J. Eukaryot. Microbiol., 48(1), 2001 pp. 38–51

Fine Structure of Clonally Propagated In Vitro Life Stages of a *Perkinsus* sp. Isolated from the Baltic Clam *Macoma balthica*

CATHLEEN A. COSS,[1] JOSÉ A. F. ROBLEDO[1] and GERARDO R. VASTA

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202, USA

ABSTRACT. We established monoclonal in vitro cultures of a *Perkinsus* sp. isolated from the baltic clam *Macoma balthica* and compared morphological features of various life stages by light and transmission electron microscopy to those of the currently accepted *Perkinsus* species: *Perkinsus marinus, Perkinsus olseni, Perkinsus atlanticus,* and *Perkinsus qugwadi*. Except that trophozoites were slightly larger than those of *P. marinus,* and that they underwent zoosporulation in culture, observation of our isolate under light microscopy did not reveal striking differences from any *Perkinsus* species. *Perkinsus* sp. from *M. balthica* shared fine structural characteristics with other *Perkinsus* species that clearly place it within this genus. Although zoospores of *Perkinsus* sp. from *M. balthica* were slightly smaller than those from other species, the ultrastructural arrangement and appearance of the apical complex and flagella seem to be identical to those of *P. marinus* and *P. atlanticus.* Our isolate also appeared, in some sections, to have cortical alveolar expansions of the plasmalemma at regions other than the anterior end and lobulated mitochondria that were reported as unique for *P. qugwadi*. Little consensus exists among authors in the assignment of taxonomic weight to any particular morphological feature to designate *Perkinsus* species. The present study of gross morphology and ultrastructure was complemented with molecular studies reported elsewhere, which propose that *Perkinsus* sp. from *Macoma balthica* is a distinct species.

Key Words. Culture, electron microscopy, oysters, parasites, protozoan, trophozoites, ultrastructure, zoospores.

THE protistan parasite, *Perkinsus marinus* (Apicomplexa, Perkinsea), causes significant mortality of the eastern oyster (*Crassostrea virginica*) along the Gulf of Mexico and Atlantic coasts of North America (Burreson and Ragone-Calvo 1985; Ford 1996; Soniat 1996). The systematic position of the genus *Perkinsus* continues to be controversial, and classification of organisms within this genus has been based on a variety of criteria for taxonomic evaluation applied by the different authors. *Perkinsus marinus* was initially described as a fungus, *Dermocystidium marinum* (Mackin, Owen, and Collier 1950), based on the nuclear position close to the cell wall, and the presence of a centrally located endosome. Re-examination of its morphological features, which in culture resembled those from water molds, led to inclusion in the protozoan phylum Labyrinthomorpha as *Labyrinthomixa marina* (Mackin and Ray 1966). Evaluation of zoospore ultrastructure (Perkins and Menzel 1967; Perkins 1969; Perkins 1976) led to reclassification in 1978 to the protozoan phylum Apicomplexa in a newly established taxon (Class: Perkinsea, Order: Perkinsida, Family: Perkinsidae, Genus: *Perkinsus*, Species: *marinus*) (Levine 1978). Later based on actin partial gene sequences, Reece et al. (1997) proposed that *Perkinsus* is more closely related to dinoflagellates than to the apicomplexans. Further re-interpretation by Siddall et al. (1997) of previously reported ultrastructural studies (Azevedo 1989; Azevedo, Corral, and Cachola 1990; Perkins and Menzel 1967; Perkins 1969; Perkins 1976) together with analysis of a few alveolate DNA sequences led them to reject inclusion of *Perkinsus* genus in the phylum Apicomplexa, and to propose a much closer relationship to the Dinozoa. This concept has recently been challenged by the detailed ultrastructural study and rigorous analysis of the SSU sequence of the parasite *Parvilucifera infestans* by Norén, Moestrup, and Rehnstan-Holm (1999), who questioned previous phylogenetic analyses for *Perkinsus* spp. and established a new phylum, Perkinsozoa, within the Alveolata that includes *Perkinsus* spp. under the class Perkinsea, and constitutes a sister group to the apicomplexans.

In addition to *P. marinus,* three *Perkinsus* species are currently accepted: *Perkinsus olseni,* found in two abalone species, *Haliotis rubra* and *Haliotis laevigata,* from Australia (Lester and Davis 1981; Lester, Goggin, and Sewell 1990), *Perkinsus atlanticus,* first identified in the clam *Ruditapes decussatus* in Portugal (Azevedo 1989), and *Perkinsus qugwadi,* described in the Japanese scallop, *Patinopecten yessoensis,* cultured on the west coast of Canada (Blackbourn, Bower, and Meyer 1998). *Perkinsus karlssoni* was described as a new species in the bay scallop (*Argopecten irradians*) from the east coast of Canada (McGladdery, Cawthorn, and Bradford 1991). However, after re-evaluation Goggin et al. (1996) concluded that it was a combination of several different organisms and therefore is no longer considered a *Perkinsus* species.

Perkinsus marinus has been found in *C. virginica* in most areas of the Chesapeake Bay. However, *Perkinsus*-like organisms also have been detected in sympatric bivalve species (Andrews 1954), although little is known about their host specificity, virulence, and taxonomic relationships to *P. marinus.* A study on the ultrastructure of zoospores of a *Perkinsus* sp. from the baltic clam *Macoma balthica* showed that it was similar to that of both *P. atlanticus* and *P. marinus* (Perkins 1968), but its species identification was not resolved. It easily undergoes zoosporulation in sea water, a feature described for *P. atlanticus* (Azevedo, Corral, and Cachola 1990) but difficult to induce in *P. marinus* (Perkins 1996). This led to the suggestion that this *Perkinsus* sp. from *M. balthica* is most likely *P. atlanticus* (Kleinschuster et al. 1994; Perkins 1996).

In order to rigorously describe this *Perkinsus* sp. from *M. balthica* [*Perkinsus* sp. (*M.b.*)], the study reported herein was designed to: (1) establish a monoclonal culture of this isolate from *M. balthica,* and (2) characterize the fine structure of the various life stages of the in vitro clonally-propagated parasite, with special focus on the zoospores, for comparison with the previously described species within the genus *Perkinsus.*

MATERIALS AND METHODS

Collection of clams, fluid thioglycollate medium assay, and establishment of *Perkinsus* sp. in vitro cultures. *Macoma balthica* were collected in August 1996 from the Rhode River, Maryland. Cultures of *Perkinsus* sp. (*M.b.*) were established using clam heart explants, and maintained in 6-well plates or culture flasks (tissue culture treated polystyrene, Corning) in 5, 10 or 50 ml of DME/F12 (1:2), 5% FBS medium, following the methods of Gauthier and Vasta (1993, 1995). The transfer of one-twentieth of the culture volume into fresh medium was designated as one passage. Clonal cultures were established by limiting dilution, as reported earlier (Gauthier and Vasta, 1995). Briefly, cell numbers were counted in a hemocytometer, cell densities adjusted to 3 cells/ml, and plated at 100 μl/well in 96-well plates. Each well received 50 μl of 0.2

Corresponding Author: G. R. Vasta—Telephone number: 410-234-8826; FAX number: 410-234-8896; E-mail: vasta@umbi.umd.edu

[1] CAC. and JAFR. contributed equally to this study.

μm-filtered spent medium. After incubation for 24 h, the wells containing single cells were identified by visual inspection on an inverted microscope. Following incubation for 5 d, wells containing single colonies were selected and transferred to single wells in a 24-well plate. Incubation for an additional 3–4 d was followed with a second round of cloning to ensure clonality of the cultures.

Cultures of *Perkinsus* sp. (*M.b.*) consisted of a mixture of life stages ranging from trophozoites, propagating from budding and palintomy, through the process of zoosporulation, to the release of mature zoospores. Monoclonal cultures derived from the same *Perkinsus* sp. (*M.b.*) culture have been maintained since then in our laboratory and continue to produce the above-mentioned life stages after 4 yr in culture.

Light microscopy. Fifty μl aliquots of the culture were used to view the different *Perkinsus* sp. (*M.b.*) life stages present in the culture. Photomicrographs were taken on a Zeiss Axioskop equipped with differential interference contrast optics interfaced with a Sony 3 CCD Video Camera (Model DXC-760MD) and Sony Video Printer (Model UP-5100).

Transmission electron microscopy. A 3-ml aliquot of a monoclonal culture of *Perkinsus* sp. (*M.b.*) was centrifuged at 380 *g* for 5 min, the supernatant removed, and the cell pellet fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.8, at 4 °C for 3 h. The pellet was then washed in 0.1 M sodium cacodylate buffer, pH 7.8, at 4 °C for 2 h, and post-fixed in buffered 2% OsO_4 at 4 °C for 3 h. After dehydration in an ethanol series the sample was embedded in Spurr's resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined in a Zeiss EM10 CA microscope. For negative staining a drop of washed zoospores was placed on coated grids and fixed with 1% potassium phosphotungstate. Fixed samples (3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.8, at 4 °C for 3 h) were also deposited directly on the grid for negative staining and observed under microscopy as above.

Terminology. Structures of the presumptive apical complex were identified according to the terminology recommended by Perkins (1996).

RESULTS

Mature trophozoites were approximately spherical and 9.8 ± 4.2 μm (n = 30) in diam. under light microscopy. A large eccentric vacuole was observed in mature trophozoites. This vacuole displaced the nucleus against the plasma membrane, giving the cell the ''signet ring'' appearance typical of *Perkinsus* species. In culture the cells divided either by budding (Fig. 1) or palintomy and often remained in clumps.

Zoosporangia were spherical and 67 ± 12 μm (n = 21) in diam. In some zoosporangia the active process of zoosporulation was observed (Fig. 2–5). The immature zoospores divided inside the zoosporangium (Fig. 2, 3). As they matured the zoospores became motile in the lumen of the zoosporangium, giving them a swarming appearance (Fig. 4), just prior to their release (Fig. 5). Zoosporangia full of zoospores or with only a few remaining zoospores were frequently observed (Fig. 5), suggesting that zoosporulation was a continuous process in actively proliferating cultures, and the process of releasing the zoospores occurred rapidly. Individual zoospores were observed simultaneously with the other stages in the culture (Fig. 6). Some swam quickly while others were tethered by one flagellum to the culture well surface and were propelled in a circular motion by the free second flagellum. After several days to two weeks, individually cloned zoospores lost the flagella and became trophozoites that continued to proliferate by budding or palintomy and zoosporulated.

Immature trophozoites were characterized by the presence of multiple vacuoles of various sizes spread throughout the cytoplasm (Fig. 7). The shape of the nucleus was often spherical, occupying a large proportion of the cell. Although *Perkinsus* sp. (*M.b.*) trophozoites propagated by either budding or palintomy, palintomy was more frequently observed in culture (Fig. 8–10). Inside the tomont, trophozoites were tightly packed against each other (Fig. 8, 9). Some of the trophozoites had a large vacuole, although the nucleus was not packed against the plasmalemma (Fig. 9). Following karyokinesis and during cytokinesis there was active secretion of components that eventually may constitute the trophozoite cell wall (Fig. 10). These components were most frequently observed at the site of division but were also present at other locations around the trophozoite and did not appear to be associated with the micropore complex described below.

Mature trophozoites were spherical and surrounded by an amorphous wall (Fig. 11, 12) beneath which the plasmalemma bore uniformly distributed micropores or pore complexes opening toward the cell wall (Fig. 11–14). Pore complexes appeared to be uniform in size, approximately 100 nm in depth, and 100 nm in diam. by cross-section with what appeared to a be small opening (20 nm in diam.) within each complex. In longitudinal section, micropores appeared to be surrounded by an electron-dense cytoplasmic collar (Fig. 12). The mature trophozoite was dominated by a large vacuole containing one or more dense inclusions or vacuoplasts (Fig. 11), which in live cells exhibited Brownian movement. The large vacuole in mature trophozoites displaced the cell organelles and the nucleus against the plasmalemma (Fig. 11). The eccentric and uniformly dense nucleus had a prominent central nucleolus, and numerous nuclear pores (Fig. 11). Scattered virus-like particles were seen in some of the nuclei (data not shown). The remainder of the cytoplasm contained lipid droplets, small vacuoles, vacuoplast precursors in the cisternae of the endoplasmic reticulum, ribosomes, and numerous lobulated mitochondria (Fig. 11).

Mature trophozoites became prezoosporangia that underwent zoosporulation by forming a discharge tube around a lens-shaped area under the cell wall. Cell division within the developing zoosporangia led to cells that matured into hundreds of motile zoospores. A plug of dense material at the base of the discharge tube probably prevented the premature release of immature zoospores (Fig. 15).

Immature zoospores inside the zoosporangia had several vacuoles that presumably merge to form a large vacuole localized at the anterior end of the mature zoospore (Fig. 16). Immature zoospores close to maturation were characterized by the presence of flagella (Fig. 16). At this stage the zoospore appears to have all the structures present in the mature zoospore (see below).

Mature zoospores were uninucleated, biflagellated, and ovoid measuring 4.4 ± 0.6 μm × 2.0 ± 0.5 μm (n = 10), as assessed by TEM. Zoospores contained organelles presumed to be an apical complex at the anterior end (Fig. 17). This complex consisted of a conoid, rectilinear micronemes, conoid-associated micronemes, and subpellicular microtubules (Fig. 17–20). The conoid was composed of helically coiled microtubules forming a cone, open at the anterior end (Fig. 18, 19). The nucleus of the zoospore was lobulated, located on the ventral (flagellar) side of the body, lacked an evident nucleolus, and usually presented highly condensed chromatin (Fig. 17, 20, 21). A series of microtubules originated at the level of the anterior polar ring and extended beneath the plasmalemma to the posterior end of the zoospore (Fig. 20). The rectilinear micronemes consisted of dense material, originated posteriorly to the polar ring, and were found to extend more than half of the length of the body. These

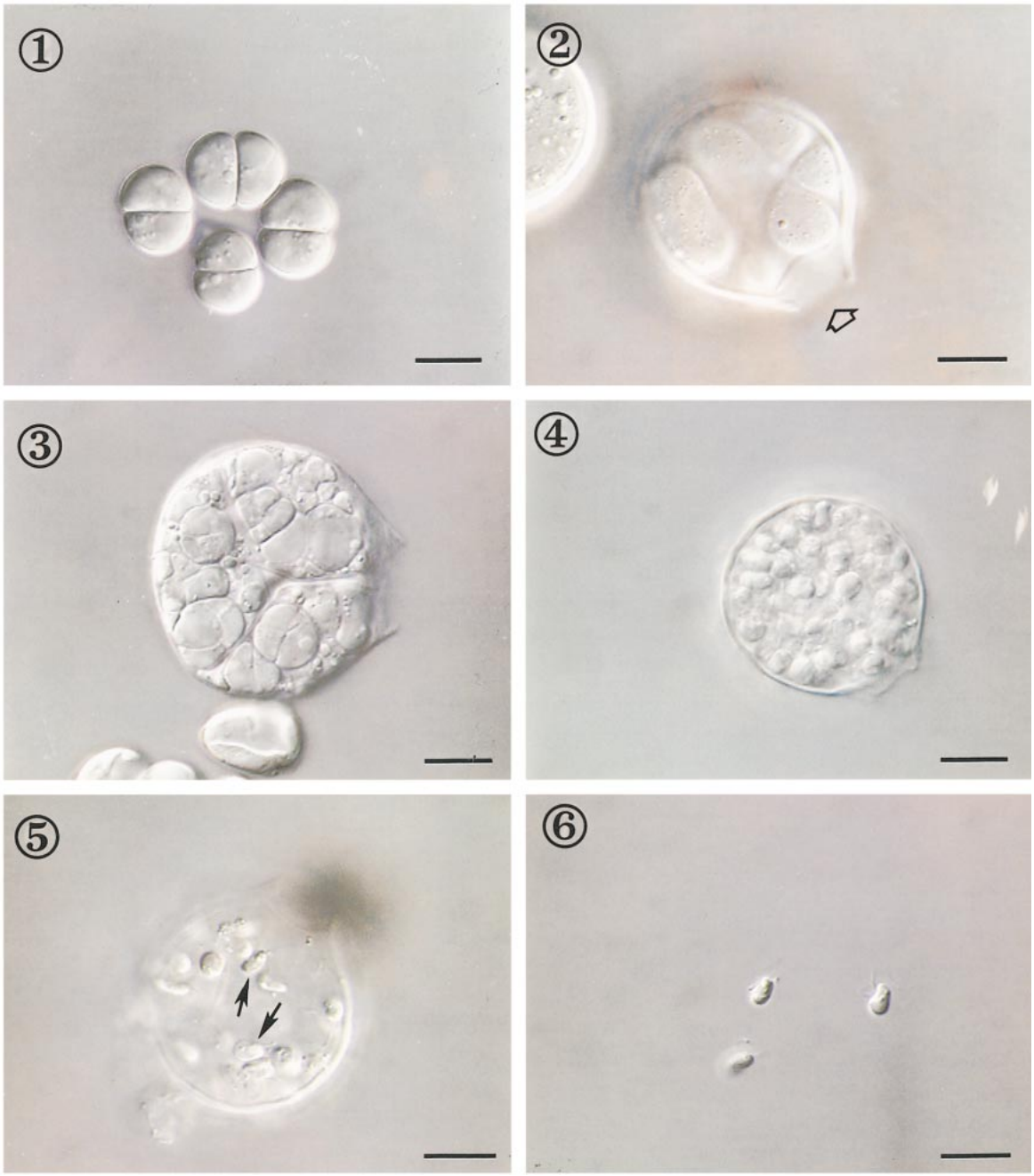


Fig. **1–6**. Life stages of in vitro cultured *Perkinsus* sp. isolated from *Macoma balthica*. **1.** Trophozoites propagating by budding. Scale bar = 10 μm. **2.** Zoosporangium containing immature zoospores. Open arrow, discharge tube. Scale bar = 10 μm. **3.** Immature zoospores dividing inside the zoosporangium. Scale bar = 10 μm. **4.** Mature zoospores inside zoosporangium prior to release. Scale bar = 10 μm. **5.** Partially empty zoosporangium with some zoospores (arrows) left in its interior. Scale bar = 10 μm. **6.** Free zoospores swimming in the culture medium after release. Scale bar = 10 μm.

structures appeared to be variable in composition and diameter even in the same zoospore (data not shown). Cortical alveolar extensions of the plasmalemma were primarily present at the anterior end of the zoospore (Fig. 17–20), but were occasionally found at other regions around the cell including the posterior end. The mitochondria were usually lobulated (Fig. 21).

Anterior and posterior flagella were inserted together ventrally in the anterior third of the zoospore. The dense body in the lumen of the kinetosome appeared as a dense cylindrical (Fig. 22), ''H''- shaped (Fig. 23), or ovoid inclusion (Fig. 24, 25) depending on the section. Accompanying the inclusion body was a cup-like structure, the terminal plate of the central pair of axoneme microtubules, the terminal helix of the central microtubular array, and presumptive microtubular organizing centers (Fig. 22–24). In some sections, there was a structure resembling the terminal plate of the central pair of axoneme microtubules below the cup-like structure (Fig. 24). The microtubules at the level of the cup-like structure consisted of a more

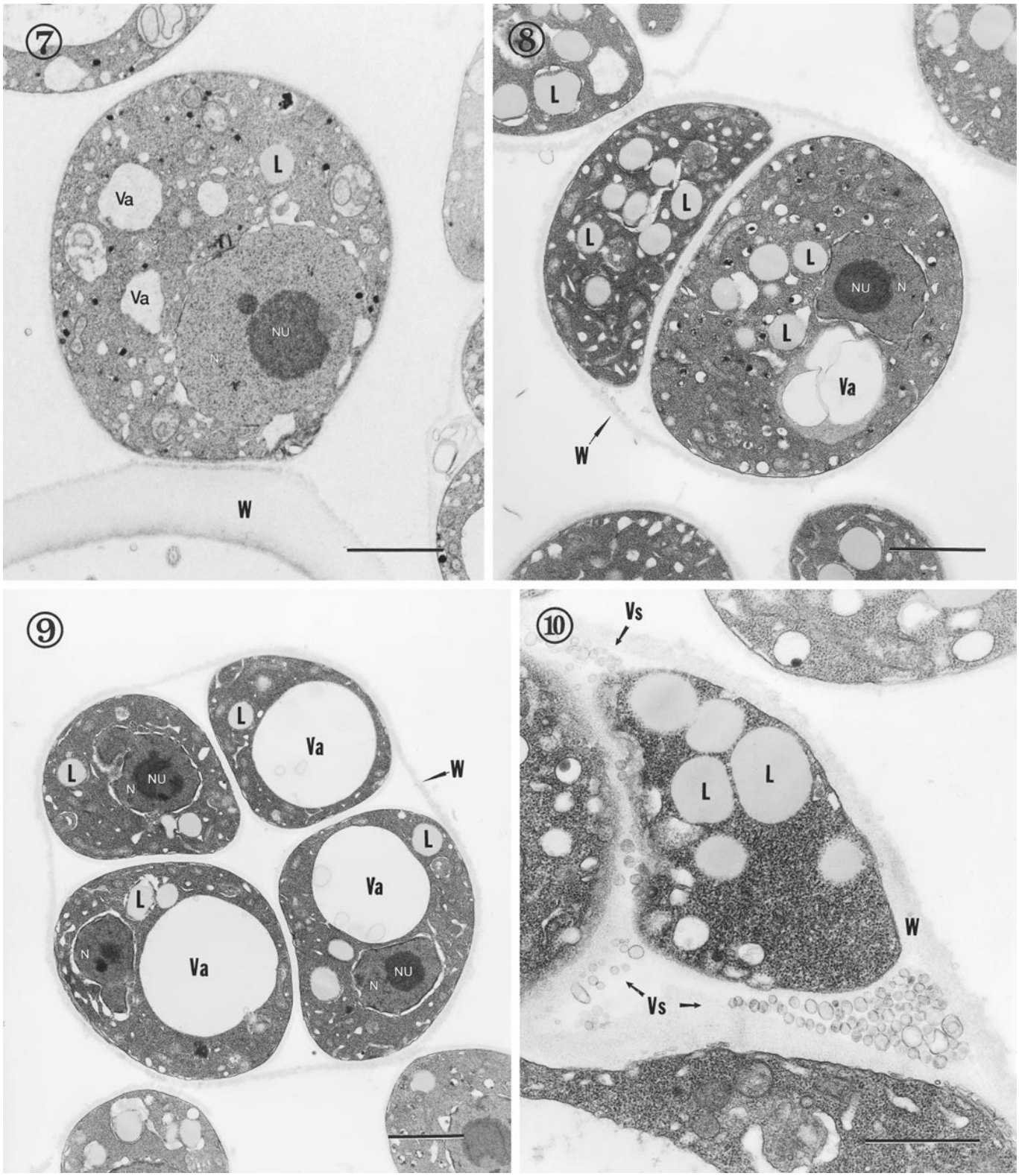


Fig. **7–10**. Electron micrographs of dividing immature trophozoites of *Perkinsus* sp. isolated from *Macoma balthica.* **7.** Immature trophozoite. Scale bar = 2 μm. **8.** Two cells stage. Scale bar = 2 μm. **9.** Four cells stage. Scale bar = 2 μm. **10.** Detail of the vesicles produced during division. Scale bar = 1 μm. L, lipid droplet; N, nucleus; NU, nucleolus; Va, vacuole; Vs, vesicles; W, wall.

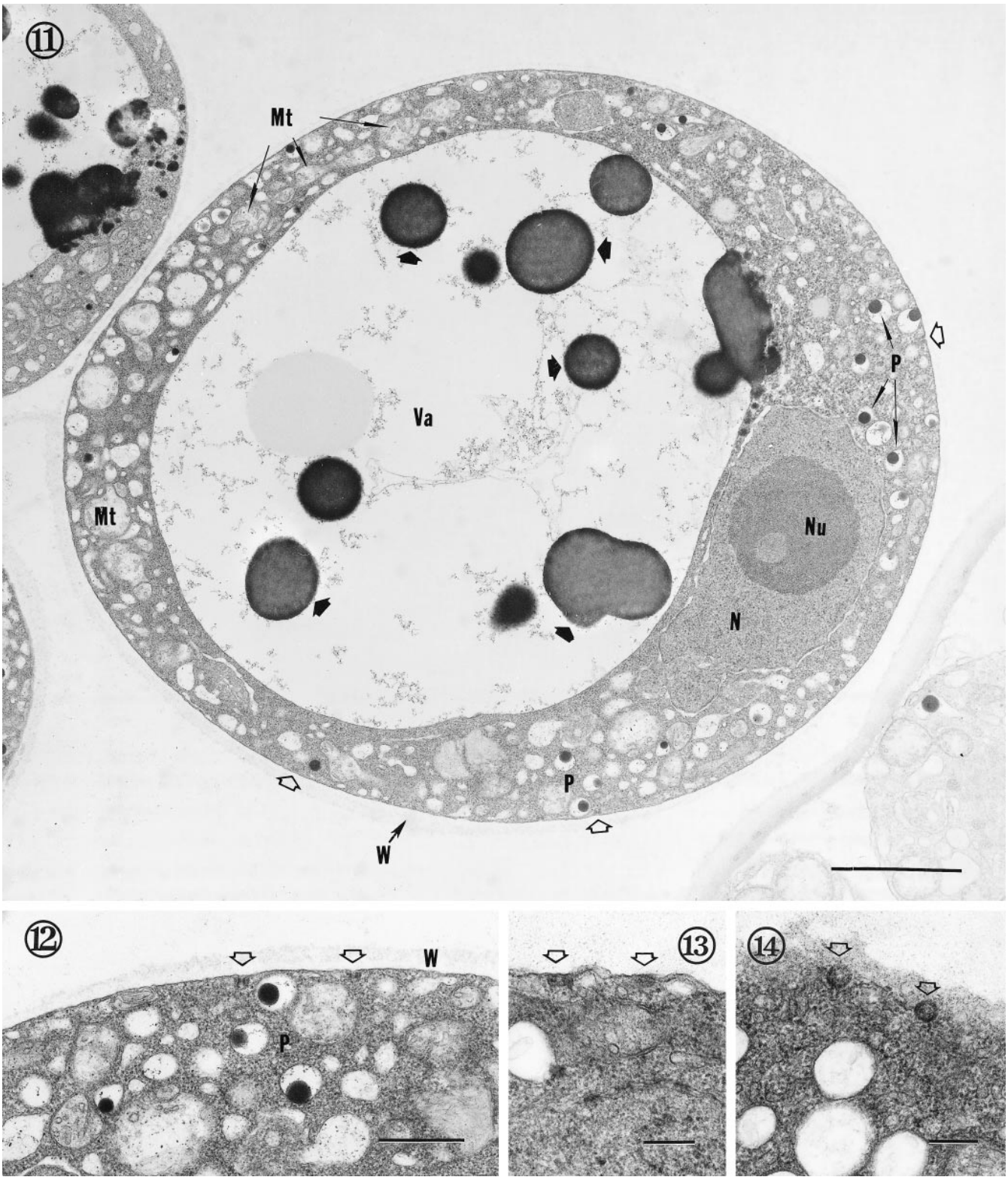


Fig. **11–14**. Electron micrographs of trophozoites of *Perkinsus* sp. isolated from *Macoma balthica*. **11.** Mature trophozoite. Scale bar = 2 μm. **12–13.** Detail of a longitudinal section through the micropores of the trophozoite. Scale bar = 1 μm. **14.** Transverse section through the micropores of the trophozoite. Scale bar = 1 μm. Solid arrows, vacuoplast; open arrows, micropores; Mt, mitochondria; N, nucleus; Nu, nucleolus; P, vacuoplast precursors; Va, vacuole; W, wall.

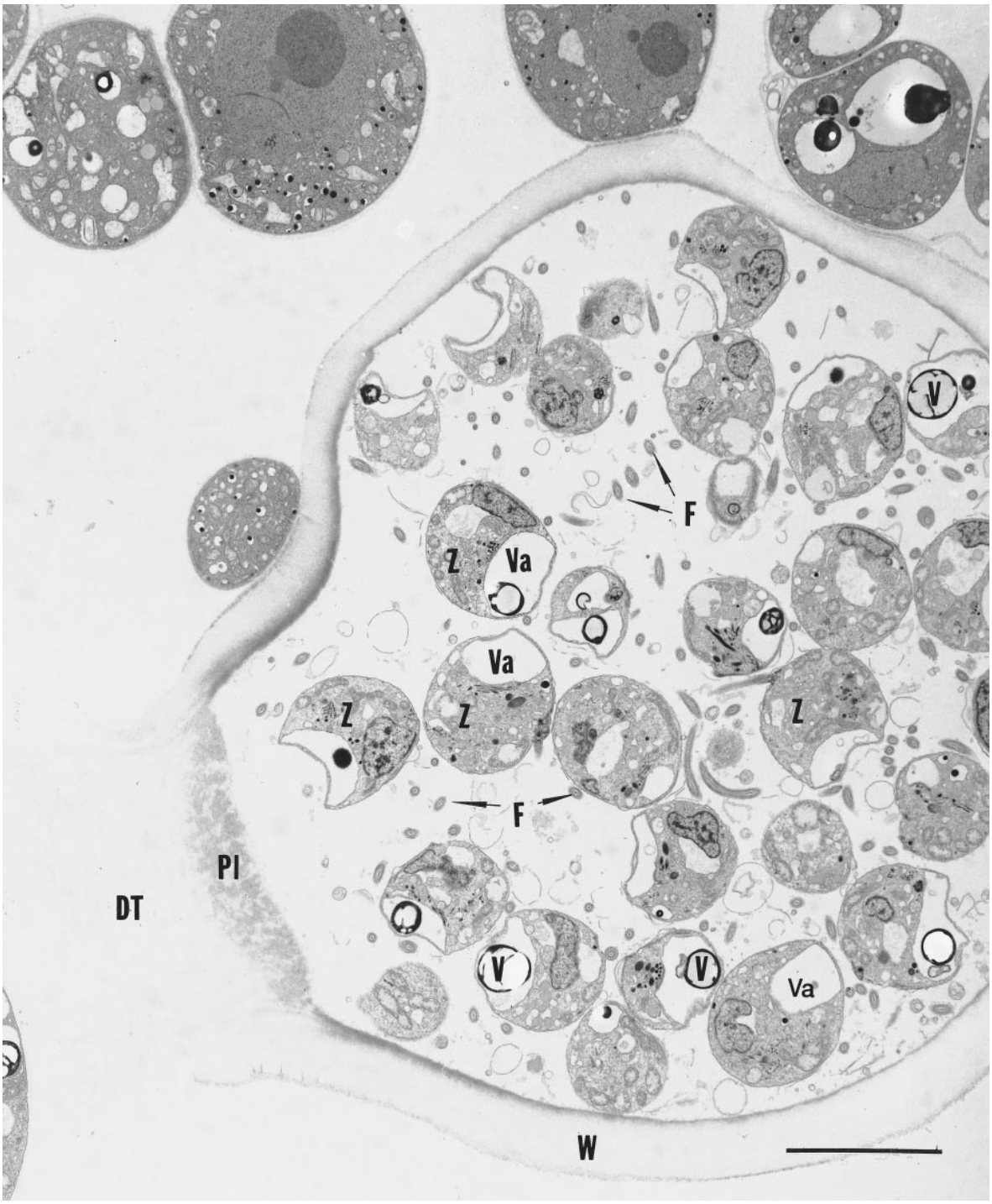


Fig. **15**. Mature zoosporangia of *Perkinsus* sp. isolated from *Macoma balthica*. Scale bar = 5 μm. DT, discharge tube; F, flagella; Pl, plug; V, vacuoplast; Va, vacuole; W, wall; Z, zoospore.

dense material that resembled the terminal helix of the central microtubular array (Fig. 22, 23), a feature not observed elsewhere in the 9-doublet microtubular array of the flagella. Transverse sections of the flagella showed the typical eukaryotic 9-doublet/2-central microtubular array surrounded by a thick cell membrane (Fig. 26). The kinetosome had the typical configuration of 9-triplets without a central microtubular array (Fig. 27).

The posterior flagellum was bare and tapered at about two-thirds of the length from the body so that the diameter of the distal end was approximately 1/3 of that at the base (Fig. 28). The anterior flagellum was characterized by the presence of thin hair-like appendages associated with spurs (usually four to five thin hair-like appendages accompanying each spur) (Fig. 29). The length of the thin hair-like appendages was 1.1 ± 0.4 μm (n = 32) and spurs were spaced 99 ± 25 nm (n = 29) apart.

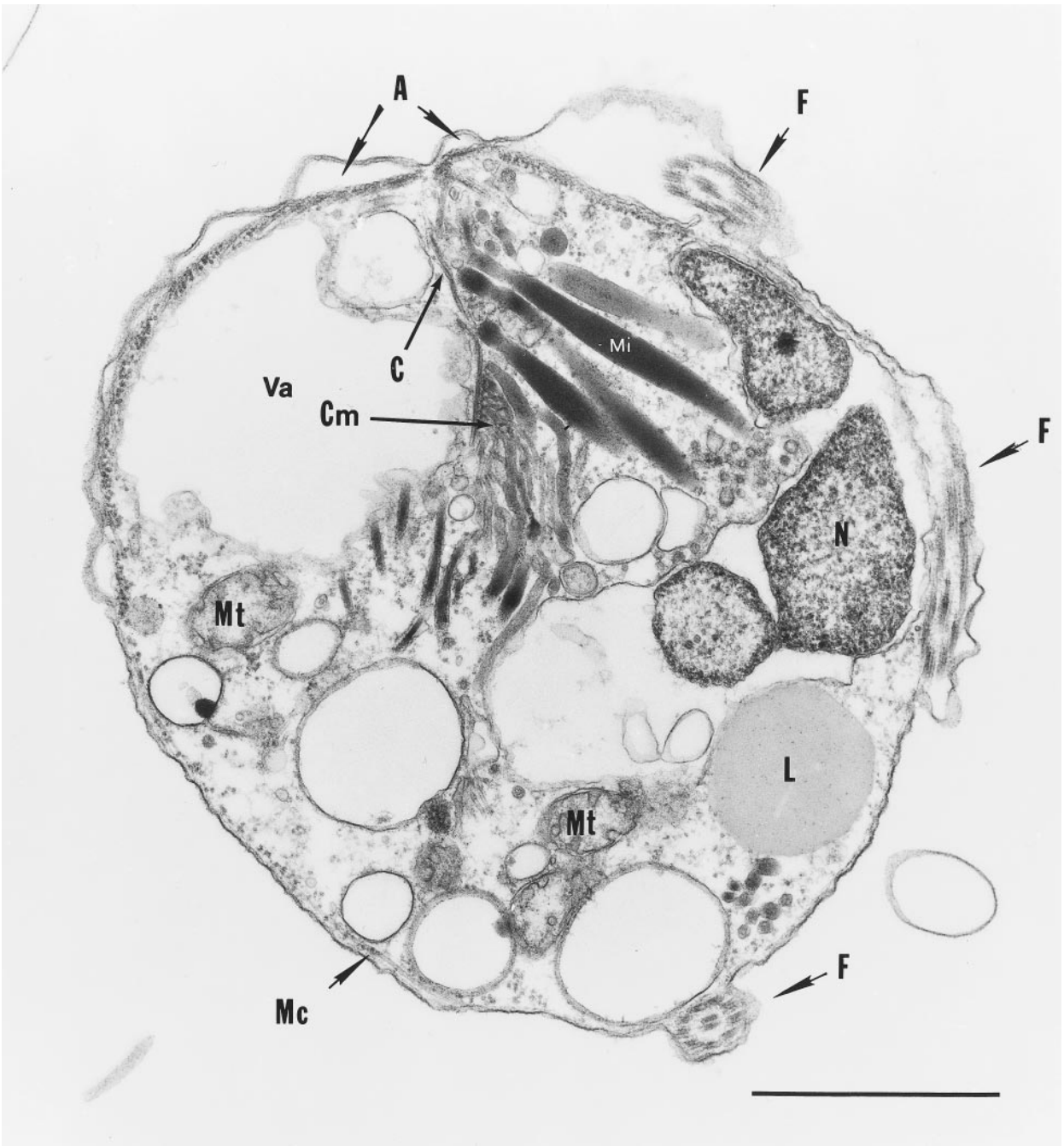


Fig. **16**. Electron micrographs of an immature zoospore of *Perkinsus* sp. isolated from *Macoma balthica*. Scale bar = 1 μm. A, cortical alveoli expansion; C, conoid; Cm, conoid associated micronemes; F, flagella; L, lipid droplet; Mc, subpellicular microtubules; Mi, rectilinear micronemes; Mt, mitochondria; N, nucleus; Va, vacuole.

DISCUSSION

The gross morphology of various life stages of the *Perkinsus* sp. was similar to *Labyrinthomyxa* sp. (Perkins 1968) and *Perkinsus* sp. (Kleinschuster et al. 1994), both from the same bivalve host (*M. balthica*), and to *P. marinus* (Perkins 1996), *P. olseni* (Lester and Davis 1981) and *P. atlanticus* (Azevedo 1989; Azevedo, Corral, and Cachola 1990). The *Perkinsus* species described in this study continuously sporulated in culture without any manipulation to induce it. This is in contrast to other *Perkinsus* species that require either incubation in sea water (*P. atlanticus*) (Azevedo, Corral, and Cachola 1990) or incubation initially in FTM and subsequently seawater (*P. marinus*) (Perkins 1996).

The *Perkinsus* sp. (*M.b.*) clone shares most of the characteristics described by Perkins (1968) for *Labyrinthomyxa* sp. except that it sporulates spontaneously in culture without prior incubation in sea water, and the zoospores are slightly larger (4.4 ± 0.6 μm, as compared to 4 ± 1 μm for *Labyrinthomyxa* sp.) and more homogeneous in size. The greater variation in dimensions of the zoospores analyzed by Perkins from either

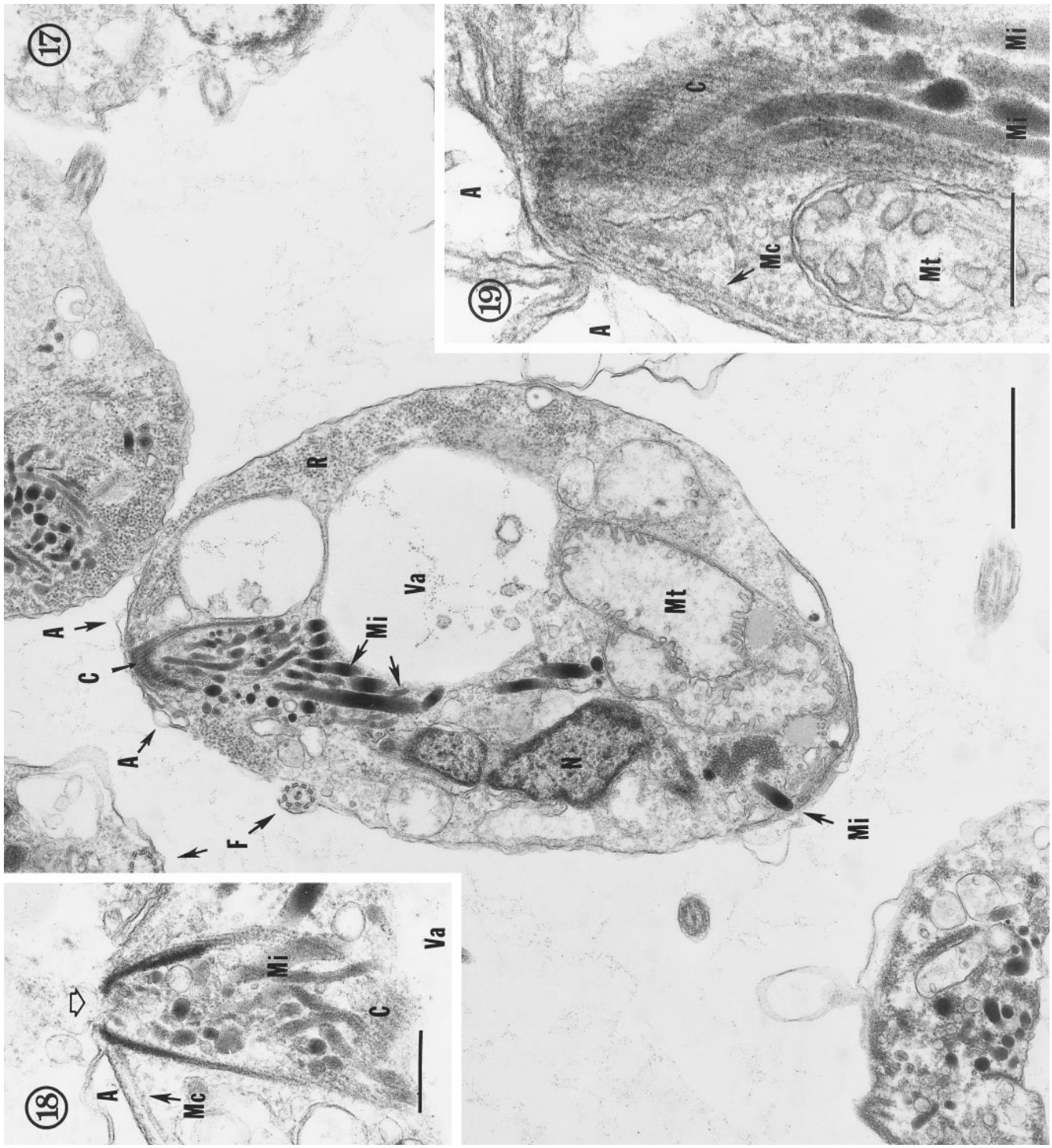


Fig. **17–19**. Electron micrographs of mature zoospores of *Perkinsus* sp. isolated from *Macoma balthica*. **17.** Longitudinal section of zoospore. Scale bar = 1 μm. Fig. **18.** Detail of anterior end of another zoospore. Scale bar = 250 nm. Fig. **19.** Truncated conoid of a third zoospore. Open arrow, truncated conoid. Scale bar = 250 nm. A, cortical alveoli expansion; C, conoid; F, flagella; Mc, subpellicular microtubules; Mi, rectilinear micronemes; Mt, mitochondria; N, nucleus; R, ribosomes; Va, vacuole.

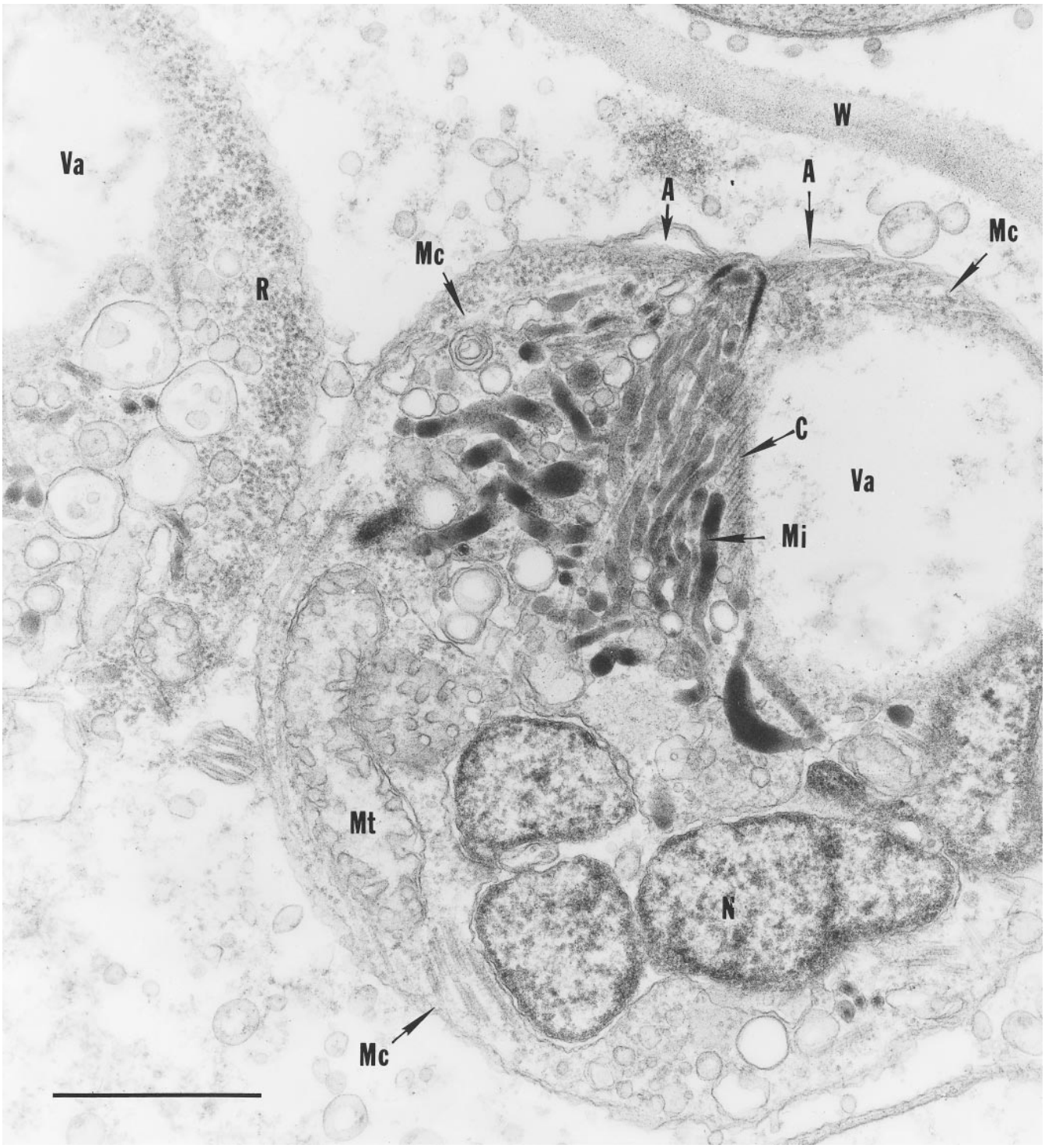


Fig. **20**. Oblique section through a mature zoospore of *Perkinsus* sp. isolated from *Macoma balthica.* Scale bar = 750 nm. A, cortical alveoli expansion; C, conoid; Mc, subpellicular microtubules; Mi, rectilinear micronemes; Mt, mitochondria; N, nucleus; R, ribosomes; Va, vacuole; W, wall.

C. virginica (Perkins and Menzel 1967) or *M. balthica* (Perkins 1968) may have been due to the presence of two or more different species in an individual host. In fact, we have found through molecular analysis that *P. marinus* and this genetically different *Perkinsus* sp. can both be detected in the same individual bivalve (Coss et al. 1999). Alternative explanations could include a polyclonal population of a single parasite species, or influences of the various host microenvironments in the multiple tissues infected. Greater consistency in the dimensions of our isolate would be expected as the analysis was carried out on a clonal culture, where all cells were genetically homogeneous and were not subject to host tissue responses. This

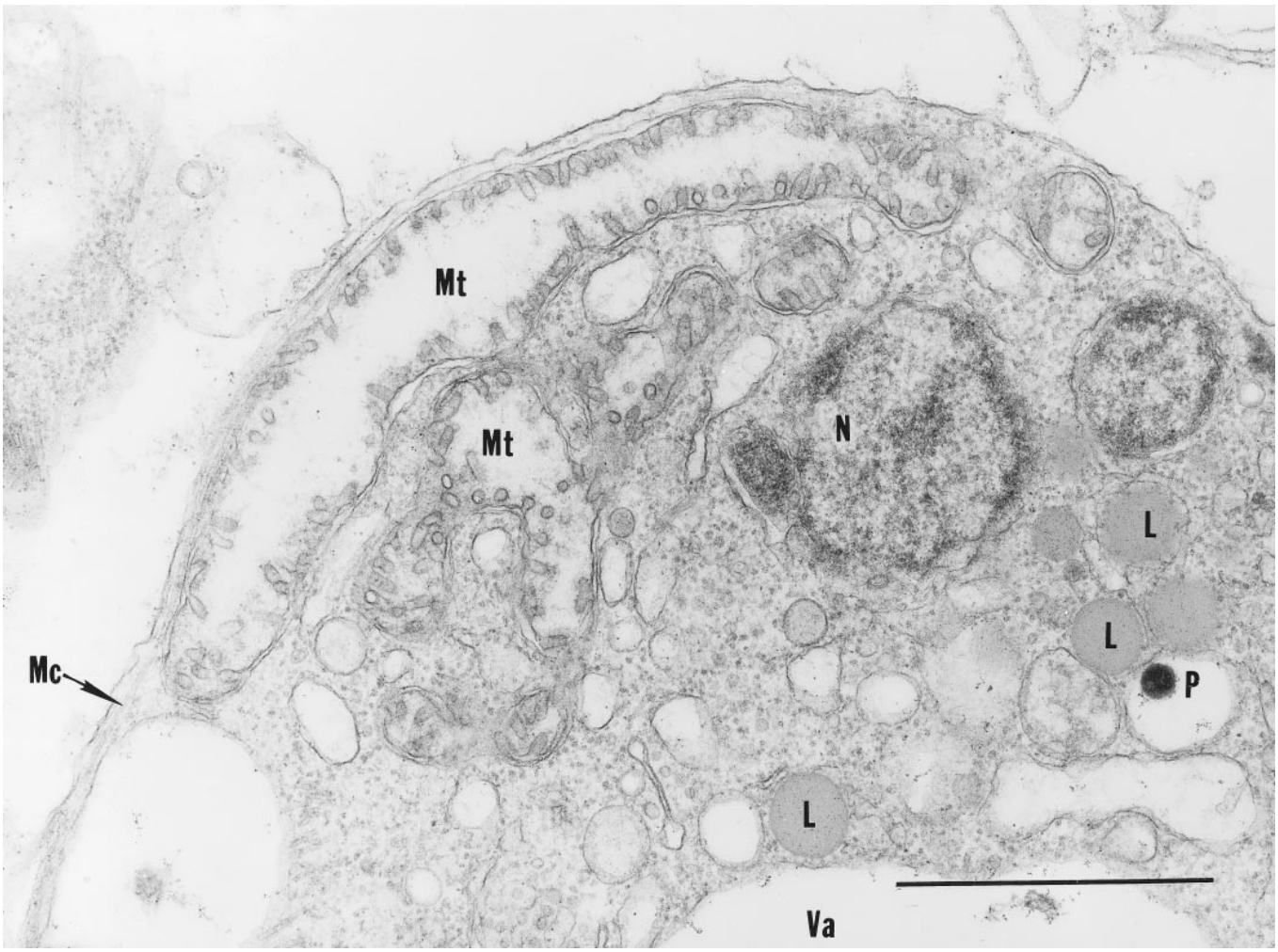


Fig. **21**. Electron micrograph of the mitochondrion of a zoospore from *Perkinsus* sp. isolated from *Macoma balthica.* Scale bar = 1 μm. L, lipid droplet; Mc, subpellicular microtubules; Mt, mitochondria; N, nucleus; P, vacuoplast precursors; Va, vacuole.

phenomenon was reported for *P. marinus* trophozoites by Gauthier and Vasta (1993), where optimization of the culture medium resulted in size homogeneity.

By light microscopy, the clonal *Perkinsus* sp. (*M.b.*) described herein is also morphologically similar to the cultured *Perkinsus* sp. isolate from *M. balthica* described by Kleinschuster et al. (1994). This was reported to differ from *P. marinus* by exhibiting higher rates of proliferation under the same culture conditions and by undergoing sporulation by direct exposure to sea water (Kleinschuster et al. 1994). No details were provided by the authors, however, as to how this isolate may have differed at the ultrastructural level from that described earlier by Perkins (1968) (*Labyrinthomixa* sp.) or *P. marinus.* Our *Perkinsus* sp. (*M.b.*) clone differs from the isolate described by Kleinschuster et al. (1994) in that it does not require placement in sea water to zoosporulate. In addition, a feature that was not reported earlier for either *Labyrinthomixa* sp. (Perkins 1968) or the cultured *Perkinsus* sp. isolate from *M. balthica* as described by Kleinschuster et al. (1994) is that cloned individual zoospores eventually lost their flagella and gave rise to trophozoites that then proceeded to proliferate by budding or palintomy and underwent zoosporulation. Kleinschuster et al. (1994) and Perkins (1996) proposed that because of the ease of sporulation of *Perkinsus* sp. from *M. balthica* it could actually be *P. atlanticus.* Our *Perkinsus* sp. (*M.b.*) isolate differs from *P. atlanticus* in that the zoospores are smaller and spontaneously zoosporulates in culture. The in vitro culture of *Perkinsus* sp. (*M.b.*) resembles the morphological changes of *Parvilucifera infestans* zoospores, which after penetrating the host cell (*Dinophysis* sp.), grow into large spherical bodies that eventually become sporangia, and produce new zooids either within the dead host cell or after their release from the host (Norén, Moestrup, and Rehnstan-Holm, 1999).

One of the major problems in the designation of species within the genus *Perkinsus* is that the examination of the gross morphology of the various life stages of *Perkinsus* isolates has not provided evidence robust enough on which to discriminate new isolates at the specific level (Perkins 1996). In many instances, gross morphological differences have been attributed to responses to environmental factors, including host reaction to parasite infection (Blackbourn, Bower, and Meyer, 1998; Lester and Davis 1981; Perkins 1988; Perkins, 1996). Therefore, it has been proposed that species designations should be based on differences in fine structure of the zoospore stage (Perkins 1996), although most reports have included other life cycle stages. For example, *P. atlanticus* differed from *P. marinus* in that: (a) the mitochondria in prezoosporangia were present in all successive bipartitions and centrioles appeared in the last

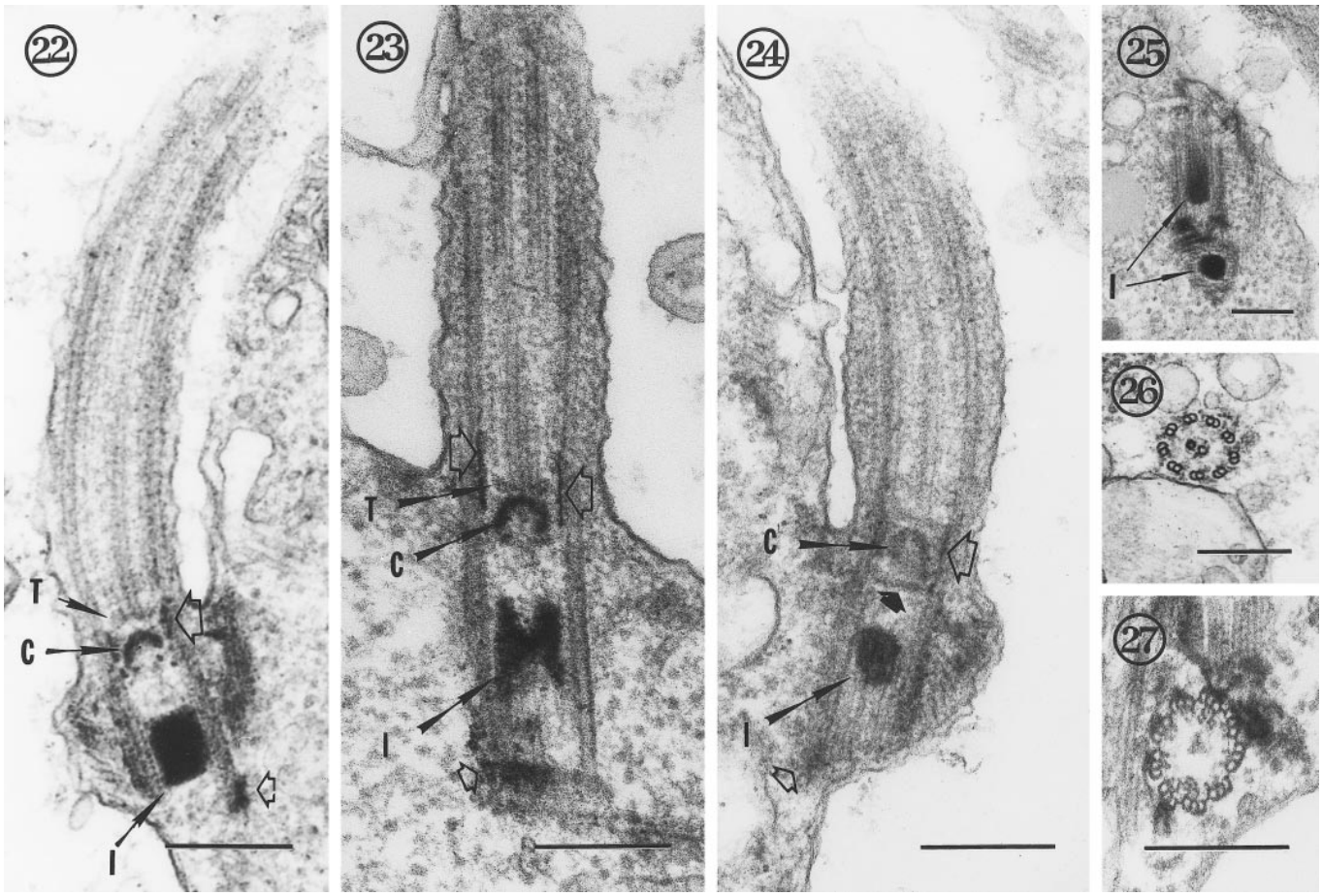


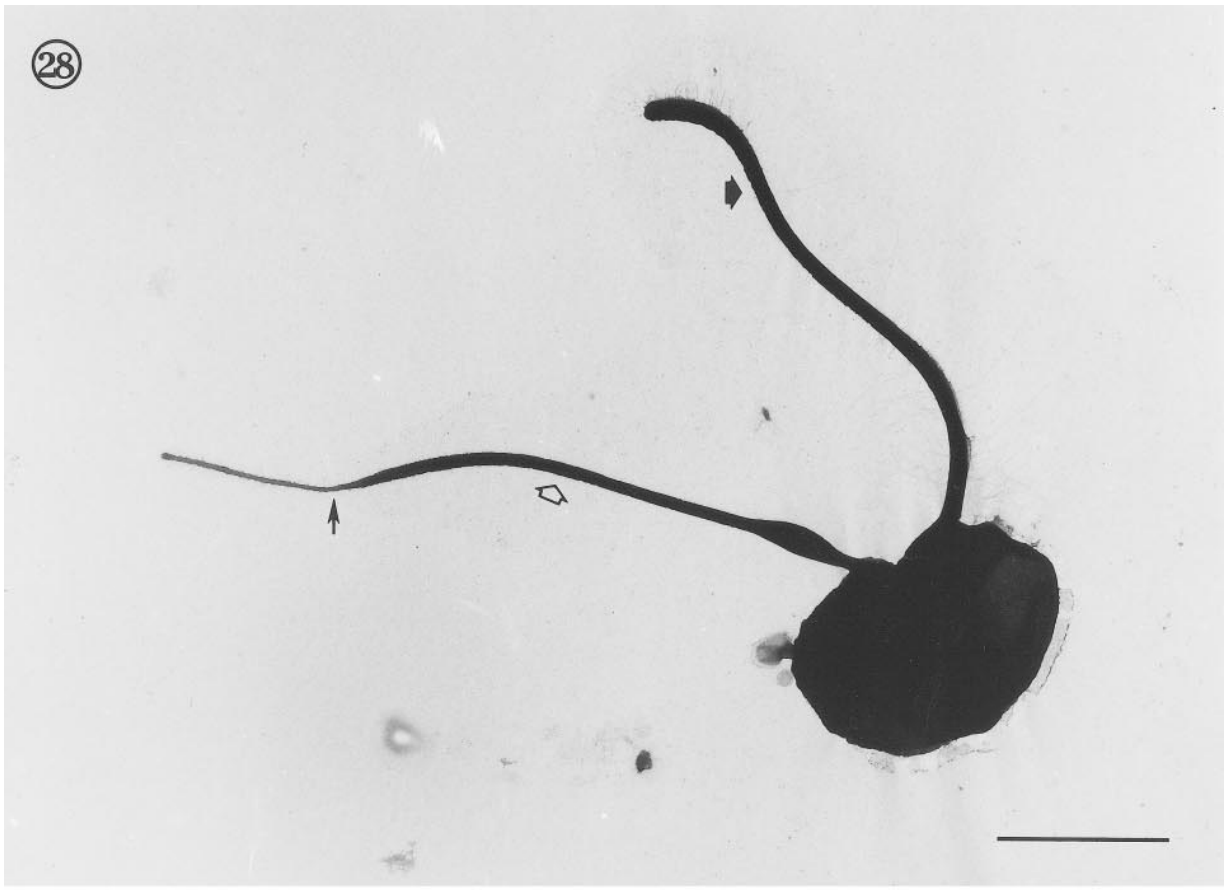
Fig. **22–27**. Details of the flagella of *Perkinsus* sp. isolated from *Macoma balthica*. **22–24.** Longitudinal sections through the anterior flagellum and kinetosome. Scale bar = 250 nm. **25.** Detail of the kinetosomes of the posterior flagellum (upper) and the anterior flagellum (lower). Scale bar = 250 nm. **26.** Transverse section through the flagellum showing the microtubules. Scale bar = 250 nm. **27.** Transverse section through the flagellum between the cup-like structure and the kinetosome. Scale bar = 250 nm. Small open arrow, presumptive microtubular organizing center. Large open arrow, electron dense material accompanying the cup-like structure, C, cup-like structure; I, inclusion body; T, terminal plate of central pair of axoneme microtubules.

cellular division; (b) it readily zoosporulated after FTM incubation and exposure to sea water; and (c) zoospores exhibited multiple cytoplasmic vacuoles and a smaller angle between flagella (65°) as compared to *P. marinus* (90°) (Azevedo 1989; Azevedo, Corral, and Cachola 1990). Blackbourn, Bower, and Meyer (1998) suggested that the angle between flagella of *P. qugwadi* was variable, and would not constitute a character with taxonomic weight. The anterior flagellum of *P. atlanticus* zoospores exhibits hairs-like appendages (mastigonemes) (Azevedo, C., pers. commun.), a feature shared with *P. marinus* (Perkins 1967).

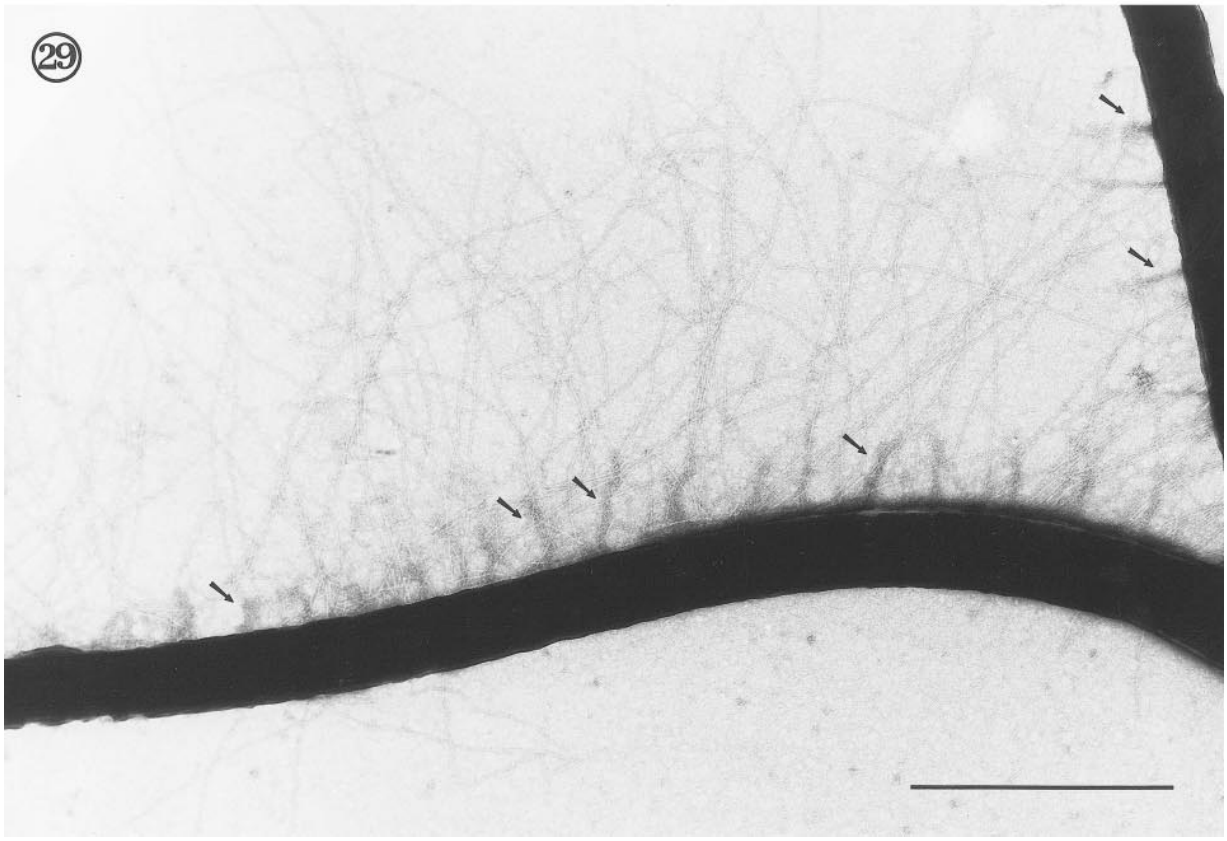
We attempted to identify ultrastructural characteristics that would determine if our isolate was *P. atlanticus*, as suggested by Kleinschuster et al. (1994) and Perkins (1996), or clearly indicate if it was a distinct species. Our *Perkinsus* sp. (*M.b.*) isolate shares most of the ultrastructural characteristics with *P. marinus* and *P. atlanticus* zoospores, such as the arrangement and appearance of the structures of the apical complex, the cortical alveolar expansions at the anterior end of the zoospore body, and the structures of the flagella (Table 1–3). Our *Perkinsus* sp. (*M.b.*) isolate also exhibits some of the characteristics that were thought to distinguish *P. qugwadi* from the other species within the genus. While we can find mitochondria that have the typical oblong or round shape, we can also see, even in the same cell, mitochondria that appear to be lobulated as was described to be unique for *P. qugwadi* (Blackbourn, Bower, and Meyer 1998). In addition, other features described as unique for *P. qugwadi* such as the presence of an elongated conoid with micronemes that wrap around the nucleus, the lack of a dense body within the lumen of the flagella kinetosome, and the presence of cortical alveolar extensions over the entire body, as well as the lobulated mitocondria, were not consistently present in all cell sections examined (Blackbourn, Bower, and Meyer 1998). Several reasons may explain the inconsisten-

→

Fig. **28–29**. Negative staining of the zoospore of *Perkinsus* sp. isolated from *Macoma balthica*. **28.** Zoospore showing the anterior (solid arrow) and posterior flagella (open arrow). Note the tapering of the posterior flagellum at its distal end (arrow). Scale bar = 2 μm. **29.** Detail of the thin hair-like appendages and spurs of the anterior flagellum (arrows). Scale bar = 500 nm.



28



29

Table **1**. Comparative chart of morphological features of *Perkinsus* species.

Perkinsus species	Host type	Source	Zoosporulation Induction	Assay by FTM/ Lugol's	Zoosporangia Diameter (μm)	Zoosporangia Discharge Tube	Zoospore Body Size (μm)
P. marinus	*C. virginica*	HT/FTM/SW	FTM/SW	Positive	45 ± 15	Present	5 ± 1 × 2.0 ± 0.5
P. olseni	*H. ruber*	Host tissue	FTM/SW	Positive	75 ± 19	Poorly developed	nr
P. atlanticus	*R. decussatus*	HT/FTM/SW	SW	Positive	nr	Present	4.5 × 2.9
P. qugwadi	*P. yessoensis*	Host tissue	Only in host	Negative	13	Absent	[d]4.5 ± 1 × 2.0 ± 0.5; [e]3.9 ± 0.3 × 2.5 ± 0.3
Perkinsus sp.[a]	*M. balthica*	HT/FTM/SW	SW	Positive	nr	Present	4 ± 1 × 2.5 ± 0.5
Perkinsus sp.[b]	*M. balthica*	Cell culture	SW	nr	nr	Present	nr
Perkinsus sp.[c]	*M. balthica*	Monoclonal culture	Culture/SW	Positive	67 ± 12	Present	4.4 ± 0.6 × 2.0 ± 0.5

[a] Perkins (1968); [b] Kleinschuster et al. (1994); [c] *Perkinsus* sp. (*M.b.*); [d] gonadal imprint; [e] whole cell; nd, not determined; nr, not reported; HT, host tissue; FTM, fluid thioglycollate media; SW, seawater.

cies, among which are the plane of the cut through the cell, artifacts resulting from the fixation process, and the possibility of multiple parasite species/strains present in the infected bivalve tissue. Thus, these characters may not be taxonomically robust enough and other features that appear to be relevant at the genus level, such as the pattern of flagellar insertion, may be incorporated (Norén, Moestrup, and Rehnstan-Holm (1999). In addition, the species designation from an infected tissue sample may have to be corroborated with fine structure information from clonal cultures.

Although most of the studies on the fine structure of *Perkinsus* isolates have focused on the zoospore stage, there has been little consensus among authors in the assignment of taxonomic weight to particular features. Furthermore, there has been little justification for the choice of those characters and their assignment to the generic, specific or sub-specific level. In addition, statements about ultrastructural observations have not always been supported by the actual electron micrographs reported (Blackbourn, Bower, and Meyer 1998), and re-interpretation (Siddal et al. 1997) of well documented published observations have recently been questioned (Norén, Moestrup, and Rehnstan-Holm 1999). Physiological aspects of the parasite, such as spontaneous sporulation in culture and enlargement in thioglycollate medium may not represent characters robust enough to be considered of taxonomic value at the generic level (Blackbourn, Bower, and Meyer 1998; Goggin et al. 1996). Finally, serological approaches for the identification or discrimination of *Perkinsus* spp. have failed so far. For example, that cross-reactivity with anti-*P. marinus* antibodies supports the inclusion of *P. qugwadi* in the genus *Perkinsus* (Blackbourn, Bower and Meyer 1998) is questionable because the aforementioned anti-*P. marinus* antibodies also cross-react with various dinoflagellate species (Dungan et al. 1998).

In order to complement the morphological studies on the *Perkinsus* sp. (*M.b.*) isolate reported herein, molecular studies were undertaken in our laboratory (Coss et al. 2001). Sequence data from the rRNA locus indicate not only that *Perkinsus* sp. (*M.b.*) is not *P. marinus*, but also that it is not *P. atlanticus, P. olseni* or *P. qugwadi,* and the degree of sequence difference is comparable to or greater than differences between accepted *Perkinsus* species. Until taxonomic weight at the specific level can be assigned to well-defined gross morphological and fine structure characteristics of *Perkinsus* morphotypes, molecular approaches may provide invaluable information to aid in species designation within the genus *Perkinsus.*

ACKNOWLEDGMENTS

This study was supported by DOC Cooperative Agreement No. NA46RG0091 (Oyster Disease Research Program, Sea Grant, NOAA) awarded by NOAA through the Maryland Sea Grant to GRV., and a Smithsonian Predoctoral Fellowship to CAC. We thank Dr. D. W. Coats of SERC for providing access to the facilities and expertise in the use of the microscope. We thank Dr. J. Endlich and T. Maugel for their technical assistance in TEM. We are grateful to Dr. S. Poynton and Dr. M. S. Quesenberry for their valuable comments on the manuscript. This work was submitted in partial fulfillment of the requirements for the Ph.D. degree for CAC at George Washington University.

Table **2**. Comparative chart of ultrastructural features of *Perkinsus* species.

	Prezoosporangium			Zoospore	
Perkinsus sp.	Mitochondria	Centrioles	Mitochondria Form	Conoid Assoc. Micronemes Location/Shape	Alveoli Expansions
P. marinus	Lost or reduced in FTM Restored in zoospore	Appear at first bipartition	nr	Diagonally to posterior	Anterior end
P. olseni	nr	nr	nr	nr	nr
P. atlanticus	Several present in all successive bipartitions	Appear at last bipartition	nr	Diagonally to posterior	Anterior end
P. qugwadi	nr	nr	Some lobulated	Elongated, around nucleus	Entire surface
Perkinsus sp.[a]	Retained	nr	nr	Diagonally to posterior	Anterior end
Perkinsus sp.[b]	nr	nr	nr	nr	nr
Perkinsus sp.[c]	nd	nd	Some lobulated	Diagonally to posterior	Primary at anterior end

[a] Perkins (1968); [b] Kleinschuster et al. (1994); [c] *Perkinsus* sp. (*M.b.*); nd, not determined; nr, not reported.

Table **3**. Comparative chart of flagellar features of *Perkinsus* species.

Perkinsus sp.	Length		Dense Kinetosome Body
	Anterior (μm)	Posterior (μm)	
P. marinus	14 ± 4	8 ± 2	Present
P. olseni	nr	nr	nr
P. atlanticus	17.2 ± 2.4	11.0 ± 3	Present
P. qugwadi	9.7 ± 2[d]	7.7 ± 1.8[d]	Absent
	9 ± 12[e]	8 ± 1.2[e]	
Perkinsus sp.[a]	15 ± 2	8 ± 1	Present
Perkinsus sp.[b]	nr	nr	nr
Perkinsus sp.[c]	nd	nd	Present

[a] Perkins (1968); [b] Kleinschuster et al. (1994); [c] *Perkinsus* sp. (*M.b.*); [d] gonadal imprint; [e] whole cell preparation; nd, not determined; nr, not reported.

LITERATURE CITED

Andrews, J. D. 1954. Notes on fungus parasites of bivalve mollusks in Chesapeake Bay. *Proc. Natl. Shellfisheries Assoc.,* **45**:157–163.

Azevedo, C. 1989. Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussatus* from Portugal. *J. Parasitol.,* **75**:627–635.

Azevedo, C., Corral, L. & Cachola, R. 1990. Fine structure of zoosporulation in *Perkinsus atlanticus* (Apicomplexa: Perkinsea) *Parasitology,* **100**:351–358.

Blackbourn, J., Bower, S. M. & Meyer, G. R. 1998. *Perkinsus qugwadi* sp. nov. (incertae sedis), a pathogenic protozoan parasite of Japanese scallops, *Patinopecten yessoensis,* cultured in British Columbia, Canada. *Can. J. Zool.,* **76**:942–953.

Burreson, E. M. & Ragone-Calvo, L. M. 1996. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. *J. Shellfish Res.,* **15**:17–34.

Coss, A. C., Robledo, J. A. F., Ruiz, G. M. & Vasta, G. R. 2001. Description of *Perkinsus andrewsi* n. sp. isolated from the baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of a species-specific PCR-based diagnostic assay. *J. Eukaryot. Microbiol.,* **48**:52–61.

Coss, C. A., Robledo, J. A. F., Vasta G. R. & Ruiz, G. M. 1999. Identification of a new *Perkinsus* species isolated from *Macoma balthica* by characterization of the ribosomal RNA locus. Evidence of its presence, simultaneously with *P. marinus,* in *Crassostrea virginica, Macoma mitchelli* and *Mercenaria mercenaria. Abstr. Nat. Shellfish. Assoc.,* **91**:36.

Dungan, C., Bushek, D. & Lewitus, A. J. 1998. Antibodies to the protozoan oyster pathogen *P. marinus* (Apicomplexa) bind to some dinoflagellates (Dinophyceae): pragmatic and phylogenetic implications. Abstract. *Third Int. Symp. Aquatic Animal Health.* p.113.

Ford, S. E. 1996. Range extension by the oyster parasite *Perkinsus marinus* into the northeastern United States: response to climate change? *J. Shellfish Res.,* **15**:45–56.

Gauthier, J. D. & Vasta, G. R. 1993. Continuous in vitro culture of the eastern oyster parasite *Perkinsus marinus. J. Invert. Pathol.,* **62**:321–323.

Gauthier, J. D. & Vasta, G. R. 1995. In vitro culture of the eastern oyster parasite *Perkinsus marinus*: optimization of the methodology. *J. Invertebr. Pathol.,* **66**:156–168.

Goggin, C. L., McGladdery, S. E., Whyte, S. K. & Cawthorn, R. J. 1996. An assessment of lesions in bay scallops *Argopecten irradians* attributed to *Perkinsus karlssoni* (Protozoa, Apicomplexa). *Dis. Aquat. Org.,* **24**:77–80.

Kleinschuster, S. J., Perkins, F. O., Dykstra, M. J. & Swink, S. L. 1994. The in vitro life cycle of a *Perkinsus* species (Apicomplexa, Perkinsidae) isolated from *Macoma balthica* (Linneaus, 1758). *J. Shellfish Res.,* **13**:461–464.

Lester, R. J. G. & Davis, G. H. G. 1981. A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber. J. Invert. Pathol.,* **37**:181–187.

Lester, R. J. G., Goggin, C. L. & Sewell, K. B. 1990. *Perkinsus* in Australia. *In*: Cheng, T. C. & Perkins, F. O. (ed.), Pathology in Marine Aquaculture. Academic Press, New York. p. 189–199.

Levine, N. D. 1978. *Perkinsus* gen. n. and other new taxa in the protozoan phylum Apicomplexa. *J. Parasitol.,* **64**:549.

Mackin, J. G. & Ray, S. M. 1966. The taxonomic relationships of *Dermocystidium marinum* Mackin, Owen and Collier. *J. Invertebr. Pathol.,* **8**:544–545.

Mackin, J. G., Owen, H. M. & Collier, A. 1950. Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). *Science,* **111**:328–329.

McGladdery, S. E., Cawthorn, R. J. & Bradford, B. C. 1991. *Perkinsus karlssoni* n. sp. (Apicomplexa) in bay scallops *Argopecten irradians. Dis. Aquat. Org.,* **10**:127–137.

Norén, F., Moestrup, Ø. & Rehnstam-Holm, A.-S. 1999. *Parvilucifera infectans* Norén et Moestrup gen. et sp. nov.(Perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. *Europ. J. Protistol.,* **35**:233–254.

Perkins, F. O. 1968. Fine structure of zoospores from *Labyrinthomyxa* sp. parasitizing the clam *Macoma balthica*. *Chesapeake Sci.,* **9**:198–202.

Perkins, F. O. 1969. Ultrastructure of vegetative stages in *Labyrinthomyxa marina* (= *Dermocystidium marinum*), a commercially significant oyster pathogen. *J. Invertebr. Pathol.,* **13**:199–222.

Perkins, F. O. 1976. Zoospores of the oyster pathogen, *Dermocystidium marinum.* I. Fine structure of the conoid and other sporozoan-like organelles. *J. Parasitol.,* **62**:959–974.

Perkins, F. O. 1988. Structure of protistan parasites found in bivalve molluscs. *Amer. Fish. Soc. Spec. Publ.,* **18**:93–111.

Perkins, F. O. 1996. The structure of *Perkinsus marinus* (Mackin, Owen and Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. *J. Shellfish Res.,* **15**:67–87.

Perkins, F. O. & Menzel, R. W. 1967. Ultrastructure of sporulation in the oyster pathogen *Dermocystidium marinum. J. Invertebr. Pathol.,* **9**:205–229.

Reece, K. S., Siddall, M. E., Burreson, E. M. & Graves, J. E. 1997. Phylogenetic analysis of *Perkinsus* based on actin gene sequences. *J. Parasitology,* **83**:417–423.

Siddall, M. E., Reece, K. S., Graves, J. E. & Burreson, E. M. 1997. ''Total evidence'' refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. *Parasitology,* **115**:165–167.

Soniat, T. M. 1996. Epizootiology of *Perkinsus marinus* disease of Eastern oysters in the Gulf of Mexico. *J. Shellfish Res.,* **15**:35–43.

Received: 04-05-00, 07-17-00; accepted 07-30-00