

C. S. Lobban · M. Schefter · A. G. B. Simpson
X. Pochon · J. Pawlowski · W. Foissner

***Maristentor dinoferus* n. gen., n. sp., a giant heterotrich ciliate (Spirotrichea: Heterotrichida) with zooxanthellae, from coral reefs on Guam, Mariana Islands**

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Abstract *Maristentor dinoferus* n. gen., n. sp., was discovered on coral reefs on Guam in 1996 and has since been found frequently, at depths of 3–20 m. It forms black clusters, visible to the naked eye, especially on *Padina* spp. (Phaeophyta) and other light-colored backgrounds. When fully extended, this sessile ciliate is trumpet-shaped, up to 1 mm tall and 300 µm wide across the cap. The ciliate is host to 500–800 symbiotic algae. The anterior cap, or peristomial area, is divided into two conspicuous lobes by a deep ventral indentation. There is a single globular macronucleus, many

micronuclei and, on average, 101 somatic ciliary rows and 397 adoral membranelles. *M. dinoferus* may be closely related to limnetic *Stentor* spp., but differs in two conspicuous features: (1) the cilia on the peristomial bottom are scattered (ordered rows in *Stentor* spp.) and (2) the paroral membrane is very short and opposite the buccal portion of the adoral zone of membranelles (in *Stentor* spp., it accompanies the entire membranelar zone). The cells appear dark due to stripes of cortical granules; the granules are more concentrated in a “black band” below the cap. The cortical pigment(s) is red fluorescent with a broad absorption peak in the blue (ca. 420–480 nm), and sharp peaks in the yellow-green (ca. 550 nm) and red (600 nm). Ultrastructural and molecular data demonstrate that the symbiont is a dinoflagellate of the genus *Symbiodinium*, the first unequivocal report of zooxanthellae in a ciliate. Phylogenetic analysis of a portion of the large subunit ribosomal RNA gene (28S rDNA) showed that the symbionts belong to *Symbiodinium* sp. clade C, a lineage that also inhabits many corals on Guam. The ciliate changes shape at night, and the symbionts, which are spread out in the cap during the day, are mostly withdrawn into the stalk at night; these changes were apparently not simply a response to darkness.

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C.S. Lobban (✉)
University of Guam,
Division of Natural Sciences, Mangilao,
GU 96923, USA

E-mail: clobban@uog.edu
Tel.: +1-671-7352787
Fax: +1-671-7341299

M. Schefter
Box 5126, UOG Station,
Mangilao, GU 96923, USA

A.G.B. Simpson
University of Sydney,
School of Biological Sciences,
Sydney, NSW 2006, Australia

X. Pochon · J. Pawlowski
University of Geneva,
Department of Zoology and Animal Biology,
1224 Chêne-Bougeries, Switzerland

W. Foissner
Universität Salzburg, Institut für Zoologie,
Hellbrunnerstrasse 34, 5020 Salzburg, Austria

Present address: A.G.B. Simpson
Dalhousie University,
Department of Biochemistry and Molecular Biology,
Halifax, NS B3H 4H7, Canada

Introduction

The ciliates and flagellates of tropical reefs are a largely invisible and unexplored component of the benthos, primarily in sediments (Patterson et al. 1989; Larsen and Patterson 1990; Lynn et al. 1991); rarely do they become apparent to divers. Wichterman (1942) reported a new genus of ciliate, *Paraeuplotes tortugensis*, with zooxanthellae from Tortugas, Florida. A macroscopic folliculinid ciliate (“*Halofolliculina corallasia*” nomen nudem) has recently been reported to form conspicuous aggregations on coral at several Indo-Pacific sites (Antonius 1999). The organism reportedly kills coral, and the dis-

ease has been termed "skeletal eroding band". Lobban and Scheffer (1996) reported macroscopic aggregations of an apparently novel *Stentor*-like ciliate on reef substrates in Guam. This organism does not appear to cause harm and contains zooxanthellae reminiscent of those in corals, other invertebrates, and foraminifera on tropical reefs (Rowan 1998; Carlos et al. 1999). In the present study we report on the morphology and behavior of the *Stentor*-like ciliate, for which we propose the new genus and species names *Maristentor dinoferus*, and we examine the identity of its algal symbiont.

Materials and methods

Maristentor dinoferus was first collected 20 m deep at "Pete's Reef" (Agat Bay) in July 1995. The principal study site was 3–5 m deep on the reef at Scuba Beach, on the north shore of Apra Harbor, Guam, Mariana Islands (13°27'N; 144°40'E), where we collected or observed populations at approximately bimonthly intervals. Occasional collections were made from other reefs on Guam: 15 m deep at "Coral Gardens" (Agat Bay), July 1996; 20 m deep at Apuntua Pt. (south side of Orote Peninsula), March 2000; 7 m deep at Dadi Beach (south side of Orote Peninsula), April 2001; and 3–5 m deep at several sites in Apra Harbor at various times since September 1998.

Populations of *M. dinoferus* were collected by placing blades of the seaweed *Padina* spp. with adherent clusters (see Fig. 1a) into plastic bags filled with ambient water. Collections were maintained for up to 1 month in culture dishes, small aquaria, or tissue culture plates. Containers were placed near a window out of direct sun, in a room without air conditioning. Room temperatures were 27–30°C, compared to 29–30°C measured for freshly collected seawater from the principal study site. Once the ciliates had left *Padina* spp., the seaweed was removed from the dishes. We have so far been unable to establish long-term cultures in controlled conditions.

Live specimens were studied with conventional and inverted microscopes using bright field, dark field, phase contrast, and interference contrast illumination. The ciliary pattern was revealed with protargol and scanning electron microscopy on cells fixed with Stieve's or Bouin's fluid, see Foissner (1991) for a detailed description of these methods. Preliminary observations of day–night changes were made both during the natural light:dark cycle and when cells were kept under lights at night and in darkness during the day.

Measurements on extended specimens were made from photographic records of live cells in various postures and orientations in tissue culture dishes; measurements on silver-prepared specimens were made on permanent slides with oil immersion using an eyepiece micrometer. Drawings of live cells were based on micrographs; those of impregnated specimens were made with a camera lucida. Numbers of symbionts were counted from cells that had burst and spread on a drying slide.

Ciliate terminology follows that of Corliss (1979) and Foissner and Wöflfl (1994). Classification in Alveolata follows Patterson (1999). By convention, the cells are illustrated and left/right identified according to how they appear in the compound microscope; this is the opposite of their appearance in the dissection microscope.

Preliminary examination of cortical pigment (and algal pigments, not reported here) was carried out using methanol extraction followed by phase partitioning with salt and ether, as described in Chapman (1988). The aqueous extract contained the cortical pigment(s), and the ether layer contained the algal pigments. Absorption spectra for the cortical pigment were made with a Hitachi Perkin-Elmer spectrophotometer.

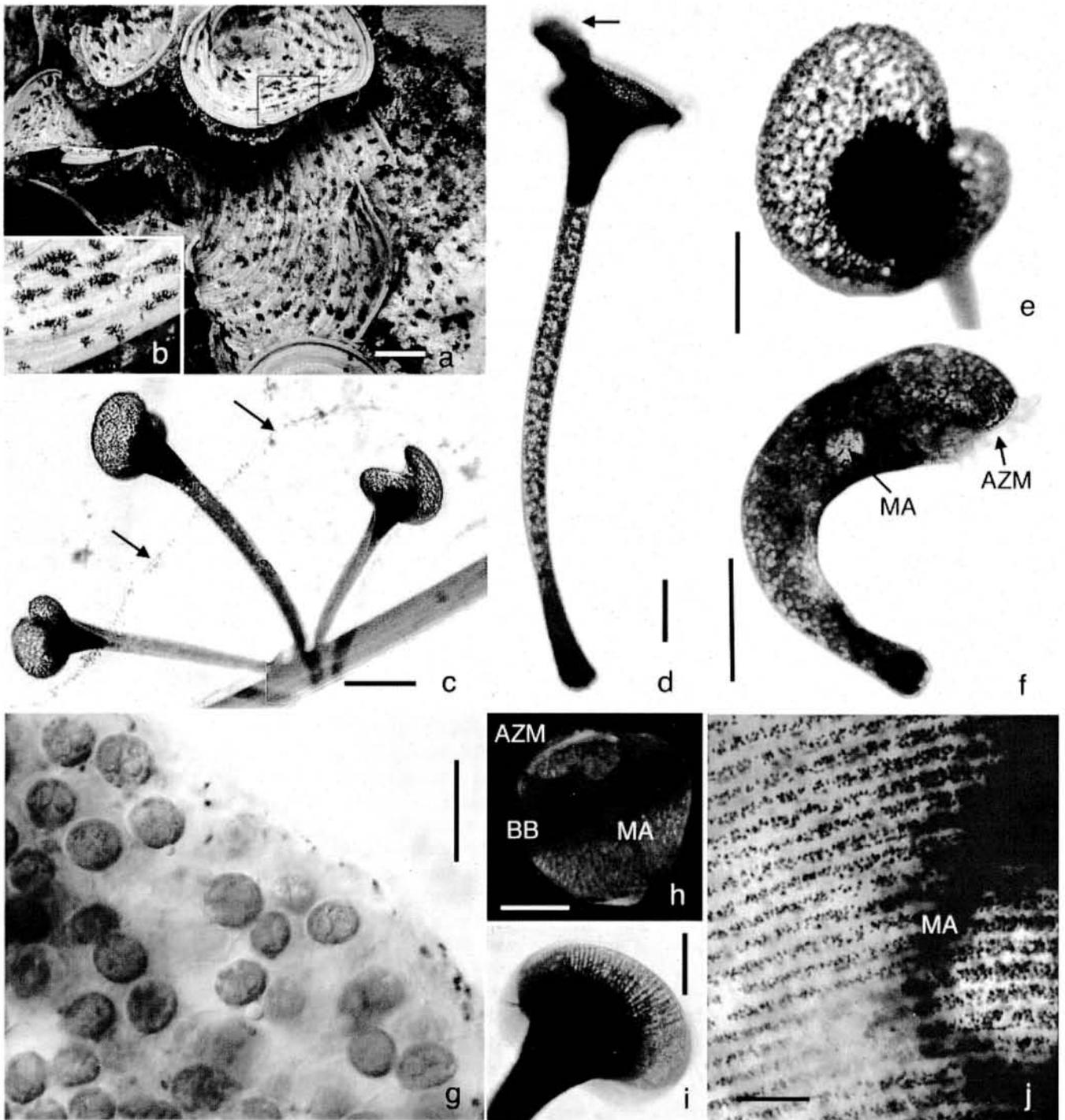
For transmission electron microscopy, individual cells were selected by fine pipette and relaxed in 7% w/v MgCl₂. Some cells were fixed for ca. 30 min in a cocktail of 2.5% v/v glutaraldehyde, 200 mM cacodylate buffer (pH 7.4), and 10% w/v sucrose. These

Fig. 1a–j *Maristentor dinoferus*. Living specimens. **a** Underwater photograph of undisturbed population shows clusters of individuals on *Padina* sp. as well as on adjacent rock and crustose coralline algae; **b** shows detail at approximately twice life size. **c** Group of three specimens in culture dish showing fully expanded posture (viewed from below with inverted microscope). Note subapical concentration of dark pigment granules and mucilage produced by cells (arrows). **d** Right lateral view of fully expanded specimen, showing display of symbiotic algae in cap and stalk. Arrow marks widely projecting left peristomial lobe. **e** Frontal view of fully expanded cap (peristomial bottom). Note distribution of zooxanthellae in cap and deep ventral indentation dividing peristomial bottom in two conspicuous lobes of unequal size. **f** Cell in crawling posture, on microscope slide, showing macronucleus, membranelle band, and zooxanthellae (differential interference contrast, flash). **g** Posterior portion of crawling cell in optical section, showing symbiotic algae; pyrenoid and nucleus visible in some zooxanthellae (differential interference contrast, flash). **h** Ventral view of fully contracted specimen, showing nuclear apparatus and subapical pigment band (dark field illumination). **i** Cap of cell at night showing distribution of symbionts and cortical pigment; compare with **e**. **j** Surface view showing stripes of cortical granules, which also surround nuclear apparatus (differential interference contrast, flash). The yellow zooxanthellae show in the background. Scale bars: 10 mm (a); 200 μm (c); 100 μm (d, e, f, h, i); 20 μm (g, j) (AZM adoral zone of membranelles; BB black pigment band; MA macronucleus)

cells were washed, then post-fixed for ca. 30 min in ice-cold 0.4% w/v osmium tetroxide. Other cells were immersed in ice-cold 5% v/v glutaraldehyde, 200 mM cacodylate buffer (pH 7.4), and 10% w/v sucrose, followed 15 s later by an equal volume of ice-cold 0.8% w/v osmium tetroxide in 200 mM cacodylate and 10% sucrose. These cells were left on ice for ca. 30 min. In both cases cells were then washed free of fixative and dehydrated through an ascending series of ethanols. After passing through 100% ethanol, the cells were infiltrated with Spurr's resin, then embedded. Silver-gold ultrathin sections were cut with a diamond knife using a Reichert (Leica) Ultracut S ultramicrotome. Sections were mounted on pioloform-coated slot grids after the technique of Rowley and Moran (1975), carbon coated, and finally stained with Reynolds lead citrate (Reynolds 1963) and saturated uranyl acetate in 50% ethanol. Sections were examined using a Zeiss 902 electron microscope.

For DNA extraction, amplification, cloning and sequencing, six samples of cells were taken from a collection from the principal site. DNA was extracted in guanidine lysis buffer (Tkach and Pawlowski 1999), precipitated with isopropanol and dissolved in distilled water. Polymerase chain reaction (PCR) amplifications were performed in a total volume of 50 μl using 40 cycles of 30 s at 94°C, 30 s at 52°C, and 120 s at 72°C, followed by 5 min at 72°C for final extension. All amplifications were done using one specific and one universal primer. Specific primers were designed according to the *Symbiodinium*-related ribosomal DNA (rDNA) sequences available in GenBank. Specific primer sequences were: S-DINO (5'CGTCTCTACCGATTGAGTGA3') and ITS_DINO (5'GTGAATTGCAGAACTCC3'). The sequence of the universal eukaryotic primer L_0 is 5'GCTATCCTGAGRGAAGAACTTCG3'. The localization of primers is shown in the electronic supplementary material (Appendix 1). The amplified PCR products were purified using a High Pure PCR purification kit (Roche Diagnostics) then ligated into the pGEM-T vector system (Promega) and cloned in XL-2 ultracompetent cells (Stratagene). Sequences were prepared with an ABI-PRISM Big Dye terminator cycle sequencing kit and analyzed with an ABI-377 DNA sequencer (Perkin-Elmer), all according to the manufacturers' instructions.

For sequence analysis, a fragment of the 28S rDNA, with 529 aligned sites, was used for phylogenetic analysis. We chose 28S rather than 18S rDNA, because the sequence variation in this gene is more suitable for analysis within the *Symbiodinium* complex



(Lenaers et al. 1991; Scholin et al. 1994; Zardoya et al. 1995; Baker et al. 1997; Wilcox 1998), and a large number of corresponding sequences are available in GenBank. Six sequences obtained from *M. dinoferus* were aligned with 32 sequences of the *Symbiodinium*-complex available in the EMBL/GenBank database (see electronic supplementary material (Appendix 2) for references). SEA-VIEW software (Galtier and Gouy 1996) was used for alignment. The rDNA sequences were analyzed using the neighbor-joining (NJ) method (Saitoh and Nei 1987), applied to distances corrected for multiple hits, and for unequal transition and transversion rates, using the Kimura two-parameter model (Kimura 1980). The reliability of internal branches in the NJ tree was assessed by

bootstrapping (Felsenstein 1988), with 1,000 resamplings. The PHYLO_WIN program (Galtier and Gouy 1996) was used for distance computations, tree-building, and bootstrapping. The six *Symbiodinium* sp. sequences have been deposited in GenBank (accession numbers: AJ278598–AJ278603), as follows: AJ278598, sten_2 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2' (ITS2), isolate sten_2; AJ278599, sten_8 28S rRNA gene and internal transcribed spacer 2' (ITS2), isolate sten_8; AJ278600, sten_10 28S rRNA gene and internal transcribed spacer 2' (ITS2), isolate sten_10; AJ278601, sten_9 28S rRNA gene and internal transcribed spacer 2' (ITS2), isolate sten_9; AJ278602, sten_10b

28S rRNA gene and internal transcribed spacer 2' (ITS2), isolate sten_10b; AJ278603, sten_12 28S rRNA gene and internal transcribed spacer 2' (ITS2), isolate sten_12.

Results

Occurrence, ecology, and behavior

To date, *Maristentor dinofereus* has been positively identified only from Guam (as listed in "Materials and methods"). There was a population at Scuba Beach continuously from July 1999 to July 2000, sparse except during July and August 1999, when it was abundant and extensive. In July 1998, we found one similar-looking patch on *Padina* spp. from a reef in the Somosomo Strait off Taveuni, Fiji. We collected the sample and examined it carefully with a hand lens but could not check microscopically. We cannot exclude the possibility of its being another species, but we are confident that it was in the genus *Maristentor*.

M. dinofereus was most commonly seen on *Padina* spp. (Fig. 1a), but also occurred on other surfaces (Fig. 1a), including coralline algae, coral rubble, and dead shell. Patches ranged from a few clusters on single blades of *Padina* spp. up to populations covering 400 cm² of substrate (Fig. 1a shows an area ~10 cm wide of a larger patch; we distinguished over 400 individuals within area of the inset, Fig. 1b). Individuals were seen gliding on the substrate between clusters, and additional observations in culture (see below) suggest that the clusters were dynamic. Patches were evident from several feet away, and at close range individual ciliates could be distinguished with the naked eye. If a blade of *Padina* sp. with clusters of *M. dinofereus* was tapped, the individuals immediately contracted. This obvious behavior was an aid to identification in the field. We have not ascertained whether portions of the population are also in the sediments or water column.

In captivity, *M. dinofereus* quickly left the seaweed. Within 30 min many had left, and within 3 h virtually all had. They collected near the water surface, either attaching to the wall of the container or hanging from the water meniscus. Freshly collected *M. dinofereus* in a daylight bag stayed on *Padina* spp. for over 1 h; whereas, in a comparable collection covered with black cloth, they swam to the top and sides of the bag within 30 min. Those in the daylight bag also left the seaweed overnight and did not return to it in the morning. However, during two night dives (2100, 0400 hours), we observed that *M. dinofereus* in situ remained on *Padina* spp.

Cells showed a strong preference to be at or near the water surface on the lighted side of a container, or even to hang from the surface meniscus. Fresh specimens showed positive phototaxis: in dim light they swam to the bottom of tubes illuminated from below. They formed dynamic clusters of some 15–30 individuals. At times, most of the cells in the dishes were in clusters; at other times, they were more uniformly dispersed. Cells

monitored over a period of a month in September 1998 were always clumped when observed during the late afternoon or evening, but were dispersed on 8 of 14 mornings; usually, all dishes were similar at a given time.

M. dinofereus exhibited distinct day–night changes (see Fig. 1e vs. Fig. 1i; Table 1). The description given under "posture one" below reflects the appearance during the day, when the stalk was fully extended: the algal symbionts in the cap were spread out in a monolayer, and very little pigment was visible in this region or in the stalk. The great majority of the algae were exposed in clear areas of the cap and stalk, but some were in the anterior pigmented band. Within about an hour after nightfall, however, the stalk contracted somewhat, few algae were visible in the cap, especially towards the margins, and stripes of cortical pigment granules were clearly visible in the cap (see Fig. 1i). The cap remained extended, however, and the membranellar band seemed as active as during the day. In contrast to the contracted stalk, the cap remained transparent, notwithstanding the pigment stripes. The changes did not appear to be simple responses to darkness; they may result from a circadian rhythm, but tests in standardized culture conditions are needed to examine this further.

Morphology

M. dinofereus exhibited four postures (shapes) in the laboratory, and we have seen three of these in nature. Furthermore, *M. dinofereus* showed pronounced day–night changes (described above). Morphometric data (Table 1) show the variation of cells under various circumstances (Figs. 1, 2, 3, 4, 5, 6).

Posture one was the sedentary, extended state, where the organism was up to 1 mm long and slenderly trumpet-shaped (Figs. 1c, d, e, 2a). It showed a wide, asymmetric peristomial bottom ("cap") and a gradually narrowing postoral portion ("stalk") attached to the substrate by a heavily ciliated holdfast (Figs. 2c, 4f). The holdfast very likely secreted the mucilage skein that was observed on the side of the dish where *M. dinofereus* had settled. We also noted mucilage around a cluster of three individuals in the tissue culture chamber (Fig. 1c). However, we never observed a mucilaginous lorica as is known from several *Stentor* species (Foissner and Wölfl 1994).

In the extended state, the peristomial area was spread out and divided into two conspicuous lobes by a deep ventral indentation. The left lobe, which contained the buccal part of the adoral zone of membranelles, was higher and larger than the right lobe, giving the cap an L-shape when seen from the left (Fig. 1c). The organism was thick and dark in the region below the cap, because most of the pigment granules were concentrated there; thus, the ciliate appeared black at low magnification (100×) and to the naked eye (Fig. 1a, b). The buccal apparatus was also obscured.

Table 1 *Maristentor dinoferus*. Morphometric data (in μm). Peristomial bottom width measured as the lateral axis and depth as the dorsoventral axis (IV in vivo; P protargol-impregnated, mounted specimens from field; N number of specimens investigated)

Characteristic	Method	Mean	SD	Median	Min.	Max.	N
Body, length fully extended (day)	IV	825.0	116.5	800	600	1000	8
Body, length fully extended (night)	IV	457.0	37.1	450	400	500	10
Peristomial bottom, width (day)	IV	253.5	23.0	250	190	290	20
Peristomial bottom, depth (day)	IV	213.0	25.2	200	150	250	20
Stalk, width near base (day)	IV	32.5	4.6	30	30	40	8
Macronuclei, number	P	1.0	0.0	1.0	1	1	21
Macronucleus, long axis	P	35.2	3.6	35.0	28	42	21
Macronucleus, short axis	P	32.6	4.4	33.0	23	40	21
Micronuclei, diameter	P	2.0	0.0	2.0	2	2	21
Pigment granules, diameter	IV	1.4	0.5	2.0	1	2	20
Ciliary rows in mid-body, number	P	101.1	7.6	102.0	80	110	21
Adoral membranelles, number	P	396.8	38.4	390.0	350	460	11
Symbiotic algae, diameter	IV	9.5	0.9	10.0	8	11	20
Symbiotic algae, long axis	P	8.0	0.7	8.0	7	9	21
Symbiotic algae, short axis	P	7.6	0.7	8.0	6	9	21

Posture two was the crawling state. Cells migrated across the substrate, crawling with the stalk almost fully extended and flattened against the substrate, but with the cap withdrawn (seen in situ and in culture dishes). This was also how they appeared when constrained on a microscope slide (Fig. 1f). In the crawling state, *M. dinoferus* resembled Fauré-Fremiet's (1936) illustration of *Condylostoma auriculatus*, except that the more prominent or sharply defined lobe was the left one, not the right. They seemed to move with the right lateral surface to the substrate (left lobe uppermost), and not with the oral (ventral) side to the substrate as Tartar (1961) reported for *Stentor* spp.

Posture three is the contracted state (Figs. 1h, 2b, 5a). When disturbed, and also spontaneously, cells contracted abruptly, becoming broadly conical or globular. Most of their algal symbionts were then obscured by a broad, subapical, transverse band of pigment (Figs. 1h, 2b), corresponding to the dark area of the extended cell.

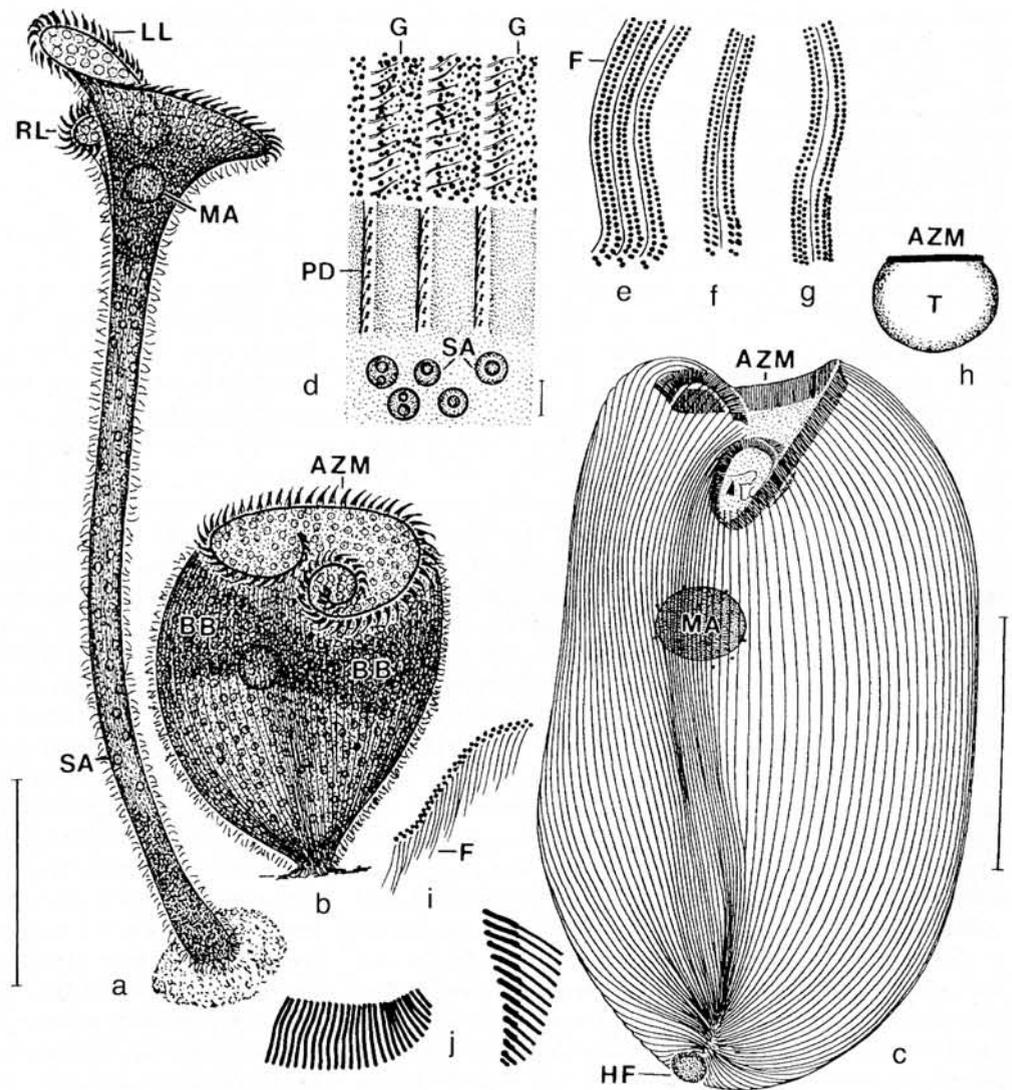
Posture four was the free-swimming state. Cells swam when first transferred to a culture dish, but under most other conditions moved by crawling. *M. dinoferus* swimming could not be observed in situ. They swam rapidly, spiraling clockwise, with the stalk and peristomial bottom contracted, becoming shorter by more than half, but not completely contracted. Swimming cells were broadly conical or bullet-shaped, tapering toward the posterior. When they contacted a surface they did so with the frontal area first, subsequently lying down and extending the stalk to take up the crawling posture.

The nuclear apparatus lay subapically below the buccal cavity and consisted of a single, globular macronucleus and many minute micronuclei. In vivo, it was often difficult to see, hidden by pigment granules and symbiotic algae (Fig. 1c, d, i). The macronucleus was about 40 μm across (Figs. 1f, 2c) and contained many minute, pale nucleoli. There were more than ten micronuclei of $\sim 2 \mu\text{m}$ diameter near and attached to the macronucleus (Fig. 4g); the exact number could be not determined because the macronucleus always hid some micronuclei.

Pigment granules occurred in both the cortical region and the ground cytoplasm. In the cortex, they were arranged in conspicuous stripes between the ciliary rows, which stood out as clear lines (Figs. 1j, 2d). The pigment granules were 1–2 μm across and dark blood red to reddish purple when observed under high magnification and transmitted light (Fig. 1g, j); under low magnification, they often appeared bluish or greenish. The aqueous phase of the pigment extract, containing the cortical pigment(s), appeared red in bulk (e.g. in a bottle 50 mm diameter) but bluish or gray in smaller quantities (e.g. a spectrophotometer cuvette). Some ciliate pigment appeared to transfer to the ether phase along with the zooxanthellar pigments and remained at the origin in preliminary thin-layer chromatography (data not shown). The absorption spectrum of the aqueous phase showed a broad peak in the blue (ca. 420–480 nm), and sharp peaks in the yellow-green (ca. 550 nm) and red (600 nm) spectra (data not shown). This extract fluoresced red under white, blue, and "black" light (electronic supplementary material – Appendix 3). The emission appeared to be in the range 600–650 nm, but we did not have the equipment to measure the fluorescence emission or excitation spectra, nor to assess fluorescence in vivo. The color of the granules as seen under the microscope, especially with flash photography and high magnification (Fig. 1g, j), may be partly due to fluorescence in vivo.

M. dinoferus was holotrichously (completely) ciliated. The cilia emerged from closely spaced (0.5–1 μm in contracted, protargol-impregnated specimens) dikinetids (pairs of basal bodies) that were ciliated on only one basal body, possibly the anterior (Figs. 2d, 6a). The ciliary rows extended longitudinally in the same pattern as in *Stentor* spp. (Tartar 1961; Foissner et al. 1992), at least posteriorly (Fig. 2c). The width of spacing of the ciliary rows graded imperceptibly around the circumference of the cell from the most widely spaced rows (separated by the broadest pigment stripes) on the left of the buccal cavity, to the most closely spaced ciliary rows (separated by the narrowest pigment stripes) near the

Fig. 2a–j *Maristentor dinoferrus*. Postures (shapes) and cytological details from life (a, b) and after protargol impregnation (c, middle part of d, e–j). **a** Left lateral view of extended specimen. The peristomial bottom is divided into two conspicuous lobes by a deep ventral indentation. **b** Fully contracted specimen. **c** Total ventral view showing main organelles. Note stripe contrast zone in cell's midline. **d** Surface view showing main structures from outside to inside: stripes of cortical granules between ciliary rows; paired basal bodies which give rise to the postciliodesmata; and symbiotic algae. **e–g** Structure of adoral membranelles in the regions marked in Fig. 3. **h** Cross section of tubular structure under adoral zone of membranelles. **i** Part of paroral membrane. **j** Distal and proximal end of adoral zone. **Scale bars:** 200 μm (a, b); 100 μm (c); 10 μm (d) (AZM adoral zone of membranelles; BB black pigment band; F fibers; G cortical granule stripes; HF holdfast; LL left peristomial lobe; MA macronucleus; PD postciliodesma; RL right peristomial lobe; SA symbiotic algae; T tubular structure underneath AZM)



midline slightly right of the buccal cavity. Along the cell's midline the most closely spaced rows abutted the most widely spaced rows at acute angles, forming a suture, the so-called "stripe contrast" (Fig. 4b). The peristomial field, which was slightly elliptical (Table 1), bore numerous dikinetids with single cilia. These cilia were very scattered (Figs. 3, 5b) in contrast to *Stentor* spp., where they form semicircular rows (Foissner et al. 1992; Foissner and Wöfl 1994).

Two fibrillar systems were recognizable after protargol impregnation in the somatic cortex. The first comprised the postciliodesmata (Figs. 2d, 6a), which were associated with the basal bodies of the cilia and extended close to the right of the ciliary rows; usually they impregnated only faintly with protargol. The second, which obviously represented the contractile (myoneme) system, also extended right of the ciliary rows and showed numerous bifurcations in the stripe contrast zone. The individual myonemes were 1–2 μm wide bands of very fine fibers and were often impregnated heavily with protargol (Fig. 4c).

The oral apparatus was composed of a prominent adoral zone of membranelles and a minute paroral membrane (Figs. 3, 4c). The adoral zone, which gradually narrowed toward both ends (Fig. 2j), performed one spiral turn on the margin of the peristomial bottom and a second in the comparatively small buccal cavity left of the cell's midline (Figs. 2c, 4d, e). In its main portion, the individual membranelles consisted of two 8–12 μm long ciliary rows and a single, oblique dikinetid proximally (Fig. 2e); in the buccal portion of the zone, the membranelles had a short third row attached and lacked the proximal dikinetid (Fig. 2f, g). Very fine fibers originated from the membranelles and formed a conspicuous tubular structure under the zone (Figs. 2h, 3). The paroral membrane extended from the proximal end of the adoral zone anteriorly on the opposite side of the buccal cavity and ended near the buccal opening (Figs. 2c, 3). The paroral membrane was difficult to recognize and composed of (ciliated?) dikinetids from which fibers (~10 μm long) originated and extended into the buccal cytoplasm (Figs. 2i, 4c).

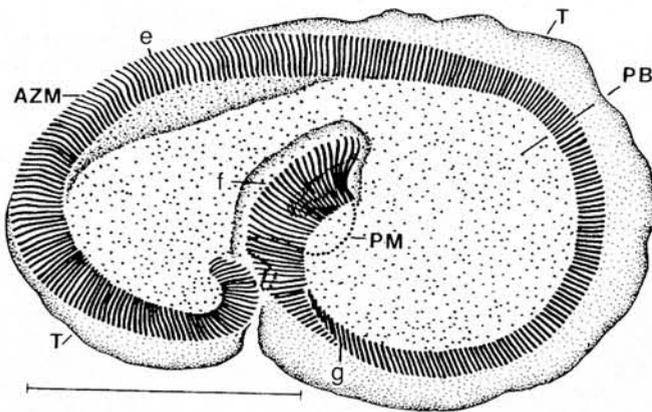


Fig. 3 *Maristentor dinofereus*. After protargol impregnation. Frontal view; note scattered basal bodies on peristomial bottom and short paroral membrane. Scale bar: 50 μm (AZM adoral zone of membranelles; PB peristomial bottom; PM paroral membrane; T tubular structure underneath AZM; e, f, g denote regions shown in Fig. 2e–g at higher magnification)

M. dinofereus contained about 500–800 zooxanthellae (Figs. 1g, 4a), mainly in the expanded cap, where they formed a single layer (Fig. 1e), and in the stalk (Fig. 1d), but also subapically, where they were almost invisible due to the concentrated pigment granules. The individual symbionts were 8–10 μm across in vivo, with an evident pyrenoid and nucleus (Fig. 1g). We observed many dividing algae, showing that they were indeed symbionts and not merely ingested food. Transmission electron microscopy showed that the symbionts belong to the Dinophyta and, probably, to *Symbiodinium* sp. They possessed the typical dinoflagellate nucleus with condensed chromosomes (Fig. 6b), triple plastid lamellae (Fig. 6e) with no girdle lamellae, and cytoplasmic channels through the dividing nucleus (Fig. 6c, d). The pyrenoid was surrounded by the plastid membrane and lacked invasive plastid thylakoids (Fig. 6b). Zooxanthellae showed no significant thickening of the amphiesmal vesicles (by thecal plate material) but were enclosed in host vacuoles (Fig. 6b, c). We do not have any further information about the vacuoles; no connection with the ciliate cytoskeleton was evident, although the zooxanthellae are moved within the cell, as noted above.

Molecular identification of the zooxanthellae: sequence data and phylogenetic analysis

For one sample, we obtained a 1,595-bp fragment which included the whole ITS rDNA region, starting at the 3' end of the 18S rDNA and terminating in the conserved region C4 of the 28S rDNA. This fragment was amplified with primers S_DINO and L_0 (electronic supplementary material – Appendix 1). For five other samples, a shorter fragment of about 1,060 bp was amplified using a pair of primers ITS_DINO and L_0. This fragment comprised only the ITS2 region and the

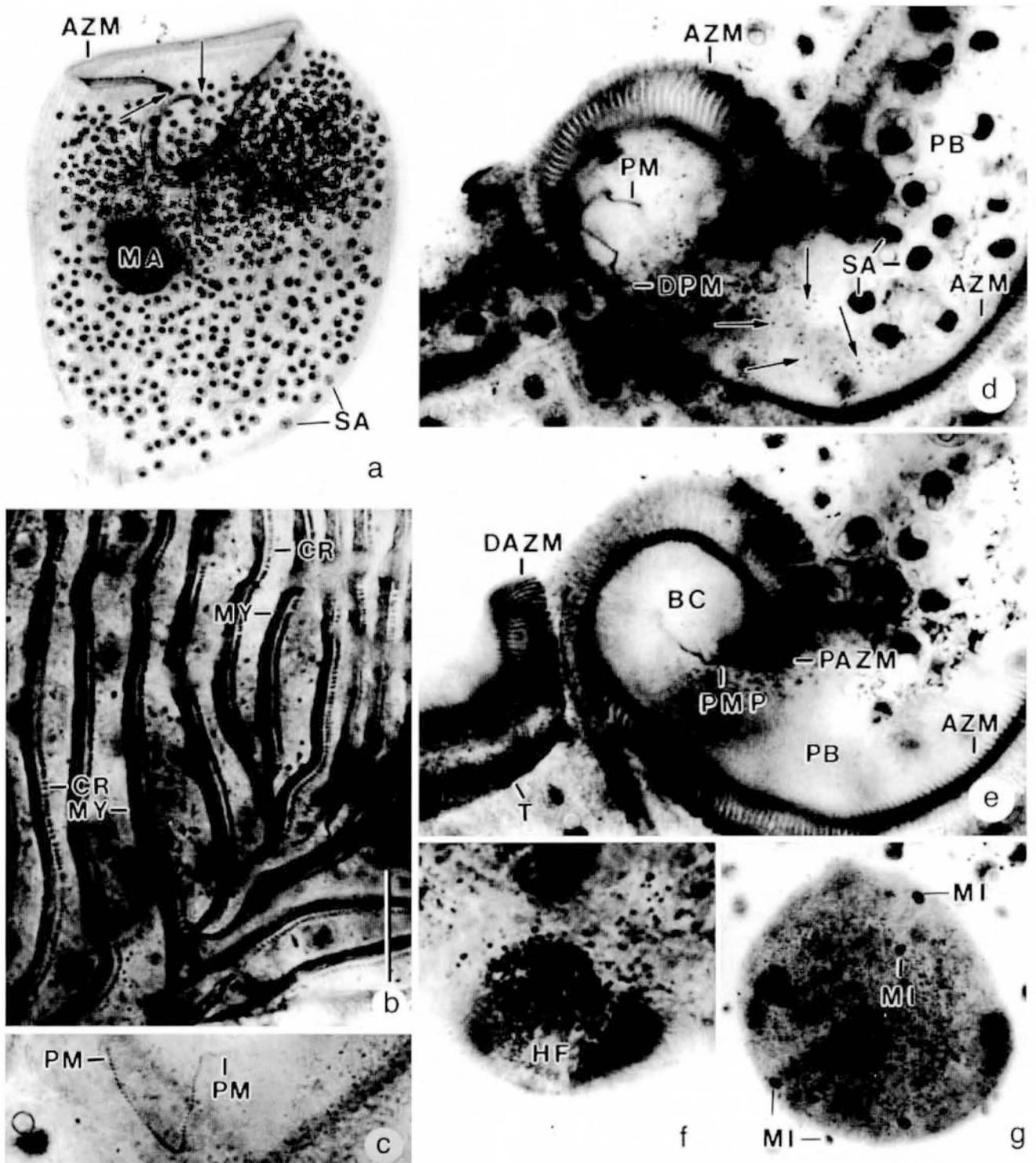
domains C1–C4 of the 28S rDNA (electronic supplementary material – Appendix 1).

One clone was sequenced for each DNA extract. Two sequences (sten_8 and sten_10) were identical. Two others (sten_12 and sten_10b) differed by only two mutations (0.175% of sequence divergence). The mean divergence between all sequences obtained from *M. dinofereus* was very low ($0.42 \pm 0.26\%$). This variation was due to three transitions, four transversions, and two insertions within the six analyzed sequences (data not shown). It corresponded to the mean sequence divergence ($0.88 \pm 0.99\%$) observed within *Symbiodinium* sp. clade C, where our sequences branched according to phylogenetic analyses (see below). For comparison, the mean sequence divergence between clade C and other clades (A, B, D) was, respectively, 22.74%, 12.75%, and 18.68%.

The phylogenetic analysis of six sequences obtained from *M. dinofereus* was compared with 32 dinoflagellate sequences (Fig. 7). All sequences grouped into five clearly distinguished clades, each supported by 100% bootstrap value. One clade, composed of *Gymnodinium simplex* and *G. beii* was chosen as the outgroup, following previous analyses with more extensive samples of dinoflagellates and other eukaryotes (Wilcox 1998). Three other clades correspond to clades A, B, and C identified previously by several authors (Rowan and Powers 1991; Rowan and Knowlton 1995; Baker et al. 1997). The structure of the tree and branching order between the clades is consistent with previous work (Wilcox 1998). Two sequences, AF170148 and AF170149 (Baker 1999), form a fourth *Symbiodinium* sp. clade, here referred to as “D”.

Diagnoses

- *Maristentor* n. gen.
- Diagnosis: Stentoridae Carus, 1863, having cilia scattered (not in rows) on peristomial bottom and paroral membrane restricted to proximal portion and opposite side of adoral zone of membranelles.
- Type species: *Maristentor dinofereus* n. sp.
- Etymology: Composite of names referring to locality (Mariana Islands) and body shape resemblance to the genus *Stentor*.
- *Maristentor dinofereus* n. sp.
- Diagnosis: Length in vivo about 800 μm , width of peristomial bottom about 250 μm . Distinctly trumpet-shaped when attached and fully extended, peristomial bottom slightly elliptical and divided into two conspicuous lobes by deep ventral indentation. Single, globular macronucleus and many spherical micronuclei. Cells dark at low magnification due to stripes of dark blood red cortical granules and 500–800 dinoflagellate endosymbionts (zooxanthellae) in the genus *Symbiodinium*. On average 101 somatic ciliary rows and 397 adoral membranelles, forming a spiral on both the margin of the peristomial bottom, and in the buccal cavity. Gregarious.



- Type location: Coral reef in Apra Harbor, Guam, Mariana Islands, 13°27'N; 144°40'E.
- Type material: One holotype slide and two paratype slides with protargol-impregnated specimens (protocol B in Foissner 1991; Wilbert's method) have been deposited in the Oberösterreichische Landesmuseum in Linz (LI), Austria, accession numbers: 2000/48–50.

- The slides contain many specimens with relevant cells marked by black ink circles on the cover glass. Also deposited at this locality are three tubes with specimens fixed as for transmission electron microscopy (see "Materials and methods").
- Etymology: Bearing dinoflagellates, with reference to the algal partner in the symbiosis.

Fig. 4a–g *Maristentor dinoferus*. After protargol impregnation. **a** Ventral view showing distal (left arrow) and proximal (right arrow) end of membranellar spiral and symbiotic algae concentrated in a broad, subapical band. **b** Stripe contrast zone, where cilia rows and myonemes of the right and left side merge (cf. Fig. 2c). **c** The paroral membrane is composed of narrowly spaced dikinetids. **d, e** Oral apparatus at two focal planes, showing the short paroral membrane, which commences at the proximal end of the adoral zone of membranelles and extends anteriorly on the opposite side of the buccal cavity. Arrows mark some of the many scattered dikinetids on the peristomial bottom. **f** The holdfast bears numerous scattered basal bodies (cilia). **g** Nuclear apparatus. Scale bar: 10 μm (in b only; no scale bars on other figures because they show heavily squashed specimens) (AZM adoral zone of membranelles; BC buccal cavity; CR ciliary rows; DAZM distal end of AZM; DPM distal end of PM; HF holdfast; MA macronucleus; MI micronucleus; MY myonemes; PAZM proximal end of AZM; PB peristomial bottom; PD postciliodesma; PM paroral membrane; PMP proximal end of PM; SA symbiotic algae; T tubular structure underneath AZM)

Discussion

The general organization of *Maristentor dinoferus* matches that of heterotrich ciliates, as defined by Kahl (1932), Corliss (1979), and Foissner et al. (1992). The familial classification is more difficult and is based on the review by Tuffrau (1994). Certainly, this organism resembles *Stentor*, a common and well-known genus of freshwater ciliates (for reviews, see Foissner 1991; Foissner and Wölfl 1994), in its sessile habit, body shape, contractility, somatic ciliary pattern, and location and

arrangement of the most prominent organelle, the adoral zone of membranelles. Even the bilobate peristomial bottom is not unique because it is found also in *Stentor barretti*, a freshwater species (Warren 1985). However, a more detailed analysis reveals two significant differences that, in the absence of a detailed phylogenetic understanding of *Stentor* and its close relatives, we consider relevant at genus level. First, the cilia on the peristomial bottom form ordered rows in *Stentor* spp., while they are scattered in *M. dinoferus*. Second, the paroral membrane accompanies the entire adoral zone of membranelles in *Stentor*, but in *M. dinoferus* consists of only a short row opposite the buccal portion of the membranellar zone.

The paroral membrane of *M. dinoferus* does not accompany the adoral zone of membranelles, as in *Stentor* spp., but extends on the opposite side of the buccal cavity. Thus, the presumed relationship between the genera *Stentor* and *Maristentor* may be questioned and demands a more detailed comparison with other heterotrichs. Specifically, the *Maristentor* pattern resembles genera like *Spirostomum*, *Blepharisma*, *Gruberia*, and *Condylostoma*, which occur in both freshwater and marine habitats. However, the peristomial bottom of these and related genera is unciliated and all are freely motile and have the adoral zone of membranelles orientated along the main body axis (Dragesco and Dragesco-Kernéis 1986; Foissner et al. 1992; Tuffrau 1994). Thus, the genus *Maristentor* is very likely more closely related to *Stentor* than to spirostomids and condylostomatids. Accordingly, we assign *Maristentor*

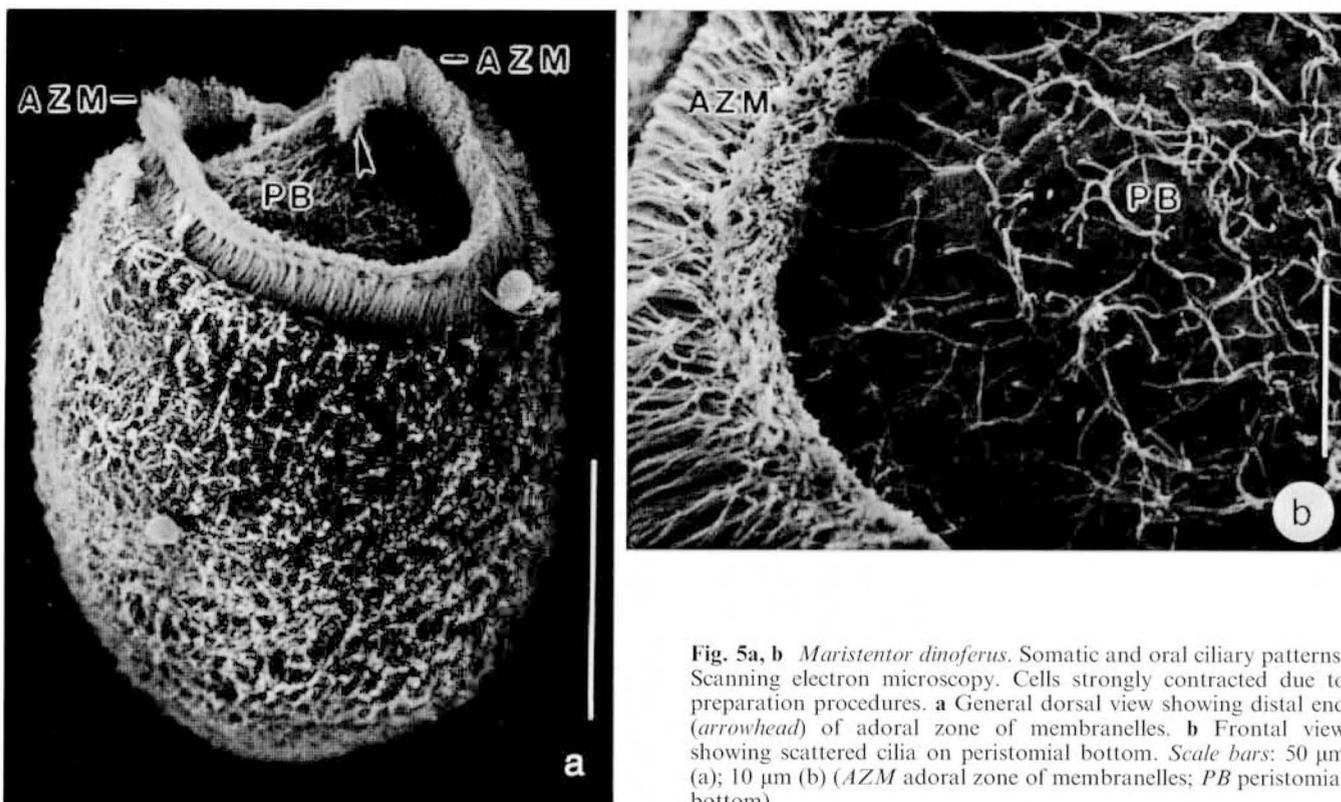


Fig. 5a, b *Maristentor dinoferus*. Somatic and oral ciliary patterns. Scanning electron microscopy. Cells strongly contracted due to preparation procedures. **a** General dorsal view showing distal end (arrowhead) of adoral zone of membranelles. **b** Frontal view showing scattered cilia on peristomial bottom. Scale bars: 50 μm (a); 10 μm (b) (AZM adoral zone of membranelles; PB peristomial bottom)

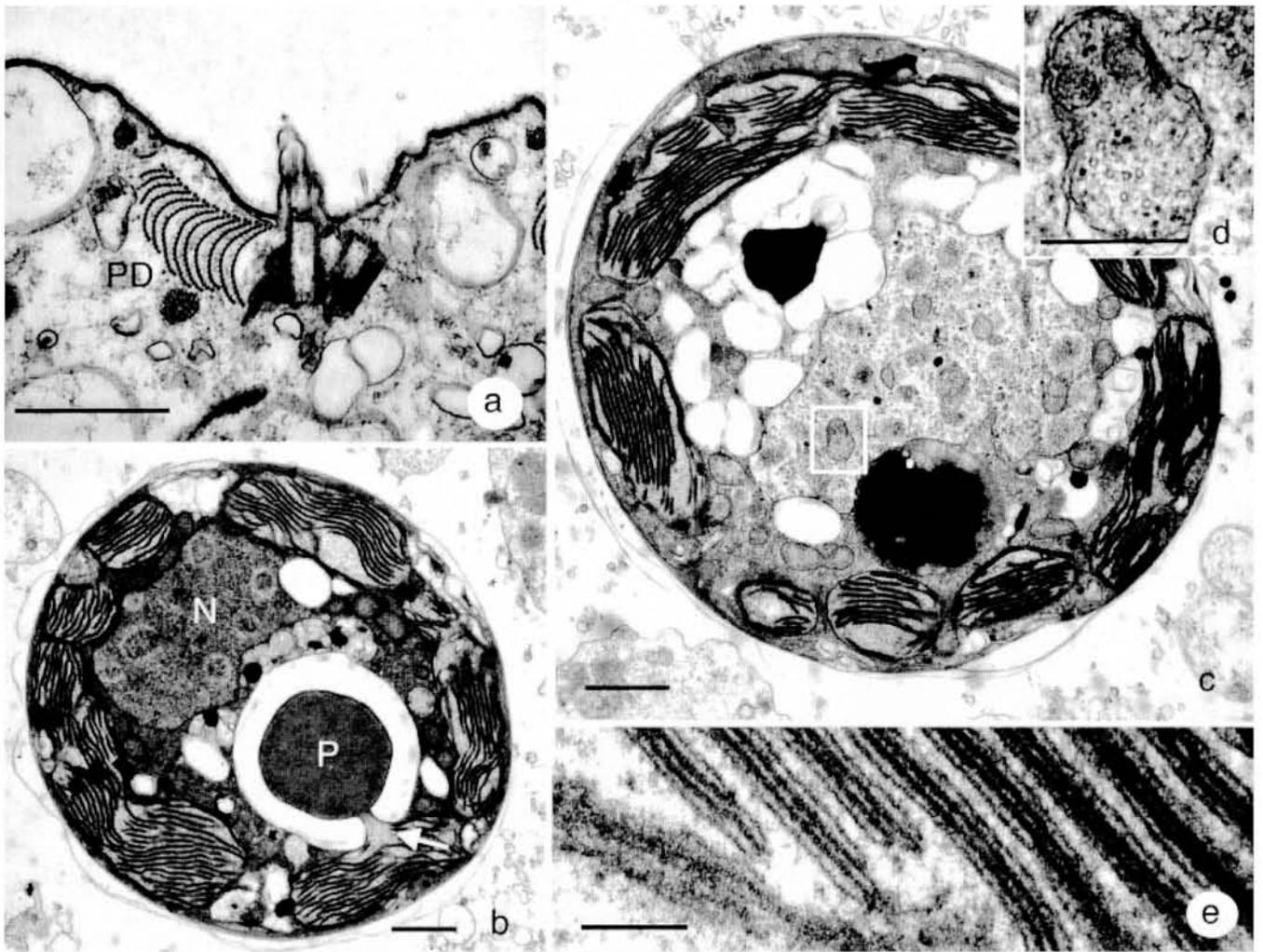


Fig. 6a–e *Maristentor dinoferus* (a), *Symbiodinium* sp. (b–e). Transmission electron microscopy. **a** Transverse section of somatic cortex showing a ciliated basal body, which gives rise to a postcilial microtubule ribbon, several of which overlap forming a conspicuous postciliodesma. **b** Symbiont showing stalked pyrenoid (arrow points to stalk). **c, d** Cell with nucleus undergoing division, bundles of microtubules in fenestrae (enlarged in d). **e** Detail of plastid showing triple lamellae characteristic of dinoflagellate. Scale bars: 1 μm (a–c); 0.5 μm (d); 0.1 μm (e) (N dinoflagellate nucleus; P pyrenoid; PD postciliodesma)

to the Stentoridae, which was previously monotypic (Tuffrau 1994).

Few *Stentor* species have been found in marine habitats (Foissner and Wöfl 1994). Of these, *S. auricula* (Kent 1880, 1881, 1882) and *S. auriculatus* (Kahl 1932) deserve special attention because Jankowski (1978) classified them in a new genus, *Condylostentor*. However, *Condylostentor* differs from *Maristentor* in having a conspicuous paroral membrane, in lacking cilia on the peristomial bottom, and in lacking pigmented cortical granules (Kent 1880, 1881, 1882; Gruber 1884; Kahl 1932; Fauré-Fremiet 1936). As mentioned by Fauré-Fremiet (1936), both *S. auricula* and *S. auriculatus* very likely belong to or are closely related to species in the

genus *Condylostoma*. These two marine *Stentor* spp. are much smaller (extended length = 350 μm) than *M. dinoferus* and are colorless (Kahl 1932).

Two other marine ciliates are very different from *M. dinoferus*. One is "*Halofolliculina corallasia*," a loricate heterotrich from the Indo-Pacific region, which appears as deep-purple to black bands on a wide variety of massive and branching corals (Antonius 1999). Similarly, *M. dinoferus* is not related to Wichterman's (1942) *Paraeuplotes tortugensis*, which is a hypotrichous ciliate (Nassophorea: Hypotrichia), for which Wichterman proposed a new family, Paraeuplotidae (not listed by Small and Lynn 1985).

Ultrastructurally, the symbiont closely resembles *Symbiodinium* spp. (Trench and Blank 1987), and molecular data confirm the generic identity. Phylogenetic analysis shows that all six sequences obtained from *M. dinoferus* fall within clade C, as do symbionts isolated from a variety of invertebrate hosts in Guam (Pochon et al., in press). Although this is the only group of *Symbiodinium*-like symbionts found in this ciliate, they do not seem to be host specific. The *M. dinoferus* symbionts do not form a single group, but are interspersed among other clade C sequences. It is difficult to

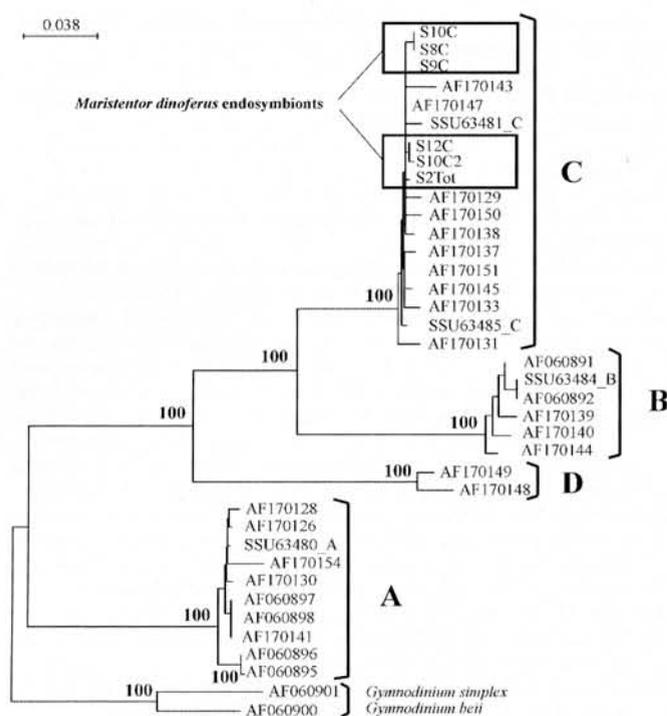


Fig. 7 *Symbiodinium* sp. Phylogenetic reconstruction of the complex, inferred from partial LSU rDNA (28S) based on the neighbor-joining method, including six sequences obtained from *Maristentor dinoferus*. Analysis based on 529 aligned sites excluding all gaps. Numbers at nodes represent percentage bootstrap support following 1,000 (neighbor joining) data resampling. *Gymnodinium simplex* and *G. beii* were chosen as the outgroup

determine if this is due to a very low sequence divergence within this clade or to a lack of specificity in these particular symbionts. The analysis of ITS sequences, which evolve faster than the 28S sequences (Lee and Taylor 1992; Baldwin et al. 1995), did not show any unique pattern for *M. dinoferus* symbionts (data not shown).

M. dinoferus is the first ciliate with zooxanthellae that are unequivocally dinoflagellates, to the best of our knowledge. The only other tropical marine ciliate with symbionts had yellowish-brown bodies that Wichterman (1942) identified as *Zooxanthella*, but his description of the zooxanthellae is derived entirely from an account of zooxanthellae from corals, and not from detailed observation of the symbionts. Green algae or cyanobacteria occur as symbionts in other ciliates, including several species of *Stentor* (Taylor 1984; Foissner and Wöflfl 1994). The gymnostome ciliate *Mesodinium rubrum* has a cryptophyte symbiont (Taylor 1982). While dinoflagellates were previously unknown in ciliates, many tropical reef invertebrates and foraminifera form symbioses with dinoflagellates, particularly *Symbiodinium* spp. (Rowan 1998; Carlos et al. 1999). The diurnal changes in morphology are also novel observations for ciliates, although a tidal rhythm in the marine oligotrich ciliate *Strombidium oculatum* was reported by Fauré-Fremiet (1948).

Metamorphosing corals of many species must re-establish their symbiosis from the benthic pool of free-living *Symbiodinium* spp. *M. dinoferus* might participate in this pool (suggested by the genetic similarity), or might maintain a separate stock through cell division. We have not observed reproduction in *M. dinoferus*, but presume that cell division would divide the algal population between the daughter cells.

Although *M. dinoferus* was most commonly seen on blades of *Padina* spp., this may be largely due to this alga being a very common substratum on Guam reefs and providing a contrasting background against which clusters of the ciliate are more readily seen. We have documented *M. dinoferus* on other surfaces, as noted above, but patches on dark backgrounds were very difficult to see. On the other hand, preliminary observations of other erect seaweeds adjacent to patches on *Padina* spp., indicated that *M. dinoferus* was not present on *Halimeda* sp. or *Tydemania expeditionis*. More work needs to be done to establish the substratum preferences (if any) of *M. dinoferus*.

The role of the cortical pigment is unknown. We are intrigued that a ciliate with endosymbiotic algae has extensive cortical pigment even at 20 m depth, and that the fluorescence of the pigment appears to be near the red absorption peak for chlorophylls. Studies of *Stentor* spp. pigments (reviewed by Tartar 1961, pp 47–48) offer no insights. However, evidence from corals that some fluorescent host pigments may enhance zooxanthellar photosynthesis (Schlichter et al. 1985, 1986, 1994; Schlichter and Fricke 1991) suggests a line of study on *M. dinoferus*. Schlichter and colleagues have found turquoise (430–500 nm) fluorescence in a variety of corals, especially the deepwater *Leptoseris fragilis*. Gastrodermal chromatophores in this coral form a layer under the zooxanthellae. Photosynthetic enhancement would be worth testing in *M. dinoferus*. Perhaps in deep water, the pigmented cortical layer of *M. dinoferus* also serves as a “light guide,” making the cells optically black, as has been found in deepwater seaweeds (Ramus 1981). That is, we envision that the upwardly oriented, funnel-shaped cells could retain almost all the light entering them, either by direct absorption by the algal pigments, or after fluorescence from the inner side of the pigment granules. In some shallow-water corals fluorescent pigments may serve a protective role, lying above the algae rather than below (Salih et al. 2000). *M. dinoferus* offers an interesting system in which to study the functional role(s) of the cortical pigment(s), because of the different postures it can adopt and its ability to redistribute zooxanthellae and pigment granules. Green fluorescent protein (GFP) and other fluorescent proteins are widely used as in vivo markers of gene expression and protein localization (Matz et al. 1999), but preliminary characterization of *M. dinoferus* extracts indicated that this fluorescent pigment is not a protein (M. Matz, personal communication).

The relationship between this novel ciliate and the *Symbiodinium* sp. is fascinating in itself, and in

comparison with other *Symbiodinium* spp. symbioses on coral reefs. Laboratory studies on the life history of *M. dinoferus* and its nutritional relationship with the symbionts, and field studies on its behavior and population dynamics are needed.

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