea appeared occasionally.

Reinfection. The gerbils previously infected with *Giardia* from patients with symptomatic giardiosis were resistant to reinfection with either homologous or heterologous *Giardia* isolates. The animals primarily infected with HP-10 *G. intestinalis* isolate were resistant even to reinoculation with *G. muris* which belongs to another morphological group typical of rodents. On the other hand, the animals previously infected with *Giardia* from man with asymptomatic giardiosis were susceptible, although not all of them, to reinfection. The patterns of primary infections and reinfections with homologous and heterologous *Giardia* isolates are shown in Figs. 2 and 3. Five out of seven HP-63-challenged gerbils and three out of five HP-34-challenged gerbils became infected. It is interesting to note that the resistance of animals to heterologous challenge (HP-34) was greater than to homologous challenge (HP-63). The number of cyst output and the period of reinfection induced with homologous *Giardia* isolate were similar to primary infection (Fig. 2). However, the period of reinfection induced with heterologous isolate was shorter and we observed a decreased number of cysts in feces in comparison with the primary infection (Fig. 3).

No *Giardia* trophozoites were detected in the small intestine of gerbils at necropsy.

DISCUSSION

Differences have been observed in the pattern of experimental primary infection induced with human *Giardia* isolates derived from different geographic regions (Belosevic et al. 1983, Aggarwal and Nash 1987, Abaza et al. 1991). Although these studies yielded valuable information they contributed little to an understanding of the epidemiology of giardiosis. The genetic diversity of human *Giardia* isolates varied from one geographic region to another (Thompson and Meloni 1993). Therefore, from the epidemiological and medical point of view, more useful are studies which identify and characterize different *Giardia* isolates originating from the same geographic area. In this study biological properties of different human *Giardia* isolates obtained from the same geographic region were compared *in vivo* and the isolates with well defined characteristics were used.

The results of experimental infection and reinfection of gerbils with *Giardia* isolates from patients with different clinical manifestations from one geographic region showed two patterns of giardiosis. The first one concerned *Giardia* isolates from patients with symptomatic giardiosis, including the isolate from a man with chronic infection. This pattern was related to a low infectivity of the parasites and a short period of infection - shorter than one month. The second pattern, which involved *Giardia* isolated from a patient with asymptomatic giardiosis, was associated with the high infectivity of the isolate and the long-term primary infection.

Epidemiological and experimental observations indicated that a primary giardiosis induced either partial or complete resistance of the host to reinfection (Moore et al. 1969, Belosevic et al. 1983, Faubert et al. 1983, Aggarwal and Nash 1987). The results of the present study revealed that different human *Giardia* isolates induced various host responses to repeated challenge of infection. This study showed that only the primary infection with human isolates from symptomatic giardiosis did protect the gerbils from reinfection both with homologous and heterologous *Giardia* isolates. However, most of the animals previously infected with an isolate from asymptomatic giardiosis were susceptible to reinfection with homologous as well as with heterologous *Giardia* isolates. As a rule, the reinfection of susceptible gerbils with homologous *Giardia* isolates was shorter than the primary infection (Aggarwal and Nash 1987). On the contrary, in the present study the pattern of secondary giardiosis with homologous HP-63 isolate from man with asymptomatic giardiosis was similar to the primary infection. It seems that isolate did not induce immunity. Earlier studies indi-

cated that *Giardia* changed its surface antigen(s) during infection (Aggarwal and Nash 1988, Nash et al. 1990b) and that the rate of the appearance of antigenic variation as well as the size of the variant antigen were different in various *Giardia* isolates (Nash et al. 1990a). It may be presumed that the surface antigens of HP-63 isolate trophozoites underwent such variation and, therefore, it is possible that the duration of the infection was long-term and did not induce any immunity. However, it is interesting that the reinfection caused with a heterologous *Giardia* isolate was very short and the numbers of cysts excreted after reinfection were lower than these observed in primary infection.

Some earlier studies showed some correlation between the definite characteristics of human *Giardia* isolates and their infectivity to gerbils, pattern of infection or the ability to induce a resistance to reinfections (Belosevic et al. 1983, Aggarwal and Nash 1987, Abaza et al. 1991). In contrast to those earlier studies, some other studies did not show such correlation (Visvesvara et al. 1988). Also in our study the attempt to prove the correlation between the infectivity, the pattern of infection in gerbils, and the susceptibility to reinfection with one or more different characteristics of human *Giardia* isolates failed. The most unexpected results of the experimental infection concerned the HP-10 isolate. Despite the unique schizodeme of this isolate (Homan et al., 1992), the general pattern of experimental infection was similar to the pattern of infection with other human isolates from symptomatic cases. Thus, the present study, like the study of Nash et al. (1987) proved that most probably there exist other factors which influence the pattern of infection with *Giardia*.

The pattern of infection in the original host differed from the experimental pattern in Mongolian gerbils. Nash et al. (1987) observed such a different pattern of infections even in individual volunteers infected with the same isolate.

In many studies of experimental giardiosis different number of cysts for inoculation as well as different methods of estimating the number of cysts excreted in feces were used. Therefore the comparison of the results of this study with the results of other studies is difficult. However, Rendtorff (1954) showed that 100 cysts of human *Giardia* were sufficient to induce the infection of volunteers. In this study the number of cysts of two *Giardia* isolates from patients with symptomatic giardiosis did not induce the infection in any of the inoculated gerbils.

In laboratory diagnosis of giardiosis a microscopic examination of feces for cysts detection is commonly used. The examination of feces seems to be simple and easy. However, the occurrence of atypical cysts sometimes makes the diagnosis difficult (Kasprzak and Mazur 1973). In that study atypical cysts in feces were observed sporadically in all experiments during the entire period of infections. Therefore, the suggestion that such cysts are excreted in the terminal phase of infection (Iwańczuk 1961) is not in accordance with the observations of the present study. One can accept that the occurrence of atypical cysts depends rather on environmental conditions in experimental host than on the dying out of the parasite population.

The general conclusion of this study is that the populations of *Giardia* (isolates) from the same geographic area differ markedly in several properties which do not correlate, and the only feature which discriminates *Giardia* populations is the ability to induce symptoms and pattern of infection.

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Comparative Studies of Experimental Giardiosis* in Mongolian Gerbils. II. Infections Induced with Different *Giardia* Isolates from Zoo Animals

Anna C. MAJEWSKA

Department of Biology and Medical Parasitology, Karol Marcinkowski University of Medical Sciences, Poznań, Poland

Summary. The aim of the studies was to compare the pattern of experimental giardiosis induced in Mongolian gerbils with different *Giardia* isolates from zoo animals (Primates and Rodentia) and to correlate this pattern of infection and the infectivity of isolates as well as the susceptibility of gerbils to reinfection with definite characteristics of *Giardia* isolates.

The Mongolian gerbils were susceptible to primary infection induced with all tested isolates, however, the infectivity of the isolates, pattern of experimental infections, and the resistance of animals to reinfection were different. The infectivity of *Giardia* isolated from a rodent was very high; all animals in all experiments became infected. On the contrary, the infectivity of both *Giardia* isolates from primates was low. The prepatent periods of the primary infections were short in all experiments with the exception of the experiment with *Giardia* trophozoites from a rodent. The experiments with *Giardia* isolates from different species of zoo animals showed two patterns of primary infection in gerbils. The first one concerned *Giardia* isolates from primates and was characterized by a continuous cysts excretion and rapid elimination of parasites. The second pattern which regarded *Giardia* isolate from a rodent, was characterized by intermittent cyst output and a long-term primary infection. The primary infection with *Giardia* isolates from primates induced in gerbils a resistance to challenge to different *Giardia* populations of the *duodenalis* group and to *G. muris*. On the other hand, in most of the gerbils infected primarily with a rodent *Giardia* isolate no resistance to reinfection with homologous or heterologous *Giardia* isolates was developed. In general, the reinfections were of shorter duration and the numbers of excreted cysts were lower. Atypical cysts observed earlier in feces of gerbils infected with human *Giardia* isolates were found only in feces of animals infected with *Giardia* from primates.

The patterns of infection with *Giardia* did not correlate with any observed characteristics of the parasite. However, the pattern of experimental infection, the susceptibility of gerbils to reinfection, and the infectivity of isolates from primates were comparable to these features of *Giardia* from symptomatic patients (Majewska 1995). On the other hand, the pattern of infection, the susceptibility to reinfection and the infectivity of the isolate from a rodent were similar to the characteristics of *Giardia* isolates from asymptomatic patient and from another rodent (Majewska 1994,1995).

Key words. *Giardia*, giardiosis, experimental infection, reinfection, Mongolian gerbil (*Meriones unguiculatus*), zoo animals.

INTRODUCTION

For a long time *Giardia* has been an object of many controversies, the main reason being the disputable taxonomic status of *Giardia* species. Among the investigators there are advocates of two extreme opinions: some authors believe that *Giardia* species are rigidly host specific and on this basis over 40 species were described (Hegner 1930, Kulda and Nohynkova 1978), and the others accepted Filice's (1952) opinion on the existence of three morphologically distinct groups (or species): *G. agilis*, *G. muris* and *G. duodenalis* (*G. intestinalis*). Most disagreements concern the *Giardia duodenalis* group, the parasites occurring in mammals including human, birds, and reptiles. Most probab-

*According to Standardized Nomenclature of Parasitic Diseases (SNOPAD) made during ICOPA, Paris, 1990

Address for correspondence: A.C. Majewska, Department of Biology and Medical Parasitology, Karol Marcinkowski University of Medical Sciences, ul. Fredry 10, PL- 61-701 Poznań, Poland; Fax: (0-61) 52-71-92

ly the birds harbour distinct *Giardia* species. Recently, Erlandsen and Bemrick (1987) described a new species *G. psittaci* from budgerigars, and showed in electrophoretic analysis that karyotypes of *G. muris* as well as of *G. duodenalis* from different host species and *G. ardeae* differed markedly from one another (Campbell et al. 1990). The wide occurrence of morphologically indistinguishable *G. duodenalis* populations in different host species complicates the epidemiology of this medically important protozoan parasite. Besides, there are several modes of transmission: a direct fecal-oral route in conditions of poor personal hygiene or between sexual partners (Laughon et al. 1988, Novotny et al 1990), and an indirect route usually after ingestion of contaminated water or food (Osterholm et al. 1981,Craun 1986). Recently, animals have also been indicated as possible sources of human giardiosis (Kasprzak and Pawłowski 1989, Meloni et al. 1993). Therefore, in the past decade the enhancement of attempts to resolve the key problem whether giardiosis is an anthropozoonosis was observed.

Despite numerous cross-transmission experiments, considerable variability of the results has been observed. Also studies using modern molecular techniques showed either great heterogeneity or some similarities among human and animal *Giardia* isolates (De Jonckheere et al. 1990, Homan et al. 1992, van Belkum et al. 1993). However, the differences between human and animals *Giardia* populations do not determine that cross transmission is impossible (Boreham et al. 1990).

Although the most reliable and conclusive observation would be the experimental infection of humans with *Giardia* from animals, such experiments are still very few (Davis and Hibler 1979, Majewska 1994). Thus, the other method is the comparison of the pattern of infections with *Giardia* from humans and animals in experimental animal models.

The aim of these studies was to compare the pattern of experimental giardiosis induced in Mongolian gerbils with different *Giardia* isolates from zoo animals and to correlate this pattern of infection and the infectivity of isolates as well as the susceptibility of gerbils to reinfection with definite characteristics of *Giardia* isolates.

MATERIALS AND METHODS

Parasite isolates. For primary infection of gerbils three axenic *Giardia* isolates of the *duodenalis* morphological group from animals of The Zoological Garden in Poznań were used: SLP-111 from a slow loris (*Nycticebus coucang*, Primates), SP-115 from siamang (*Symphalangus syndactylus*, Primates), and CP-117 from cuis (*Galea musteloides*, Rodentia) (Majewska and Kasprzak 1990). For reinfection studies apart from the above quoted isolates, two further *Giardia* isolates were used; GGPRP-114 - the axenic isolate of *duodenalis* type from a Gambian giant pouched rat (*Cricetomys gambianus*, Rodentia) (Majewska and Kasprzak 1990), and the *G. muris* isolate from a naturally infected gerbil. *G. muris* was determined on the ground of the morphology of the trophozoites obtained from the gerbils' intestine.

Characteristics of *Giardia* of *duodenalis* type isolates. The isolates were defined by the following characteristics: (1) RFLP analysis, isoenzyme patterns, total protein patterns, and the hybridization results with specific molecular probe pGI7 showed that all the isolates but SLP-111 differed from 15 Polish human isolates (De Jonckheere et al. 1990); (2) the dsRNA virus was found only in the trophozoites of SLP-111 isolate (De Jonckheere et al. 1990); (3) the serological survey showed that the isolates did not react with rabbit antiserum against *G. intestinalis* from man (Kasprzak et al. 1991); (4) the *in vitro* study revealed different sensitivity of the isolates to ornidazole and metronidazole (Majewska et al. 1991); (5) the morphometric analysis of the axenic cultured trophozoites showed a similarity between SLP-111 and SP-115 *Giardia* isolates, however, the CP-117 constituted a separate group (Majewska et al. 1993); (6) a mixed erythrocyte-trophozoite test showed that these isolates differed as concerned the activity of a surface membrane-associated lectin(s) from the human isolates (unpublished data); (7) the lectin induced agglutination showed - similarly as in the human *Giardia* isolates - little of representative carbohydrate moieties on the surface of trophozoites of all isolates (unpublished data).

Preparation and administration of inocula. The preparation of trophozoite and cysts inocula was described elsewhere (Belosevic et al. 1983, Majewska 1995). The volume of the inocula was 0.1 ml; the number of trophozoites was $2x10^5$, and the number of viable cysts was $1x10^2$ or $1x10^5$. The slightly anaesthesised gerbils were inoculated with trophozoites or cysts intragastrically.

Experimental animals. Six- to eight-week-old Mongolian gerbils (*Meriones unguiculatus*) from the same *Giardia*-free colonies were used in all experiments. The conditions of animal maintenance were the same as in earlier experiments (Majewska 1995).

Experimental design. Primary experimental infection. In each experiment one group of 7-8 animals was inoculated with trophozoites and two groups of 5 gerbils were inoculated with two different inocula of cysts.

Reinfection. The gerbils primarily infected with trophozoites were reinoculated with $5x10^4$ cysts of the homologous *Giardia* isolate on the 21st day after elimination of primary infection. The animals primarily infected with $1x10^5$ of cysts of SLP-111, SP-115, or CP-117 *Giardia* isolates were reinoculated on the 21st day after elimination of primary infection with $5x10^4$ cysts of a heterologous *Giardia* isolate (*G. muris*, SP-115, and GGPRP-114 respectively).

Quantitative examination of feces for cysts. The monitoring of the cyst release pattern during primary infection and reinfection was described elsewhere (Majewska 1995).

Autopsy examination. After the observations had been completed, the gerbils were killed with overdoses of ether and then their small intestines were examined. Necropsy was performed after 20 consecutive negative coproscopic examinations.

Uninoculated and untreated gerbils were maintained in the same room throughout the experiments as a control.

RESULTS

Table 1 summarizes the results of all primary experimental infections in gerbils. The Mongolian gerbils were susceptible to primary infection induced with all tested isolates, however, the infectivity of the isolates, pattern of experimental infections, and the resistance of animals to reinfection were different.

The infectivity of CP-117 *Giardia* isolate from cuis was very high; all animals in all experiments became infected. In contrast to that, the infectivity of both *Giardia* isolates from primates was low, particularly after inoculation of the Mongolian gerbils with trophozoites or small inoculum of cysts.

The prepatent periods of the primary infections were short in all experiments with the exception of the ex-

Table 1

Results of experimental infections of Mongolian gerbils inoculated with different zoo animals *Giardia* isolates

Isolate	Inoculum of trophozoites (T) or cysts (C)	No. of animals inoculated/ infected	Incubation peroid (d. p. i.)	Elimation of parasites (d.p.i)	Maximal excretion rates of cysts ($\bar{x}$ 10^3) released in faces (x±SE)	
					during 2 h	per 1 g
SLP-111	200000 T	7/3	4-7	25-29	259.1±152.9	1438.8±849.6
	100000 C	5/5	4-6	16-21	237.5±92.0	2566.2±618.3
	100 C	5/4	6-7	24-28	275.4±82.7	2797.1±586.4
SP-115	200000 T	7/2	4-7	31-33	371.1±195.4	2532.4±1266.8
	100000 C	5/5	4-6	20-26	191.4±57.3	1831.1±431.8
	100 C	5/2	6	26-28	287.5±27.4	1855.4±111.4
CP-117	200000 T	8/8	10-14	35-47	114.0±44.4	1640.9±592.2
	100000 C	5/5	4-5	33-49	127.0±9.4	598.0±29.5
	100 C	5/5	5-7	18-63	278.0±19.8	942.0±64.5

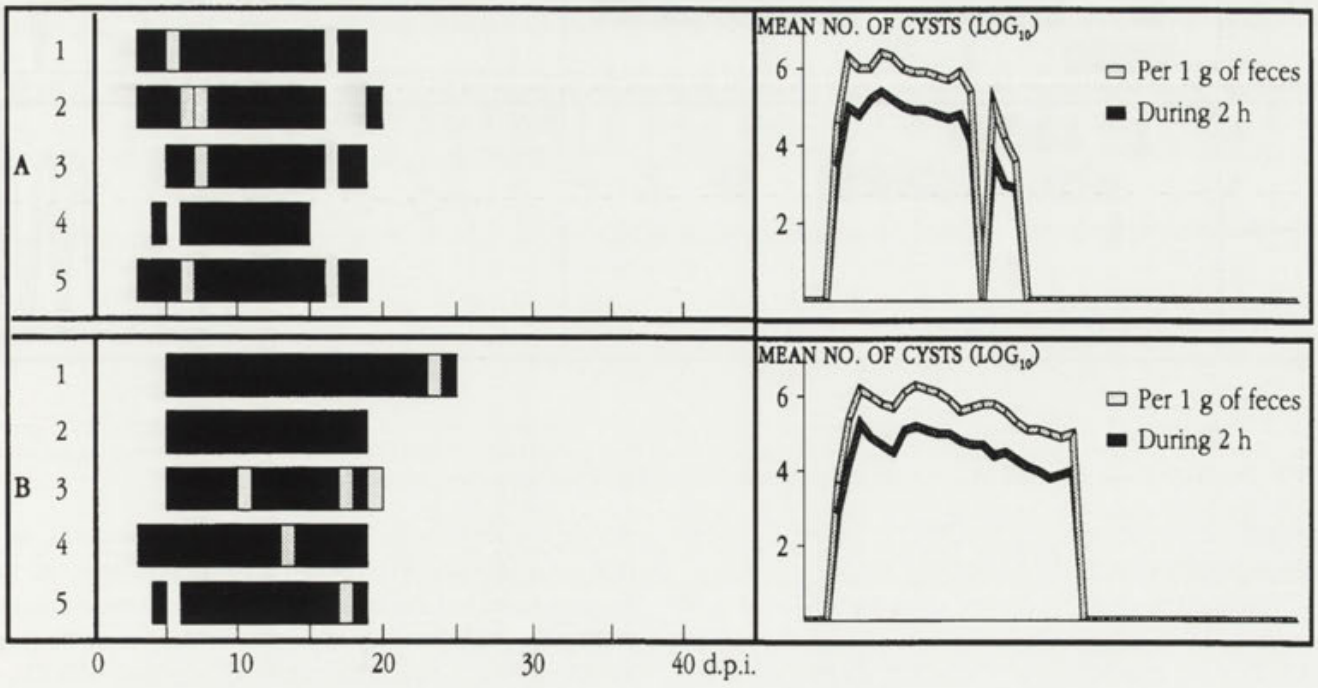

Fig. 1. Patterns of experimental infections in gerbils inoculated with *Giardia* isolate from primates. A - pattern of cysts excretion by 5 gerbils inoculated with 10^5 cysts of SLP-111 isolate; B - pattern of cysts excretion by 5 gerbils inoculated with 10^5 cysts of SP-115 isolate; ▮ - cysts in formed feces; □ - atypical cysts in feces

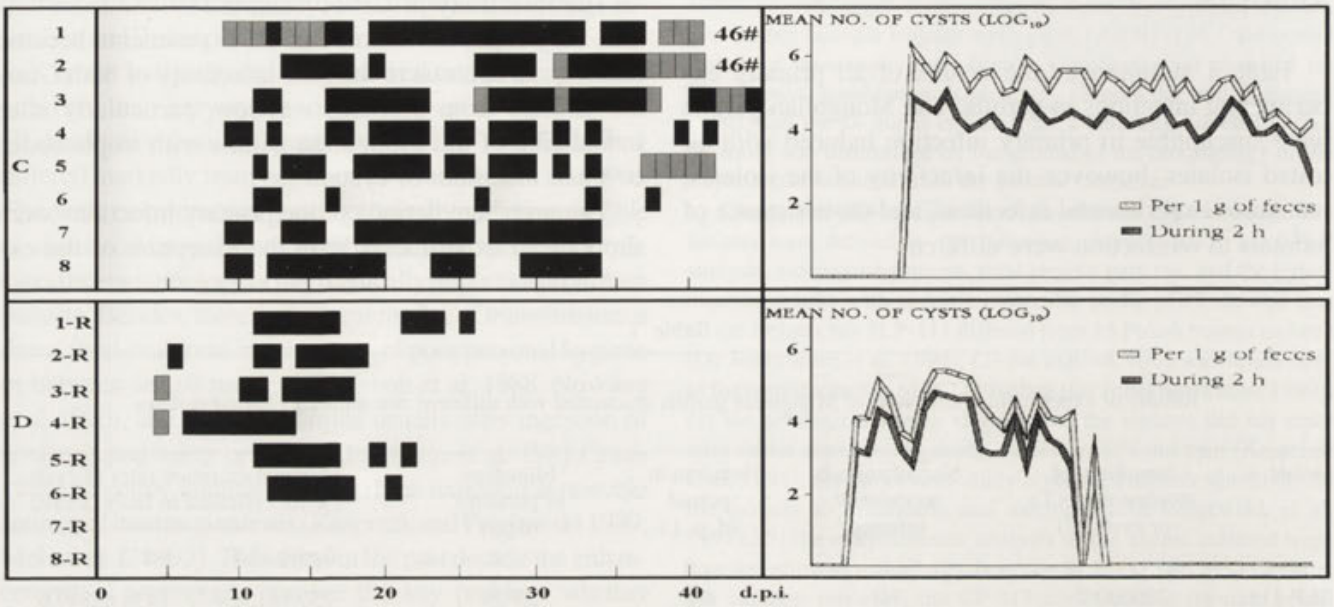

Fig. 2. Patterns of experimental infections in gerbils inoculated with *Giardia* isolate from cuis. C - pattern of cysts excretion by 8 gerbils inoculated with $2x10^5$ trophozoites CP-117; D - pattern of cysts excretion by 8 gerbils reinoculated (R) with $5x10^4$ cysts of homologous isolate; ▓ -cysts in loose feces; # - the last day of cysts release; for other explanations see Fig. 1

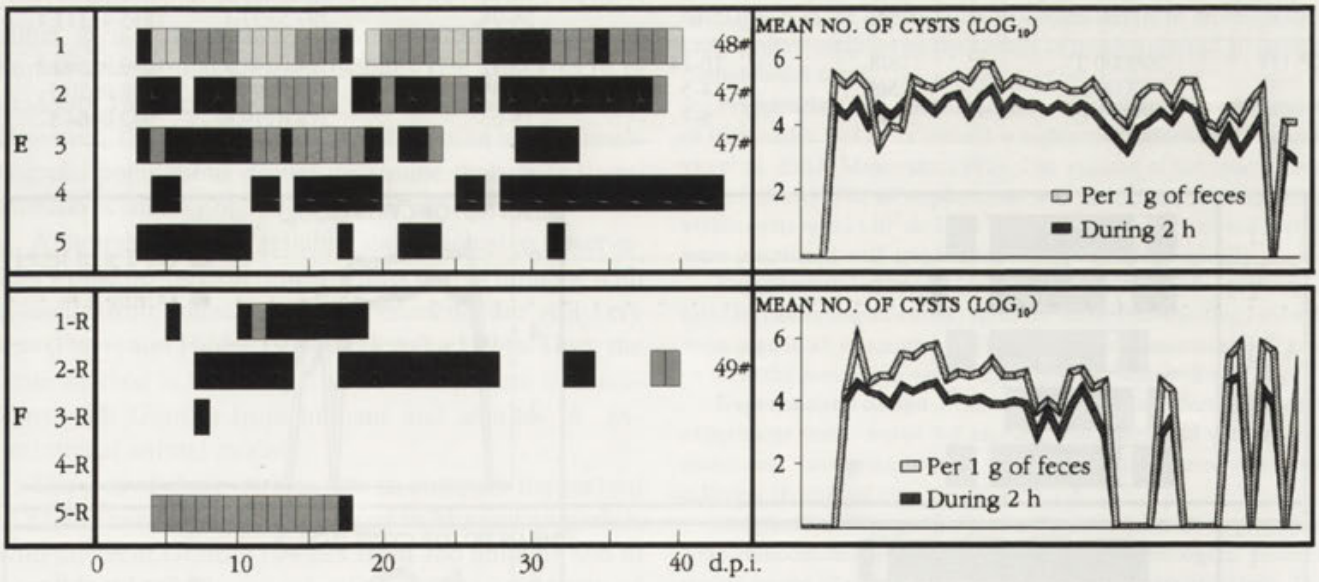

Fig. 3. Patterns of experimental infections in gerbils inoculated with *Giardia* isolate from cuis. E - pattern of cysts excretion by 5 gerbils inoculated with 10^5 cysts CP-117 isolate; F - pattern of cysts excretion by 5 gerbils reinoculated (R) with $5x10^4$ cysts of heterologous isolate (GGPRP-114); ▒ - cysts and diahorrea; # - the last day of cysts release; for other explanations see Fig. 1

perimental inoculation with CP-117 *Giardia* trophozoites from a cuis.

The pattern of the primary infection induced with *Giardia* isolated from a rodent differed from the pattern of infections caused by the isolates from primates. The gerbils infected with CP-117 *Giardia* isolate from a cuis excreted intermittently cysts in the feces during 5-7 weeks (Figs. 2, 3), while cyst excretion in the animals infected with *Giardia* isolates from primates was, as a rule, continuous. The isolates from primates, at the beginning and in the terminal phase of patent infection, released cysts intermittently in individual animals (Fig. 1). Moreover, a rapid resolution of infection with *Giardia* from primates was observed (Table 1). As a rule, the gerbils infected with *Giardia* from primates released greater numbers of cysts in feces (Table 1). Diarrhoea

and loose feces occurred only in some gerbils infected with *Giardia* from rodent (Figs. 2, 3). More often the diarrhoea and loose feces occurred in gerbils inoculated with a greater dose of cysts of CP-117 isolate. Solely in gerbils infected with *Giardia* isolates from primates a sporadic occurrence of atypical cysts in feces during the entire infection was observed (Fig. 1).

All gerbils previously infected with *Giardia* isolates from primates (SLP-111 and SP-115) were resistant to reinfection with either homologous or heterologous *Giardia* isolates. The animals primarily infected with SLP-111 isolate from a slow loris were resistant also to *G. muris,* a parasite typical for rodents and isolated from a spontaneously infected gerbil. On the contrary, most of the gerbils previously infected with the CP-117 isolate from a rodent (cuis) were susceptible to reinfection both with homologous and heterologous *Giardia* isolates (Figs. 2, 3). The pattern of reinfection induced with homologous *Giardia* isolate differed from the pattern of primary infections. Only six out of eight gerbils became infected. The incubation period of reinfection with homologous *Giardia* isolate ranged between 5 and 13 days, and the duration of reinfection was shorter than the duration of primary infection (Fig. 2). As a rule, the cyst output during reinfection was much lower than during the primary infection. The maximal mean number of cysts released during 2 h was 64 100 ± 18100 and in 1 g of feces was 276 300 ± 85 700. Diarrhoea was observed only in some gerbils at the beginning of the patent period of reinfection (Fig. 2). The resistance of animals reinoculated with heterologous GGPRP-114 *Giardia* isolate was lower than that of gerbils reinoculated with homologous isolate (CP-117) (Fig. 3). The incubation period of reinfection was a little longer than in the primary infection. Also the duration of reinfection in most animals was shorter than the duration of primary infection. In general, the reinfected gerbils excreted cysts intermittently, and the number of cysts at the beginning and in the late phase of reinfection was higher than in primary infection (Fig. 3). The maximal mean number of cysts released during 2 h was 43 000 ± 5000 and in 1 g of feces was 1 256 100 ± 279 900. In some gerbils diarrhoea or loose feces was observed.

After the observation of the pattern of infection or reinfection had been accomplished, the *post mortem* study showed no *Giardia* trophozoites in the small intestine.

Daily microscopic examination of feces of control animals during the entire period of the cross-transmission experiments showed that they were free from intestinal parasites. Also at *post mortem* examinations intestinal parasites were not found.

DISCUSSION

A recent experiment showed clearly that animal *Giardia* population may induce infection of man (Majewska 1994). Further studies would constitute a reliable direct evidence of *Giardia* cross-transmission from animal to man. In the present study, which supports and extends previous observations (Majewska 1995), differences in the infectivity of *Giardia* isolates from zoo animals were found. Whereas the isolate from a rodent induced the infection of all gerbils, both isolates from primates caused the infection only in a part of the animals. As concerns the differences in trophozoites infectivity they may suggest the existence of a diverse susceptibility of the trophozoites to the conditions of a gerbil stomach. The results of this study, as well as those of Wallis and Wallis (1986), showed that *Giardia* trophozoites from rodents were more infective than the trophozoites from other mammals. On the other hand, an earlier study proved that the trophozoites from an asymptomatic patient might also induce the infection in most of inoculated gerbils (Majewska 1995).

The experiments with *Giardia* isolated from different species of zoo animals showed two patterns of primary infection in gerbils. The first one concerns *Giardia* isolates from primates. That pattern was characterized by rapid elimination of parasites which was comparable to the period of infection caused by *Giardia* from symptomatic patients (Majewska 1995). The second pattern regarded *Giardia* isolated from a rodent and was characterized by a long-term primary infection, similar to the period of primary infection with *Giardia* isolates from an asymptomatic patient and from another rodent (Gambian giant pouched rat) (Majewska 1994, 1995).

The ability of the isolates tested in this study to induce the resistance of the gerbils to reinfection was also varied. The self-limited primary infection with *Giardia* isolates from primates induced in gerbils a resistance to challenge to different *Giardia* populations of *duodenalis* group and to *G. muris*. Such general resistance was observed in gerbils infected with *Giardia* from symptomatic patients (Majewska 1995). On the other hand, in most of the gerbils infected primarily with a rodent *Giardia* isolate no resistance to reinfection with homologous or heterologous *Giardia* isolates was developed. Similar susceptibility to reinfections was

observed in an earlier study on gerbils primarily infected with *Giardia* from asymptomatic patient (Majewska 1995). In general, the reinfections were of shorter period and the number of excreted cysts was lower.

In general, the infectivity of *Giardia* isolates from primates and the isolate from a rodent, the pattern of infection and the ability of the isolates to induce a resistance of gerbils to reinfection were very similar to the features of *Giardia* isolated respectively from symptomatic patients or from an asymptomatic person in the same geographic region. The patterns of infection with *Giardia* did not correlate with any observed characteristics of the parasite.

The present and previous studies (Majewska 1995) proved that different *Giardia* populations (isolates) independently of the primary host (source of isolation) may reveal similar infectivity potential, may cause the infection of similar pattern which induces corresponding resistance to reinfection. From the epidemiological point of view these data imply that the infectivity potential of different *Giardia* populations may be the same for different host species, and that in consequence there are possible cross-infections with *Giardia*. However, we may observe infections of different duration with variable output of cysts in feces, and different degree of resistance to reinfection. Thus, we may suppose that the challenge to *Giardia* may result either in an easy spread between hosts of the same or close related species or in a weak transmission of the parasite between wider range of unrelated species of animals and man.

Acknowledgments. This work was supported by grants from U. S. - Poland Maria Skłodowska-Curie Joint Fund II (MZ/HHS/91/76) and from the State Committee for Scientific Research, Warszawa, Poland (PB/1200/P2/92/02).

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Formation and Orientation of Skeletal Elements During Development of Oral Territory in a Ciliate, *Dileptus*

Krystyna GOLIŃSKA

Department of Cell Biology, Nencki Institute of Experimental Biology, Warszawa, Poland

Summary. Cytostomal field in *Dileptus* occupies a subapical position in the cell, at the base of a long anterior processus termed the proboscis. During stomatogenesis primordia for the cytostomal field appear on the ventral side of the cell, while on the left and right sides appear primordia for left and right margins of an extension of the cytostomal field (located along the ventral side of proboscis). Stomatogenesis is of a holotelokinetal type, i.e. oral primordia form all around the cell, within each row of locomotor cilia. Head-to-tail sealing of primordia results in one continuous row of oral ciliature. Different sectors of this row develop in different ways, giving rise to different oral structures. This is very unusual for a haptorid ciliate. Another peculiarity of stomatogenesis in *Dileptus* lies in the orientation of its ciliary components. Oral basal bodies, both paired and single, orientate with their posterior side (where oral fibre arise) towards the anterior cell pole, and retain this orientation throughout late stage of cell shaping. Oral fibres are at first directed anteriorly, later on the fibres on right and left cell sides curve ventrally, while fibres on ventral side still point anteriorly and gradually become centered at future cytostome. The change in orientation of oral fibres during stomatogenesis is interpreted as shifting of center of oral structure from apical to subapical position during stomatogenesis in *Dileptus*.

Key words. Ciliate, *Dileptus*, kinetid orientation, stomatogenesis.

INTRODUCTION

In most ciliates the oral kinetids (basal bodies with cilia and root fibres - terminology after Lynn 1988) are orientated towards the cytostome, while somatic kinetids are orientated towards the anterior and posterior cell poles. Somatic kinetids are usually orientated in such a way that they face the anterior cell pole with their anterior side. At this side a new basal body arises during formation of a new kinetid. Typical microtubular rootlets of somatic kinetid are: the transverse fibre anchored at left-anterior side of basal body, and the postciliary fibre anchored at right posterior side. Oral kinetids are orientated towards the cytostome. It is generally accepted that in ciliates two main groups can be distinguished: Rhabdophora with oral kinetids that face the cytostome with left-anterior side (with the transverse fibre), and two other subphyla with oral kinetids that face the cytostome with right-posterior side, i.e. with the postciliary fibre (Small and Lynn 1985). Such fundamental difference in general

Address for correspondence: K. Golińska, Department of Cell Biology, Nencki Institute of Experimental Biology, ul. Pasteura 3, PL-02-093 Warszawa, Poland; Fax: (4822) 22 53 42

organization of oral structure is the more amazing since it often concerns organisms otherwise very similar in organization.

Recently, however, it becomes more and more probable that the oral fibre of rhabdophoran ciliates may in reality represent rather a kind of postciliary fibre. Some rhabdophorans, when their stomatogenesis became better known, were transferred to another taxon (i.e. *Coleps*, see Huttenlauch and Bardele 1987). For *Homalozoon* (Leipe et al. 1992) authors present evidence that a new oral fibre first appears at the posterior side of a basal body, although they prefer to take it for transverse rather than for postciliary fibre. In *Dileptus*, the typical rhabdophoran ciliate, a new basal body in oral monokinetids assemble on the side opposite to oral fibre attachment. On this basis the oral fibre was considered to represent a postciliary rather than a transverse fibre (Golińska 1991). In this study early stages of formation of oral kinetids in *Dileptus* were investigated, to find out the nucleation site of oral fibre when it assembles within the somatic kinety. Special attention was also paid to changes in orientation of kinetids during stomatogenesis. The stomatogenesis in *Dileptus* is of a holotelokinetal type (Hiller 1992), and new oral kinetids are at first orientated towards the anterior cell pole. In mature cell the cytostomal area is subapical, and during advanced stages of stomatogenesis the gradual shifting in orientation of oral kinetids was observed, towards the ventral side. Thus in *Dileptus* the separation of oral orientation center from somatic one can be visualized through changes in orientation of oral kinetids during stomatogenesis.

MATERIALS AND METHODS

Dileptus margaritifer (formerly known as *Dileptus anser*) was used in this study. Stock cultures were kept in glass vessels and fed twice a week with *Colpidium* sp. Further details of culture methods are published elsewhere (Golińska and Jerka-Dziadosz 1973). In this study clones belonging to two mating types (out of three mating types known for this species - Afon'kin and Yudin 1986) were used. *Dileptus* is a ciliate that undergoes a pregamic cell division (see review by Raikov 1972), and mixing of complementary mating types was used to induce relatively synchronous cell division. Sequence of stages in cell division was found for light microscopy on preparations made 2 and 3 h after mixing the clones, for electron microscopy preparations were made 1.5, 2 and 3 h after mixing the clones. Cells enter division either before or after pair formation, and whole spectrum of divisional stages was found both on preparations made 2 and 3 h after mixing the clones.

Silver proteinate (protargol) produced by Merck was used for light microscopic preparations, according to slightly modified procedure after Dragesco (1962). Electron microscopic procedures were standard.

RESULTS

The fine structure of *Dileptus* mouthparts has been described previously (Grain and Golińska 1969; Golińska 1972, 1978, 1988), and it is only briefly reminded here. A circular cytostomal field surrounds the cytostome (Fig. 1A), and its extension occupies the ventral part of anterior cell processus, termed the proboscis. Cytostomal field together with its extension (named the ventral band) is contoured by single continuous row of oral kinetids. Composition of oral kinetids is different in different regions of this continuous row (Fig. 1B). Three types of oral kinetids can be distinguished, namely those on right margin of ventral band, on left margin of ventral band, and on margin of cytostomal field. Oral kinetids from these regions differ not only in their fibrous equipment but also, as will be shown, in ways of development of their primordia. Both the structure of oral kinetids and way of their development will be described separately for three regions of oral structure.

Oral kinetids at right margin of ventral band

Right portion of ventral band is the place where toxicysts are docked to the cell membrane (Fig. 1B).

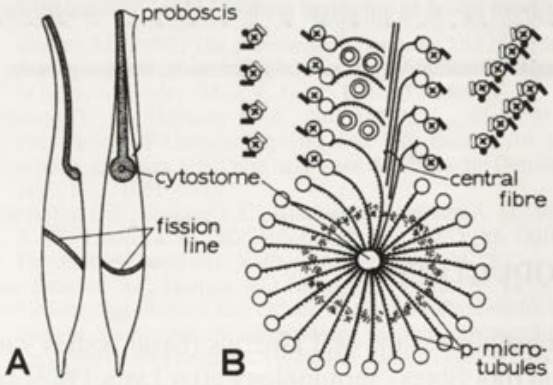

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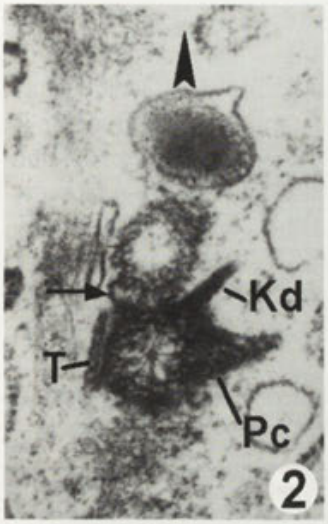
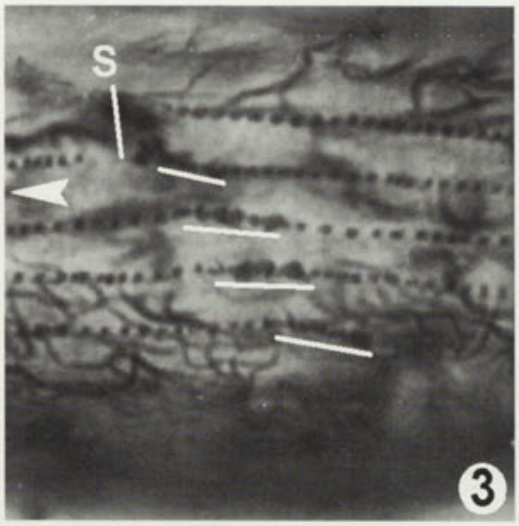
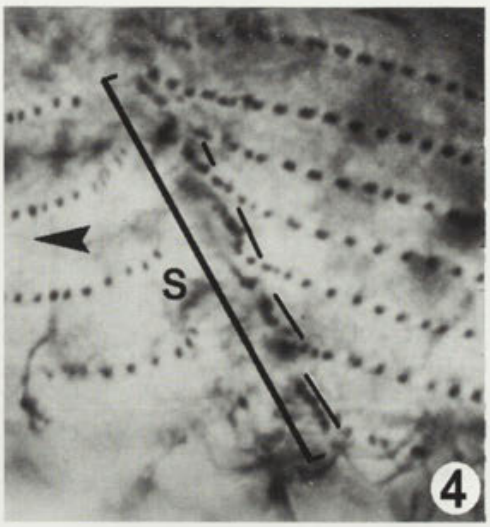
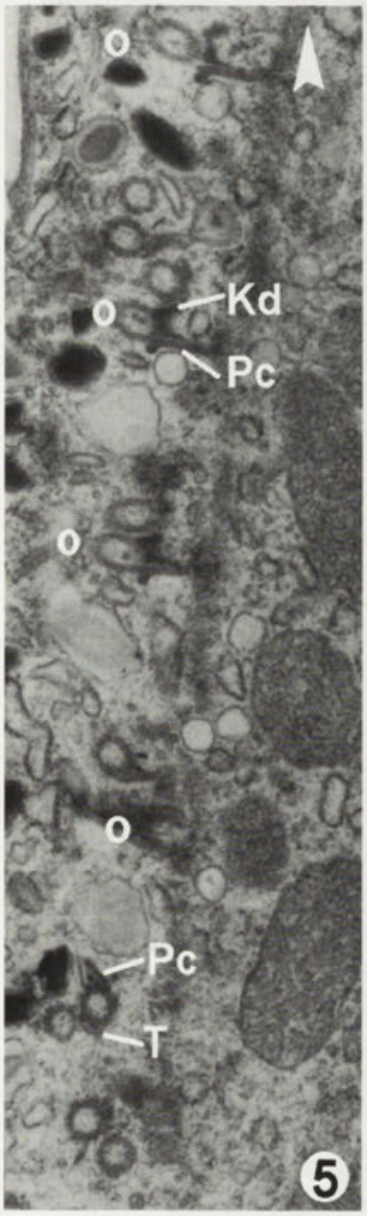
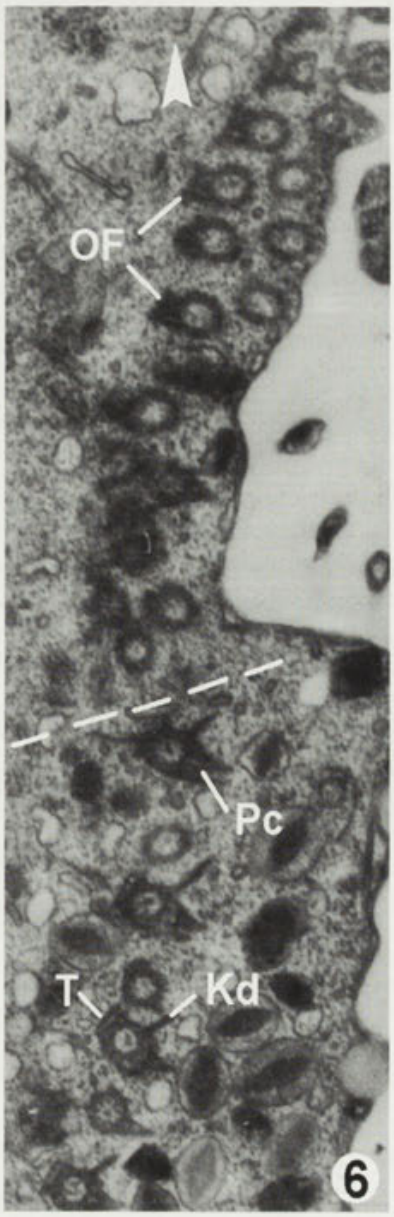
Fig. 1. A - side- and ventral view of dividing *Dileptus*. Oral structure is the dotted area. B - mature oral structure and accompanying somatic kinetids. This and all the other Figures are viewed from the outside of the cell. Open circles are barren basal bodies, open circles with an "x" inside are basal bodies bearing cilia. Concentric circles represent toxicysts, black circles are kinetodesmal fibrils, small dots are p-microtubules forming also inner pharyngeal basket on cytostomal field. Heavy lines are oral fibres, open bars are transverse fibres and black bars are postciliary fibres

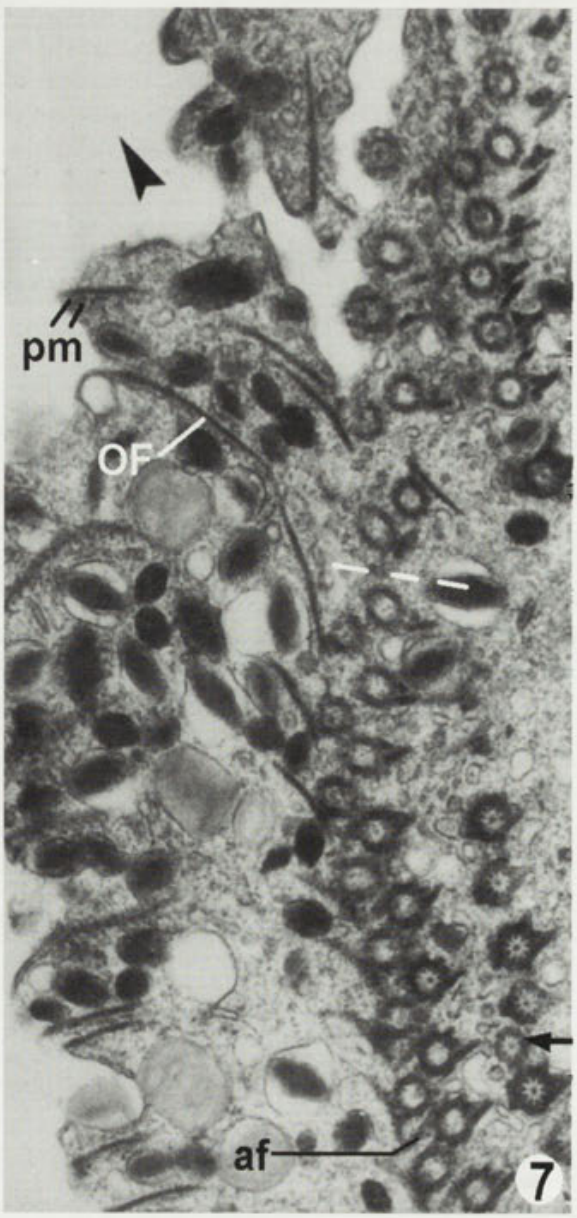












Toxicysts are mounted in between oral fibres (OF) supplied by oral dikinetids. An oral dikinetid consists of one nonciliated basal body which anchors OF and nematodesma, and ciliated basal body which anchors postciliary fibre (Pc). Right oral dikinetids are linked together with an additional fibre (Figs. 14, 15, 21B), anchored at ciliferous basal body of one dikinetid and at basal part of OF of another dikinetid. Oral fibres in the right portion of ventral band are accompanied by perpendicular microtubules (p-microtubules) which are anchored at subpellicular alveoli and run deeply into the endoplasm of proboscis (Golińska 1978). Identical microtubules were described in other haptorids (in *Homalozoon* by Kuhlmann et al. 1980) and named the bulge (?) microtubules.

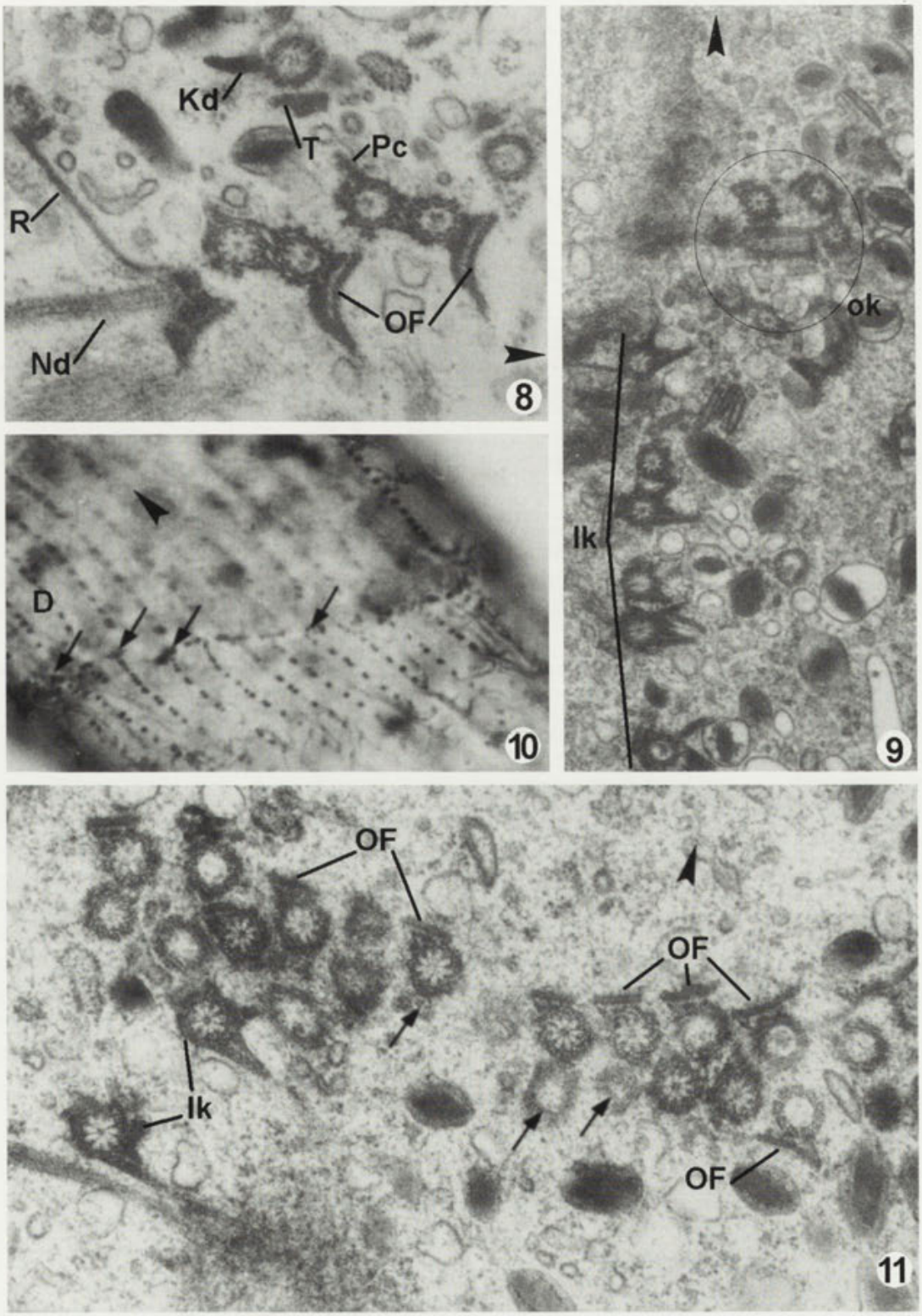
Alongside the row of oral dikinetids runs the so-named dense kinety (Golińska and Kink 1976) which consists of densely packed somatic monokinetids (Fig. 1B). The dense kinety is in continuation of somatic kinety on right-ventral side of the cell, where somatic kinetids are normally spaced. Each monokinetid of dense kinety provides filamentous root fibre that usually contacts material between basal bodies of oral dikinetid (Fig. 8). This rootlet resembles deep fibre of a somatic kinetid (Golińska 1991), but is deprived of microtubular component of a deep fibre.

The right margin of ventral band is the first part of oral structure to be made during cell fission. It is formed on right side of cell body (Golińska 1972). The earliest sign of division is crowding of kinetids within somatic kineties in midbody dorsal-right region (Fig. 3). Segments of densely packed kinetids form an oblique patch, the segments situated more ventrally being formed more posteriorly. In front of crowded segments appears an empty space, where Pc fibres of somatic kinetids belonging to anterior daughter support the cell surface. Intense proliferation during formation of crowded segments differs from proliferation of somatic kinetids because of rotation of newly formed basal bodies. This rotation may precede the formation of rootlets. In this case rotation is inferred from the curvature of a linker between old and new basal body (Fig. 2). In later stages the rotation is inferred from position of Pc fibre on new kinetid (Fig. 5). New oral kinetid when rotated and equipped with Pc and small transverse (T) fibres, edifies new basal body on its side opposite to Pc fibre, regardless its orientation in a kinety (Fig.5).

Intense proliferation of basal bodies lasts until a crowded segment of kinety is formed, like that on Fig. 3. In next stage it contains oral dikinetids only (Fig. 6) with all OFs on left side of a kinety. The segment of crowded oral kinetids separates from the rest of its parental somatic kinety, thus becoming a kinetofragment. During this stage of stomatogenesis all kineties on the right side of the cell curve dorsally, both oral kinetofragments and posterior parts of somatic kineties in anterior daughter (Fig. 4). The rotation of oral kinetofragments lasts until they are positioned alongside a fission line, with all OFs directed anteriorly (Fig. 7). The bent kinetofragments are long enough to seal together into continuous row without additional proliferation of oral kinetids.

During rotation of kinetofragments and longtime after formation of continuous oral row, intense proliferation continues in somatic kineties close to oral primordia (Figs. 6, 7). The subsequent elongation of somatic kineties in posterior daughter leads to bending of the anterior ends of these kineties, alongside the bent oral kinetofragments. The curved ends of somatic kineties represent a future dense kinety. A final stage (during elongation of proboscis after fission) is sealing of bent ends of somatic kineties into a dense kinety. Thus in

Figs. 2-7. Oral primordia on the right side of the cell. Large arrowhead points to anterior cell pole. 2 - formation of new oral kinetid in front of somatic one. An arrow points to the link between old and new basal body: its curvature indicates direction in which the new basal body rotates (x 51 000). 3 - early oral primordia, protargol impregnated preparation. Dorsal side of the cell is in the upper part of the photograph. Heavy lines are drawn along crowded segments of kineties, where numerous oral kinetids are already formed (x 5 000). 4 - next stage in oral primordium formation, protargol impregnated preparation. Dorsal side of the cell is in the upper part of the photograph. Oral kinetids are grouped in kinetofragments (heavy lines), and the kinetofragments are bent toward the dorsal side. Note that kineties in anterior daughter also curve towards the dorsal side (x 5 000). 5 - early stage in formation of right oral kinetids within a somatic kinety. Old somatic kinetids bear transverse fibres, postciliary fibres, kinetodesmal fibrils and contain dark granules inside basal body. New oral kinetids have T and Pc fibres, no Kd fibril, and edify new basal bodies on their side opposite to Pc fibre. Note that oral kinetids in the lower part of the photograph are rotated, as indicates position of their Pc fibre and site of new basal body formation (x 21 500). 6 - oral primordium in stage of development corresponding to that shown on Fig. 3. Broken line separates region of oral kinetids bearing oral fibres from row of somatic kinetids with kinetodesmal fibrils, postciliary fibres, and transverse fibres (x 24 500). 7 - late stage in formation of oral primordium. Oral fibre accompany oral dikinetids, some additional fibres are already present. Two kinetofragments (here separated by broken line) are almost sealed into a common row. Note one dikinetid just above the broken line, which is not yet rotated. Alongside each row of oral dikinetids run curved ends of somatic kineties, where proliferation of basal bodies still proceeds (arrow) (x 23 500). Abbreviations used: af - additional fibres; cf - central fibre; D - dorsal side of the cell; dk - oral dikinetids; eb - external pharyngeal basket; lk - somatic kinetid; Kd - kinetodesmal fibril; Nd - nematodesma; O - old somatic kinetids; OF - oral fibres; ok - oral kinetids; Pc - postciliary fibres; pm - p-microtubules; R - filamentous rootlet of somatic kinetid; S - a gap in ciliary rows between primordia of posterior daughter and somatic kineties of anterior daughter; sc - region of sensory cilia; sr - short rows of oralized somatic kinetids; T - transverse fibres; t - toxicyst; vb - ventral band



Figs. 8-11. Oral primordia on the left side of the cell. Large arrowheads point to the anterior cell pole. 8 - structure of oral dikinetids and of accompanying somatic kinetids on left side of ventral band in mature oral structure. Note that the accompanying somatic kinetids face anterior cell pole with their left posterior side. Filamentous rootlet of somatic kinetid contacts material between basal bodies in oral dikinetid (x 45 000). 9 - early oral primordium on left side of the cell. Small group of oral kinetids is formed near the end of disrupted somatic kinety (x 22 500). 10 - early oral primordia on left side of protargol impregnated cell. Groups of oral kinetids (arrows) form near ends of disrupted somatic kineties (x 5 000). 11 - left oral primordia in stage of development corresponding to that shown on Fig. 10. Note that group of oral kinetids is spreading both to left and right sides of its parental somatic kinety. Almost all oral fibres face the anterior cell pole. Intense proliferation of basal bodies (arrow) takes place on this side of oral kinetids that is opposite to OFs (x 47 000). Abbreviations as in Figs. 2-7

origin of dense kinety are kinetofragments of somatic kineties.

Another than kinetids components of the right portion of ventral band, like toxicysts, p-microtubules, appear along OFs when rotation of oral kinetofragments is completed (Figs. 7, 14, 15). Also an additional fibre appears in this stage of right primordium formation.

Oral kinetids at left margin of ventral bend

Oral dikinetids situated along left margin of ventral band (Fig. 18) are slightly different from right oral dikinetids. Oral fibres of left dikinetids contain more microtubules than right ones (Golińska 1984), no toxicysts or p-microtubules are present in left part of ventral band, and no additional fibres link together oral dikinetids. Left OFs curve posteriorly on ventral band (Fig. 1B), and their long distal segments form a stack of ribbons named the central fibre. The central fibre separates wide right portion of ventral band from narrow left portion in mature oral structure. During division, the left row of dikinetids is formed by especially numerous primordia that appear on the dorsal and left sides of the cell (Fig. 21A).

Early stage in formation of left oral kinetids is the appearance of small groups of basal bodies near endings of disrupted somatic kineties (Figs. 9, 10). In this region, unlike on the right side, no formation of crowded sectors occurs within somatic kineties. Oral kinetids in early left primordia point with OFs to different directions, like on Fig. 19. Thus during this stage rotation of oral kinetids takes place.

Later on, while intense proliferation of basal bodies lasts within small groups, they spread to both right and left sides from their parental somatic kinety (Figs. 9, 10, 11). During this spreading most OFs are directed anteriorly. Intense proliferation of basal bodies lasts within spreading groups (Fig. 11), and both monokinetids and second basal bodies for dikinetids are forming. The proliferation lasts until all segments of oral dikinetids are sealed into one row. From this stage on, no proliferation can be found next to oral dikinetids (Figs. 14, 15).

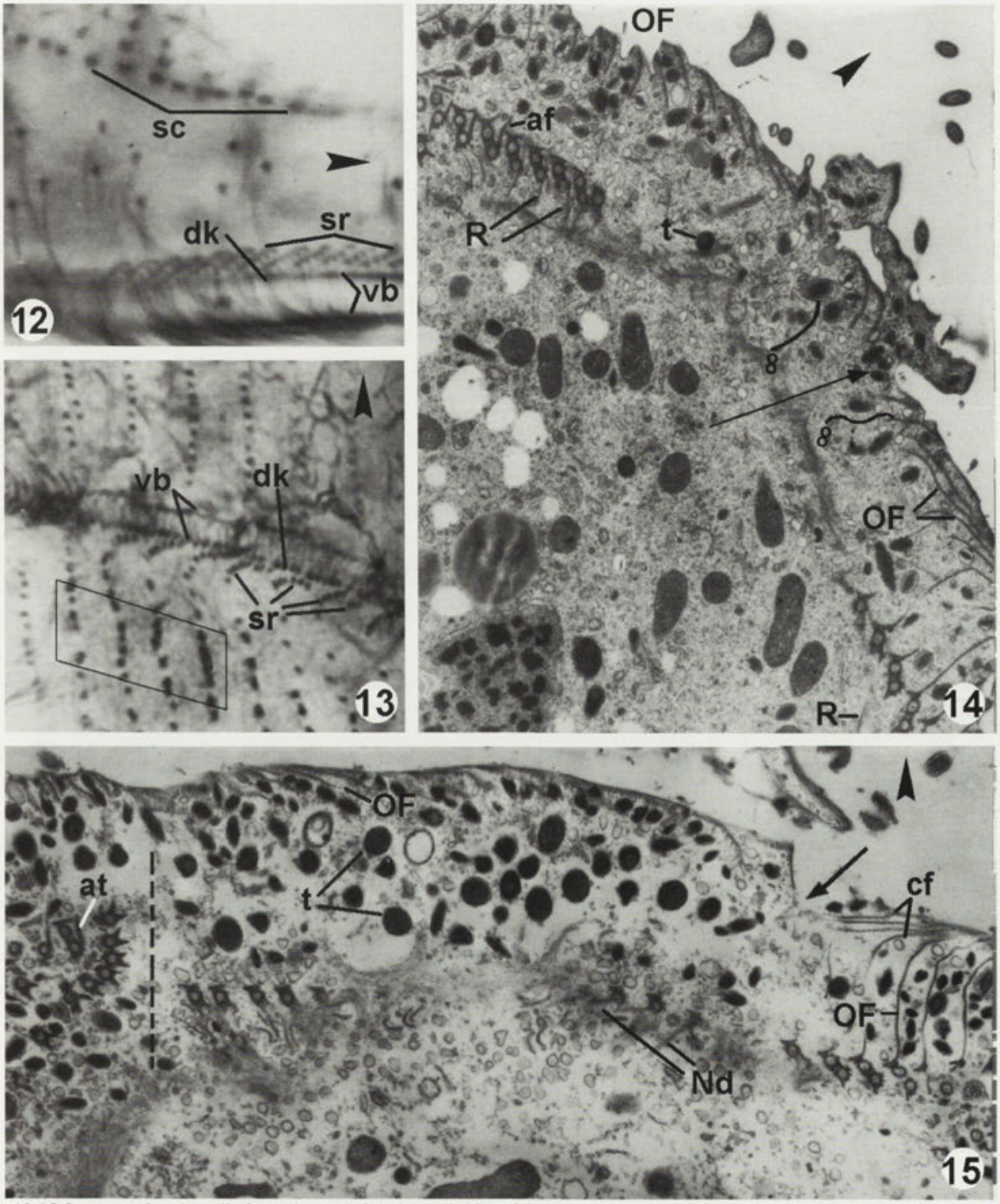
Proliferation in somatic kineties close to left oral primordium occurs during early stage of stomatogenesis (Fig. 9), then top parts of somatic kineties curve to the right, in the same direction like those on right side of the cell (Fig. 13). Proliferation in somatic kineties stops early, and curved endings become the only part of kineties where basal bodies are densely packed (Fig. 13). The isolated curved endings of kineties develop root fibres directed to oral dikinetids and become oralized somatic ciliature that accompanies oral structure. The left kinetofragments subdivide into two or more short rows, and their number becomes higher than the number of their parental somatic kineties. Thus on the left side of the cell, the oralized somatic ciliature does not seal into single row like right one, but in contrary, it disjuncts into numerous short rows (Fig. 12).

On the left side of the cell a second round of somatic basal bodies proliferation takes place, leading to formation of the so-named sensory cilia (Golińska 1983). Sensory cilia form within several somatic kineties at some distance from short rows (Fig. 13). In light microscope it looks like an appearance of big dark dots in somatic kineties. Each dot represents a pair of sensory cilia, the posterior one being the modified old somatic kinetid, the anterior ones being a new basal body formed during second round of proliferation on the left side of the cell (Golińska 1983).

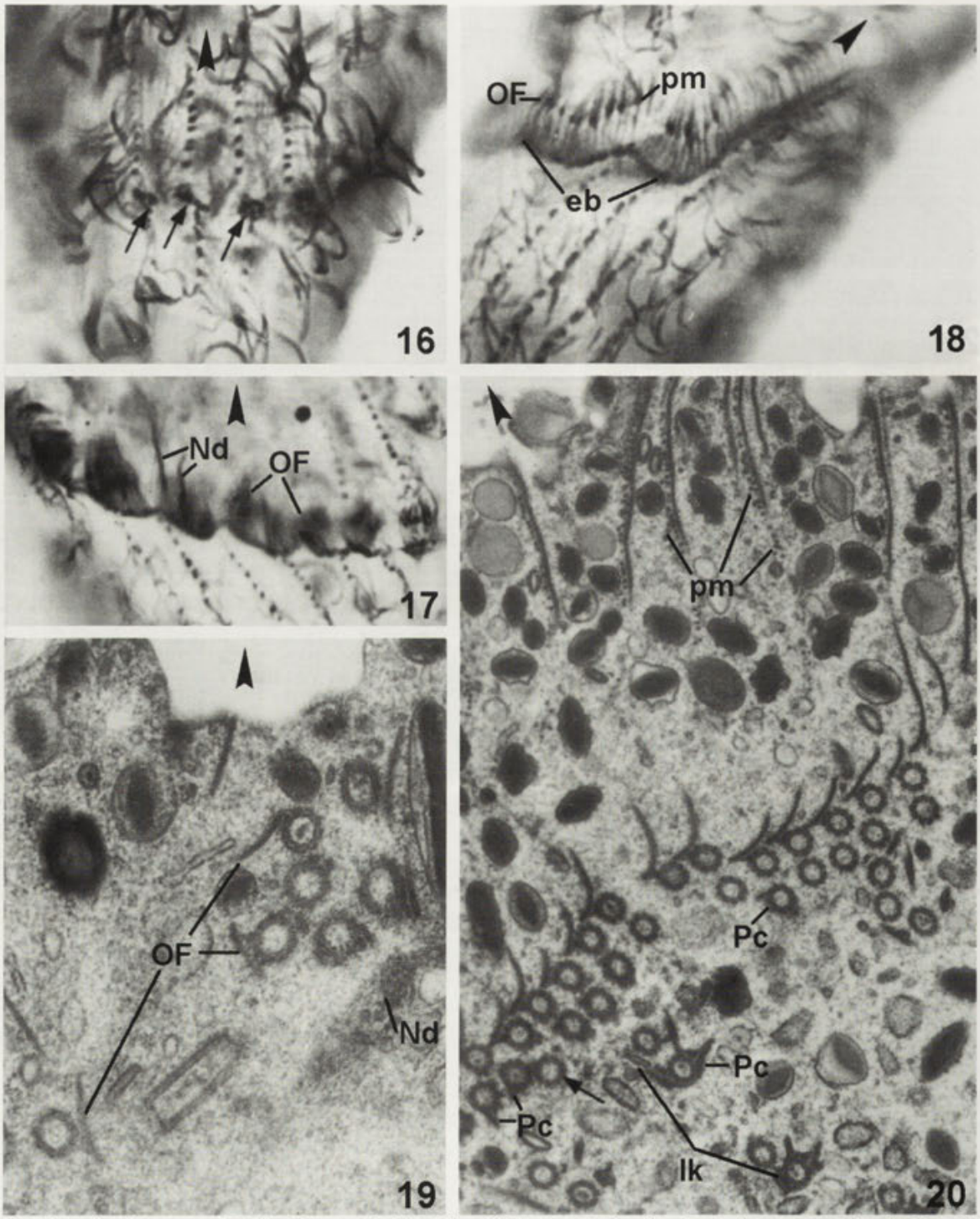
In mature cells, sensory cilia occupy the dorsal side of proboscis, while short rows of oralized somatic kinetids line up the left margin of ventral band (Fig. 12). Between short rows and sensory rows only few somatic kinetids are present, and only in basal part of proboscis. This confirms the observation that during stomatogenesis proliferation of somatic kinetids occurs firstly in region of short rows, secondly in region of sensory cilia, but it is weak or absent in the remaining territory on the left side of the cell.

During advanced stage of stomatogenesis, the orientation of oral and oralized kinetids on the dorsal side of the cell was observed. The place where right oral primordia abut upon the left ones, represents the future apex of proboscis. The orientation of right and left dikinetids is the same (Figs. 14, 15), oblique towards the A-P axis of cell body. Different is the orientation of their OFs: on the right side OFs simply arch towards the ventral side, while on the left side there is a S-shaped bending of OF ribbons, to allow them to curve towards the ventral side of the cell (Fig. 14). There is a sharp boundary between right and left primordia both in orientation of OFs and in kind of other structures for the future halves of ventral band. In the right part are already present numerous toxicysts, p-microtubules accompany OFs, additional fibres link together oral dikinetids. In the left part distal portions of OF ribbons stack one upon the other, thus forming a central fibre (Fig. 15).

Orientation of oralized somatic kinetids is very similar on right and left cell sides: they are tilted about 90° when compared to somatic kinetids in longitudinal



Figs. 12-15. Late stage in primordia formation on left and dorsal sides of the cell. Large arrowheads point to the anterior cell pole. 12 - pattern of kinetids on left side of proboscis in mature cell. Region of sensory cilia on the dorsal side of proboscis. Short rows of oralized somatic kinetids accompany oral dikinetids (x 5 000). 13 - formation of structures shown on Fig. 12, dorsal-left side of dividing cell, protargol impregnated specimen. Region of forming sensory cilia is contoured (x 5 000). 14 - dorsal part of oral primordium. The place where right oral dikinetids meet left oral dikinetids, is shown with an arrow. On both sides of this arrow the schematic representation of oral dikinetids is drawn, to visualize the orientation of dikinetids which is the same for left and right ones, while the orientation of left and right oral fibres is different (x 10 000). 15 - the same stage as shown on Fig. 14. An arrow indicates the place where left and right primordia meet. Central fibre made up of curved ends of left oral fibres. Nematodesma in this most-dorsal part of right primordium is already properly orientated. Broken line separates two segments of right primordium, not yet sealed into continuous row (x 8 500). Abbreviations as in Figs 2-7



Figs. 16-20. Primordia of cytostomal field, right ventral side of the cell. Large arrowheads point to the anterior cell pole. 16 - early ventral primordia, protargol impregnated preparation. Groups of oral kinetids form near anterior ends of disrupted somatic kineties, on both left and right sides (arrows) (x 4 500). 17 - stage corresponding to that shown on Fig. 20. The row of oral kinetids is almost continuous, although composed of numerous arc-shaped segments. All oral fibres are directed anteriorly and so are nematodesmata. Note that OFs of every arch converge at common point (x 4 500). 18 - late stage in formation of cytostomal field, protargol impregnated preparation. Oral kinetids form continuous meandering row. In this stage they bear large nematodesmata of the external pharyngeal basket. Oral fibres are directed anteriorly. Dark deposits situated in half of their length are bundles of p-microtubules, which later on will arrange into the internal pharyngeal basket (x 5 000). 19 - early ventral primordium, corresponding to that in Fig. 16. Oral fibres point to different directions, basal bodies are tilted at different angles. Nematodesma is already present (x 37 500). 20 - advanced stage in primordium formation, region where merge two arc-shaped segments of oral row. Crowded groups of p-microtubules accompany oral fibres. Numerous new basal bodies accompany oral monokinetids, and somatic kinetids. Oral kinetids may proliferate before their OFs are developed (arrow) and still look like postciliary fibre of somatic kinetid (x 22 000). Abbreviations as in Figs. 2-7

rows, and they face oral structure with their transverse fibres (T fibres). From under the T ribbon their deep rootlets emerge, and some of these come into contact with oral dikinetids (Fig. 8). The difference between right and left oralized kinetids lies in their overall pattern: right ones form dense kinety parallel to oral row, left ones form numerous short rows oblique towards the oral row. During elongation of proboscis and sealing together of right and left longitudinal halves of ventral band, all these structures retain their orientation and this results in 180° difference in left and right kinetids orientation on both sides of ventral band (Grain and Golińska 1969).

Oral kinetids at the margin of cytostomal field

In mature oral apparatus the cytostome is encircled by a circular cytostomal field, situated at the base of proboscis (Fig. 1A). The margin of the field is surrounded by single row of oral monokinetids (Fig. 1B), continuous with rows of dikinetids on right and left margins of ventral band. Oral monokinetids are composed of nonciliated basal bodies bearing large nematodesmata and OFs. This OF is similar to that of right dikinetid in number of constituent microtubules and in presence of p-microtubules along the OF ribbon, although no toxicysts are mounted to cell surface in this region. The OFs of oral monokinetids are all directed towards the cytostome and some of them enter the cytostomal depression. Large nematodesmata attached to oral kinetids form the outer pharyngeal basket, extending deeply into the endoplasm. Inside this basket there is much smaller inner pharyngeal basket, made up of numerous microtubular bundles held together and linked to cell surface by filamentous ring (Grain and Golińska 1969). Rows of somatic kinetids which abut the cytostomal field curve to the right, thus the row of oral monokinetids is accompanied by curved endings of somatic kineties. Kinetids within the bent ends face oral ones with T fibres, and rootlets from under T fibres are directed towards oral monokinetids. No contact, however, was observed between the rootlets and oral monokinetids.

The cytostomal field develops on the ventral side of the cell, and it is the last oral structure to form. Early primordia resemble left oral primordia, because they are small groups of oral kinetids formed at tops of disrupted somatic kineties (Figs. 16, 19). Basal bodies within small groups not only point with OFs in all possible directions, but are also tilted at different angles towards the cell surface. In this stage already

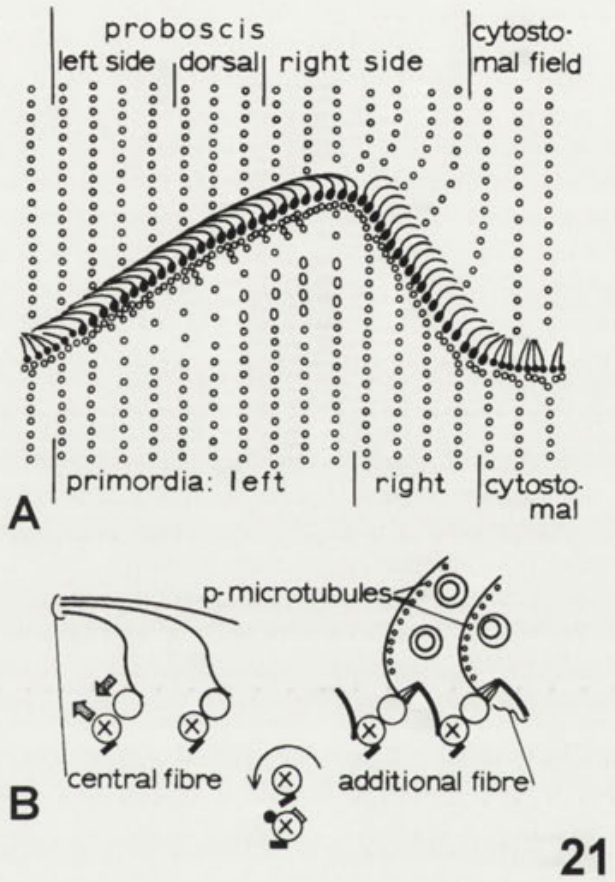


Fig. 21. A - schematic representation of the whole area of fission furrow. In the upper part the latitudes of oral parts in anterior daughter are shown, in the lower part the latitudes of different kinds of oral primordia are shown. Number of kineties bearing left oral primordia is much lower than in nature. Open circles are somatic kinetids, open ovals are pairs of sensory kinetids. Black circles are oral monokinetids, black ovals are oral dikinetids. Lines represent oral fibres. It is evident that during maturation of oral structure numerous left somatic kineties end on dorsal and left sides of the cell, not reaching the proboscis region. B - two dikinetids of left primordium and two dikinetids of right primordium are shown. In the middle is an early stage in formation of oral kinetid in front of somatic one. An arrow shows the direction in rotation of new basal body. Large open circles are basal bodies, open circles with an "x" inside are basal bodies bearing cilia, concentric circles represent toxicysts. The black circle is a kinetodesmal fibril, open bars are transverse fibres and black bars are postciliary fibres. Heavy lines are oral fibres. Dotted arrows in vicinity of exterme left oral dikinetid indicate the orientation of basal bodies within a dikinetid

nematodesmata are forming (Fig. 19). During the next stage, ventral primordia spread both to left and right sides, forming several arc-shaped rows (Fig. 17). In this stage all OFs are directed anteriorly, and so are nematodesmata (Figs. 17, 20). All OFs of one arc converge at common focal point, their distal parts do not curve in any direction. The number of arcs initially corresponds to the number of somatic kineties that participate in their formation. During further develop-

ment, when primordia join each other, the number of arcs diminishes. When OFs acquire orientation towards anterior cell pole, p-microtubules appear along them. Later on, the number of p-microtubules especially increases in mid-length of OF ribbons giving rise to elongated crowdings of microtubules (Fig. 20). During the next stage the microtubular crowdings gather into bundles (Fig. 18) for the future internal pharyngeal basket. In this stage primordia are sealed into a continuous row, and the number of arcs reduces until all OFs focus at common point, the future cytostomal depression.

During development of ventral primordia, the proliferation of basal bodies does not stop after the continuous row of oral kinetids is formed. It is known that proliferation of basal bodies occurs even in the mature oral structure of *Dileptus* (Golińska and Kink 1976). It is not known, however, whether whole offspring of oral kinetids develops into oral kinetids: some of them could develop into somatic kinetids, both during stomatogenesis and in mature life.

Spatial relationship between old and new oral structure

The oral primordia of left and right margins of ventral band and of cytostomal field, engage in their development different number of somatic kineties, i.e. they are of uneven length. The shortest and of the most steep slope are primordia for the right margin of ventral band (Fig. 21A). Usually only 3 or 4 primordia develop into this part of oral structure. Right primordia occupy kineties that are in prolongation of kineties on the right side of proboscis. The so-named dense kinety which accompanies the right oral row on proboscis is continuous with a kinety where the primordium for right margin of the cytostomal field is formed.

Primordia for cytostomal field are formed in 4 to 5 kineties. All the rest of somatic kineties (cells in growing culture have 36 to 61 kineties, Drzewińska and Golińska 1987) edify primordia for the left margin of ventral band. This indicates that during elongation of proboscis, somatic kineties on its left side do not elongate, leaving only few scattered kinetids in basal part of proboscis (Fig. 12). It also confirms that basal body proliferation on the left side is weak or absent when oral and oralized kinetids are already developed. Sensory cilia develop within 4 to 7 somatic kineties on dorsal side of the cell. During elongation of proboscis these kineties apparently end at different levels, because on mature proboscis only 3 kineties are left in prolongation of rows of sensory cilia.

DISCUSSION

Three main topics emerge from the presented observations. Firstly, oral primordia for three oral regions differ in their organization from the very beginning of development. Secondly, development of oral kinetids comprises their rotation. Thirdly, the presented observations on spatial orientation of kinetids during stomatogenesis allow to analyse the formation and shifting of orientation centre for oral structure.

Development of three regions of oral structure

It is interesting that for each of three main parts of oral apparatus in *Dileptus* an another developmental pathway is taken. This special pathway concerns not only the oral kinetids, but also their somatic associates, termed the oralized somatic kinetids (Foissner and Foissner 1988). This is very unusual for an haptorid ciliate, since in other members of this group the oral kinetids are formed in the same fashion all around the cytostomal field.

Right oral primordia of *Dileptus* form within somatic kineties, then bend to the right side and separate from somatic kineties. Later on, these kinetofragments join each other to form the continuous row of oral kinetids. This way of formation of oral kinetids was found in *Homalozoon* (Leipe et al. 1992). During later stages of stomatogenesis in *Dileptus* right somatic kineties elongate due to intense proliferation of somatic kinetids, and bend alongside the oral row. Such ciliary pattern is found around oral structure in mature cells of *Spathidium, Homalozoon,* or *Bryophyllum* (Fryd-Versavel et al. 1975, Leipe et al. 1992). The further development of right oral primordia in *Dileptus* is, however, unique for it. The formation of additional fibres and head-to-tail sealing of oralized kinetofragments, are so far found only in *Dileptus*. The left oral primordia of *Dileptus* form as small groups of oral kinetids in front of disrupted somatic kineties. Apparently the left primordia, unlike the right ones, do not rely in their organization upon a somatic kinety. The formation of oralized somatic kineties at first occurs similarly to that on the right side, because distal ends of somatic kineties bend rightward alongside the oral row. Further development of the left kinetofragments comprises, however, its segmentation into two or more pieces (instead of sealing into continuous row like on the right side). This pattern of circumoral ciliature: multiple short oblique rows of oralized kinetids that accompany the oral row of dikinetids, was found in many gymnostome ciliates, like

Lacrymaria (Bohatier 1970), *Acropisthium* (Bohatier and Detcheva 1973), or *Monodinium* (Rodrigues de Santa Rosa and Didier 1975). The central fibre and lack of p-microtubules, that characterize left side of ventral band in *Dileptus*, were described in *Litonotus* (Bohatier and Njiné 1973).

The ventral primordia of *Dileptus* form similarly to the left ones, as small "anarchic fields" in disrupted ventral kineties. When they spread to left and right sides, however, they form arc-shaped short rows, which join each other and gradually straighten their multi-arc outline until all OFs point to a common center where a cytostomal depression is formed. Characteristic for ventral oral primordia is the formation of monokinetids, accompanied by their crowded offspring. Oral monokinetids were described by Foissner and Foissner (1985) in *Enchelydium*. The formation of arc-shaped oral primordia was described in *Spathidium* (Berger et al. 1983).

It appears that all the parts of mature oral apparatus of *Dileptus*, and stages of its formation, can separately be found in different related ciliates. What is unusual in *Dileptus* is the diversity in structure of different regions of mature oral apparatus, and in ways the regions are formed. It can be supposed (until some other data on rhabdophorean stomatogenesis are available), that *Dileptus* resembles some ancestral form of a haptorid ciliate, its relatives retaining only different portions of the complex ancestral oral apparatus.

Rotation of oral kinetids

The rotation of oral kinetids during stomatogenesis in *Dileptus* is especially well documented for right oral primordia, because the right oral kinetids form within a somatic kinety with an A-P axis easily discernible. The early 180° rotation results in OFs position on this side of kinetofragment where somatic kinetids have their T fibres. In a next stage rotation of the kinetofragment change the orientation of oral kinetids until their OFs face the anterior cell pole. Oral primordia of left and ventral sides, which develop later than the right ones, are not formed within a kinetofragment. The rotation of kinetids in "anarchic fields" seems to lead directly to orientation with OFs facing the anterior cell pole.

The rotation does not represent a regular stage in formation of oral kinetids, since only the first generation of basal bodies rotate. Their offspring generate OFs in proper orientation, and take place in between neighbor oral kinetids until the primordium is sufficiently crowded. New basal bodies destined to develop into the second component of a dikinetid, form their Pc fibre at this side of basal body where oral monokinetids form OF ribbon. Thus a rotation of the second basal body must take place, in order to establish proper structure of the dikinetid (Figs. 18, 21B), similar to that described for paroral dyads in *Furgasonia* (Eisler 1988, 1989).

In other than *Dileptus* ciliates, the rotation of oral kinetids forming in a kinetofragment was reported for *Coleps* (Huttenlauch and Bardele 1987). Rotation of the whole kinetofragment was reported for many other cyrtophorean ciliates, like *Trithigmostoma* (Hofmann and Bardele 1987), *Chilodonella* (Hofmann 1987), and for rhabdophorean *Homalozoon* (Leipe et al. 1992). Rotation of oral kinetids within anarchic fields, although widespread among ciliates, is rather unusual for gymnostome ciliates which rely upon the kinety as a basis in oral primordia organization (see discussion by Peck 1974). In *Dileptus*, however, a kind of anarchic field was found both in left and in ventral primordia.

The bending of anterior ends of somatic kineties alongside the oral row, is frequently encountered amongst haptorid ciliates. This bending is always to the right side, the somatic kinetids coming to face the oral row with their T fibres. The separation of the bent kinetofragment from its parental kinety, and subsequent sealing of kinetofragments into continuous row, or their segmentation into numerous short rows, was so far observed only in *Dileptus*. These peculiarities in stomatogenesis of *Dileptus* are most probably related to formation of proboscis, a peculiar part of *Dileptus* body.

Orientation of oral kinetids in *Dileptus*

When the surface of a ciliate is regarded as a morphogenetic field, stomatogenesis is the process by which a new territory arises with skeletal elements orientated towards another orientation centre than skeletal elements elsewhere on cell cortex. In ciliates with oral apparatus situated on the ventral side, stomatogenesis starts with positioning of oral primordia (reviewed by Frankel 1989). In ciliates with apically located oral apparatus and stomatogenesis of a holotelokinetal type (Hiller 1992) formation of anterior cell pole and of oral center for the posterior daughter, are inseparable.

This is the first detailed description of holotelokinetal stomatogenesis in a ciliate with subapical oral area. Stomatogenesis in *Dileptus* is holotelokinetal, because oral primordia form in vicinity of all somatic kineties, and constriction of fission furrow is executed through the development of oral structure (Golińska 1972). As shown in this study, during early stages of

stomatogenesis all oral kinetids attain (due to rotation) the same orientation: with their OFs facing the anterior cell pole. It can be speculated that in this time the orientation centre for oral kinetids is situated at the anterior cell pole, thus oral structure is truly apical.

Oral kinetids retain this once acquired orientation through the elongation and maturation of proboscis and cytostomal field. This results in reverse position of left and right dikinetids on both sides of the ventral band (Grain and Golińska 1969). The shifting of oral orientation centre towards the ventral side can be deduced from observations on orientation of OFs all around the fission line. On both right and left sides of dividing cell OFs are bending towards the ventral side, and a sharp boundary appears between left and right OFs on dorsal side of the cell. There is no bending of OFs on ventral side. Instead, OFs of separate arcs center at separate loci (orientation centers?). During further development the number of these loci diminishes until a single cytostomal depression is formed. It can be imagined that the bending of OFs and reduction in number of "cytostomes" represents the shifting of oral orientation centre from the anterior pole to subapical ventral position.

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Polynemic Structures in the Differentiated Macronucleus of the Ciliate *Bursaria ovata* Beers 1952

Galina I. SERGEJEVA and Nina N. BOBYLEVA

Laboratory of Cytology of Unicellular Organisms, Institute of Cytology, Russian Academy of Sciences, St.Petersburg, Russia

Summary. Polynemic loci in differentiated interphase macronuclei have been detected in starved and fed vegetative *Bursaria ovata* by chromatin spreading in low ionic strength conditions. In both cases a spatial relation has been found between polynemic and non-polynemic loci. In starved animals, polynemic cords about O.5 μm wide are found, which mainly consist of bundles of fibers about 50-70 nm and 20-35 nm thick (when rotary shadowed with Pt/Pd). Occasionally, polynemic cords alternate with superdense segments, in some of which polynemy is supposed. In fed cells the polynemic loci are represented by bundles of almost parallel nucleosomal and non-nucleosomal sister fibrils, in identical sites of which lateral loops of nucleosomal fibrils arise. In polynemic loci of fed *B. ovata* primary, secondary, etc. branching points are found on sister fibrils. In some cases the branching regions are clearly dichotomous structures with gradual increase in the number of sister fibrils. In others, several secondary sisters fibrils seem to branch off from one point on the axial non-polynemic fibril or on the some sister fibrils, giving the impression of "umbrella" or "fan" pattern. The computer image analysis of branching zones allowed us to suggest that, in the former case, the process of dichotomy is due to stepwise replication whereas the fan-like branching is an extremely "steep" dichotomy. We regard the local polynemic structures in the chromatin of differentiated macronuclei of vegetative *B. ovata* as a special case of polyteny. The earlier data and the data of this article lead us to a conclusion about presence of the hidden local polyteny of a non-classical type in polyploid macronuclei of two known species of *Bursaria - B. ovata and B. truncatella*.

Key words. Polyteny, polynemy, macronucleus, ciliates.

INTRODUCTION

It has been established that the development of the new somatic nucleus (macronucleus) during the sexual process of some ciliates includes a period of polytenization (reviews: Raikov 1982, Ammermann 1987). Giant chromosomes are found during development of the maconucleus anlagen in Hypotricha, *Nyctotherus, Chilodonella* and some other ciliates. The species with giant chromosomes do not belong to one systematic group of the ciliates, but are scattered throughout the entire phylum. The giant chromosomes of the macronuclear anlagen of Hypotricha have a structure similar to classical polytene chromosomes of Diptera, at least under the light microscope (review: Raikov 1982). The banded polytenic (oligotenic) loci were also detected in *Bursaria truncatella* at the late spireme stage of the macronuclear anlagen and on this grounds the polytenic (oligotenic) nature of the anlagen was supposed (Poljansky and Sergejeva 1981). Nothing is known about

Address for correspondence: G. I. Sergejeva, Institute of Cytology, 4, Tikhoretsky Avenue, 194064 St. Petersburg, Russia; Fax: (812) 247-03-41; E-mail: cytol!burs@dfa.spb.su

polyteny in the adult or differentiated macronucleus of the vegetative ciliates. But the polytene origin of some chromatin elements in the differentiated macronucleus is presumed to occur in *Chilodonella* (Radzikowski 1973), *Tetrahymena* (Seyfert 1979), *Paramecium* (Samuel et al. 1981). The presumption about the existence of polytene loci in the differentiated macronucleus of vegetative *Bursaria truncatella* has been made before (Sergejeva 1977). It was shown that in ultrathin sections of the differentiated macronucleus of vegetative *B. truncatella*, chromatin bodies are often joined into chains by either single DNP fibers or bundles of them. In the latter case the bundles of DNP fibers extended through several chromatin bodies, being more loosely packed in the regions between the bodies. Later, bundles of parallel nucleosomal fibers were visualized in spread chromatin preparations of macronuclei isolated from interphase vegetative *B. truncatella* (Martinkina et al. 1983, Vengerov et al. 1983), encysting bursarias (Sergejeva et al. 1987) and cysts (Tikhonenko et al. 1984). However, it still remained doubtful whether or not this polyteny is only apparent, as in the case observed by Mullinger and Johnson (1980) in mitotic chromosomes of HeLa cells. In addition, studies of spread chromatin as well as of ultrathin sections failed to yield convincing evidence of the presence of local polyteny in the differentiated macronuclei of *B. truncatella*. This might only be proved by electron microscopic demonstration of transitory zones from polynemic to non-polynemic chromatin regions.

An alternation of polynemic and non-polynemic loci has been found by us in the macronucleus of another species of the genus *Bursaria, B. ovata* Beers (Sergejeva and Bobyleva 1989). In this paper we present a larger spectrum of the structural variants of polynemic loci as observed by electron microscopy in spread chromatin preparations of differentiated macronuclei isolated from both fed and starved interphase *B. ovata*.

MATERIALS AND METHODS

For the study of the supramolecular chromatin organization of the interphase macronucleus of *Bursaria ovata* (Beers 1952) we used laboratory clones and subclones of natural strains isolated from temporary pools in the vicinity of St. Petersburg and in Siberia (near lake Baikal). Some of the strains have been collected for us by Dr. A. V. Jankovsky of the Zoological Institute of St. Petersburg. In some experiments we used clones obtained by excystment of resting cysts of the above mentioned strains. Cells were cultivated and made to excyst using a slightly modified technique earlier described for *B. truncatella* (Sergejeva 1977).

Preparation of cells for chromatin spreading and electron microscopic studies. The cells were used either soon after feeding, the cytoplasm still filled by food vacuoles (fed cells) or the day after feeding when the cells were completely free of food vacuoles (starved cells). In both cases we selected vegetative cells of maximum size with a well developed oral apparatus, and in which no signs of preparation for division were visible with the light microscope. The worm-shaped macronucleus of such cells is stretched to the maximum. We suppose that these cells were at late S and G_2 stages of the cell cycle in accordance with the data of Nilova (1968) and Ruthmann (1964).

From several variants of the modified Miller's spreading method (Miller and Bakken 1972, Sergejeva et al. 1987) the following appeared to be the most effective: cells were placed into a lysing solution previously cooled to 7-8°C. Two variants of the solution were used: (A) 0.5% Triton X-100 (1 part), 0.1 mM sodium tetraborate containing 0.1 mM EDTA (Serva) (1 part), deionized water (3 parts); (B) 0.5% Triton X-100 (2 parts), 0.1 mM sodium tetraborate with 0.1 mM EDTA (Serva) (5 parts) and 0.1 mM sodium tetraborate (10 parts). Immediately after disrupting the cells, the isolated macronuclei were transferred first into 0.1 mM sodium tetraborate containing 0.1 mM PhMSF (phenyl-methyl-sulfonyl-fluoride, Böehringer), then into 0.1 mM sodium tetraborate containing 0.1 mM EDTA, and finally, into 0.1 mM sodium tetraborate. Spreading of the nuclei was performed in a microchamber of siliconized glass for about 5 min, the pH was 8.5-9.0 in all the solutions. Spread nuclei were layered on the solution containing 4% formaldehyde and 0.1 M sucrose (Serva), at 7-8°C, pH 9.0. Spread chromatin was centrifuged for 15 min at 3500 rpm at 4°C onto an electron microscopic grid freshly ionized in 0.001% Alcian blue solution and previously covered with a collodion-carbon supporting film. The specimens were then washed in 0.4% Kodak Photo-flo solution, air-dried and rotary shadowed with platinum-palladium (1:4) at the angle of 9°. Preparation of *B. ovata* for obtaining ultrathin sections was carried out as previously described for *B. truncatella* (Sergejeva 1980). The spread preparations and fine sections were examined in JEM-7A and JEM-100CX electron microscopes at an accelerating voltage 50 and 80 kV and screen magnifications from 5000 to 15000x. The sizes of all structures are calculated taking into account the platinum-palladium shadowing.

RESULTS

In ultrathin sections, the macronucleus of *Bursaria ovata* (Fig. 1) exhibits the same structure as polyploid macronuclei of most ciliates studied (see review: Raikov 1982) including that of *B. truncatella* (Sergejeva 1976, 1977, 1980; Livolant et al. 1993). The nucleus, limited by a two-membrane envelope perforated with numerous pores, is filled with small chromatin bodies (size about 70-100 nm), rather often linked in *B. ovata* into short chains. There are numerous nucleoli of fibrillar type which differ in size and shape. In starved individuals, the nucleoli are very

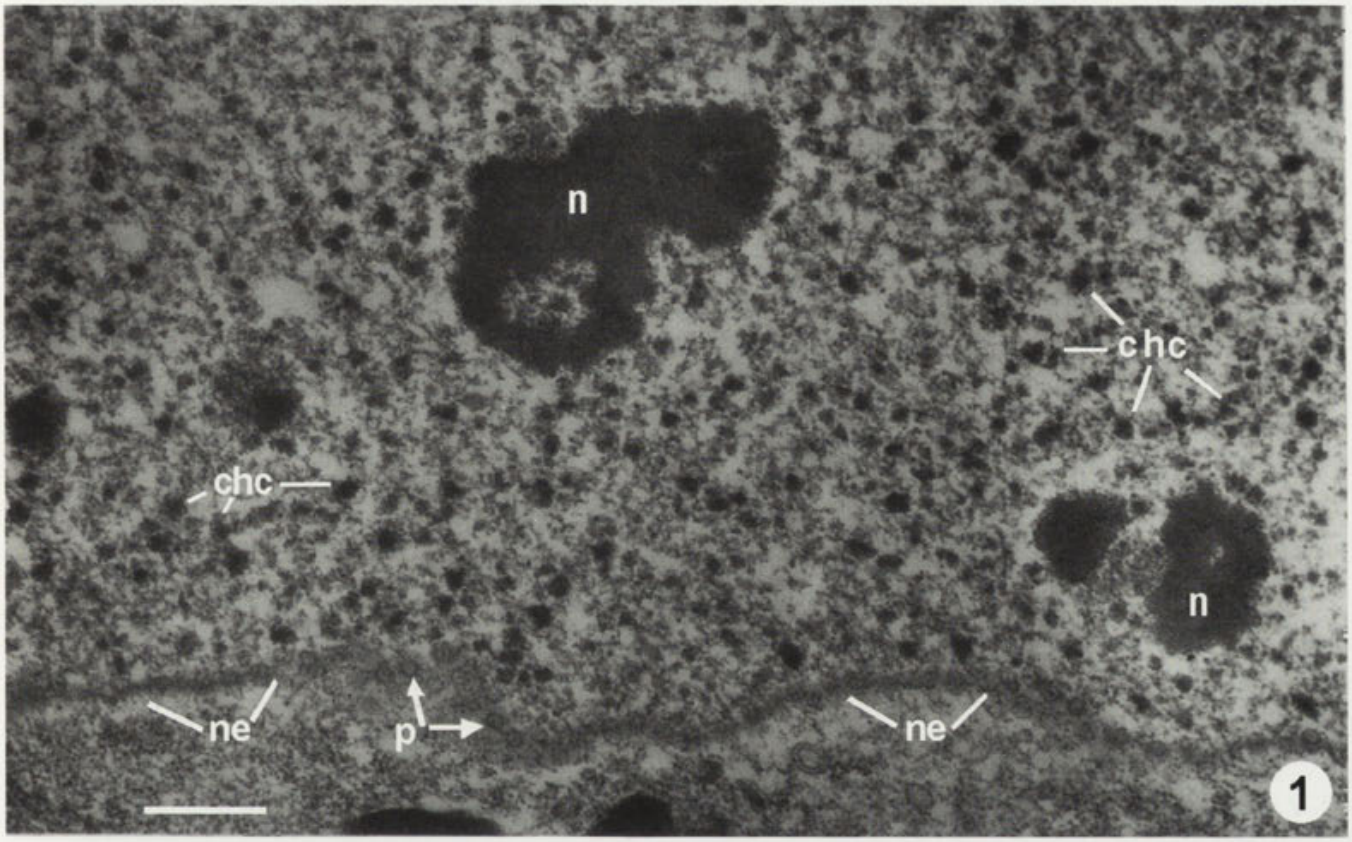


Fig. 1. Fragment of an ultrathin section of the macronucleus of starved *B. ovata*. The macronucleus is limited by a nuclear envelope (ne) perforated with numerous pores (p) with a central granule (at arrows). The karyoplasm is filled with fibrogranular material in which clearly discernible are nucleoli (n) of fibrillar type and chromatin clumps (chc) typical of polyploid macronucleus of ciliates. Bar - 0.5 μm

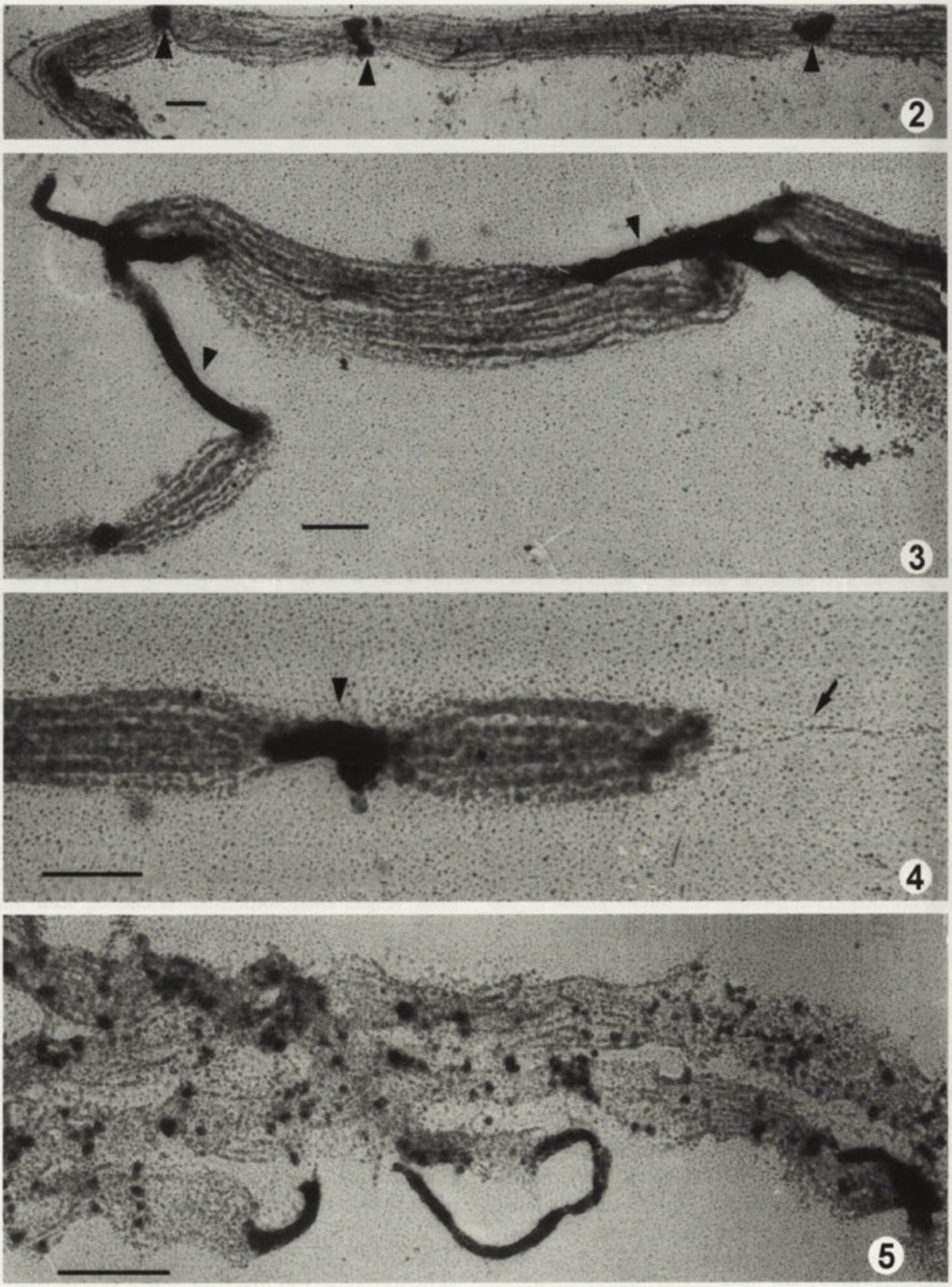
compact, often of complex shape, or joined in groups of 2-3 or more; they are devoid of the granular component, and sometimes ring-shaped. Numerous small granules about 18 nm in diameter and fibrils about 25 nm thick are dispersed through the nucleoplasm of the macronucleus of both starved and fed *B. ovata*.

The mosaic pattern of chromatin decompactization in spread preparations of interphase macronuclei of *B. ovata* is the same as demonstrated earlier for *B. truncatella* (Martinkina et al. 1983, Sergejeva et al. 1987). The main part of the macronuclear interphase chromatin is organized in compact chromatin bodies or clumps which have a loop-packed structure of the "rosette" type. Compact chromatin bodies and "rosettes" are interconnected by one or several nucleosomal fibers. In addition to the above described structures, spread preparations of macronuclear chromatin of both starved and fed cells show polynemic loci of different length alternating with non-polynemic two-thread (two-chromatid) fibrils.

The main task of this paper is to give a detailed description of these structures.

In starved cells, the bulk of the chromatin is represented by very long ribbon-like polynemic cords of different density (Figs. 2-5). Such ribbon-like cords consist of densely packed parallel fibrils about 50-70 nm thick (Figs. 2-4); sometimes it can be seen that these fibrils are formed of two interwoven thinner fibrils 20 to 35 nm thick. The width of the ribbon-like polynemic cords is about 0.5 μm, and each of them counts from 8 to 11 fibrils 50-70 nm thick (Figs. 2-4). The cords are interspersed by superdense chromatin segments, the length of which can vary significantly; for example, in the Figs 2-4 the length of them ranges from 0.25 μm to 1.5 μm. The length of polynemic regions may also vary significantly and reach 5.5 μm. If one takes into account that the width of single sister fibrils is supranucleosomal (about 30 nm),which corresponds to a compaction factor of about 40 times (see review: Pienta and Coffey 1984), each sister fibril in the 5.5 μm long polynemic locus must be about 220 μm long, when recalculated for the double helical DNA.

Polynemic regions in the spread chromatin preparations sometimes seem to be torn across the long axis of



Figs. 2-5. Regions of polynemic structures detected in preparations of decondensed chromatin of the macronucleus isolated from starved *B. ovata*. Ribbon like polynemic cords consisting of densely packed parallel fibrils alternate with superdense segments (at arrowheads). The transformation of dichotomically branching two-chromatid DNP-fibril into a polynemic cord is shown at arrow in Fig 4. Bars - 0.5 μm

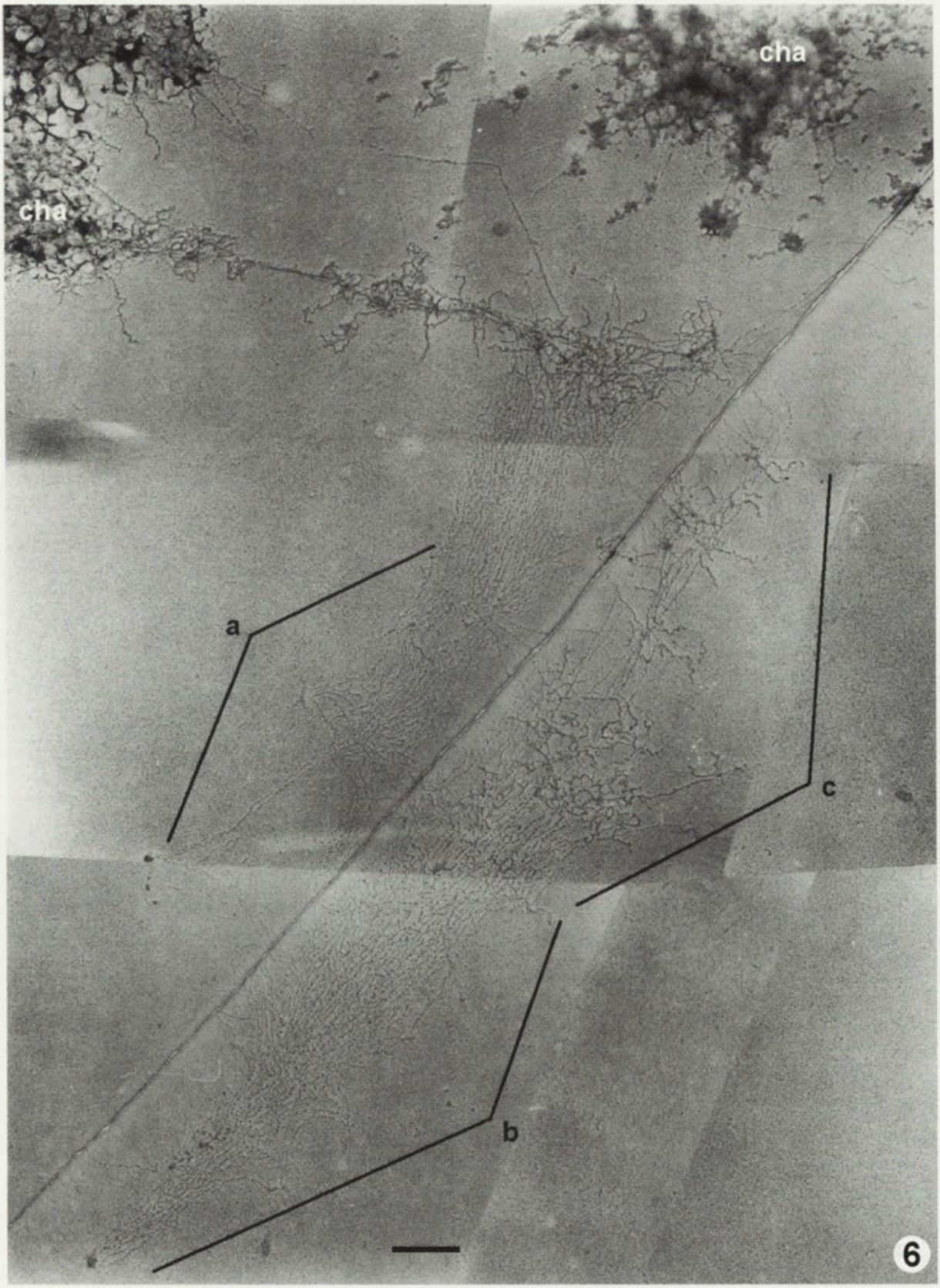


Fig. 6. Region of macronuclear chromatin of Ma isolated from fed *B. ovata*. Clearly are seen three polynemic loci (a,b,c) of a giant lateral loop formed by an axial chromatin fibril connecting two large chromatin agglomerations (cha). Bar - 0.5 µm

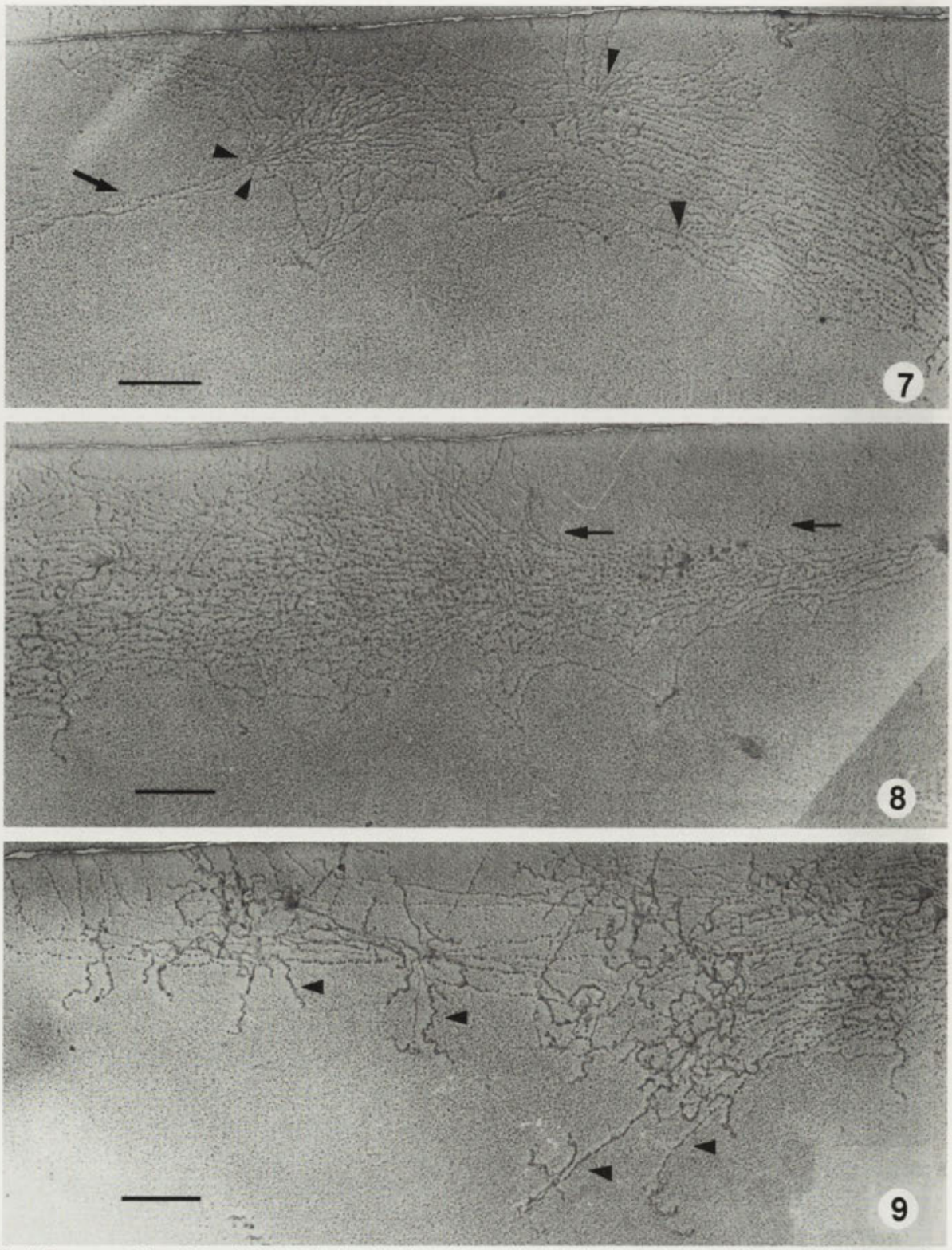
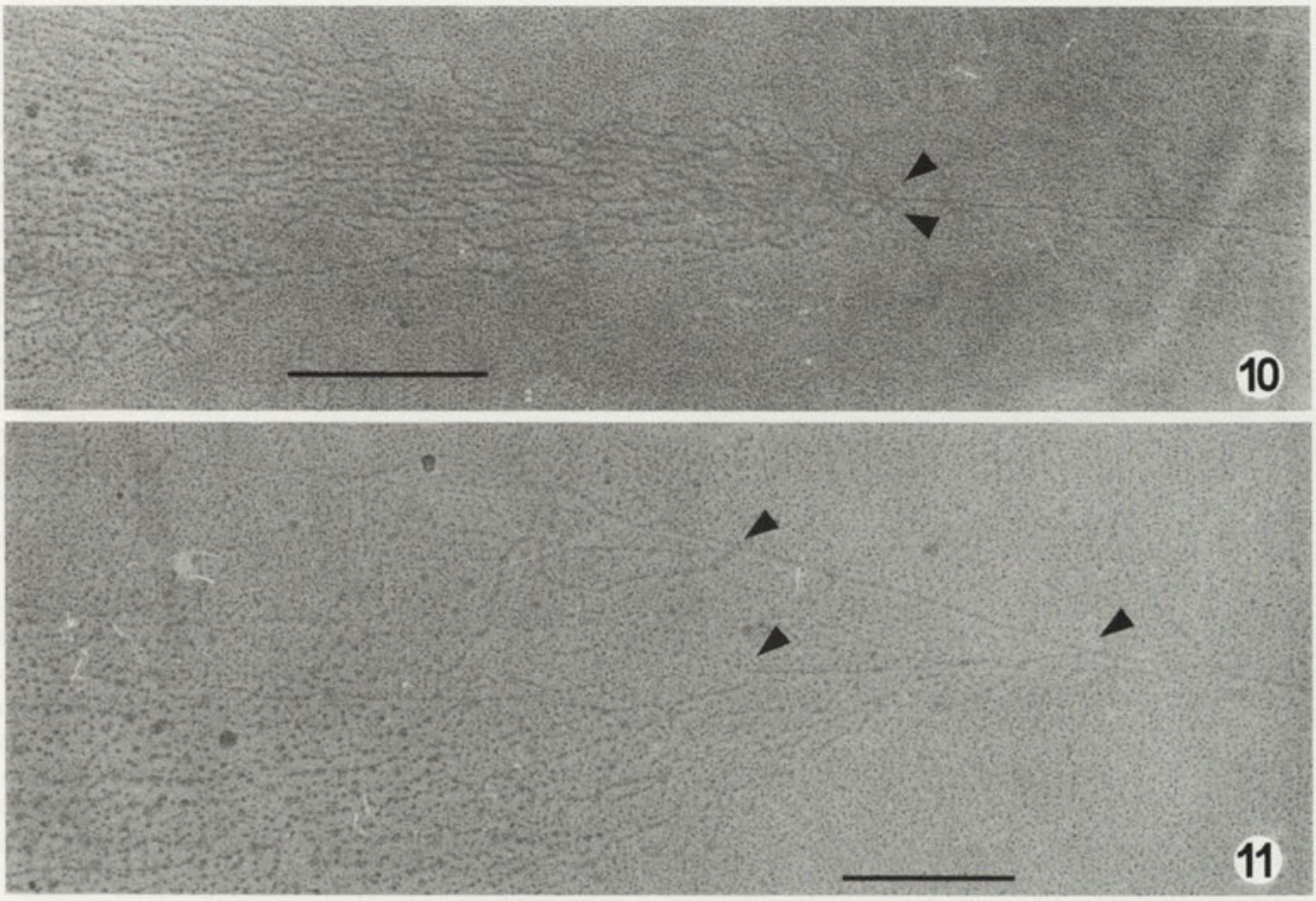


Fig. 7. Fragment of the polynemic locus (a) of the giant loop represented on Fig. 6. A two-chromatid DNP fibril (at arrow), from which the sister fibrils branch off forming a structure of "umbrella" or "fan" pattern. Primary and secondary branching points are shown by arrowheads. Bar - 0.5 µm

Fig. 8. Fragment of the polynemic locus (b) of the giant loop represented on Fig. 6. Polytene region with gradually increasing number of sister fibrils (at arrows). Bar - 0.5 µm

Fig. 9. Fragment of the polynemic locus (c) of the giant loop represented on Fig. 6. The lateral DNP loops diverging from parallel nucleosomal sister fibrils (at arrowheads). Bar - 0.5 µm

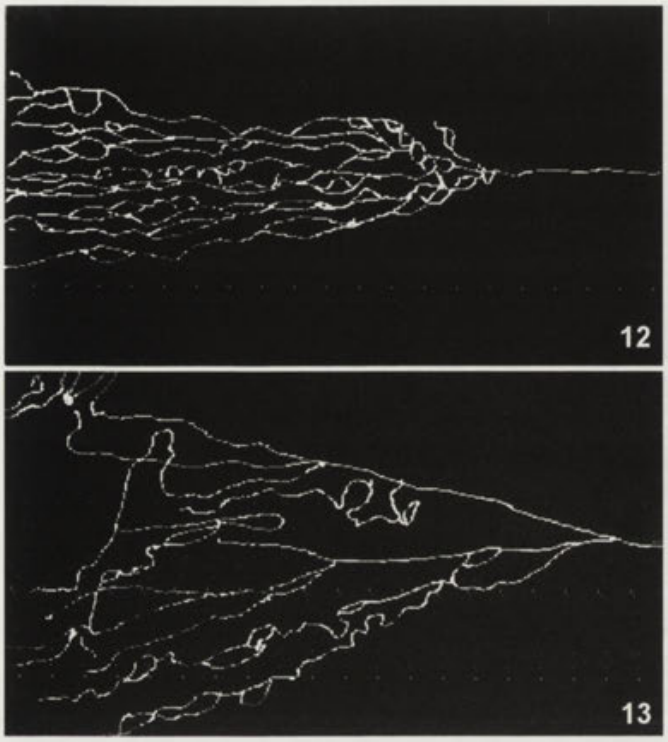


Figs. 10-11. Zones of transition from non-polynemic to polynemic regions (at arrowheads) with well expressed fan-like (Fig. 10) and dichotomic (Fig. 11) pattern of branching of chromatin fibrils. Bars - 0.5 µm

them. But we did not see the polynemic structures torn across the superdense regions. The Fig. 2 shows very well that several polynemic loci disposed between superdense regions of chromatin have a similar number of DNP threads. But in Fig. 3, a significant difference in the number of DNP fibers at the two sides of a superdense region can be seen. Only in rare cases, we observed direct transition of the polynemic cords into two fibrils in starved cells (Fig.4). Figure 4 clearly shows the dichotomic branching of a double-stranded fibril and the transformation of a dichotomic branching region into a polynemic cord about 0.5 µm thick. This fragment of polynemic structure is also peculiar in that one individual thin DNP thread is seen stretching both beside the polynemic regions (arrowhead) proper and along the superdense region connecting them. Continuity of the DNP fibrils in the superdense regions and in adjoining polynemic structures is still more clearly seen in Fig. 2. In this case the thickness of sister fibrils at the level of superdense loci does not differ from their thickness in polynemic regions.

Finally, in starved ciliates we have found polynemic cords with both non-parallel packing of chromatin threads 20 to 70 nm thick and with regions which in their structure resemble minibands and poorly puffed loci of classical polytene chromosomes of Diptera (Fig. 5). In such polynemic cords the individual threads are clearly seen on very short distances, or sometimes represented as aggregations of granules of different size (from 15-25 nm to 40-50nm). In these cases, single granules often contact very short fragments of threads 25 to 30 nm thick. The level of chromatin compactization can be different in identical regions of sister fibrils in such loose polynemic structures.

In starved *B. ovata* large aggregations of fibrils about 70 nm thick are often disposed in contact with polynemic cords very strongly resembling nucleoli in their structure. It can frequently be seen too, that nucleoli are connected to several sister fibrils of the polynemic cord, not to a single one. A detailed description of the structure of nucleoli on spread chromatin preparations of *B. ovata* will be given elsewhere.



Figs. 12-13. Computer images of chromatin fragments represented in Figs. 10 and 11, obtained by the image analyzer Magiscan 2 AR (Joyce-Loeble, England). The operations of the noise averaging, of the sharpening, of the finding edges and of the segmentation were made for improving images after enlargement

In fed *B. ovata*, the polynemic structures on spread preparations of the macronucleus look quite different in comparison with the starved cells. It should be noted also that the chromatin of fed individuals after a short-term spreading is mostly represented by clumpy or rosette-like and fine-fibrillar chromatin with numerous inclusions of nucleoli decompacted to a different extent, rather than by dense ribbon-like cords. Long electron-dense chromatin fibers and polynemic cords comparable with those represented in Figs. 2-4 are very rare in fed ciliates. In agglomerations of clumpy and fibrillar chromatin or on their margins, some polynemic structures can be seen. However, they differ in supramolecular organization from the polynemic loci of starved cells. Fig. 6 shows several polynemic regions of one giant microloop, formed by the axial chromatin fibril connecting two agglomerations of the clumped chromatin. In one of the polynemic segments of this giant loop (a), the sister fibrils in the transition point are organized into "fan" or "umbrella" like structures, i.e. it seems that numerous sister threads diverge from one point of a double-stranded fibril (Fig. 7). On some of the primary sister fibrils there are secondary, tertiary, etc. points of polytenization, also with a "fan"-like type of branching (Fig. 7, arrowheads). It seems that the localization of secondary and following "fan"-like structures on sister fibrils of the polynemic region is asymmetric. In an other polynemic region (b) of the same giant loop, the DNA strands exhibit a dichotomic branching pattern, producing the impression of gradual increase in polyteny (Fig.8). Thus, in the chromatin loop, about 20 μm long,represented in Fig. 6, there are polynemic regions with both fan-like and dichotomic type of organization of sister fibrils. In both cases nucleosomal packing appears only at some distance from the point of transition of a non-polynemic region into a polynemic one. The polynemic segment (c) of the giant loop, represented in Fig. 9, has a large number of lateral mini-loops and thick fibers diverging from sister fibrils. Some of these lateral fibers, at higher magnifications, represent nucleosomic loops coiled upon itself. Such loops are about 20 nm to 35 nm thick, sometimes granules can be seen attached to them, the size of single or double nucleosomes. The sister fibrils, from which lateral fibrils diverge, also show a nucleosomal structure. Figs. 10 and 11 represent transitory zones of the polynemic regions that are not segments of the above-mentioned giant loop but parts of a very long polynemic region of a macronucleus from fed *B. ovata*. In these zones one can also see two types of branching pattern: the fan-like branching (Fig.10) and the typical dichotomy (Fig.11). For receiving additional information about the types of branching pattern, we obtained a computer image of these polynemic regions on the image analyzer Magiscan-2AR. The computer images are given in Figs. 12 and 13, accordingly. The computer images show only a part of the fibers to facilitate the analysis of their arrangement. We found numerous forks and "eyes"of replication in transitory zones of both types of polynemic segments.

DISCUSSION

We consider the local polynemic structures present in the chromatin of differentiated macronuclei of vegetative *B. ovata* as a particular case of the general phenomenon of polyteny. On the basis of the data on polyteny available in the literature (reviews: Beermann 1972, Korge 1987, Zhimulev 1992), the type of polyteny that we have revealed in differentiated somatic nuclei of *B. ovata* should be considered as hidden local polyteny, since it can only be detected by electron microscopy. Moreover, the structure of polytene loci of differentiated

macronuclei of *Bursaria* allow to attribute them to polytene structures of a type other than the classical polytenic chromosomes of Diptera as well as the giant chromosomes in the macronuclear anlagen of hypotrichids (reviews: Raikov 1982, Ammermann 1987, Zhimulev 1992). Polytene structures in differentiated macronuclei of *B. ovata* should undoubtedly be attributed to the non-classical type. But polytene loci of *B. ovata* are not similar in structure with non-classical polytene (oligotene) chromosomes of some plants (review: Nagl 1981). We suggest that in the differentiated macronucleus of vegetative *Bursaria* presents another type of non-classical polyteny, perhaps specific for polyploid differentiated somatic nuclei of ciliates. We must to remark that the fine structure of the oligotene chromosomes of developing macronuclear anlagen of *Bursaria* remains unknown.

Analysis of DNA replication and determination of DNA content of nuclei using autoradiographic and cytophotometric methods have shown that the polytene chromosomes of many Diptera are regionally underreplicated (reviews: Sorsa 1976, Laird 1980, Korge 1987, Zhimulev 1992). There is a well proved hypothesis that the giant chromosomes in developing macronuclear anlagen of some ciliates are products of unequal replication (Ammermann 1987). Local polynemy found in somatic polyploid nuclei of vegetative *B. ovata* is the first case of electron microscopic visualization of disproportionally replicated regions in differentiated macronuclei of ciliates. To the best of our knowledge polyteny and polyploidy are interconnected phenomena. Both polyteny and polyploidy perform comparable functions in the cell - the enlargement of genetical material. A strong similarity in the ultrastructural organization of many polyploid macronuclei of ciliates (reviews: Raikov 1982) with the macronucleus of the genus *Bursaria* permits us to believe that it is worthwhile to look for hidden polyteny in differentiated somatic nuclei of other ciliate species too.

It should be pointed out that the polytene loci detected in macronuclei of *B. ovata* are characterized by a low level of polyteny; they should rather be called oligotene loci. Low level of polyteny has also been previously supposed for the macronucleus of *B. truncatella* (Sergejeva 1977). As has been shown earlier, the DNA amount in the differentiated macronucleus of *B. truncatella* is more than 2600 times higher than in the micronucleus (Ruthmann 1964), and the quantity of DNA in the macronucleus of this species reaches 38.000 pg (Winkler et al. 1982). It is also known that 50% of DNA in the differentiated macronucleus of *B. truncatella* is represented by repetitive sequences (Borchsenius and Sergejeva 1979). Since the average size of vegetative cells of *B. ovata* exceeds that of *B. truncatella* at least 1.5 times (with a proportional increase in macronuclear size), it is reasonable to assume that the average DNA content and repetition of polytene structures in the macronucleus of *B. ovata* described above can even be higher than in the macronucleus of *B. truncatella*. However, more experiments and alternative approaches are necessary to prove that.

As it is shown above, the polytene loci in macronuclei from fed and starved *B. ovata* essentially differ in their structure. The thickness of the sister fibers in polytene loci from starved animals is about 30 nm or between 50 and 70 nm. Hence, we can suppose that these polytene loci contain the histone H1 (reviews: van Holde 1988, Georgiev 1989) and they are not active transcriptionally.

The polytene loci from macronuclei of fed *B. ovata* are formed by nucleosomic and by thinner non-nucleosomic fibers. Moreover, some of these loci contain numerous forks and "eyes" of replication. Hence polytene loci in fed *B. ovata* are more active at least in replication than the ones from starved animals. The arrangement of the replication forks and "eyes" along these polynemic structures lead us to suppose that the two types of branching pattern reflect temporal differences in the replication rate in different parts of the macronuclear genome. The dichotomic pattern would be due to a stepwise replication whereas the fan-like branching is an extremely "steep" dichotomy, reflecting a more rapid rate of replication. A part of polynemic loci in macronuclei of fed *B. ovata* with secondary, tertiary, ets., points of polytenization may be apparent ones and only reflect the asynchrony of reduplication of sister fibrils in the amplified regions of chromatin.

We should like to emphasize that only the method of chromatine spreading has given us the possibility to identify the hidden locally limited polyteny of the non-classical type in the differentiated polyploid macronucleus of vegetative cells of *B. ovata* and to observe distinct differences in structural organization of these hidden polytene loci in fed and starved animals.

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Characterization of Low and High Molecular Weight DNA in the Macronucleus of the Ciliate *Chilodonella steini*

Günther STEINBRÜCK[1], Stefan RADZIKOWSKI[2], Małgorzata GOŁEMBIEWSKA-SKOCZYLAS[2] and Beata SAPETTO-REBOW[2]

[1]Zoologisches Institut, Abteilung Zellbiologie, Universität Tübingen, Tübingen, Germany; [2]Department of Zoology, Institute of Zoology, Warsaw University, Warszawa, Poland

Summary. The macronucleus of the ciliated protozoan *Chilodonella steini* (*Trithigmostoma steini*) is heteromeric, consisting of regions with different chromatin organization. Filter hybridization experiments with α-tubulin and histone H4 gene probes showed that the protein-coding sequences are found in the low molecular weight fraction of *Chilodonella* macronuclear DNA. From this we conclude that the macronucleus of *C. steini* consists in part of short gene-sized DNA pieces, which is very similar to the genome organization known from hypotrichous ciliates. Using in situ hybridization, we have confirmed that the low molecular weight DNA occurs in the outer zone - the "orthomere", and in the "endosome" of the heteromeric macronucleus.

Key words. Ciliates, *Chilodonella steini*, heteromeric macronuclei, gene-sized DNA fragments, in situ hybridization.

INTRODUCTION

Genome organization in certain ciliates is fundamentally different from that found in other eukaryotic organisms. Every ciliate has two kinds of nuclei: one or more small generative micronuclei with high molecular weight DNA, organized in chromosomes, and one or more vegetative macronuclei which show an unusual chromosomal organization in some groups of ciliates. The most extraordinary form of macronuclear genome organization is found in hypotrichous ciliates where DNA occurs in the form of short gene-sized pieces (for review see Steinbrück 1990).

The object of our study, *Chilodonella steini,* has one generative and one vegetative nucleus. The macronucleus belongs to the heteromeric type (Radzikowski 1965, 1973, 1976, 1985, 1990; Raikov 1982). In nuclei of this type, two spheres can be discerned: an outer "orthomere" with late replicating chromatin, and an inner "paramere" with early replicating chromatin (Radzikowski 1976, 1979, 1985).

The formation of a new macronucleus is a complex sequence of events, occurring after the sexual process (conjugation). These events include formation of polytenic chromosomes, their fragmentation, and elimination of about 35% of the total chromatin content of the anlage (Radzikowski 1973). As repeatedly

Address for correspondence: S. Radzikowski, Institute of Zoology, University of Warsaw, Krakowskie Przedmieście 26/28, PL - 00-927/1 Warszawa, Poland; Fax: (4822) 26-86-24; E-mail: ZOOL@PLEARN

reported, the mature macronucleus contains only chromosome fragments (Radzikowski 1973, 1977, 1979).

This unusual macronuclear organization is convenient for studies of the genome, enabling experimental approaches to the analysis of different fractions of the macronuclear DNA in particular.

Metenier and Hufschmid (1988), and Lahlafi and Metenier (1991) have shown that macronuclear DNA of the ciliate *Chilodonella cucullulus,* a sibling species of *C. steini,* consists of 70-80% of low molecular weight DNA.

Our investigations (Radzikowski and Steinbrück 1990) showed that labelled rDNA genes hybridize to a single size class of low molecular weight DNA in *C. steini.*

The aim of this investigation was to characterize the low molecular weight fraction of macronuclear DNA, and in particular to establish whether protein-coding genes, e.g. histone H4 and α-tubulin genes, occur in the form of short gene-sized DNA molecules. We were also interested to see whether this size class of DNA molecules is confined to specific regions of the heteromeric macronucleus.

MATERIALS AND METHODS

Cultivation

A clone of *Chilodonella steini (Trithigmostoma steini),* immature for conjugation, was used. The methods of cultivation were described earlier (Radzikowski and Gołembiewska-Skoczylas 1977).

Isolation of nuclei

Cells were collected by low speed centrifugation (5 min, 1000 x g). Concentrated cells were lysed at 4°C in an equal volume of a solution containing 0.1% Triton X-100 and 200 mg/l spermidine-HCl. Nuclei were further purified by centrifugation through a 2M sucrose layer (10 min, 1.000 x g, 4°C). Fractions of parameres were obtained by shaking nuclei with glacial acetic acid for few seconds and putting them on the slide covered with a mixture of ethanol/acetic acid (3:1).

In situ hybridization

For *in situ* hybridization, samples of the low and high molecular weight DNA fractions were labeled with ^{3}H-TTP by the random primed DNA labeling method (Feinberg and Vogelstein 1983). The specific activity of the labeled probes was 6 x 10^6 cpm/μg as determined by liquid scintillation counting.

Purified nuclei of *C. steini* were spread onto microscope slides, fixed in a solution of ethanol/ acetic acid (3:1) for 20 min and dried. Denaturation of the nuclear DNA was performed by incubation of the slides in 0.07N NaOH for 1.5 min (see Radzikowski and Steinbrück 1990), followed by dehydration in a series of ethanol (50, 70, 96%) and drying in a desiccator.

The hybridization reaction was carried out in 10 μl hybridization solution under a siliconized 18 x 18 mm coverslip for 20 h at 37°C in a moist chamber.

The hybridization solution contained 50% formamide, 2 x SSC, 200 μg/ml denatured salmon sperm DNA as carrier and 50.000 cpm ^{3}H-labeled DNA per slide. Just before adding the solution to the slide the mixture was heated to 70°C and quickly cooled down. After hybridization the slides were washed in four changes of 2 x SSC for 30 min at room temperature. Finally, the slides were dried and covered with Ilford 5KD liquid emulsion, exposed for 6 weeks and then developed.

Electron microscopy preparation

Ciliates were fixed according to Shigenaka et al. (1973). Fixing time was 20 min at approximately 22°C. The material was embedded in Epon 812. Sections were stained with uranyl acetate and examined using a Siemens Elmiscop electron microscope.

Fractions of high and low molecular weight DNA were spread for electron microscopy according to the microversion of the method of Lang and Mitani (1970).

DNA isolation and fractionation

DNA was isolated from concentrated living cells or from cells fixed in 70% ethanol essentially as described earlier (Steinbrück et al. 1981).

Carefully isolated DNA was fractionated according to size by electrophoresis in a 1% low melting agarose gel. After electrophoresis the DNA was stained with ethidium bromide in the gel. In order to get a low molecular weight fraction which is essentially uncontaminated by breakdown products of the high molecular weight fraction, we separated the DNA molecules into three fractions (see Fig. 1): a high molecular weight fraction with molecules larger than 5 Kb, a medium-sized fraction with molecules ranging from 2 Kb - 5 Kb, and a low molecular weight size class containing the molecules smaller than 2 Kb. The three fractions were cut out of the low melting gel, and the agarose then melted at 65°C for 10 min. The agarose was removed from the liquefied solution by digestion with the enzyme gelase (0.2 units gelase per 100 mg gel, 2 h at 40°C in gelase reaction buffer). The DNA was recovered by ethanol precipitation.

Filter hybridization experiments

Samples of purified DNA of *Stylonychia lemnae* and *C. steini* were loaded onto a 1% agarose gel and separated according to size by electrophoresis at 50 V for about 6 h. DNA of the phage lambda, digested with the restriction enzymes Eco RI and Hind III, was co-electrophoresed in one lane of the gel as a size marker. After electrophoresis the DNA was transferred to a nylon membrane (Pall Biodyne B) by capillary transfer (Southern 1975). The transferred DNA molecules were fixed to the membrane by UV irradiation (UV crosslinker Fluo-Link from Biometra, 0.375 J/cm^2).

Gene-sized DNA fragments containing known protein-coding sequences were detected in hybridization experiments using digoxigenin-labeled gene probes. A cloned α1-tubulin gene of *S. lemnae* (Helftenbein 1985) and a cloned histone H4 gene (clone H4K, Wefes

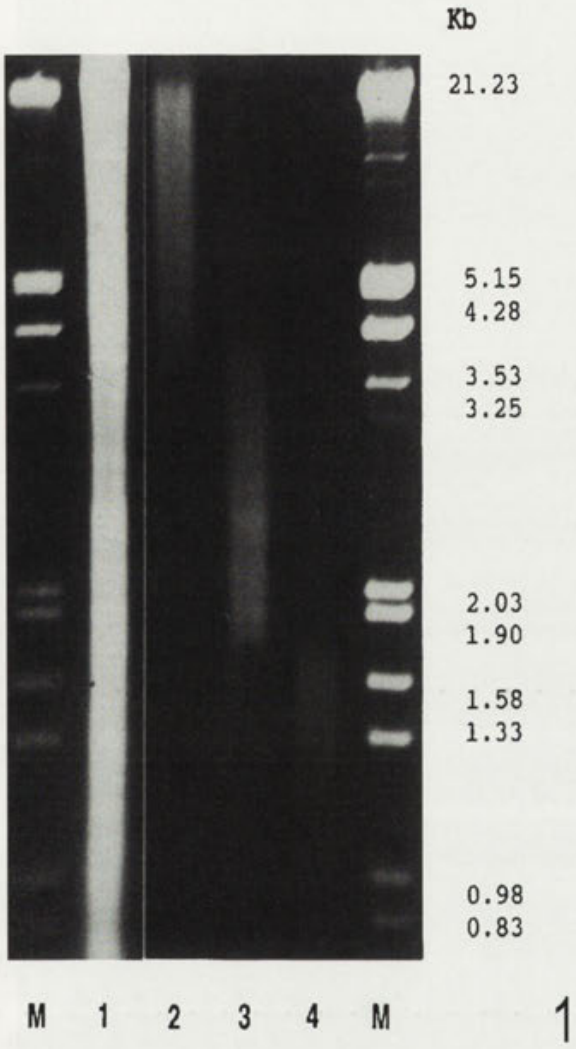


Fig. 1. Fractionation of *Chilodonella* DNA; lane 1 - unfractionated total DNA of *C. steini*, lane 2 - high molecular weight fraction (> 5 Kb), lane 3 - medium-sized fraction (2Kb - 5 Kb), lane 4 - low molecular weight fraction (< 2 Kb), M - size markers (phage lambda DNA digested with the restriction enzymes Eco RI and Hind III)

and Lipps 1990) of *S. lemnae* were used as probes. The cloned genes were labeled by random primed incorporation of digoxigenin-labeled dUTP using the DNA Labeling and Detection Kit of Boehringer Mannheim. Hybridizations were done in two steps. In a first round, the dig-labeled histone gene probe was hybridized to the membrane. After visualization of the hybridization signals the hybridized molecules were melted off by incubating the membrane in 0.1 x SSC, 1% sodium dodecylsulfate for 30 min at 96°C, and then washing the membrane several times in the same solution at 65°C and finally in distilled water. Then, a second hybridization was carried out with the dig-labeled α-tubulin gene and with dig-labeled DNA of phage lambda in one reaction mixture. The conditions of the hybridization reactions and signal detection procedure were as suggested in the Boehringer manual, except that the hybridization temperature as well as the temperature of the stringent washing step was 60°C. The detection of the hybridization signals was performed using anti-digoxigenin antibodies conjugated with alkaline phosphatase and the chemiluminescent substrate Lumigen PPD (Boehringer) for the phosphatase reaction. Finally, the membrane was exposed to Kodak X-OMAT S X-ray film for 30 min.

RESULTS

Ultrastructure of the macronucleus

The macronucleus of *C. steini* is of the heteromeric type, consisting of two discernible spheres: an outer "orthomere" and an inner "paramere" together with a centrally located "endosome" (Fig. 3). This unique configuration develops within two hours after cell division (Radzikowski 1985, Radzikowski and Steinbrück 1990).

Figure 2 shows an electron micrograph of a fragment of a macronucleus less than two hours after cell division. In the "orthomere" sphere, electron-dense structures can be seen, corresponding to chromatin grains (Chr). The inner electron-light area corresponds to the inner sphere "paramere". In this stage, no "endosome" is detectable, as this develops in a later stage of the cell cycle.

Two hours after cell division, both spheres of the heteromeric nucleus are fully developed (Figs. 2, 3); moreover, underneath the nuclear envelope, electron-dense granules can be seen, whose ultrastructure corresponds to nucleoli (Nu) (Fig. 3). In addition, in the central region of the "paramere", a condensation of electron-dense material forms the "endosome". Linearstructures (Lin) of medium electron density can be seen "paramere" at any time (Figs. 2, 3).

Evidence for gene-sized DNA fragments in the macronuclear genome of *C. steini*

In order to test whether in *C. steini* genome the DNA is organized in a similar manner as in the genomes of hypotrichous ciliates, we performed filter hybridization experiments with digoxigenin-labeled gene probes. It is well known that macronuclear genomes of hypotrichous ciliates consist of short gene-sized DNA fragments. These fragments carry the coding region for one protein in most cases, flanked by short non-coding regions. All molecules are terminated by short identical telomere sequences (for review see Steinbrück 1990).

Since micronuclear DNA of all ciliate species investigated so far is of high molecular weight and since the

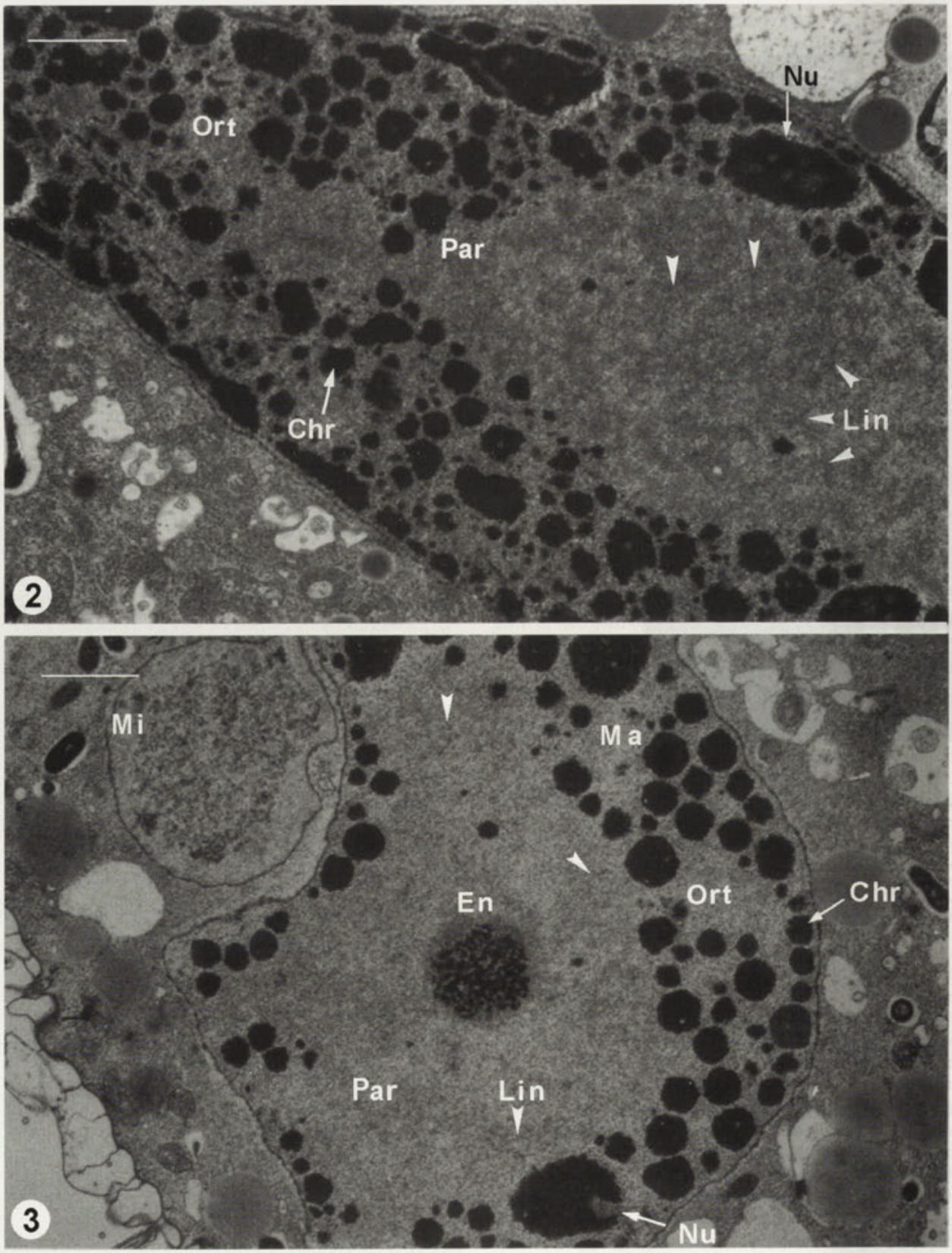


Fig. 2, 3. Electron micrographs of macronuclei of *C. steini;* 2 -0-2 h after division, 3 - more than 2 h after division. Bar - 0.6 μm (x 30 000). Abbreviation: En - endosome, Nu - nucleoli, Ort - orthomere, Par - paramere, Chr -chromatin granules, Lin - linear structures, Ma - macronucleus, Mi - micronucleus

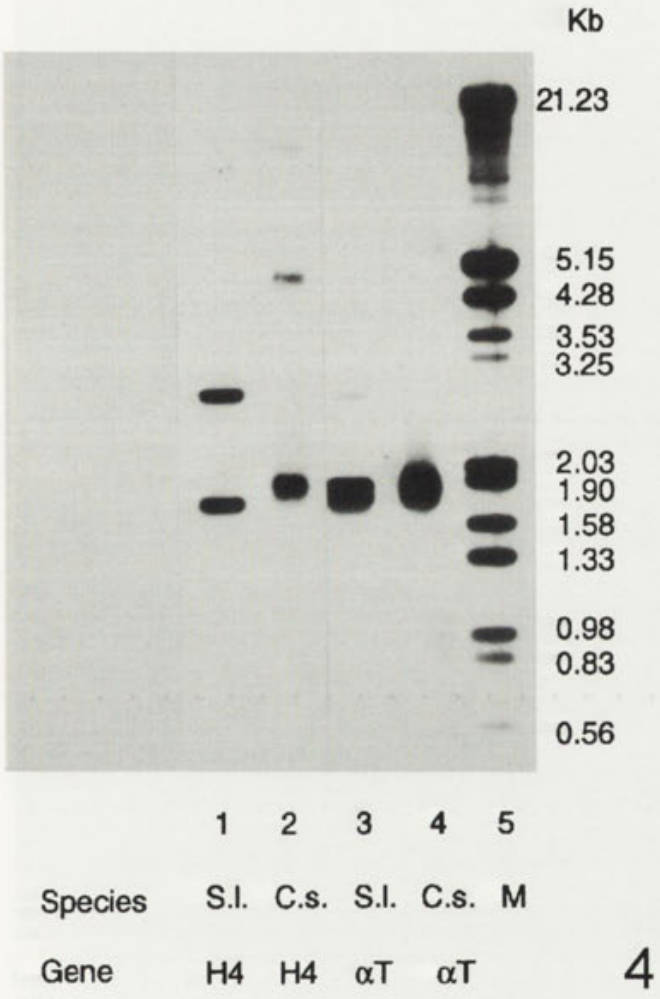


Fig. 4. Evidence for gene-sized DNA fragments carrying protein-coding genes in *C. steini* genomes. 10 µg DNA of *C. steini* (C.s.) and for comparison 2 µg DNA of the hypotrichous ciliate *Stylonychia lemnae* (S.l.) were separated according to size in a 1% agarose gel. After electrophoresis, the DNA was transferred to a nylon membrane by Southern blotting. The membrane-bound DNA molecules were hybridized first with a digoxigenin-labeled a-tubulin gene probe (aT) and in a second reaction with a dig-labeled histone H4 gene probe (H4). Both gene probes show strong hybridization signals on the *Chilodonella* DNA. The hybridizing fragments in *Chilodonella* DNA are of similar size as the signals on DNA of the hypotrichous species *S. lemnae*. M size marker as in Fig.1

amount of micronuclear DNA is much lower than that of macronuclear DNA, the hybridization signals should preferentially reflect the organization of the macronuclear genomes. High molecular weight native micronuclear DNA does not migrate far in a 1% agarose gel, and, therefore, will stay on top of each lane. On the other hand, if the macronuclear DNA consists of gene-sized DNA molecules, these molecules should be separated according to size over the whole length of the gel, as known from hypotrichous ciliates.

The hybridization signals shown in Fig. 4 clearly demonstrate that in *C. steini* a similar genome organization is found as in the macronuclear genome of the hypotrichous species *Stylonychia lemnae*. The hisone H4 gene probe hybridizes with fragments of 1.8 Kb, 6.3 Kb, and about 13.5 Kb of the *Chilodonella* DNA. Since the larger fragments of 6.3 Kb and 13.5 Kb show weaker signals than the 1.8 Kb fragments there may be either differences in the underlying copy numbers, or the sequence divergency towards the labeled probe may be different. The size of the 1.8 Kb fragments is very similar to the size of the 1.65 Kb histone H4 gene fragment of the hypotrichous ciliate *S. lemnae*. The α-tubulin gene probe gives a prominent signal at about 1.75 Kb in the hybridization reaction with *Chilodonella* DNA. The signal obtained with *S. lemnae* DNA consists of two closely spaced bands of 1.8 Kb and 1.65 Kb as can be seen more clearly on gels with different agarose concentrations and under different running conditions (not shown). We were not able to further resolve the single band shown in the *Chilodonella* lane by modifying the agarose concentration of the gels or the running conditions. Therefore, we assume that macronuclear DNA of *C. steini* contains a single size class of about 1.75 Kb molecules with α-tubulin coding sequences.

In situ hybridization experiments

In situ hybridization experiments were performed with low and high molecular weight fractions of *C. steini* DNA. In order to prevent DNA breakage during fractionation as far as possible, we used the gelase-digestion procedure for recovering DNA fractions from low melting point agarose gels. Since even in the most careful isolation and fractionation procedures some breakage of large DNA molecules cannot be ruled out completely, we collected three fractions from the gel (Fig. 1) in order to minimize contamination of the low molecular weight fraction with breakdown products of the high molecular weight fraction. The medium-sized fraction, as well as the low molecular weight fraction, consist preferentially of gene-sized DNA molecules, as shown by the filter hybridization experiments. However, the medium-sized fraction might be contaminated by a small percentage of broken molecules of the high molecular weight fraction. Therefore, only samples of the low and of the high molecular weight fractions were used for in situ hybridizations. Macronuclei isolated within a period of 0-2 h after division showed a strong labeling over the "orthomere" and a very weak signal over the "paramere"

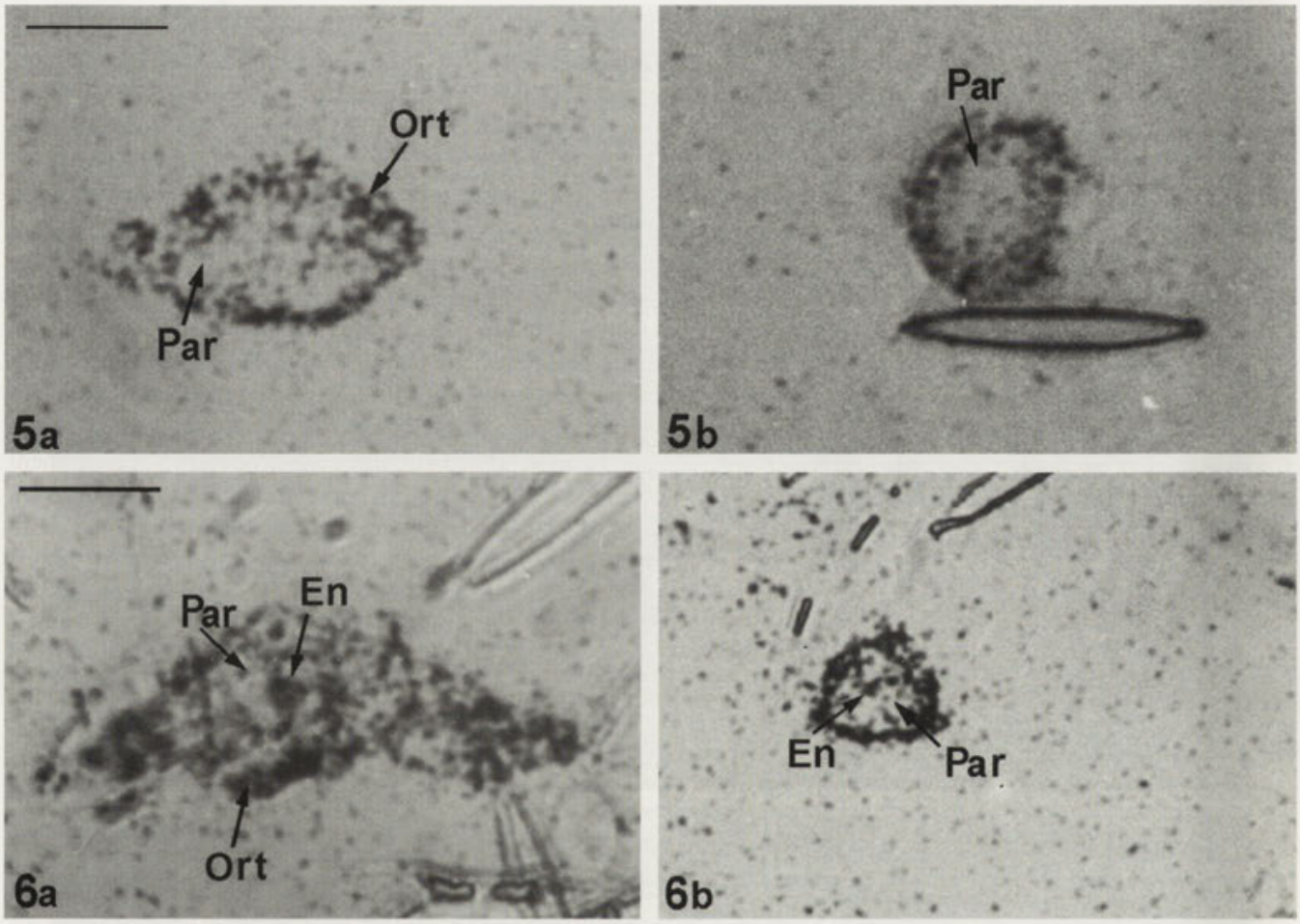


Fig. 5. In situ hybridization of low molecular weight DNA of *C. steini* with whole macronuclei (a) or isolated parameres (b), which were isolated 0-2 h after division. Ort - orthomere, Par -paramere. Strong labeling can be seen over the orthomere, weak labeling over the central part of the paramere. Signals over the edge of the isolated paramere probably result from slight contamination with orthomere DNA. Bar - 6 µm (x 3 200)
Fig. 6. In situ hybridization of low molecular weight DNA of *C. steini* with whole macronuclei (a) or isolated parameres (b), which were isolated more than two hours after division. En - endosome, Ort - orthomere, Par - paramere. Strong labeling over the orthomere and over the endosome can be seen. Bar - 6 µm (x 3 200)

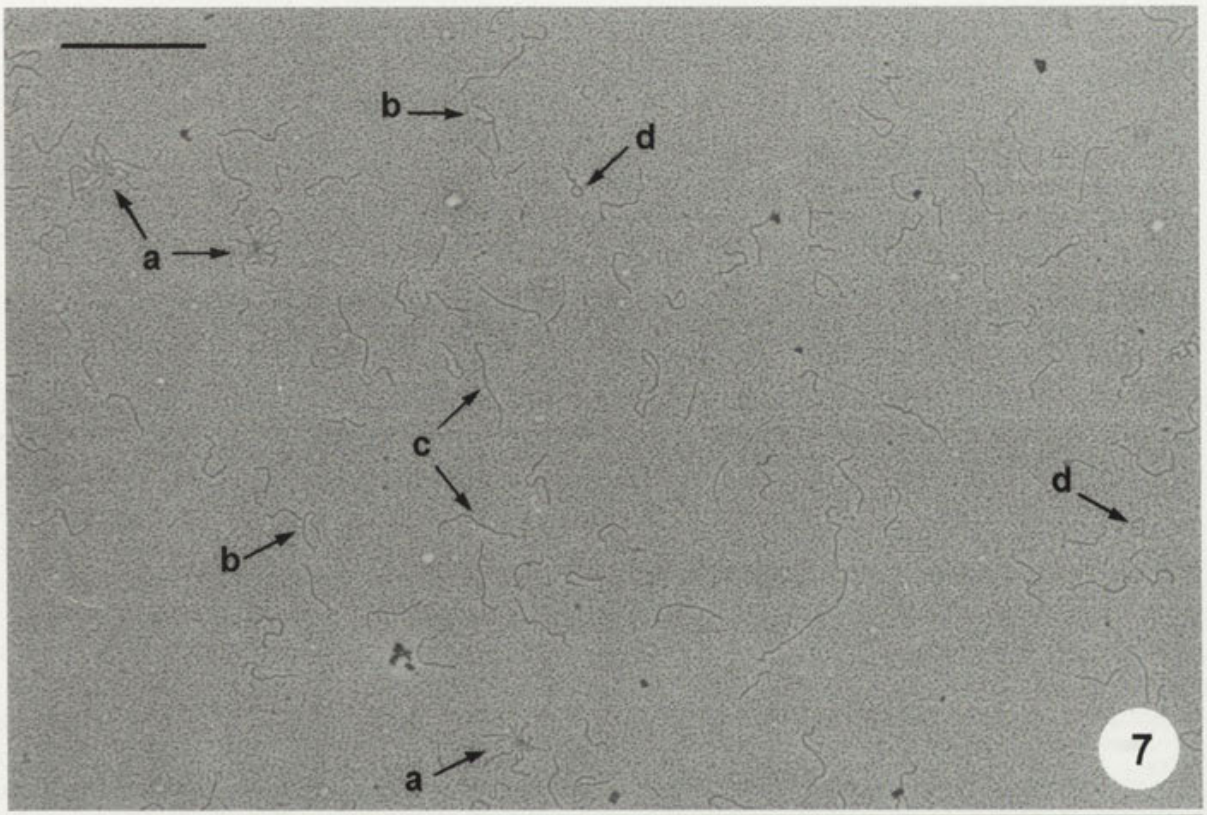
after in situ hybridization with low molecular weight DNA (Fig. 5a). Two hours after division, macronuclei showed a very intense labeling of the "orthomere" and the "endosome", and a much weaker labeling in the "paramere" (Fig. 6a). The localization of low molecular weight DNA was also analyzed using isolated "parameres", obtained from nuclei isolated within two hours after division (Fig. 5b), and from nuclei isolated more than two hours after division (Fig. 6b). In "early" nuclei, there was a weak labeling over the middle region of the "paramere". In the "late" nuclei, the "endosome" was clearly labeled. In both cases, an intense labeling was observed on the periphery.

In situ hybridization reactions of whole macronuclei and isolated "parameres" with high molecular weight DNA did not show any labeling in any of the spheres above background intensity (not shown).

Electron microscopic analysis of high and low molecular weight DNA fractions

DNA of the low and high molecular weight fractions was spread for electron microscopic analysis. The low molecular weight fraction showed short molecules ranging in size from a few hundred basepairs up to about 3.5 Kb with a majority of about 1.8 Kb molecules as expected from the fraction used. Most of the molecules are linear - c (Fig. 7); a few forked structures - b (Fig. 7) and rarely circular DNA molecules - d (Fig. 7) could also be found. In some cases, rosettes of DNA molecules could be seen containing a central origin, to which one or (less frequently) both ends of DNA molecules are bound - a (Fig. 7).

Spread preparations of the high molecular weight fraction showed much larger DNA molecules (Fig. 8). The largest molecules found are more than 20 Kb in length.



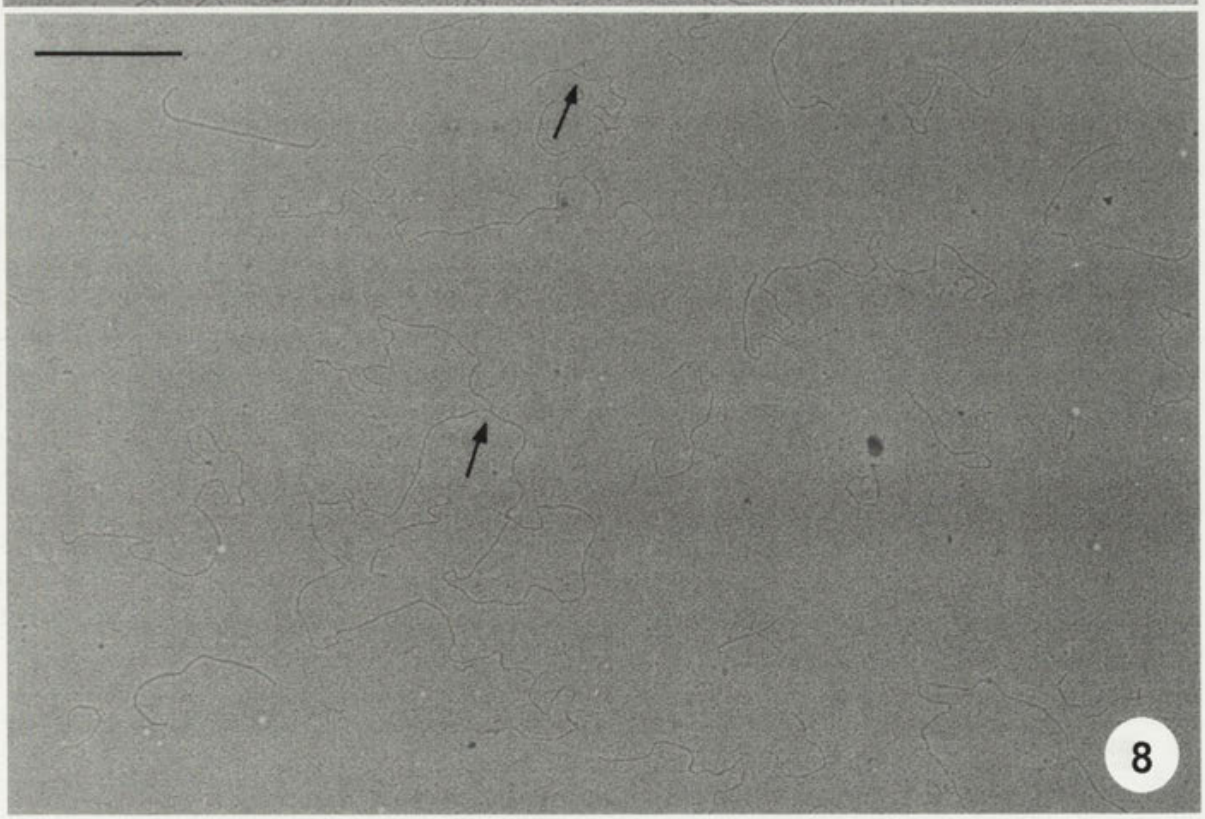


Fig. 7. Electron micrograph of spread DNA preparation. Low molecular weight fraction of *C. steini* DNA. Forms of gene-sized DNA fragments: a - rosettes, b -replication forks, c - linears, d - circles. Bar - 1 µm (x 20 000)
Fig. 8. Electron micrograph of spread DNA preparation. High molecular weight fraction. Molecules larger than 20 Kb can be seen. Replication forks can be found also in this fraction (arrow). Bar - 1 µm (x 20 000)

Also in this fraction putative replication forks could be found (arrows).

DISCUSSION

Cytological studies on ciliates belonging to the Hypotricha, initiated in the 1960's by Golikova (1964), Alonso and Pérez-Silva (1965), Ammermann (1965), Kloetzel (1970), and on ciliates belonging to the Gymnostomata (Radzikowski 1967, 1973) considerably changed our views on the structure of micronuclei and macronuclei. Studies on the development of the anlage of the new macronucleus established the existence of polytenic chromosomes, their fragmentation, and the elimination of large amounts of chromatin into the cytoplasm. These results formed the basis for the hypothesis of a non-chromosomal genome organization in macronuclei of those groups of ciliates.

Intensive studies employing molecular biology techniques centered on hypotrichous ciliates. They established that their micronuclear genomes are characterized by high molecular weight DNA and conventional chromosomal organization. Macronuclear DNA, in contrast, consists mostly of short, single gene-sized fragments, an organization not observed in any other organism (for reviews see Raikov 1982, Prescott 1983, Steinbrück 1986).

Investigations on the molecular genome structure of gymnostomous ciliates by Metenier and Hufschmid (1988) and Lahlafi and Metenier (1991) confirmed the earlier hypothesis of non-chromosomal genome organization in macronuclei of *C. cucullulus* (Radzikowski 1973, 1979). These authors found that 70-80% of macronuclear DNA belongs to a low molecular weight fraction. By investigating DNA of *C. steini,* a sibling species of *C. cucullulus*, Radzikowski and Steinbrück (1990) found that labeled rDNA probes hybridized to a single size class of macronuclear DNA molecules when separated by gel electrophoresis. By in situ hybridization experiments these authors showed that rDNA hybridizes to DNA of the outer zone, the "orthomere", and to the central "endosome" of the heteromeric macronucleus.

In this paper we have demonstrated that a low molecular weight DNA fraction hybridizes preferentially with the "orthomere" and "endosome" regions of the macronucleus of *C. steini.* Much less labeling is observed in the "paramere" region (see Figs. 5b, 6b). We conclude from these hybridization patterns that the low molecular weight DNA is mainly present in the "orthomere" and "endosome", and that the weak label over the "paramere" might result from a transfer of low molecular weight DNA from the "orthomere" through the "paramere" into the "endosome". Intense labeling over the periphery of isolated "parameres" seems to be due to remnants of "orthomere" chromatin, since both regions cannot be separated into absolutely pure fractions.

The analysis of the low molecular weight fraction of *C. steini* DNA using an electron microscopic spreading technique supports our hypothesis that the DNA of the heteromeric macronucleus consists in part of short linear fragments with an average size of about 2 Kb, substantiating the non-chromosomal character of macronuclear genome organization. The majority of the molecules are linear forms, but quite a number of forked and circular forms, and some rosette structures, could also be found. Studies on hypotrichous ciliates (Prescott et al. 1971, Ammermann et al. 1974, Swanton et al. 1980) have demonstrated that the macronuclear DNA of these ciliates exists as short linear molecules, as documented by sedimentation analysis, electron microscopy, and agarose gel electrophoresis. In *Oxytricha nova*, macronuclear DNA molecules range in size from 0.4 to 20 Kb (Prescott 1983). Similar values were reported for a number of other hypotrichous species (Swanton et al. 1980, Steinbrück et al. 1981). Replicating forms of DNA molecules were isolated from *O. nova* macronuclei containing replication bands (Murti and Prescott 1983), from *Euplotes eurystomus* (Allen et al. 1985), and from *Stylonychia lemnae* (Steinbrück 1990). These authors showed that replication is initiated at one or both ends of the linear molecules. Although we found only single-fork replicating forms in *C. steini*, we cannot exclude the possibility that origins of replication occur, at both ends of the linear molecules of *C. steini.* Time course studies of replicating macronuclear DNA molecules should answer this question.

In macronuclei of *C. steini* chromatin granules (Chr) of unknown function are visible on electron micrographs (Figs. 2, 3). In spread preparation of macronuclear DNA, rosette structures - a (Fig. 7) can be found, which might originate from the chromatin granules. Klobutcher and Prescott (1986) noticed similar structures in *Holosticha sp.* and judged them to result from proteins bound to the telomeres and aggregations of protein-telomere complexes with each other. Their interpretation was corroborated by the finding that extensive protease digestion destroyed the rosettes, leaving free, separate DNA molecules. In

macronuclear DNA of hypotrichous ciliates, all gene-sized DNA fragments are terminated by short identical telomere sequences (for reviews see Sundquist and Klug 1989, Orias and Higashinakagawa 1990, Steinbrück 1990, Kang et al. 1992). Lipps (1980) gives the following interpretation for the existence of circular structures in macronuclear DNA of the hypotrichous species *Stylonychia mytilus:* At sufficiently high concentrations the gene-sized DNA molecules form circles by end-to-end aggregation of the 16-bases single-stranded termini of the telomeres. Aggregation could conceivably occur by GT base pairing of the single-stranded G_4T_4 tails. Thus, the presence of rosettes and circles could be an indirect evidence for the existence of similar telomere sequences in the low molecular weight fraction of *C. steini* DNA.

In situ hybridization experiments conducted with high molecular weight DNA did not yield unequivocal results. The presence of high molecular weight DNA in specific regions of the macronucleus could not convincingly be demonstrated. An explanation for the fact that hybridization signals did not exceed background labeling might be based on a lower level of amplification in this fraction.

This view is supported by the filter hybridization experiments with protein-coding DNA sequences (Fig.4). Strong hybridization signals can be seen at about 2 Kb on lanes containing *C. steini* DNA using histone H4 and α-tubulin gene probes. Much weaker signals are found in the higher molecular weight range (Fig.4, lane 2). No signal is visible in the position where the majority of the high molecular weight molecules is found (compare Fig. 1 with Fig. 4). Therefore, the conclusion can be drawn that in the macronuclear genome of *C. steini* protein-coding sequences are found in a fraction of highly amplified gene-sized DNA molecules, similar to the situation found in macronuclear genomes of hypotrichous ciliates (Steinbrück 1983).

Studies concerning the amplification process of macronuclear genes of ciliates have been restricted mainly to the rDNA of *Tetrahymena thermophila*, *Paramecium tetraurelia*, and hypotrichous species (for reviews see Blackburn 1982, Yao 1986). Differential amplification of rDNA has been found in all ciliate species investigated, and on many other eukaryotic species. Differential amplification of protein-coding genes occurring on short gene-sized DNA molecules has been found only in hypotrichous ciliates and in this study in the cyrtophorid species *C. steini*.

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Organization of Transcriptionally Inactive Chromatin of the Interphase Macronucleus of the Ciliate *Didinium nasutum*

Bella P. KARAJAN[1], Vladimir I. POPENKO[2] and Igor B. RAIKOV[1]

[1]Institute of Cytology, Russian Academy of Sciences, St.Petersburg; [2]Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

Summary. The organization of the transcriptionally inactive chromatin of the interphase macronucleus of the ciliate *Didinium nasutum* has been studied in ultrathin sections of fixed cells and in preparations of the chromatin spread in a low-salt solution (Miller's method). In preparations of weakly dispersed chromatin and in sections, almost all macronuclear chromatin is organized throughout the interphase into electron-dense bodies, 100-200 nm in diameter, connected with one another with one or several fibrils. During spreading, unwinding of chromatin bodies occurs, and loops formed by nucleosomal fibrils can be observed around a dense core. There is a structural connection of the artificially decondensed chromatin with elements of the nuclear envelope. Fully decondensed chromatin consists of long nucleosome fibrils. At the stage of mid-interphase, agglomerations of granules 19-25 nm in size which were not detected previously in other organisms, occur alternating with agglomerations of nucleosomal chromatin fibres. The two types of agglomerations are inter-connected but spatially separated. Continuity of 19-25 nm granules with nucleosome fibrils has been observed, which argues for the DNP nature of these granules. The pattern of spreading obtained can indicate a cooperative transition of the chromatin bodies (or single chromatin loops) from a compact into a decondensed state and back. The presence of differently compacted chromatin in the macronucleus of *Didinium nasutum* may reflect differential activity of certain regions of the macronucleus during mid-interphase.

Key words. Chromatin spreading, macronucleus, inactive chromatin, nucleosomes, chromatin fibre loops.

INTRODUCTION

The ciliates are a useful model object for studying the problem of structural and functional organization of the genetic apparatus of eukaryotes, in particular for investigating structural changes of the chromatin during activation and inactivation of the transcription. This is related to the fact that ciliates (1) have nuclei of two types, a polyploid, actively transcribing somatic one (the macronucleus) and a supposedly diploid generative nucleus (the micronucleus), which is fully or almost fully in the state of repressed genic activity, (2) have a comparatively short cell cycle which involves morphological reorganization and changes in the functional activity of the macronucleus, and (3) often have a complex life cycle including encystment (transition into a functionally inert state) and excystment. However, up to now the organization of the transcriptionally inactive and active chromatin of the macronucleus has been studied in a limited number of ciliates only (Lipps et al. 1978; Samuel et al. 1981; Martinkina et al. 1983; Vengerov et al. 1983; Tikhonenko et al. 1984, 1985;

Address for correspondence: I. B. Raikov, Institute of Cytology, 4,Tikhoretsky Avenue, 194064 St.Petersburg, Russia; Fax:(812) 247-03-41; E-mail: root@cell.spb.su

Borchsenius et al. 1988; Popenko et al. 1988; Schlegel et al. 1990).

The aim of the present study was to reveal the structural organization of the transcriptionally inactive chromatin of the ciliate *Didinium nasutum* at various stages of the interphase by using the method of chromatin spreading in a low ionic strength solution (Miller's method, Miller and Beatty 1969). This method, first used for studying the structural organization of the nucleolar chromatin in amphibian oocytes (Miller and Beatty 1969, Miller et al. 1970), has been successfully applied later to the investigation of organization of the chromosomes and of the interphase chromatin of various plant and animal cells (reviews: Franke et al. 1979, Scheer and Zentgraf 1982, Scheer 1987, Van Holde 1989).

MATERIALS AND METHODS

The laboratory strain of *Didinium nasutum* used in the present work has been cultivated at room temperature in lettuce medium and fed with *Paramecium caudatum* cultivated separately. The macronuclei at various stages of the interphase were isolated by hand from groups of logarithmic growth phase individuals synchronized by selecting dividing cells and putting them into a vial with fresh culture medium. As shown elsewhere (Karajan 1987), the generation time of the clone used is 5 h. Under the same culture conditions that were used in the present work, only 3-4% of the individuals have a longer or a shorter cell cycle. For each series of experiments, six to eight macronuclei per grid were taken. The macronuclei were hand isolated with needles in a solution containing 0.5% triton X-100 (Serva, Germany) in 0.1 mM borate buffer, pH 8.7-9.0. Isolated macronuclei were washed in 0.1 mM borate buffer and transferred for 5, 10, 20, 30, 60, 90 or 120 min into a drop of the same buffer for lysis and spreading of the chromatin. The spread chromatin was layered on top of a solution containing 4% formaldehyde and 0.1 M saccharose, pH 8.7, and centrifuged for 10 min at 3000 g to deposit the chromatin onto a freshly ionized grid covered with a parlodion-carbon support film. The grids were washed in a 0.4% solution of Kodak Photoflo, pH 7.8, air-dried and shadowed with a platinum-palladium alloy (4:1) at an angle of 6°.

For ultrathin sections, the ciliates were fixed with 1% osmium tetroxide in a 0.025 M sodium cacodylate buffer. The sections were routinely stained and studied with a JEM-100C electron microscope operated at 80 kv.

Since preparations obtained from macronuclei at different stages of the interphase are generally similar, we shall specify the stage of the cell cycle only in cases when the pattern of spreading is characteristic for this stage only.

RESULTS

The macronuclear chromatin of *Didinium nasutum* is represented in ultrathin sections by many compact electron dense bodies of irregular shape, which are distributed more or less evenly throughout the nucleus. The matrix of the macronucleus consists of winding filaments which connect the chromatin bodies with each other and with the nucleoli (Fig. 1).

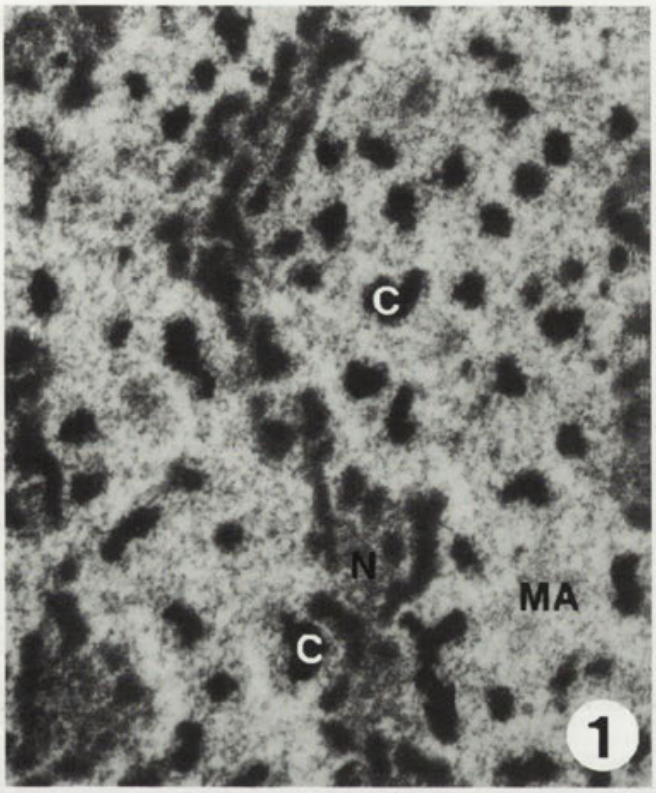
In preparations of spread chromatin from macronuclei incubated in a low ionic strength solution for 5-10 min, large accumulations of chromatin bodies occur. At the periphery of the accumulations the chromatin bodies are surrounded by loops of fibrils carrying beaded granules of nucleosome (10-12 nm) or nucleomere (19-25 nm) size (Fig. 2). Chromatin bodies corresponding in shape and size (100-200 nm) to the bodies seen in ultrathin sections occur in the central parts of the chromatin accumulations. The surfaces of large chromatin bodies is uneven, and globular particles 10-25 nm in size can be distinguished in them (Fig. 2, white arrows). The same preparations show that the chromatin bodies are interconnected by strands (Fig. 2, black arrows).

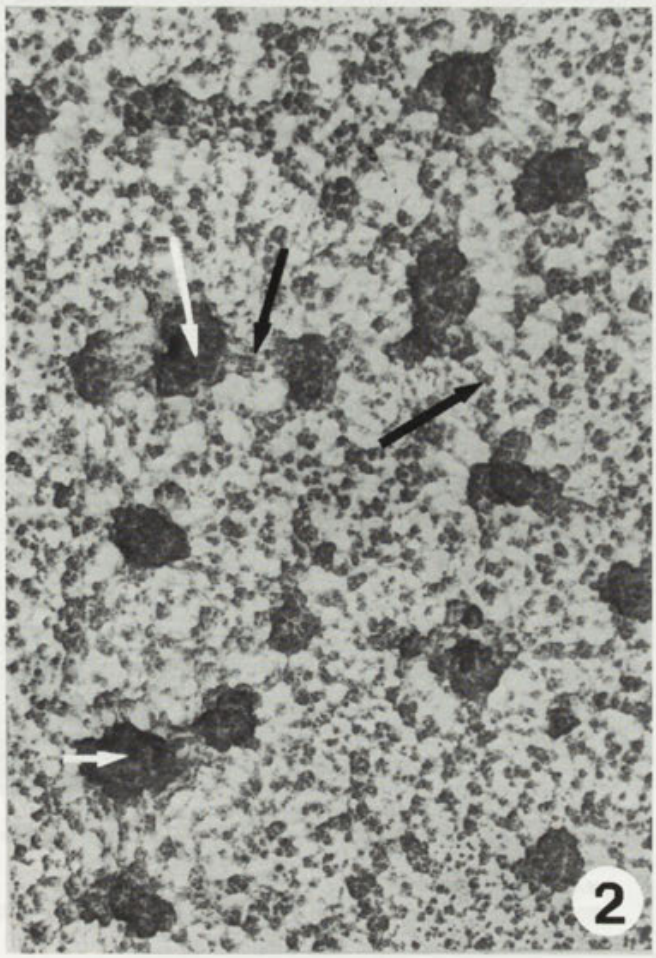
In preparations of chromatin from macronuclei incubated for 45 min in 0.1 mM borate buffer, the accumulations of chromatin bodies appear looser (Fig. 3). All compact chromatin bodies are surrounded by halos of chromatin fibrils. They carry granules 10-25 nm in size. Larger granules are nearer to the central compact core of the chromatin bodies, whereas more peripheral loops of the chromatin fibrils carry nucleosome-sized granules (Fig. 3, arrows). With the same incubation time, some preparations show pore complexes with distinct central granules which are closely adjacent to the macronuclear chromatin (Figs. 4, 5). In many cases, a structural connection of the dispersed chromatin with pore complexes of the nuclear envelope can be observed (Fig. 4, arrows). The pore diameter in both ultrathin sections (Fig. 6) and preparations of spread chromatin is 86-95 nm, the size of the central granule, 20-25 nm.

After incubation of the chromatin in 0.1 mM borate buffer for 60 min, most chromatin bodies are fully decondensed or are substantially smaller than in preparations of chromatin incubated for 20-45 min.

In preparations of the chromatin obtained from macronuclei at the mid-interphase stage and spread in 0.1 mM buffer for 90 min, homogeneous accumulations of granules 19-25 nm in size, alternating with accumulations of nucleosomal chromatin fibrils, are observed (Figs. 7, 8). The two types of accumulations are connected with each other but spatially are separate. Granules (19-25 nm) are in many cases seen to be continuous with nucleosomal fibrils (Fig. 8, arrows).

After incubation of the macronuclear chromatin in a low-salt solution during 120 min, the part of the chromatin





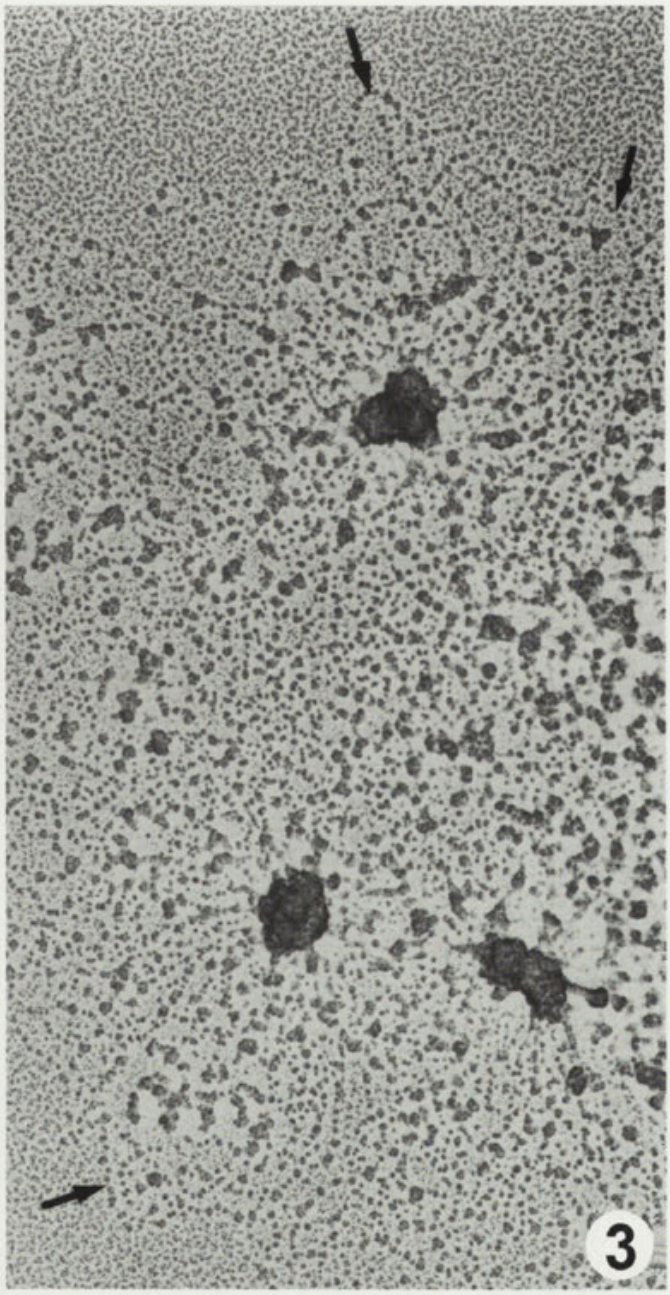
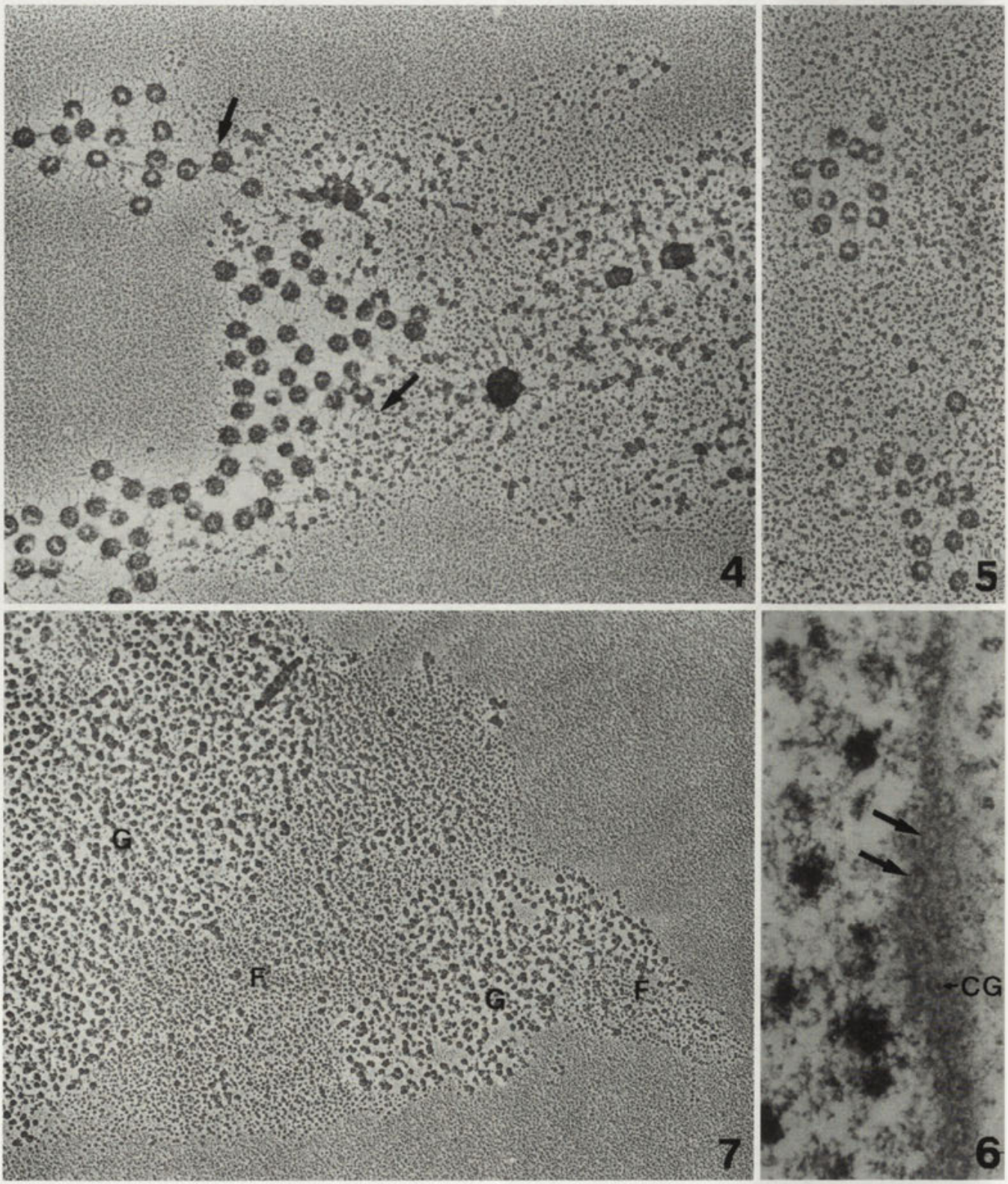


Fig. 1. Ultrathin section of a region of the interphase macronucleus (MA) showing chromatin bodies (C) and nucleoli (N). x 21 000
Fig. 2. Macronuclear chromatin incubated in 0.1 mM borate buffer for 5-10 min. Globular structures on the surfaces of chromatin bodies at *white arrows*, strands interconnecting the chromatin bodies at *black arrows*. x 33 000
Fig. 3. Chromatin loops formed by nucleosome fibrils (*arrows*) at the periphery of aggregations of partly decondensed chromatin bodies, incubated in 0.1 mM borate buffer for 45 min. x 36 500



Figs. 4, 5. Connections between macronuclear chromatin (*arrows*) incubated in 0.1 mM borate buffer for 45 min (Fig. 4) or 60 min (Fig. 5) and pore complexes of the nuclear envelope. x 26 300 and x 28 000, respectively

Fig. 6. Ultrathin section of the macronuclear envelope showing pores (*arrows*) with central granules (CG). x 36 000

Fig. 7. Macronuclear chromatin at the stage of mid-interphase, incubated in 0.1 mM borate buffer for 90 min, showing alternating accumulations of granules 19-25 nm (G) and nucleosome fibrils (F). x 24 500

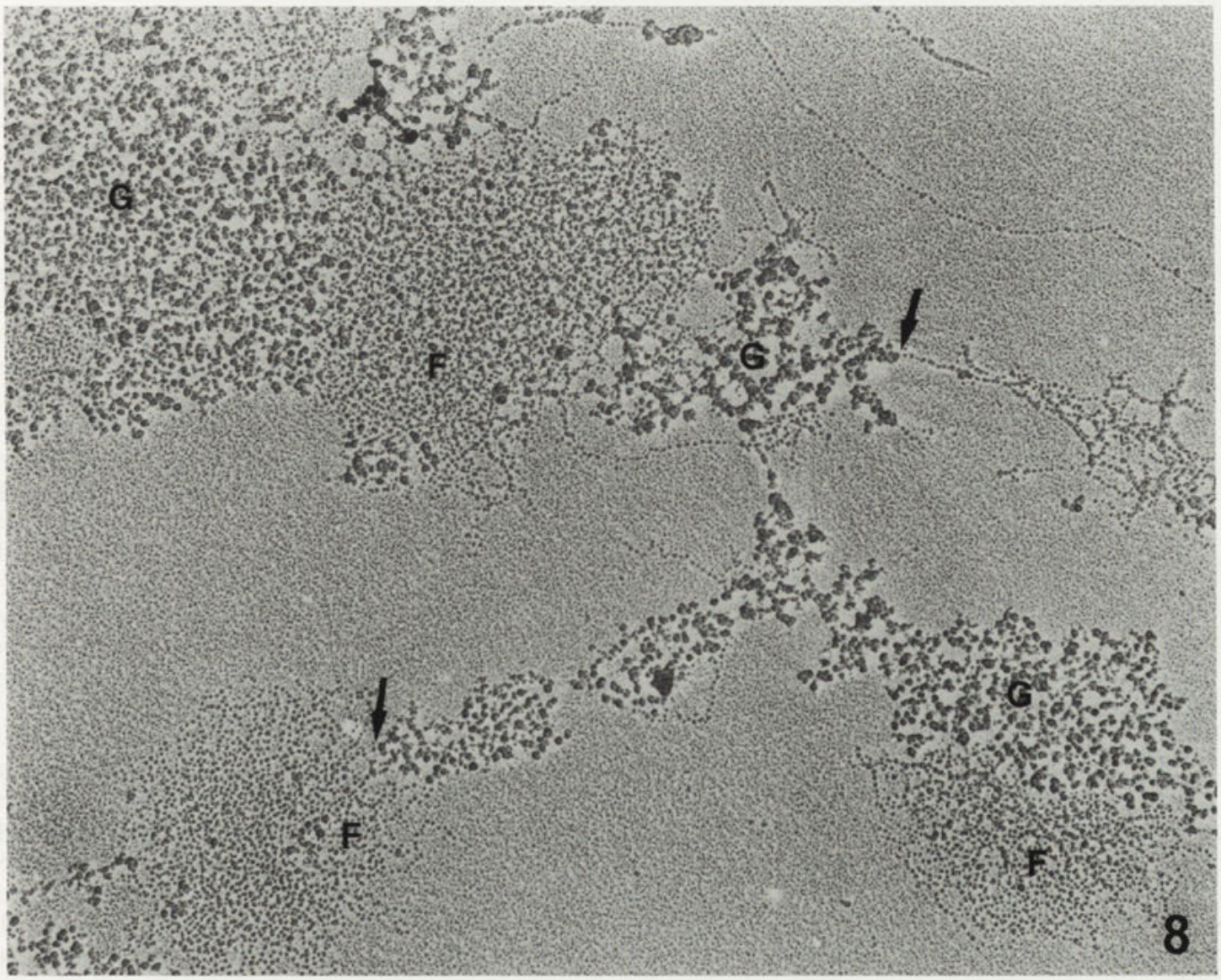


Fig. 8. Continuity of 19-25 nm granules and nucleosome fibrils (*arrows*) at the boundary of alternating accumulations of granules (G) and fibrils (F) during mid-interphase. x 25 000

consisting of 19-25 nm granules progressively decreases, and almost all chromatin consists of long nucleosome-bearing fibrils. The size of the nucleosomes themselves (10-12 nm) and the density of their disposition along the fibril (27-32 nucleosomes per 1 μm) is similar to nucleosome chains typical for the inactive chromatin of other cells (Lohr and Van Holde 1979, Zentgraf et al. 1980, Martinkina et al. 1983, Miller 1984, Zentgraf and Franke 1984, Borchsenius et al. 1988, Gaginskaya and Tsvetkov 1988, Kamaka and Thomas 1990).

DISCUSSION

In ultrathin sections, the interphasic chromatin of the macronucleus of *D. nasutum* has the structure similar to that of DNA-rich macronuclei of other ciliates (Raikov 1982). In chromatin preparations obtained by the Miller's method from the macronucleus of the ciliate *Bursaria truncatella*, it has been shown that chromatin bodies are formed by densely packed loops of chromatin threads which can be decondensed in vitro with formation of radial loops after incubation in a solution of low ionic strength (Martinkina et al. 1983). The same occurs *in vivo* upon natural chromatin activation, e.g. during excystment (Tikhonenko et al. 1984). The data obtained in the present work on the macronuclear chromatin of *Didinium nasutum* are well compatible with these results. Thus, we observed an increase of the halo of chromatin loops around bodies of condensed chromatin upon increase of the time of incubation in 0.1 mM borate buffer. Partly decondensed chromatin bodies, obtained

with chromatin incubation for 45 min in the low-salt solution, are surrounded with halos formed by chromatin fibrils carrying granules 10-12 nm and 19-25 nm in size, the larger granules being localized nearer to the central compact cores of the chromatin bodies. It is probable that granules 19-25 nm in size represent intermediate structures in the process of decondensation of chromatin bodies. The following facts argue in favour of this supposition: (1) the larger granules lie nearer to the core of the chromatin bodies, forming a halo; (2) nucleosome chains predominate farther from the core of the bodies (i.e. where decondensation goes quicker); (3) 19-25 nm granules are completely absent in fully decondensed chromatin accumulations which consist of nucleosome chains only.

A structural connection of the chromatin with free pore complexes was revealed with medium (45-60 min) times of chromatin dispersion. These complexes can be elements of the macronuclear envelope destroyed during spreading. Structural connection of the chromatin and elements of the nuclear envelope, first discovered by Du Praw (1965) in embryonal cells of the honey bee, has later been observed in a number of eukaryotic cells (Comings and Okada 1970, 1973; Engelhardt and Pusa 1972). However, since these and other papers have not demonstrated that the chromatin was connected with exactly the pore complexes of the nuclear envelope, many authors maintain that the chromatin is connected with the nuclear lamina underlying the inner nuclear membrane rather than with the pore complexes (Franke et al. 1973, Franke 1974). Anyway, the association of the DNP with residual structures of the nuclear envelope is considered to be important. It is supposed that interphase chromosomes so become "anchored" to the nuclear envelope, and in this sense the function of the latter is analogous to the function of the plasma membrane of prokaryotic cells. It is also admitted that the nuclear envelope is an important factor determining the higher levels of organization of the chromatin. Some authors suppose that the sites of fixation of the chromatin to the nuclear envelope can function as sites of DNA synthesis initiation (Gerace and Burke 1988, Scheer et al. 1988, Dabauville and Scheer 1991, Miller et al. 1991, Akey 1992, Forbes 1992).

Apparently the amount of the DNA connected with the nuclear envelope and/or the soundness of this connection depends in the macronucleus of *D. nasutum* on the period of the cell cycle, since we have found no association of the chromatin with pore complexes in preparations of spread chromatin prepared from dividing cells (unpublished data). It is probable that such an association is related to the process of nuclear DNA replication.

After spreading the macronuclear chromatin of *D.nasutum* in 0.1 mM borate buffer for 90 min at the stage of mid-interphase, we have observed structures which were up to now not described in nuclei of other cells. These are large accumulations of granules 19-25 nm in size which alternate with regions filled with a mass of nucleosome chromatin fibrils, the two types of accumulations being spatially distinct but interconnected at the boundary. In some cases there is direct continuity between the granules and the nucleosome chains, which argues for the DNP nature of the granules. Possibly, the granules are an intermediate step of decondensation of the compact chromatin. The pattern of spreading obtained argues for a co-operative nature of transition of the dense chromatin bodies via the stage of chromatin 19-25 nm granules to decompactized chromatin (nucleosome chains) and back. The possibility of such a transition was experimentally demonstrated in chick erythrocyte nuclei depending on changes in the conditions of chromatin spreading (Zentgraf et al. 1980).

It is probable that during prolonged incubation in low ionic strength solution, accumulations consisting of a mass of nucleosome chains are formed from compact chromatin bodies, the structure of which is weakened due to chromatin transition into an active state, while the 19-25 nm granules correspond to the inactive chromatin. In this case, the simultaneous presence of chromatin at different stages of decompaction can reflect differential activity of some domains in the macronucleus during the stage of mid-interphase. But it must be pointed out that at least some granules 19-25 nm in size can be RNP structures. Consequently, it seems important to carry out further a detailed cytochemical analysis of the structures described here.

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Uronema gallicum sp. n. (Protozoa: Ciliophora) a New Marine Scuticociliate from the Coastal Area of Calais

Blanca PÉREZ-UZ[1] and Weibo SONG[2]

[1]Microbiology Group, Department of Zoology, The Natural History Museum, Cromwell Road, London, U.K.; [2]College of Fisheries, Ocean University of Qingdao, Qingdao, P.R. China

Summary. *Uronema gallicum* sp. n. was isolated from a sample from the beach near Wimereux (France). Its morphology, silverline system and infraciliature were studied *in vivo* and in specimens impregnated with silver carbonate, silver nitrate and protargol. This new species measures 21-28 x 9-14 µm with a narrow anterior end. The number of kinetal rows is generally 14. The cytostome is situated sub-equatorially. The buccal area is large for the size of the cell. M1 is long and nonciliferous with 6 to 7 widely spaced kinetosomes. M2 is composed of three rows of 5, 4 and 2 kinetosomes.

Key words. Marine ciliate, Scuticociliatida, morphology, infraciliature, *Uronema gallicum* sp. n.

INTRODUCTION

Since the creation of the genus *Uronema* Dujardin, 1841, numerous small "closely related" ciliates have been included in it (Maupas 1883; Bütschli 1887; Cuénot 1891; Schewiakoff 1893; Martini 1910; Penard 1922; Kahl 1926, 1931; Noland 1937; Wenzel 1961; Tucolesco 1962). However it was not until the development of silver staining techniques that the taxonomic status of this genus was defined. The redefinition of species in this genus was based on detailed studies of the somatic and oral infraciliatures (Párducz 1939; Borror 1963a, b; Thompson 1964; Czapik 1968; Thompson and Evans 1968; Thompson and Kaneshiro 1968; Foissner 1971; Wilbert and Kahan 1981). This has led to the recognition of the four main species unquestionably included in this genus: *U. marinum*, *U. nigricans*, *U. elegans* and *U. filificum*.

The present work deals with a new species described on the basis of a detailed morphological and morphometrical study using different staining techniques and live observations. A comparison with the four extant species justifies the taxonomic status of the new organism.

Address for correspondence: B. Pérez-Uz, Microbiology Group, Department of Zoology, The Natural History Museum, Cromwell Road, London, SW7 5BD, U.K; Fax: +44-71-938-8754; E-mail: BPU@NHM.AC.UK

MATERIALS AND METHODS

Ciliate isolation and identification. *Uronema gallicum* (clone SWi1) was isolated from a seawater sample taken from the beach at Wimereux (France) in March 1991. Cells were cloned by successive washing of single cells followed by culture in a bacterial suspension. After a three step cloning procedure, ciliates were grown in autoclaved 0.2 µm filtered seawater plus a suspension of the bacterium *Vibrio natriegens* (strain NCIMB#857).

Identification was carried out by live observations with bright field, phase contrast and interference contrast microscopy. Infraciliature and silverline system were revealed by three silver staining techniques: pyridinated silver carbonate (Fernández-Galiano 1976), Chatton-Lwoff (Roberts and Causton 1988) and protargol (Wilbert 1975).

Morphometric analysis. Morphometrical data were collected with the help of a computer program to digitalize images (DIGIT). Live cells were recorded on a video recorder (Sony VO-9850P) and high resolution video camera (JVC, model KY-30) attached to a microscope (Olympus BH-2), and linked to a TV screen and a computer terminal (IBM 6151-130). Single frames of live or stained cells were grabbed and then measured. All morphometrical data were measured in a minimum of 30 cells. Data analysis was carried out with the statistical package SYSTAT 5.2.

Terminology is mostly based on Corliss (1979).

RESULTS

Uronema gallicum sp. n. (Figs. 1-15, Table 1)

Diagnosis: small marine bacterivorous *Uronema* with 13 to 15 somatic kineties and a single macro- and micronucleus; *in vivo* about 20-30 x 8-11 µm, cytostome constantly sub-equatorial; membranelle 1 (M1) long with 6 to 7 kinetosomes; meridian SK_n passing through the caudal cilium complex connected dorsally between the SK_9 and SK_{10}.

Type location: beach at Wimereux (France).

Type specimens: a holotype and a paratype as two slides of silver nitrate impregnated specimens have been deposited in The Natural History Museum, London with the registration No. 1994:08:27:1 and 1994:08:27:2.

Derivatio nominis: "gallicum" is Latin and means "from Gallia", Latin denomination for the area of France.

Description: body shape usually elongated as shown in Figs. 1 and 9, whereas in well nourished cells often ovoid with the width almost twice of that in normal ones (Figs. 3, 10). Anterior pole somewhat pointed, small, naked and typically truncated as in other *Uronema* spp.

Pellicle thin, slightly indented. Extrusomes fine and rod-like, about 2 µm long, situated between consecutive cilia (often easily overlooked even in interference contrast observations!) (Fig. 9). Endoplasm transparent and colourless with several large refractive crystal inclusions (Figs. 9, 10). Food vacuoles *ca* 5 µm, being of granular appearance. Macronucleus large and rounded, usually situated in anterior half of the cell with the micronucleus attached anteriorly (Fig. 5). Contractile vacuole small, pulsing frequently. Cilia about 6-7 µm long, motionless when cell is stationary, distributed irregularly.

In culture, cells usually motionless on the surface of the plates or tubes walls. Swimming fast, rotating around main axis of cell.

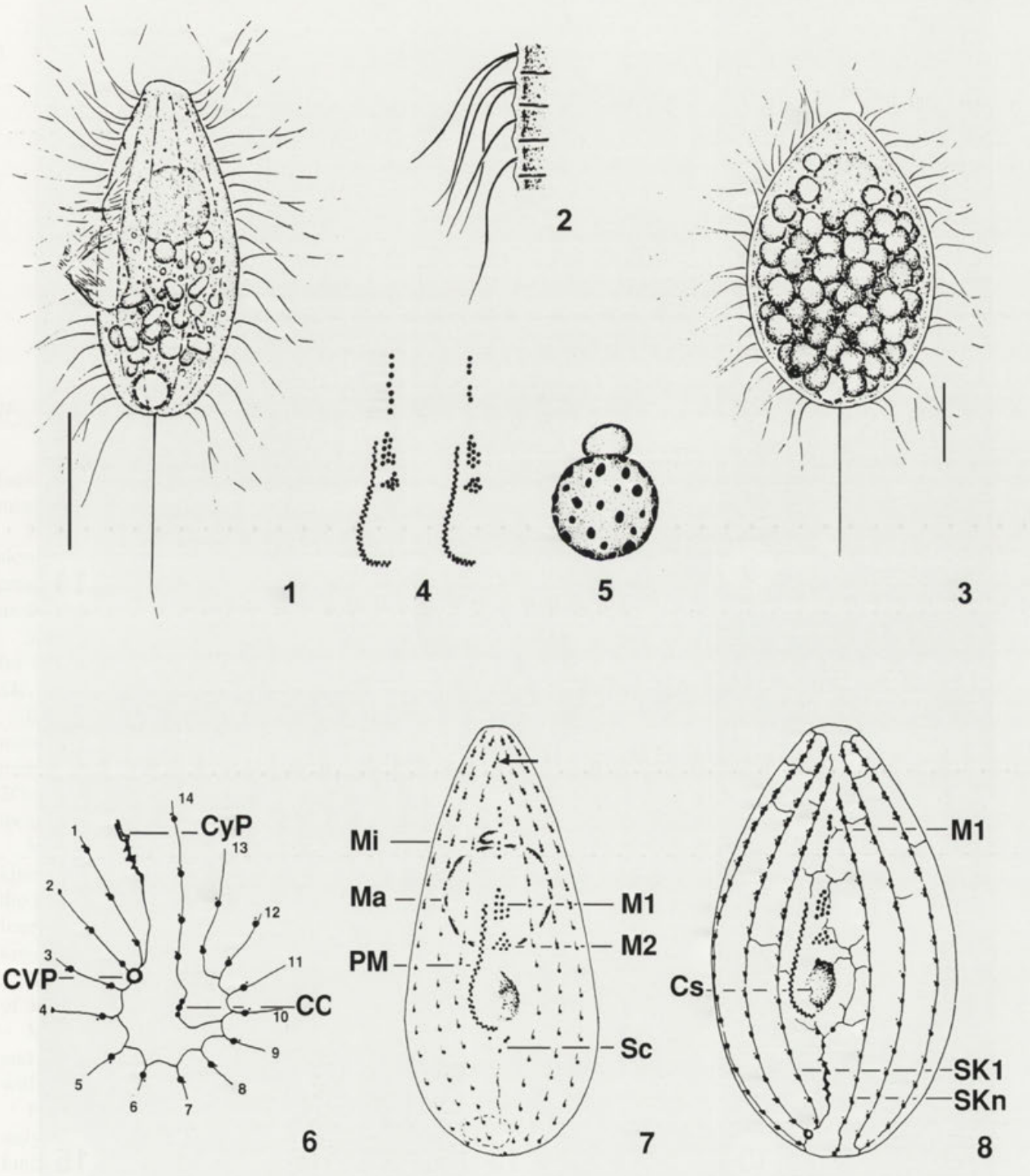
Infraciliature: number of somatic kineties (SK) between 13 and 15, usually 14 (Table 1, Fig. 16). The anterior end of SK_1 formed by a complex of three kinetosomes (Fig. 7, arrow), located at the same level of the second kinetosomic position of the other SKs. All somatic kineties (except SK_1 and SK_n) converging anteriorly to a narrow circle, connected by an argentophilic fiber SK_n extending posteriorly one kinetosome

Table 1

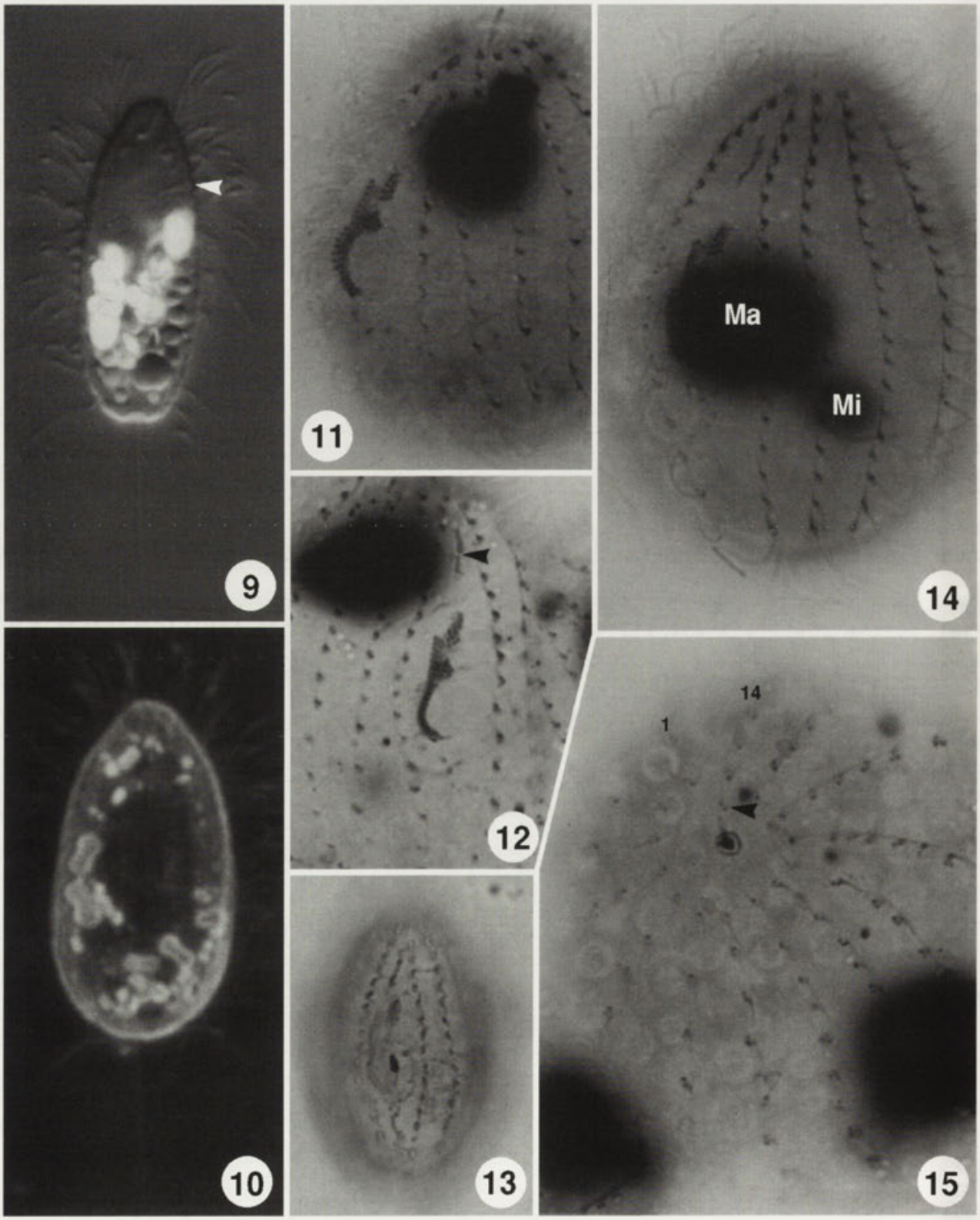
Uronema gallicum sp. n. *morphometric characterization

	Min	Max	Mean	SD	SE	n
Body, length[1]	20.2	29.5	24.2	2.12	0.39	30
Body, width[1]	7.6	11.7	8.9	0.88	0.16	30
Body, length	21.2	27.9	23.9	1.81	0.33	30
Body, width	9.0	13.9	10.8	1.14	0.21	30
M1, length	1.9	3.8	3.2	0.49	0.09	30
M2, length	2.0	2.8	2.4	0.22	0.04	30
M3, length	0.8	1.4	1.1	0.14	0.03	30
PM, length	6.7	7.8	7.2	0.29	0.05	30
Buccal area	10.9	13.7	12.4	0.76	0.14	30
Distance from apical pole to M1	3.4	5.8	4.6	0.76	0.14	30
Distance between M1 and M2	0.5	1.4	0.9	0.19	0.03	30
Distance from PM to caudal pole	4.5	9.9	7.2	1.31	0.24	30
No. of SK	13	15	14	0.42	0.05	74
No. of Kinetosomes in SK_1	15	23	19.3	2.06	0.47	19
No. of Kinetosomes in SK_n	15	21	18	1.96	0.43	21
Macronucleus, number	1	1	1	0	0	>100
Micronucleus, number	1	1	1	0	0	>100

*Data based on cells after Chatton-Lwoff impregnation except [1] that were live measurements. All measurements in µm; Min- minimum; Max- maximum; SD- standard deviation; SE- standard error; n- size of samples.



Figs. 1-8. Morphology and infraciliature of *Uronema gallicum* sp. n. after live observations (1-3), silver carbonate (4, 5, 7) and silver nitrate impregnations (6, 8). 1 - left view, typical individual; 2 - part of pellicle; 3 - individual in exponential phase; 4 - buccal apparatus; 5 - macro- and micronucleus; 6 - caudal view, silverline system; 7 - ventral view, infraciliature; arrow indicates three kinetosomes complex in SK_1; 8 - ventral view, silverline system. Bars - 10 µm. Abbreviations: CC - caudal cirri, Cs - cytostome ,CVP - contractile vacuole pore, CyP- cytopyge, Ma - macronucleus, Mi - micronucleus, M1 - membranelle, PM - paroral membranelle, Sc - scutia, SK - stomatic kinetics



Figs. 9-15. Photomicrographs of *Uronema gallicum* sp. n. *in vivo* (9, 10), silver carbonate (11, 12, 14, 15) and silver nitrate impregnation (13). 9 - typical individual in stationary phase (interference contrast), arrow indicates an extrusome; 10 - individual in exponential phase (phase contrast); 11, 12, 14 - ventral views showing the oral area, arrow in Fig. 12 marking the gap in the membranelle 1; 13 - ventral view indicating the silverline system; 15 - caudal view, numbers indicate first and last kineties; note the darker caudal complex circular structure; arrow marks extra kinetosome in last kinety

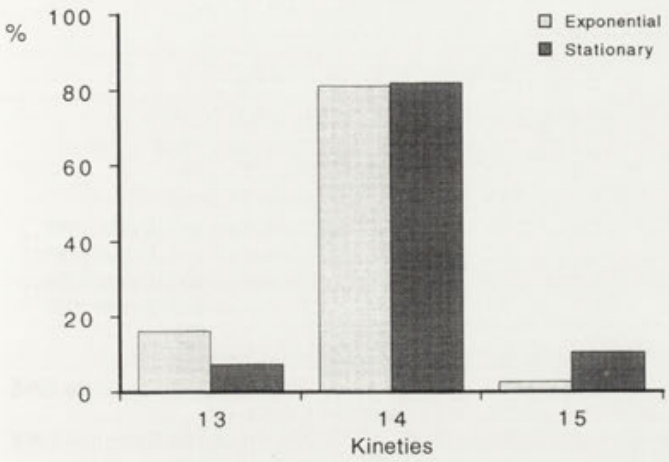


Fig. 16. Diagram showing frequencies on the number of somatic kineties in different growth phases

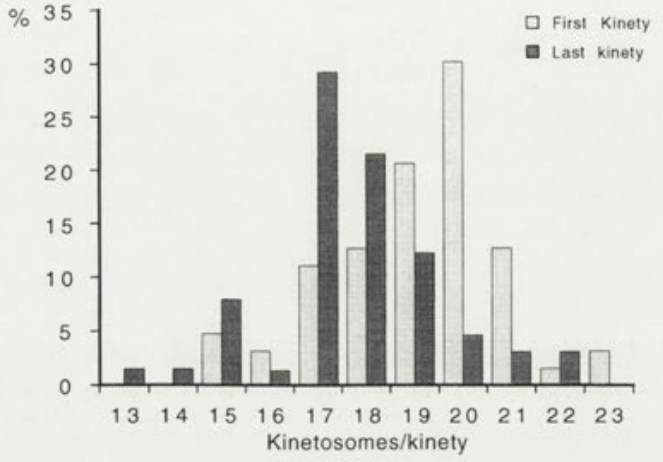


Fig. 17. Frequencies in the number of kinetosomes for the first and the last somatic kineties

further than the rest of the kineties and therefore terminating closer to the caudal complex.

The caudal cilium complex located in a small depression surrounded by a fiber which may be strongly impregnated when stained using the silver carbonate method (Fig. 15).

Somatic kineties with a high number of kinetosomes for the size of this ciliate, between 15-23 in the case of SK_1 and 15-21 in the case of SK_n (Table 1, Fig. 17).

Buccal infraciliature typical for *Uronema* with three membranelles (M1, M2 and M3) and a paroral membrane (PM). Buccal area large and extending about 2/3 of the cell length (Fig. 7). In exponential phase the location of the buccal area is more central.

M1 long, entirely non-ciliated with 6-7 widely spaced kinetosomes in a row that sometimes seems to break in the middle (Figs. 4, 8), situated between the second and fourth kinetosomic positions of SK_n, inclining anteriorly towards the last kinety.

M2 bearing long cilia, and composed of three rows of 5, 4 and 2-3 kinetosomes respectively (Figs. 7, 8).

M3 triangular in shape, bearing three lines with 4, 3 and 2 kinetosomes respectively, obliquely orientated with respect to M2 (Figs. 7, 8).

PM with two rows of kinetosomes in zig-zag pattern; only external one ciliated. Segment (A) with 8 kinetosomes, anterior end almost at same level as the posterior end of M2. Segment (B) with 10 or 11 kinetosomes.

Scutica (Sc) non-ciliated; difficult to observe with silver carbonate impregnation except in those cells already in division; it is composed of 3 or 4 kinetosomes arranged in an inverted triangular shape beneath the posterior end of the paroral membrane (Figs. 7, 8).

Contractile vacuole pore (CVP) opening at the posterior end of the second somatic kinety (Figs. 6, 8).

Cytopyge (CyP) short, situated between SK_1 and SK_n beneath the scutica (Figs. 7, 8).

Silverline system as shown in Fig. 6, with an argentophilic fiber extending from the end of SK_n to the caudal complex, and joining the argentophilic circle between meridians 9 and 10.

DISCUSSION

Uronema gallicum sp. n. presents typical characteristics of the genus *Uronema* as defined by Thompson (1964,Thompson and Evans 1968). Nine species have been newly described or redescribed as *Uronema* using silver impregnation methods (Borror 1963a, 1965; Thompson 1964; Czapik 1968; Thompson and Evans 1968; Thompson and Kaneshiro 1968; Stein and Hamilton 1969; Foissner 1971; Téllez 1980; Fernández-Leborans and Zaldumbide 1984; Pérez-Uz 1993), but of those only four are widely recognized in this genus. Detailed comparison of morphology, infraciliature and silverline system with those obtained for *U. gallicum* failed to identify this new species with any of those previously described (Table 2).

Compared to those species with the same body shape and behaviour, *U. gallicum* is characterized by the larger buccal area with respect to the size of the cell, the sub-equatorial position of the cytostome and the longer

Table 2

*Uronema** species descriptions

	Habitat	Body size	SK	M1	Ap-M1	FBC	Author
U. marinum	Marine	33.1 x 17.4	13-16(15)	2.8	5.0	9-11	Thompson 1964
	Marine[1]	26.5 x 10.7	9-11(10)	1.8	5.2	...	Pérez-Uz 1993
	Marine[2]	25.8 x 11.2	12-15(14)	2.2	5.1	...	Pérez-Uz 1993
U. nigricans	Freshwater	25.6 x 12.4	12	1.6	3.4	6-7	Thompson and Evans 1968
	Freshwater	25.5 x 11.0	13	1.8	3.0	7-8	Thompson and Evans 1968
	Freshwater	25.1 x 11.7	11-12(11)	1.6	3.3	6-7	Thompson and Evans 1968
	Freshwater	24.0 x 11.5	11-12(11)	1.5	3.1	6-7	Thompson and Evans 1968
	Freshwater[3]	22.9 x 9.9	12-13(12)	1.6	3.0	...	Pérez-Uz 1993
	Marine[4]	22.3 x 12.8	10-12(11)	1.9	3.4	...	Pérez-Uz 1993
U. elegans	Marine	46.3 x 27.6	23-24(24)	...	10	...	Thompson and Kaneshiro 1968
	Marine	40.0 x	21-22	...	...	...	Czapik 1968
U. filificum	Marine	27.0 x 16.9	16-19(17)	...	4	11	Thompson and Kaneshiro 1968
	Marine	30.0 x	18	...	3	11-12	Wilbert and Kahan 1981
U. gallicum sp. n.	Marine[5]	23.9 x 10.8	13-15(14)	3.2	4.6	9-10	Original

*Data based on cells after Chatton-Lwoff impregnation
SK- range of somatic kineties, usual number in parentheses; M1- membranelle 1; Ap-M1- distance from apical pole to anterior end of M1; FBC- meridians where the fiber crossing over basal body complex finishes. Measurements of body size, M1 and Ap-M1 in µm.
[1] Clone U.m. from CCAP#1986/2, [2] Clone Cil7, [3] Clone SRBM, [4] Clone AD1, [5] Clone SWi1

M1 with a higher number of kinetosomes. Based on these characteristics *U. gallicum* most closely resembles *U. filificum*. However the latter has a significantly different body shape, behaviour (Kahl 1931, Thompson and Kaneshiro 1968, Wilbert and Kahan 1981) and silverline system around the caudal pole (Wilbert and Kahan 1981). *Homalogastra binucleata* Song, 1993 is also morphologically similar to *U. gallicum* although this species has two macronuclear-segments and, like *U. filificum*, differs in body shape and silverline structure around the posterior pole.

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Articles from books:

Allen R. D. (1988) Cytology. In: Paramecium, (Ed. H.-D. Görtz). Springer-Verlag, Berlin, 4-40

Zeuthen E., Rasmussen L. (1972) Synchronized cell division in protozoa. In: Research in Protozoology, (Ed. T.T. Chen). Pergamon Press, Oxford, **4:** 9-145

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