

## ZOOSPORULATION OF A NEW *PERKINSUS* SPECIES ISOLATED FROM THE GILLS OF THE SOFTSHELL CLAM *MYA ARENARIA*

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### Summary :

A gill-associated *Perkinsus* sp. isolated from the softshell clam (*Mya arenaria*) is described as a new species, *P. chesapeaki* sp. nov. Examination of the parasite in seawater cultures revealed life cycle stages and zoosporulation processes similar to those described for other species of the genus *Perkinsus*. Prezoosporangia developed thickened cell walls upon contraction of the cytoplasm and development of a distinctive clear area between the cell wall and the protoplast. Successive bipartition of the protoplast led to the formation of hundred's of zoospores within mature sporangia. Zoospores were released into seawater through one or more discharge tubes. Ultrastructural studies revealed an oblong zoospore possessing two flagella that arose from a concave side located in the upper third of the zoospore body. The anterior flagellum possessed a unilateral array of hair-like structures. A large anterior vacuole and basolateral nucleus dominated the cytoplasm of the zoospore body. The presence of a rudimentary apical complex including an open-sided conoid, rhoptries, micronemes, and subpellicular microtubules were also discerned. Differences in zoospore morphology, and sequence analyses of two genes previously reported, support the designation of the gill-associated *Perkinsus* from the softshell clam as a new species.

**KEY WORDS :** *Perkinsus* spp., softshell clam (*Mya arenaria*), zoosporulation, zoospores, ultrastructure.

**Résumé :** ZOOSPORULATION D'UNE NOUVELLE ESPECE DE *PERKINSUS* ISOLÉE DES BRANCHIES DU MOLLUSQUE *MYA ARENARIA*

Description de *Perkinsus chesapeaki* n. sp. associée aux branchies du mollusque bivalve *Mya arenaria*. L'examen du parasite dans les cultures en eau de mer révèle différentes phases du cycle biologique et un processus de zoosporulation semblable à celui déjà décrit chez d'autres espèces de *Perkinsus*. Les prézoosporanges acquièrent des parois cellulaires épaissies à la suite de la rétraction du cytoplasme et de la formation d'une zone claire distincte entre paroi cellulaire et protoplasme. Les bipartitions successives du protoplasme aboutissent à la formation de centaines de zoospores à l'intérieur de sporanges mûrs. Les zoospores sont déchargées dans l'eau de mer à travers un ou plusieurs tubes d'expulsion. L'étude ultrastructurale montre que les zoospores, de forme oblongue, possèdent deux flagelles insérés au niveau du tiers antérieur, sur le côté concave du corps. Le flagelle antérieur présente une zone unilatérale ornée de formations filiformes. Une grande vacuole antérieure et un noyau basolatéral surplombent le cytoplasme du corps de la zoospore. Un complexe apical rudimentaire avec conoïde ouvert latéralement, rhoptries, micronèmes et microtubules sous pelliculaires est discernable. Les particularités morphologiques de la zoospore et les analyses séquentielles de deux gènes publiés précédemment conduisent à considérer le *Perkinsus* associé à *Mya arenaria* comme espèce nouvelle.

**MOTS CLÉS :** *Perkinsus* spp., palourde à coquille lisse (*Mya arenaria*), zoosporulation, zoospores, ultrastructure.

## INTRODUCTION

Protozoa of the genus *Perkinsus* cause significant mortalities of bivalve mollusks worldwide. Recent increases in *Perkinsus* spp. infections in the Chesapeake Bay have been associated with decreased abundances of ecologically and economi-

cally important bivalve species, including the eastern oyster *Crassostrea virginica* (Burrison & Ragone Calvo, 1996) and the softshell clam *Mya arenaria* (McLaughlin & Faisal, in press). Our previous studies have demonstrated the presence of two distinct *Perkinsus* spp. in hemolymph and gill tissues of infected softshell clams (McLaughlin & Faisal, 1998a; Kotob *et al.*, 1999a, b). The softshell clam hemolymph isolate was identified as *P. marinus* based upon its morphologic, phenotypic and genetic characteristics. The morphology and sequences of two genes, small subunit ribosomal RNA and internal transcribed spacer regions, demarcated the gill-associated *Perkinsus* sp. isolate from other *Perkinsus* species (McLaughlin & Faisal, 1998a; Kotob *et al.*, 1999a, b).

Levine (1978) established the class Perkinsea based upon ultrastructural characteristics of the zoospores of the type species *P. marinus*, a deadly pathogen of *C. virginica*, first described as *Dermocystidium marinum* Mackin,

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Owen, and Collier (Mackin *et al.*, 1950). Characteristics of the genus *Perkinsus* included the lack of sexual reproduction and the presence of biflagellated zoospores possessing an anterior vacuole, an array of unilateral hair-like structures along the anterior flagellum, and a structure resembling the apical complex characteristic of apicomplexan protists. The rudimentary apical complex of *P. marinus* contains an incomplete, or open-sided, conoid instead of the complete truncated cone present in apicomplexan species (Levine, 1970; Perkins, 1976). Recently, however, the inclusion of the class Perkinsea in the subphylum Apicomplexa has been refuted based upon molecular evidence of a phylogenetic affinity with dinoflagellates and the presence of an incomplete conoid structure (reviewed by Siddall *et al.*, 1997).

Presently, the genus *Perkinsus* includes four species, namely *P. marinus* of the eastern oyster (Mackin *et al.*, 1950; Perkins, 1976), *P. olseni* of the blacklip abalone *Haliotis ruber* (Lester & Davis, 1981), *P. atlanticus* of the carpet shell clam *Ruditapes decussatus* (Azevedo, 1989), and *P. qugwadi* of cultured Japanese scallops *Patinopecten yessoensis* (Blackbourn *et al.*, 1998). In these studies, description of a new *Perkinsus* species was premised upon morphology of life cycle stages, ultrastructure of zoospores, host affected, and host response to infection. Zoospore morphology, in particular, has been thought important in differentiating among *Perkinsus* species. For example, *P. atlanticus* zoospores are tapered both anteriorly and posteriorly while *P. marinus* zoospores are rounded anteriorly and tapered posteriorly (Perkins, 1976, 1996; Azevedo, 1989). In addition, zoospores of *P. atlanticus* are more uniform in dimension and structure than other reported species (Azevedo, 1989). Characteristics unique to *P. qugwadi* include the presence of both vegetative and zoosporulation forms in host tissues, lack of zoosporulation in seawater, and an inability to form hypnozoospores in Ray's fluid thioglycollate assays (Blackbourn *et al.*, 1998). Differences in host species and size of trophozoites provided the basis for the description of *P. olseni* as a new species (Lester & Davis, 1981). Herein, we report morphologic characteristics of the zoospores and zoosporangia of gill-associated *Perkinsus* sp. of the softshell clam.

## MATERIALS AND METHODS

### CLAMS

Fifty softshell clams were collected by hydraulic escalator dredge on September 7, 1998, from Swan Point in the Chester River, Maryland. The clams were allowed to purge for 24 h in a 10-L container of aerated, artificial seawater (Forty Fathoms,

Marine Enterprises Inc., Baltimore, MD) prepared at 12 ppt and maintained at 5°C.

### ZOOSPORULATION IN ARTIFICIAL SEAWATER

Pieces of gill (~ 6 mm) from each clam were excised and incubated for six days in tubes containing 6 ml of Ray's fluid thioglycollate medium (RFTM, Becton Dickinson Microbiology Systems, Cockeysville, MD) supplemented with 8 µl nystatin (Sigma Chemical Co., St. Louis, MO) as described by Ray (1952). A small piece of incubated gill was macerated on glass slides and stained with Lugol's iodine solution. Hypnozoospores staining a blue-black color pathognomonic to *Perkinsus* sp. were enumerated. The remaining gill tissues of infected clams were mashed against the walls of the tubes using cell scrapers or sterile inoculating loops. Following thorough maceration, 1 ml of the medium was transferred to 25 cm<sup>2</sup> flasks (Corning Glass Works, Corning, NY) containing 5 ml JL-ODRP growth media prepared as described by LaPeyre & Faisal (1996) using sterile techniques. Cultures were further incubated for 24 h at 27°C, and transferred to artificial sterile seawater (22 g/L) in 25 cm<sup>2</sup> tissue culture flasks. Seawater cultures were maintained at room temperature and the zoosporulation process was followed daily using an inverted microscope.

### ELECTRON MICROSCOPY

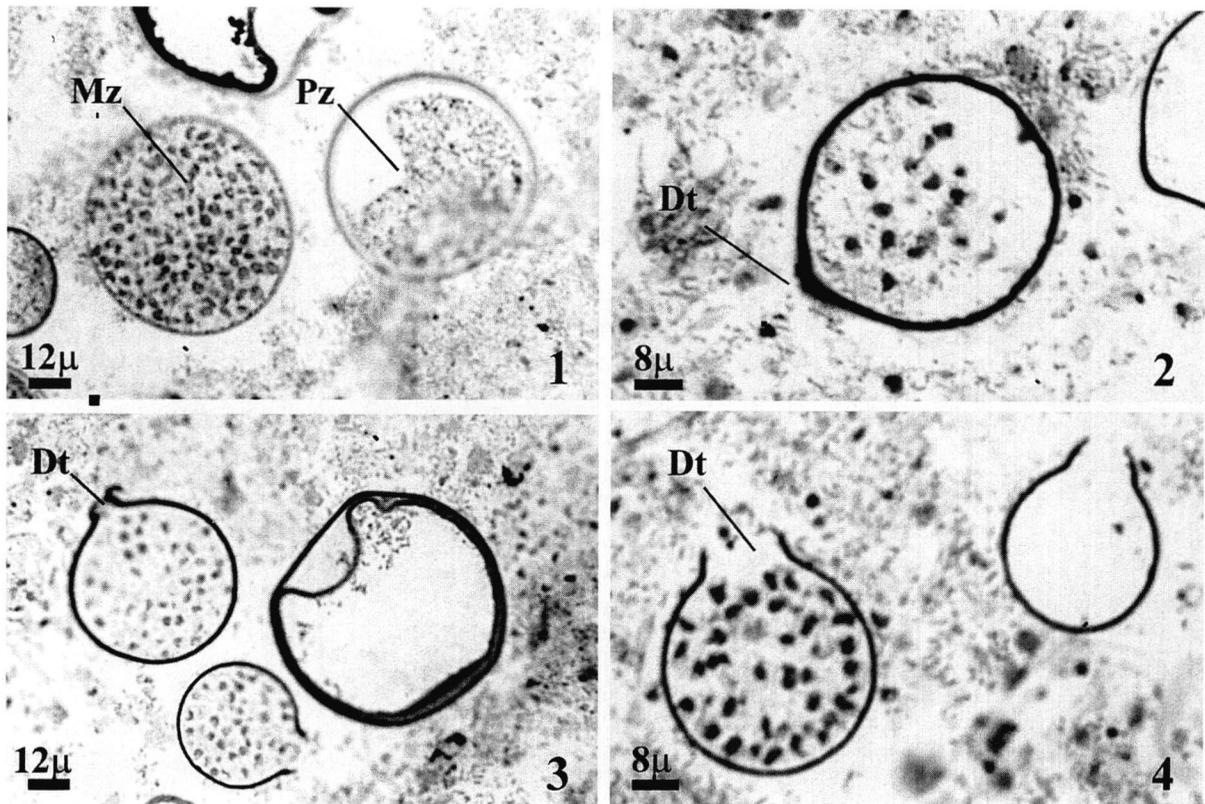
Zoosporulation preparations were fixed in 2.5 % glutaraldehyde, 0.1 M sodium cacodylate (pH 7.2) at room temperature, post-fixed in 1 % osmium tetroxide, and embedded in Eponate 812. Ultra-thin sections were stained with uranyl acetate and the sections examined with an electron microscope.

Semi-thin sections were stained with toluidine blue (Sigma) and examined by light microscopy. Dimensions of the zoospore body and flagella were taken from the stained semi-thin sections.

Despite the disputed phyla classification (Siddall *et al.*, 1997), terminology and classification criteria utilized in this report parallel previously published descriptions of *Perkinsus* species for purposes of simplicity and consistency.

## RESULTS

Examination of seawater cultures revealed an active zoosporulation process. Large prezoosporangia developed 24 hrs post-incubation and ranged in size from 20 to 135 µm (mean = 69.3 µm ± 29.3) (Fig. 1). Cell walls of the prezoosporangia thickened upon contraction of the cytoplasm with the formation of a clear area between the cell wall and the proto-



Figs 1-4. – Zoosporulation of *Perkinsus chesapeaki* sp. nov. 1: Prezoosporangia (Pz) and mature zoosporangia (Mz) containing several zoospores. 2-4: Discharge tube outgrowth formation (Dt) through which zoospores are released. (Semithin sections stained with toluidine blue.)

plast. Intermediate stages took place with successive bipartitions of the protoplast. Cells within prezoosporangia changed from spheroidal to smaller rod-like forms. Mature zoosporangia which contained numerous biflagellated zoospores with prominent anterior vacuoles were seen as early as 24 hours post-incubation. Tubular outgrowths (Figs. 2-4) ranging from 2.5-20  $\mu\text{m}$  in length were apparent 48 h post-incubation. The beginning of zoosporulation was recognized by the active movement of zoospores within the zoosporangia. Zoospores swam out of the zoosporangium through a discharge tube in an oscillatory movement, leaving behind empty sporangia (Fig. 4). Occasionally, more than one discharge tube was observed in a single zoosporangium. The release of zoospores from different zoosporangia continued for 7-8 days. The zoosporangia showed no synchronization in their development.

Mature zoosporangia had thickened cell walls composed of distinctive inner and outer layers (Fig. 5). Lamosomes were observed along the periphery of the zoosporangium cell wall (Fig. 5). Lipid-like droplets were also embedded in the inner cell wall (Fig. 5).

The bodies of free zoospores were usually oblong with rounded anterior and posterior regions. The posterior region was narrower than the anterior in some zoo-

spores (Fig. 6). Zoospore body length ranged from 2.2 to 4.7  $\mu\text{m}$  (mean =  $3.73 \pm 0.48$ ) and the width from 1.4 to 2.5  $\mu\text{m}$  (mean =  $2.41 \pm 0.36$ ). Zoospores were biflagellated (Fig. 6) with one flagellum longer than the other ( $5.51 \mu\text{m} \pm 1.19 \times 3.0 \mu\text{m} \pm 0.65$ ). The flagella

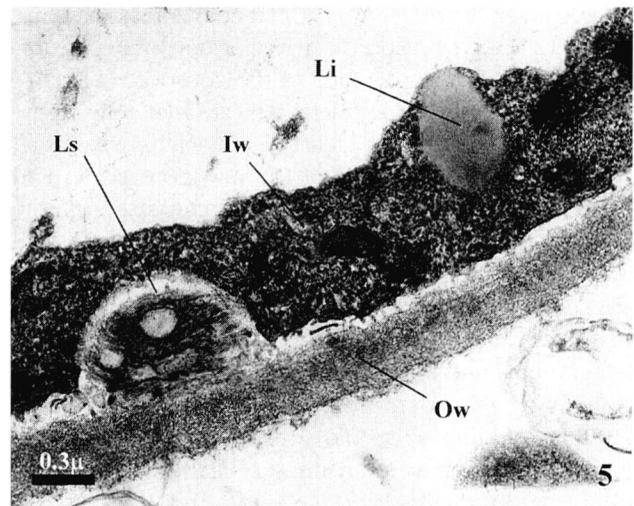


Fig. 5. – Ultrastructural details of the peripheral zone of a zoosporangium showing the bilayered wall (outer wall = Ow; inner wall = Iw), a lamosome (Ls), and lipid-like bodies (Li).

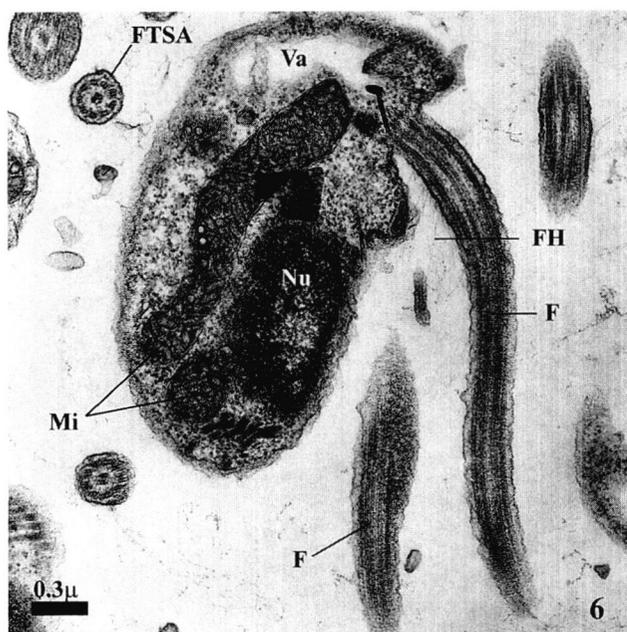
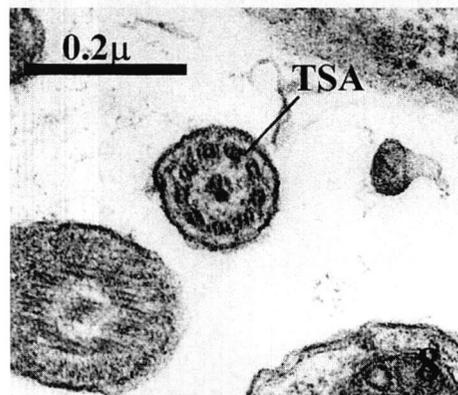
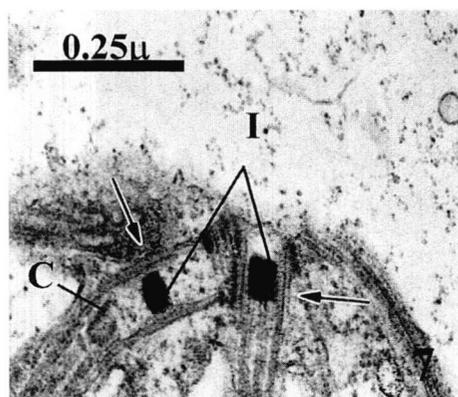


Fig. 6. – Longitudinal section of a free zoospore of *Perkinsus chesapeaki* sp. nov. showing nucleus (Nu), mitochondria (Mi), and vacuole (Va), flagella (F), and a transverse section of the axenome (FTSA). Note the unilateral array of hair-like structures on the anterior flagellum (FH).

originated from the upper third of the body and arose from a region that appeared as a concave impression. The anterior flagellum possessed an array of unilateral hair-like structures measuring 2–4 nm in diameter (Figs. 6 and 9). The basal bodies of the two flagella were oriented at an acute angle and an electron-dense body was observed within the lumen of the kinetosomes (Fig. 7). The longitudinal bundle of microtubules forming the flagellar axenome was organized into an arrangement of doublets with a nine plus two array (Fig. 8). A large anterior vacuole was apparent in zoospores (Figs. 6, 10, 12, and 13). Cortical alveoli were located externally on the anterior surface of the zoospore (Fig. 10). Zoospores had a single nucleus located laterally in the posterior portion of the cells. The nucleus was large, occupied nearly one-third of the zoospore body, and possessed an uneven condensation of chromatin (Figs. 6 and 10). Mitochondria either appeared as a longitudinal organelle (Fig. 6) or as multiple round to oval bodies (Fig. 11). Lipid-like droplets were occasionally observed posteriorly in the cytoplasm (Fig. 10).

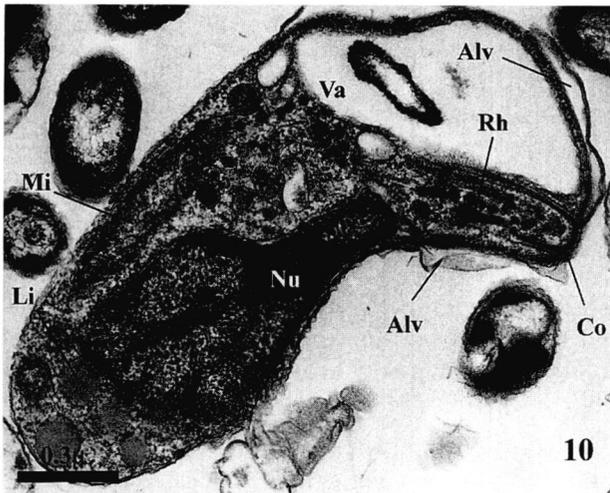
Longitudinal and transverse sections revealed structures resembling the apical complex described by Perkins (1976) including a conoid, conoid-associated micronemes, rectilinear micronemes (called the apical ribbon, ribbon-associated vesicles, and toxicysts by Siddall *et al.*, 1997), rhoptries, and subpellicular microtubules (Figs. 10–13). The conoid was open-sided and located anteriorly at the apical pole (Fig. 10). The rhop-



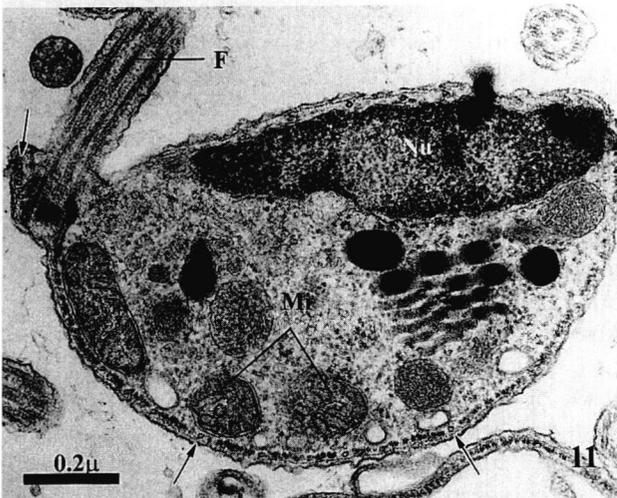
Figs 7–9. – Ultrastructural details of the flagella in a free zoospore of *Perkinsus chesapeaki* sp. nov. 7: Kinetosome of both flagella (arrows) showing a cylindrical inclusion (I) and a cup-like structure (C). 8: Transverse section of axenome (TSA) showing typical microtubular arrangement (9 + 2 array). 9: Hair-like structures (FH) located unilaterally along the anterior flagellum.

tries were electron dense and often vase-shaped with the narrower neck portion extending into the conoid region (Fig. 10).

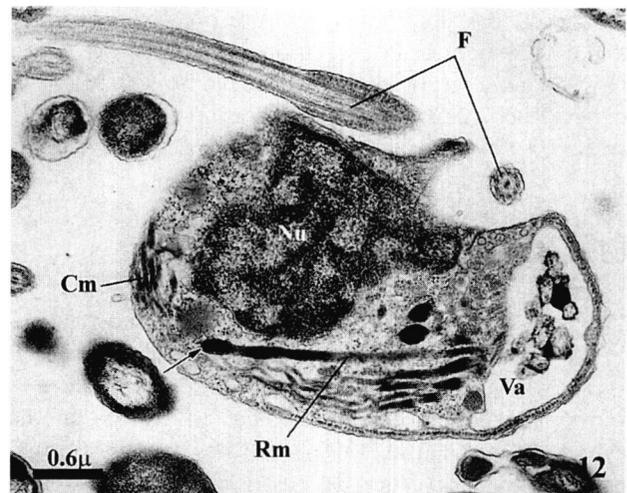
Rectilinear micronemes extended vertically from the conoid to the posterior region of the cell in a straight line and ended in a small bulb (Fig. 12). The more convoluted and curvilinear conoid-associated micronemes extended posteriorly from the conoid, wrapped around the nucleus, and then extended anteriorly (Figs. 12 and 13). Subpellicular microtubules were also observed (Fig. 11).



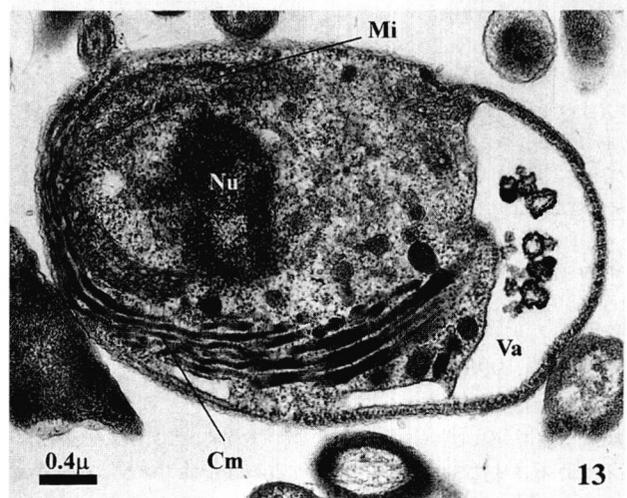
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11



12



13

Fig. 10. – Longitudinal section of a mature zoospore showing vacuole (Va), alveoli (Alv), nucleus (Nu), mitochondrion (Mi), lipid-like bodies (Li), conoid (Co), and rhoptries (Rh).

Fig. 11. – Transmission electron micrograph of a free zoospore showing subpellicular microtubules (arrows), several oval or round mitochondria (Mi), and the kinetosome of a flagellum (F).

Figs 12-13. – Longitudinal sections of free zoospores showing micronemes associated with conoid. 12: Rectilinear microneme (Rm) extending from the conoid to the nucleus (Nu) and terminating in bulb-like structure (arrow). Note the longitudinal and transverse sections of the flagella (F). 13: Conoid-associated micronemes (Cm) extending posteriorly from the conoid, curving around the region of the nucleus (Nu), and then extending anteriorly.

### *PERKINSUS CHESAPEAKI* SP. NOV.

#### Description

Uninucleated, transparent trophozoites ellipsoid to spherical, containing an eccentric nucleus with a large, single nucleolus and eccentric vacuole. Morphology of trophozoites *in vitro* and within host tissues are detailed in McLaughlin & Faisal (1998a, b). Prezoosporangia cultured in RFTM and JL-ODRP media undergo successive binary fissions and produce numerous zoospores. Zoospores are uninucleated, biflagellated, slightly oblong ( $3.7 \mu\text{m} \pm 0.48 \times 2.41 \mu\text{m} \pm 0.36$ ) and possess a large laterobasal nucleus and a single, anterior vacuole. Flagella are unequal in length ( $5.51 \mu\text{m} \pm 1.19$  and  $3.0 \mu\text{m} \pm 0.65$ ) and arise from a concave side located in the upper third of the zoospore body.

#### Taxonomic summary

*Type host*: Trophozoites in softshell clam *Mya arenaria* (Mollusca, Bivalvia).

*Type Locality*: Chesapeake Bay, Maryland.

*Prevalence*: Seasonal with peak prevalences occurring in late summer and fall.

*Site of infection*: Gills appear to be the target tissue although abscesses occur in other organs in advanced infections.

*Etymology*: *Chesapeakei* derived from Chesapeake Bay

*Specimens deposited*: Stained slides of infected softshell clam gills were deposited in the Registry of Tumors in Lower Animals, George Washington University, Washington DC (Accession number 7121). Cloned cultures were deposited in the laboratory of Dr.

Mohamed Faisal, Virginia Institute of Marine Science (VIMS), Gloucester Point, Virginia. The SSU rRNA and ITS-5.8S gene sequences are accessible from GenBank at retrieve@ncbi.nlm.nih.gov (Accession numbers AF042707 and AF091541, respectively).

## DISCUSSION

The processes of prezoosporulation and zoosporulation of *Perkinsus chesapeaki* sp. nov. in seawater were identical to those reported during *in vitro* propagation of the parasite in culture media (McLaughlin & Faisal, 1998a). Further, prezoosporulation and zoosporulation processes of *P. chesapeaki* sp. nov. in seawater were similar to those reported for *P. marinus* and *P. atlanticus* (Perkins & Menzel, 1967; Azevedo *et al.*, 1990; Auzoux-Bordenave *et al.*, 1995; Ordas & Figueras, 1998). The life cycle of *P. chesapeaki* sp. nov. is characterized by schizogony of vegetative forms with multiple divisions of mother cells into several daughter cells and successive bipartition of the cytoplasm of prezoosporangia into hundred's of zoospores. The softshell clam *Perkinsus* species appears to be unique in its ability to undergo both schizogony and active zoosporulation in culture media (McLaughlin & Faisal, 1998a).

Examination of the ultrastructure of *P. chesapeaki* sp. nov. in this study showed the zoospores to possess characteristics typical of other members of the genus *Perkinsus*. The zoospores are biflagellated with the anterior flagellum possessing unilateral hair-like extensions of the flagellar membrane. The presence of an open-sided conoid-like structure and associated vesicles further support the classification of the softshell clam parasite in the genus *Perkinsus*. Zoospores of *P. marinus*, *P. atlanticus*, and *P. quqwadi* possess similar morphological features (Perkins 1976; Azevedo, 1989; Azevedo *et al.*, 1990; Blackburn *et al.*, 1998). Detailed morphology of *P. olseni* zoospores has not been reported; however, zoosporulation studies produced biflagellated zoospores (Lester & Davis, 1981).

Differences observed in the ultrastructure of *P. chesapeaki* sp. nov. zoospores compared with other described species include the larger size of the nucleus, the presence of a single, large anterior vacuole, and multiple mitochondria of varying shapes. Moreover, the zoospore body of *P. chesapeaki* sp. nov. ( $3.7 \mu\text{m} \pm 0.48 \times 2.41 \mu\text{m} \pm 0.36$ ) differs in his dimension from *P. marinus* ( $4\text{--}6 \times 2\text{--}3 \mu\text{m}$ ), *P. atlanticus* ( $4.5 \mu\text{m} \pm 0.6 \times 2.9 \mu\text{m} \pm 0.4 \mu\text{m}$ ), and *P. quqwadi* ( $3.86 \mu\text{m} \pm 0.31 \times 2.47 \mu\text{m} \pm 0.2 \mu\text{m}$ ). Interestingly, the dimensions of the zoospore body of *P. chesapeaki* sp. nov. bear some resemblance to those of a *Perkinsus* sp. reported in the Baltic clam *Macoma balthica* (Andrews, 1954; Perkins, 1968; Kleinschuster

*et al.*, 1994). In *P. chesapeaki* sp. nov., the zoospore body ranges in length from 2.2 to 4.7  $\mu\text{m}$  and in width from 1.4 to 2.5  $\mu\text{m}$  while the Baltic clam *Perkinsus* sp. zoospores measure from 3 to 5 and 2 to 3  $\mu\text{m}$ , respectively (Perkins, 1968). Similarly, the zoospores of the *Perkinsus* sp. of Baltic clams and *P. chesapeaki* sp. nov. of softshell clams share a slightly oblong morphology. On the contrary, *P. marinus* zoospores are rounded anteriorly and tapered posteriorly while zoospores of *P. atlanticus* are more irregularly ellipsoidal and tapered at both ends. Further, the zoospores of *P. quqwadi* are typically ovoid and have a tapered posterior.

Flagella of *P. chesapeaki* zoospores are inserted at an acute angle to one another and are located in the anterior third of the zoospores, a position similar to that observed in *P. marinus* (Perkins, 1969) and *P. atlanticus* (Azevedo, 1989). On the other hand, flagellar bases of *P. quqwadi* are parallel in some zoospores and at right angles in others (Blackburn *et al.*, 1998). Both flagella of *P. chesapeaki* sp. nov. ( $5.51 \mu\text{m} \pm 1.19$  and  $3.0 \mu\text{m} \pm 0.65$ ) appear to be shorter than those described in other *Perkinsus* species including the *M. balthica* isolate (Perkins, 1968, 1969; Azevedo, 1989; Blackburn *et al.*, 1998) based upon stained thin sections. Anterior flagella measure 10–18  $\mu\text{m}$  in *P. marinus*,  $12.7 \pm 2.4 \mu\text{m}$  in *P. atlanticus*,  $9.01 \pm 1.2 \mu\text{m}$  in *P. quqwadi*, and 13–17  $\mu\text{m}$  in *Perkinsus* sp. of *M. balthica*. Posterior flagella measure 6–10  $\mu\text{m}$ ,  $10.7 \pm 3.2 \mu\text{m}$ ,  $7.95 \pm 1.2 \mu\text{m}$ , and 7–9  $\mu\text{m}$ , respectively. Electron-dense bodies in the lumen of the kinetosome of *P. chesapeaki* sp. nov. were observed in *P. marinus* (Perkins, 1969; 1988; 1996) and *P. atlanticus* (Azevedo, 1989) but were missing in *P. quqwadi* (Blackburn *et al.*, 1998). Multiple round to oval mitochondria and single bar-shaped mitochondria were observed with similar frequency in *P. chesapeaki* sp. nov. and occurred more frequently than other described species. Further, most mitochondria of *P. marinus* occurred singly and were often bar-shaped (Perkins, 1996) while *P. quqwadi* mitochondria were lobulated (Blackburn *et al.*, 1998). The cortical alveoli is located anteriorly in *P. chesapeaki* sp. nov., *P. marinus*, and *P. atlanticus* while the entire surface of *P. quqwadi* is surrounded by alveoli (Perkins, 1976; Azevedo, 1989; Blackburn *et al.*, 1998).

A new species of *Perkinsus* isolated from softshell clams is proposed based upon characteristics of trophozoite morphology, ultrastructure of organelles in zoospores, shape and size of zoospore cell body, and length of flagella. This proposal is supported by molecular evidence (Kotob *et al.*, 1999a, b). Further, the ability of *P. quqwadi* to develop at temperatures below 20°C (Bower *et al.*, 1998; Blackburn *et al.*, 1998) was similarly observed in the softshell clam isolate by McLaughlin & Faisal (in press). Prevalences of *Perkin-*

*sus* spp. infections in softshell clams collected from low salinity sites indicate the softshell clam isolate is also able to develop at salinities below 12-15 ppt (McLaughlin & Faisal, in press). Virus-like particles reported in the nucleus and cytoplasm of *P. marinus* collected from Virginia (Perkins, 1969) were lacking in *P. chesapeakei* sp. nov. In addition, *P. marinus* infections in oysters become rapidly systemic (Mackin, 1951) while the spread of infections in clams by *P. atlanticus* and *P. chesapeakei* sp. nov. appear to be delayed by initial encapsulating responses of the hosts (Chagot *et al.*, 1987; McLaughlin & Faisal, 1998b).

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