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# Continuous Culture of *Perkinsus mediterraneus*, a Parasite of the European Flat Oyster *Ostrea edulis*, and Characterization of Its Morphology, Propagation, and Extracellular Proteins in Vitro

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ABSTRACT.   Continuous in vitro cultures of *Perkinsus mediterraneus* were established from tissues of infected European flat oysters, *Ostrea edulis*. The parasite proliferated in protein-free medium and divided by schizogony in vitro. Cell morphology was similar to that observed for *P. mediterraneus* in tissues of naturally infected *O. edulis* and for other *Perkinsus* spp. cultured in vitro. Parasite cells enlarged approximately 8-fold when placed in alternative Ray's fluid thioglycollate medium, and stained black with Lugol's iodine solution, a response characteristic of *Perkinsus* spp. DNA sequences matched those determined previously for *P. mediterraneus*, and phylogenetic analyses on three different data sets indicated that this was a *Perkinsus* species with a close relationship to another recently described species, *Perkinsus honshuensis*. Parasite viability was high (>90%) in vitro, but the proliferation rate was low, with densities generally increasing 2-to-6-fold between subcultures at 6-wk intervals. Enzyme analysis of cell-free culture supernatants revealed protease-, esterase-, glycosidase-, lipase-, and phosphatase-like activities. Incubation with class-specific protease inhibitors showed that *P. mediterraneus* produced serine proteases, and eight proteolytic bands with molecular weights ranging from 34 to 79 kDa were detected in the supernatants by gelatin sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Key Words.   Actin, bivalve mollusk, extracellular enzymes, in vitro proliferation, ITS, LSU, protistan parasite, rRNA, serine proteases.

S PECIES of the genus *Perkinsus* are parasites of a wide range of marine mollusks worldwide, and have been responsible for heavy mortalities and economic losses (Dungan and Reece 2006; Goggin and Lester 1987; Villalba et al. 2004). *Perkinsus marinus*, the agent of dermo disease in *Crassostrea virginica*, causes extensive mortalities of oyster populations along the Atlantic and Gulf of Mexico coasts of North America (Burreson and Ragone Calvo 1996; Ford 1996; Soniat 1996). *Perkinsus olseni* has been associated with elevated mortalities of clams along the coasts of Portugal, Spain (Azevedo 1989; Ruano and Cachola 1986; Santmartí et al. 1995) and Asia (Park, Choi, and Choi 1999; Park et al. 2005), as well as among abalones in Australia (Lester and Davis 1981; O'Donoghue, Phillips, and Shepherd 1991). Several new *Perkinsus* species have been recently described, including *Perkinsus mediterraneus*, a parasite infecting European flat oysters (*Ostrea edulis*) from the Mediterranean coast of Spain (Casas et al. 2004) and *Perkinsus honshuensis* infecting Manila clams (*Venerupis = Tapes philippinarum*) from Japan (Dungan and Reece 2006). Except for their presence in these identified host species, little is known about these new parasites and the diseases they produce.

The development of continuous cultures of *Perkinsus* species has led to a better understanding of this group of parasites. Cultures of *P. marinus* and *P. olseni* have been used in a wide range of studies to address environmental tolerance (Burreson et al. 1994; La Peyre, Casas, and La Peyre 2006; O'Farrell et al. 2000), host specificity (Casas, Audemard, and La Peyre 2005; Dungan et al. 2007a), genetic composition (Reece et al. 2001), metabolism (Chu et al. 2002; Elandalloussi et al. 2005b; Lund et al. 2007), drug sensitivity (Calvo and Burreson 1994; Elandalloussi et al. 2005a), and virulence factors (Ahmed et al. 2003; Garreis, La Peyre, and Faisal 1996; La Peyre et al. 1995). Of particular interest are mechanisms of pathogenicity of *Perkinsus* species, which

remain largely unexplored. A number of potential virulence factors have been proposed for *P. marinus* (La Peyre et al. 1995; Schott et al. 2003; Volety and Chu 1997; Wright et al. 2002), but their specific roles in pathogenesis have not been demonstrated; while virulence factors of other *Perkinsus* spp. are not reported. Nonetheless, several findings suggest that extracellular proteins (ECP) of *P. marinus* contribute to the pathogenesis of dermo disease (Anderson 1999; Garreis et al. 1996; La Peyre, Yarnall, and Faisal 1996). Parasite body burdens in oysters fed with liposomes containing ECP (i.e. >10 kDa) in conditioned medium and then challenged with *P. marinus* were significantly higher than in oysters fed with liposomes containing fresh culture medium (La Peyre et al. 1996). Among the ECP of *P. marinus* are proteases that degraded extracellular matrix proteins (i.e. fibronectin, laminin). This may explain the extensive tissue lysis observed in heavily infected oysters, and provides a possible mechanism by which *P. marinus* can gain access to connective tissues (La Peyre et al. 1995; Mackin 1951; Ray, Mackin, and Boswell 1953). There are also some indications that *P. marinus* proteases or other ECP suppress humoral and cellular parameters of host oyster defenses (Anderson 1999; Garreis et al. 1996). However, the contributions of proteases and other ECP in pathogenesis of the diseases caused by *P. marinus* and other *Perkinsus* spp. remain to be ascertained.

This study describes the continuous in vitro culture of *P. mediterraneus* and provides confirmatory evidence that the cultured protistan parasites are *P. mediterraneus*. In addition, the growth characteristics of *P. mediterraneus* cultures were determined and an initial analysis of extracellular enzymes in cell-free culture supernatants of this parasite was done using the API ZYM system and by gelatin sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The ability to propagate *P. mediterraneus* in vitro will provide a versatile system to study the biology of this parasite.

## MATERIALS AND METHODS

**Oysters.** European flat oysters (*O. edulis*) were obtained from the Balearic Island of Menorca (Mediterranean Sea, Spain) in

October 2003, and were maintained for 10 d in a 75-L tank containing 0.22 μm filtered seawater at 35 ppt and 17 °C at the Centro de Investigacións Mariñas in Galicia, Spain. Water in the tank was aerated, and circulated through an ultraviolet light sterilizer to reduce oyster bacterial loads.

**Culture medium.** The protein-free culture medium JL-ODRP-2F (1,025 mOsm/kg, ~ 35 ppt seawater salinity equivalence, pH 7.4) was used to establish and maintain *P. mediterraneus* cultures. This medium was similar in composition to the culture medium JL-ODRP-2A (775 mOsm/kg, ~ 25 ppt seawater salinity equivalence) used to propagate *Perkinsus olseni* in vitro (Casas et al. 2002) except for the increase in salt concentrations in the buffered balanced salt solution (concentrations per liter: 1,646 mg $CaCl_2 \cdot 2H_2O$; 3,168 mg $MgSO_4$; 4,916 mg $MgCl_2 \cdot 7H_2O$; 1,788 mg KCl; 28,076 mg NaCl; 336 mg $NaHCO_3$). All chemicals were tissue culture grade from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

**Initiation and maintenance of cultures.** Ten oysters were opened and shucked from their shells. Heart and fragments of gills (about 5 mm²) were excised with sterile scissors in a laminar flow hood, and added to 50-ml tubes with 30 ml of 35 ppt, 0.22 μm filtered artificial seawater (ASW). Oyster tissues were rinsed 10 times in 30 ml of ASW and decontaminated with two, 30-min incubations in an antibiotic solution consisting per liter of 400,000 U penicillin G, 400 mg streptomycin sulfate, 200 mg gentamicin, 400 mg kanamycin A, 0.2 mg neomycin, 200 mg polymyxin B, and 400 mg erythromycin in sterile ASW. Tissues were rinsed 10 times in 30 ml ASW and cut into 2–3 mm³ fragments with a sterile razor blade. Each tissue fragment was placed directly in individual wells of a sterile 24-well tissue culture plate (Corning Inc., Corning, NY), containing 1 ml of JL-ODRP-2F medium. All plates were incubated in a humidified chamber at 28 °C. Once *P. mediterraneus* isolates started to proliferate, cells were transferred to 25-cm² flasks (Corning Inc.), and were subcultured every 6 wk using a seeding density of approximately $5 \times 10^4$ cells/ml and a 1:1 mixture of fresh and conditioned culture media. For the analysis of ECP, flasks were seeded with $5 \times 10^5$ cells/ml in fresh culture medium and incubated at 28 °C for 6 wk. The conditioned medium was then centrifuged at 1,000 g for 10 min to collect the culture supernatants. Culture supernatants were filtered through low protein-binding 0.22-μm filters, and stored at − 20 °C for later analysis.

**Morphology of cultured cells.** The morphology of the cultured cells following subculture was observed with a light microscope using differential interference contrast optics (Zeiss Axiovert 25, Carl Zeiss Inc., Thornwood, NY). Ultrastructures of *P. mediterraneus* isolate cells established from heart and gill fragments were examined on day 21 after subculture. Cells were harvested and pelleted by centrifugation at 1,000 g for 10 min at 25 °C. Cell pellets were fixed for 2 h with 2.5% (w/v) glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2 at 4 °C. The pellets were washed twice in cacodylate buffer and post-fixed for 2 h in 2% (w/v) $OsO_4$ in cacodylate buffer at 4 °C. Cells were enrobed in 1.5% (w/v) low melting point agarose solution. The blocks were dehydrated in an ethanol series and embedded in Epon. Ultra-thin sections were double stained with uranyl acetate and lead citrate and examined with a JEOL 100 CXII transmission electron microscope operated at 60 kV.

**Enlargement of cultured cells in alternative Ray's fluid thioglycollate medium.** *Perkinsus mediterraneus* isolate (Pmed-Ht2) was incubated in alternative Ray's fluid thioglycollate medium (ARFTM) prepared using alternative fluid thioglycollate medium supplemented with 16 g/L of hw-Marine Professional sea salts (Hawaiian Marine Imports Inc., Houston, TX) and 0.5% (v/v) lipid mixture 1,000x (La Peyre et al. 2003; Nickens et al. 2002). Isolate Pmed-Ht2 was established from the

heart of an infected *O. edulis*. Cells of the isolate were washed with ASW, suspended in ARFTM at a density of $1 \times 10^4$ cells/ml, and added to two wells of a 24-well tissue culture plate. The plate was incubated for 1 wk at room temperature in the dark. The diameters of 50 cells were measured with a Zeiss Axiovert 25 inverted microscope on day 0 and day 7. After 1 wk in ARFTM, the enlarged cells or hypnospores were harvested, and Lugol's iodine solution was added to determine if they stained blue-black, a characteristic response of *Perkinsus* species hypnospores (Ray 1952).

**PCR assays, DNA sequencing, and phylogenetic analyses.** DNA was isolated from cultured cells of two *P. mediterraneus* isolates established during this study, Pmed-Ht2 and Pmed-G3. Isolate Pmed-G3 was established from the gills of an infected *O. edulis*. A 0.5–1.0 ml sample of cells at a concentration of $1–5 \times 10^8$ cells/ml was centrifuged at 200 g to pellet the cells. Cell pellets were washed with 1 ml of 12 ppt ASW to remove media, and were resuspended in lysis buffer for extraction using the Qiagen™ DNeasy Tissue kit (Qiagen, Carlsbad, CA) following the manufacturer's protocol. Primers targeting the internal transcribed spacer (ITS) region of the ribosomal RNA complex of *Perkinsus* spp. were used to amplify an approximately 670-bp fragment, as described previously (Audemard, Reece, and Burreson 2004; Casas et al. 2002). Primers targeting dinoflagellate large subunit ribosomal RNA (LSU rRNA) (Lenaers et al. 1989) and *Perkinsus* spp. actin genes (Reece et al. 2004) were used to amplify LSU rRNA and actin gene fragments. Amplification products of the ITS region were cloned and sequenced according to Reece and Stokes (2003), while the LSU rRNA and actin gene amplification products were cloned and sequenced as described in Moss, Burreson, and Reece (2006). Resulting sequences were imported into MacVector 8.1.2 Sequence Analysis Software (Oxford Molecular Ltd., Oxford, UK) for trimming vector and primer sequences, and for alignment with GenBank-deposited sequences of accepted *Perkinsus* spp. Identification of the amplified nucleotide fragments involved BLAST (Altschul et al. 1990) searches of the National Center for Biotechnology Information GenBank database and phylogenetic analyses. For the phylogenetic analyses, sequences were aligned using the CLUSTAL-W algorithm (Thompson, Higgins, and Gibson 1994) in the MacVector 8.1.2, using a multiple alignment gap penalty of 7, and an extend gap penalty of 3. Neighbor joining and parsimony analyses were conducted using PAUP*4b10.0 (Swofford 2002). Bootstrap analyses were done with 100 random additions of 1,000 jackknife replicates for parsimony. Gaps were treated as missing data in all parsimony analyses.

**Characterization of cultured cells proliferation.** *Perkinsus mediterraneus* growth in culture was characterized by measuring the viability, density, metabolic activity, diameter, and percentage of cells in schizogony of two *P. mediterraneus* isolates (Pmed-Ht2 and Pmed-G2). Isolate Pmed-G2 was established from the gills of an infected *O. edulis*. At the start of the experiment (day 0), 6-wk-old cells were harvested, passed twice through a 25-gauge needle attached to a 10-ml syringe to remove clumps, and re-suspended in a 1:1 mixture of fresh and conditioned (i.e. 6-wk-old) media at a density of approximately $5 \times 10^4$ cells/ml. One-hundred microliters of each isolate suspension were added to each well of a 96-well tissue culture plate (Costar Inc., Corning, NY) and incubated in a humidified chamber at 28 °C for 30 d. Two replicate wells for each isolate and day were sampled to measure cell viability, density, diameter, and percentage of cells in schizogony, following the timetable outlined in Table 1 and according to procedures of La Peyre et al. (2006). Metabolic activity was also measured for each isolate as described previously by La Peyre et al. (2006), following the schedule in Table 1 and using the AlamarBlue assay (Alamar Biosciences Inc., Sacramento, CA), with five replicate wells for each isolate.

Table **1.** Sampling schedule by day, represented by x, for the five parameters measured in this study: *Perkinsus mediterraneus* viability, diameter, percentage of schizogony, density, and metabolic activity (MA).

| | Day | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 3 | 4 | 5 | 6 | 8 | 10 | 12 | 18 | 24 | 30 |
| Viability | x | | | | | x | | | x | x | x | x |
| Diameter | x | | | | | x | | | x | | | |
| % Schizogony | x | | | | | x | | | x | x | x | x |
| Density | x | | | | | x | | | x | x | x | x |
| MA | | x | x | x | x | x | x | x | | | | |

**Enzymatic activities of culture supernatants.** The culture supernatants of isolates Pmed-Ht2 and Pmed-G3 were concentrated 5-fold (5x) with Centriprep YM-10 concentrator (Millipore Corporation, Medford, MA). The enzymatic profiles of un-concentrated (1x) and concentrated culture supernatants were then characterized using the API ZYM system (BioMérieux Vitek Inc., Hazelwood, MO) according to the manufacturer's instructions. Fresh medium (control) and *P. mediterraneus* culture supernatants (1x and 5x) were added to 20 cupules (65 μl/cupule) of individual plastic strips. For each strip, 19 cupules contained dehydrated chromogenic substrates with appropriate buffers, while one cupule did not contain substrates and was used as a negative control. The plastic strips were then placed in moist chambers for 24 h at 35 °C. After incubation, each cupule received one drop each of Zym A and Zym B reagents. After 15 min, the color intensity in each cupule was rated on a scale from 0 to 5 using a color chart provided by the manufacturer: a score of 0 represented no reaction while a score from 1 to 5 corresponded to 5–40 nm of hydrolyzed substrate under the assay conditions.

**Detection and characterization of proteases in culture supernatants.** Proteases in cell-free culture supernatants were detected using gelatin SDS-PAGE with an 8% (w/v) acrylamide resolving gel containing 0.2% (w/v) gelatin as described by La Peyre et al. (1995). Briefly, the culture supernatants of two *P. mediterraneus* isolates, Pmed-Ht2 and Pmed-G3, were mixed 1:1 with SDS-PAGE sample buffer containing 5% (w/v) SDS and were loaded at 1 μg/lane into a 3% (w/v) stacking gel. Electrophoresis was run at 30 mA constant current and the resulting gel was incubated in 2.5% (v/v) Triton X-100 in distilled water for 30 min at 4 °C to remove SDS. Lysis of the substrate-impregnated gels by *P. mediterraneus* proteases was then allowed to progress by incubating the gel in 0.1 M Tris-HCl, pH 8.0 for 4 h at 35 °C. The gel was fixed, stained with Coomassie Blue for 2 h, and destained (40% methanol, 10% acetic acid) until clear bands appeared where proteolysis of the gelatin substrate had occurred. Quantity-One software version 4.5.0 (Bio-Rad Laboratories Inc., Hercules, CA) was used to quantify the molecular weight and number of proteolytic bands, and to determine the relative quantity of each band. Molecular weights were estimated using protein standards (SDS-PAGE broad range molecular weight standards, Bio-Rad).

To determine the class specificity of *P. mediterraneus* proteases, 20x concentrated culture medium supernatant was incubated with 4-(2-aminoethyl) benzenesulfonylfluoride (AEBSF), an inhibitor of serine proteases; *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), an inhibitor of cysteine proteases; pepstatin A, a specific inhibitor of aspartic proteases; *o*-phenanthroline (1,10-phenanthroline monohydrate), an inhibitor of metalloproteases; and protease inhibitor diluent controls (distilled water or DMSO). Protease activities were compared. Protease inhibitors were purchased from Calbiochem® (San Diego, CA) and stock solutions were prepared in distilled water

(100 mM AEBSF) or DMSO (1 mM E-64, 1 mM pepstatin A, 100 mM *o*-phenanthroline). In a 96-well microtiter plate, 10 μl of 20 mM of AEBSF, 20 μM of E-64, 10 μM of pepstatin A, 20 mM of *o*-phenanthroline or control protease inhibitor diluent solutions were added to 20 μl of 20x-concentrated *P. mediterraneus* supernatant, and incubated for 30 min at 25 °C. The inhibitor concentrations chosen were within their respective effective concentration ranges (Benyon and Salvesen 1989). After incubation, 100 μl of 2.5% (w/v) hide powder azure (HPA) suspension prepared in HPA buffer (150 mM Tris base, 30 mM $CaCl_2$, 0.05% [w/v] Brij 35, 20% [w/v] sucrose, pH 7.5) were added to each well. Plates were incubated at 37 °C for 6 h with continuous shaking. After incubation, 100 μl of 50 mM Tris/150 mM NaCl pH 8.0 solution at 4 °C were added to each well to stop the reaction. Plates were centrifuged at 3,000 *g* for 15 min at 4 °C, and 100 μl of the supernatant from each well were transferred to a new plate. Absorbances were read at 595 nm on a microplate reader (Benchmark Plus, Bio-Rad). Results were expressed as the percentage of the protease activity of 20x-concentrated *P. mediterraneus* culture supernatant incubated with the corresponding protease inhibitor diluent. All measurements were performed in triplicate.

**Statistical analysis.** Cell diameters before and after incubation in ARFTM were compared using the Kruskal–Wallis non-parametric test. Cell viability, density, diameter, metabolic activity, and percent schizogony data were analyzed using a two-factor (i.e. day and isolate) analysis of variance (ANOVA). All data are reported as mean ± SD. Cell density and metabolic activity data were log transformed to achieve normality and homogeneity of variances. The effects of protease inhibitors on culture supernatant proteolytic activities were tested using a one-factor ANOVA. Least-square means with a Tukey adjustment were used following significant ANOVA results ($P < 0.05$) to examine the differences among treatments.

RESULTS

**In vitro isolates.** Cultures of 17 isolates were established from four infected oysters. Five isolates were obtained from heart tissues and 12 isolates were obtained from gill tissues. *Perkinsus mediterraneus* isolate Pmed-G2 was deposited at the American Type Culture Collection (ATCC, Manassas, VA) as ATCC PRA-238 and isolate Pmed-Ht2 was deposited as ATCC PRA-239 (www.atcc.org).

**Morphology of cultured cells.** Using light microscopy, cultured cells were mostly spherical, although some oval-shaped cells were observed after schizogony. Trophozoite diameters were 12–38 μm; cells had a dense cytoplasm containing numerous refractile bodies (presumably lipid droplets) and a prominent vacuole, which occasionally enclosed a vacuoplast (Fig. 1). The vacuole occupied most of the volume of the cell, displacing the nucleus to an eccentric position. Trophozoite proliferation was by schizogony. Schizont diameters were 25–58 μm. Typically each schizont produced four daughter trophozoites, although some schizonts produced larger numbers of daughter trophozoites (Fig. 1). The cell walls of most schizonts appeared to rupture long after cytokinesis, and large trophozoites were observed within the persistent wall of the schizont. In vitro zoosporulation of *P. mediterraneus* isolates was observed only once over several years since the establishment of our isolate cultures. Ultrastructurally, trophozoites had thick cell walls surrounding cytoplasm containing numerous ribosomes, lipid droplets, and mitochondria with tubular cristae (Fig. 2). Each trophozoite had a prominent vacuole and a nucleus with a prominent nucleolus. Schizonts had thick cell walls, and typically produced a few large daughter trophozoites (Fig. 3, 4) that enlarged and developed thick walls inside of the schizont wall (Fig. 4). Daughter trophozoites had large nuclei and
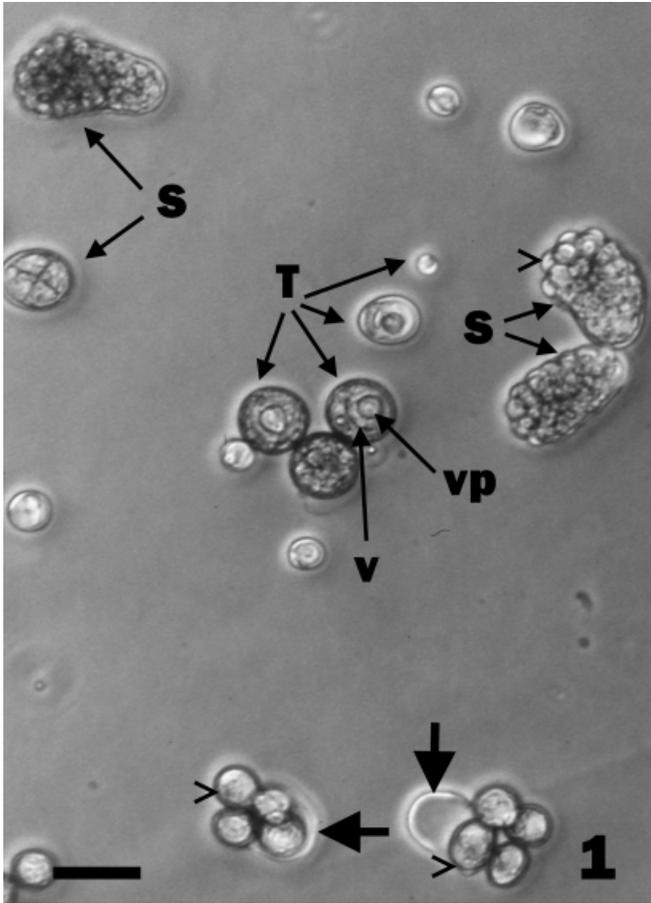
Fig. 1. Light micrograph of cultured *Perkinsus mediterraneus* cells in JL-ODRP-2F medium. Trophozoites (T) showing a granular cytoplasm with a large vacuole (v) and a prominent vacuoplast (vp). *Perkinsus mediterraneus* divides by schizogony, and schizonts (S) vary in size and in the number of daughter trophozoites (>) produced after rupturing of the schizont wall (arrows). Scale bar = 20 µm.
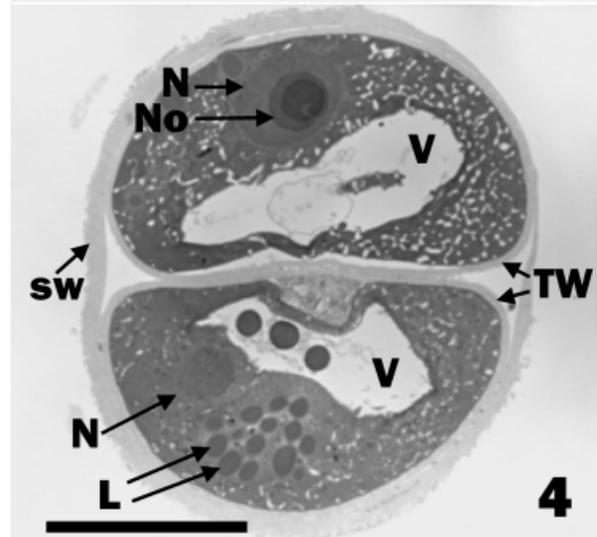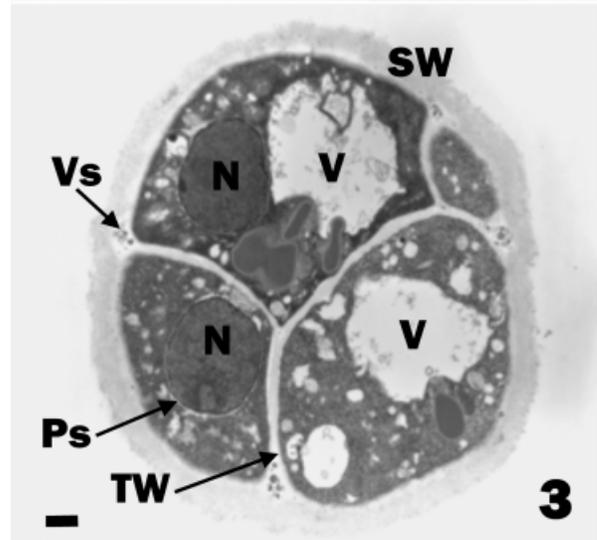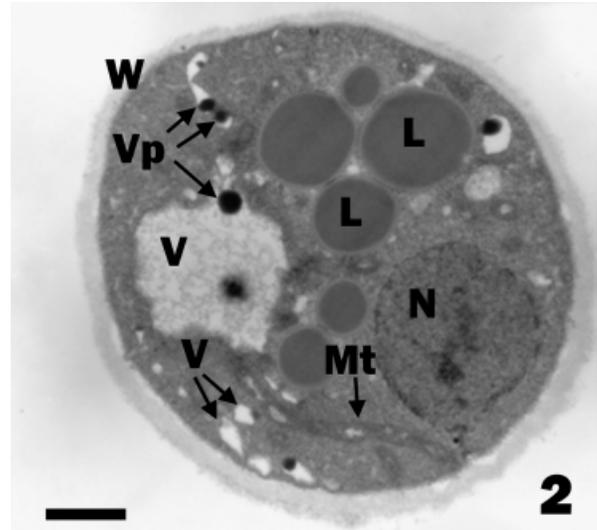
wide perinuclear spaces that were contiguous with nuclear pores (Fig. 3).

**Enlargement of cultured cells in ARFTM.** There was a significant increase in the diameter of cultured cells that were incubated in ARFTM. Mean diameter ± SD of isolate Pmed-Ht2 cells increased from $14.5 \pm 3.1$ µm on day 0 to $120.9 \pm 36.4$ µm on day 7. All enlarged cells stained blue-black with Lugol's iodine solution.

**PCR assays, DNA sequencing, and phylogenetic analyses.** The ITS region sequences from the culture Pmed-G3 were deposited in GenBank under Accession No. EU068096 and EU068097, and sequences from isolate Pmed-Ht2 under Accession No. EU068098 and EU068099. BLAST results and phylogenetic







Fig. 2–4. Transmission electron micrographs of *Perkinsus mediterraneus* cells cultured in JL-ODRP-2F medium. **2.** Trophozoite with a dense cytoplasm containing several lipid droplets (L), vacuoles (V), and mitochondria (Mt). Vacuoles contained dense bodies termed vacuoplasts (Vp). Trophozoite has thick cell wall (W) and a large nucleus (N). Scale bar = 1 µm. **3.** Dividing schizont with four daughter trophozoites tightly packed. Scale bar = 1 µm. **4.** More advanced stage in the division with larger trophozoites surrounded by a thicker cell wall (TW) inside of the schizont wall (SW). Scale bar = 10 µm. No, nucleolus; Ps, perinuclear space; Vs, vesicle.

analyses of the ITS region indicated that these cultures were *P. mediterraneus*. The highest scoring sequences in the BLAST search were previously deposited ITS region sequences of *P. mediterraneus* (GenBank Accession No. AY487840, DQ370490, and DQ370492). The ITS region sequences from the in vitro isolate cultures fell into a clade in both neighbor joining (tree not shown) and parsimony analyses (Fig. 5) with the *P. mediterraneus* sequences obtained previously from infected *O. edulis* tissue. There was 97% bootstrap support in the parsimony analysis for the clade with all the *P. mediterraneus* ITS region sequences, which included those obtained for this study from the in vitro isolate cultures (Fig. 5).

Nucleotide sequences of the LSU rRNA gene fragments amplified from *P. mediterraneus* Pmed-G3 culture DNA were deposited in GenBank under the Accession No. EF204095–204107. The *P. mediterraneus* LSU rRNA gene sequences and those from *P. honshuensis* grouped together in both distance and parsimony analyses. There was moderate bootstrap support (72%) in the parsimony tree for this clade. Sister to the *P. mediterraneus/P. honshuensis* clade were monophyletic species clades of *P. marinus* and *P. olseni* LSU rRNA gene sequences. In the LSU rRNA gene analysis, *Perkinsus chesapeaki* was the *Perkinsus* species most distantly related to *P. mediterraneus* (Fig. 6).

Nucleotide sequences of actin gene fragments amplified from *P. mediterraneus* Pmed-G3 culture DNA were deposited in GenBank under the Accession No. EF204112–204115. The four *P. mediterraneus* actin gene sequences obtained for this study were all of type 1 as designated in Burreson, Reece, and Dungan (2005), and formed a monophyletic and highly supported (100%) species clade within type 1 actin gene sequences. The *P. mediterraneus* type 1 actin gene nucleotide sequences were highly supported as sister to a highly supported (98%) monophyletic species clade containing type 1 actin gene sequences of *P. honshuensis* (Fig. 7). Although type 2 sequences were not found at this time, this does not preclude the possibility that type 2 actin sequences are in the *P. mediterraneus* genome, as they are in other *Perkinsus* spp. genomes, and would likely be found by more extensive screening and sequencing.

**Characterization of cultured cells proliferation.** No significant differences were detected between the isolates Pmed-Ht2 and Pmed-G2 relative to cell viability, density, diameter, or metabolic activity. Viability was always over 90% for both isolates during the growth study (Pmed-Ht2: 92.1% ± 3.9%; Pmed-G2: 90.9% ± 2.7%). The cell densities significantly increased from their initial densities at day 0 (Pmed-Ht2: $7.3 \pm 0.9 \times 10^4$ cells/ml; Pmed-G2: $6.2 \pm 1.9 \times 10^4$ cells/ml) to day 12 (Pmed-Ht2: $15.5 \pm 8.0 \times 10^4$ cells/ml; Pmed-G2: $16.1 \pm 9.1 \times 10^4$ cells/ml), but no further increase was found from day 12 to day 30 (Pmed-Ht2: $16.7 \pm 6.5 \times 10^4$ cells/ml; Pmed-G2: $16.6 \pm 6.5 \times 10^4$ cells/ml). Mean ± SD of trophozoite diameter of isolate Pmed-Ht2 was 19.1 ± 4.3 μm and for isolate Pmed-G2 was 19.2 ± 3.8 μm, and did not vary throughout the incubation period. Both isolates had high initial metabolic activities (day 2 >1,500 fluorescence units [fu]) (Fig. 8). Pmed-Ht2 isolate had a significant increase in metabolic activity by day 4 (4,610 ± 1,209 fu), and completely reduced the redox indicator by day 5. Isolate Pmed-G2 showed significant increase in cumulative metabolic activity on day 3 (2,846 ± 1,043 fu), and again on day 5 (4,270 ± 1,006 fu), and reduced the redox indicator by day 6 (Fig. 8).

Significant interactions between day and isolate were found when analyzing schizont size. Schizont diameters of both isolates decreased from day 0 (Pmed-Ht2: 32.3 ± 7.9 μm; Pmed-G2: 33.1 ± 6.3 μm) to day 12 (Pmed-Ht2: 27.3 ± 4.1 μm; Pmed-G2: 31.4 ± 6.4 μm). The percentage of cells in schizogony at the beginning of the study (day 0, Pmed-Ht2: 42.1% ± 5.1%; Pmed-G2: 54.8% ± 8.1%) as well as throughout the study was high. The
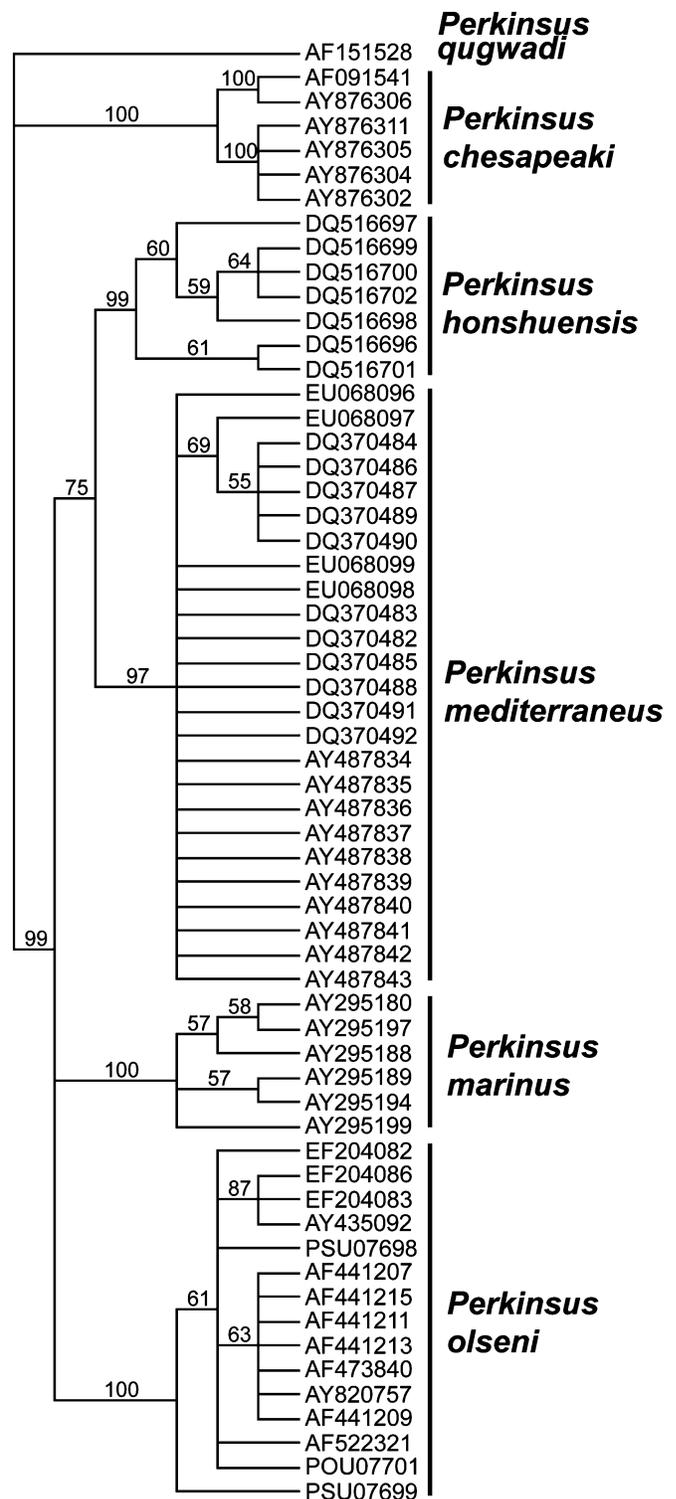


Fig. **5.** Parsimony bootstrap tree of *Perkinsus* spp. based on the ITS rRNA region. The tree was rooted with the *Perkinsus qugwadi* sequence. Bootstrap support values above 50% are indicated at nodes. Sequences from the individual species are labeled, and GenBank accession numbers are given as the taxon labels for each branch.

mean diameter of isolate Pmed-G2 schizonts was significantly larger than that of isolate Pmed-Ht2 schizonts at each sampling time (days 0, 6, and 12).
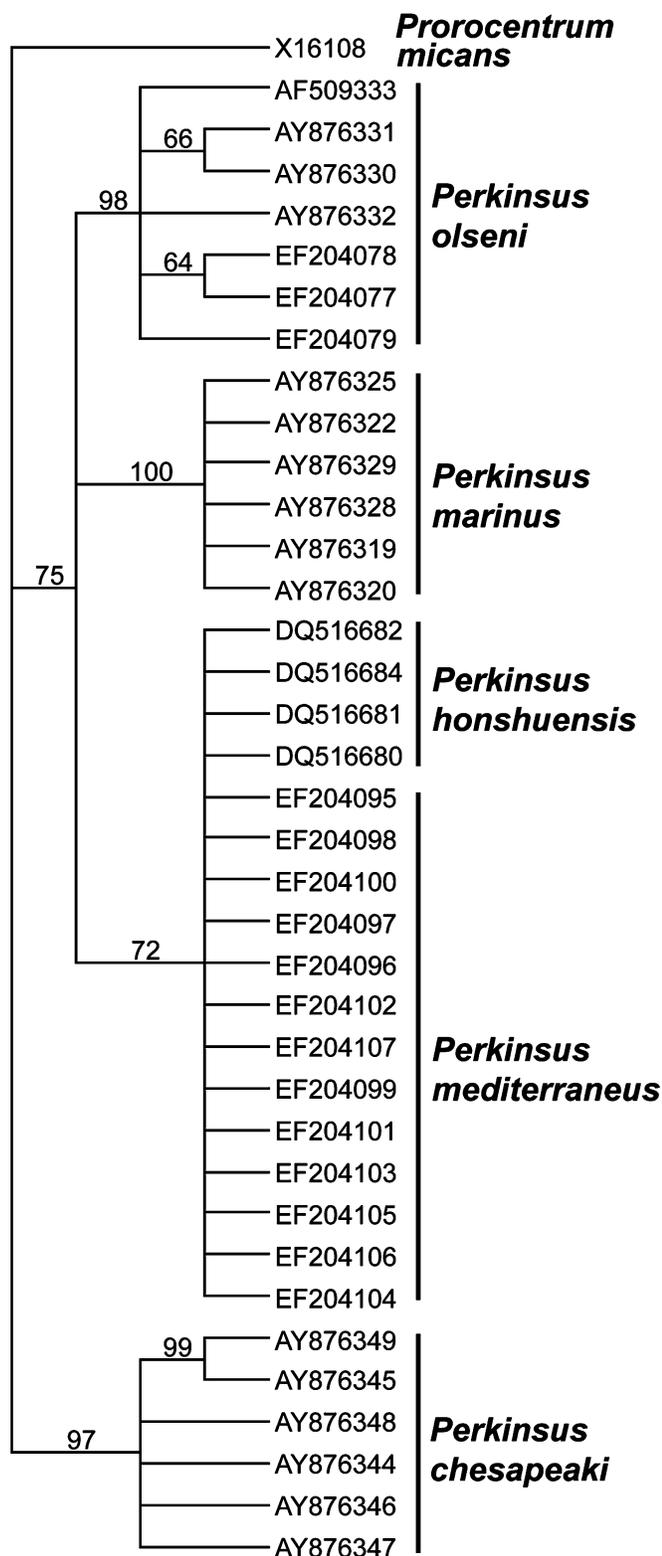
Fig. **6.** Parsimony bootstrap tree of *Perkinsus* spp. and *Prorocentrum micans* based on the large subunit rRNA gene sequences. The tree was rooted with *P. micans*. Bootstrap support values above 50% are indicated at nodes. Sequences from the individual species are labeled, and GenBank accession numbers are given as the taxon labels for each branch.



Fig. **7.** Parsimony bootstrap tree of *Perkinsus* spp. *Prorocentrum minimum* and *Amphidinium carterae* based on actin gene sequences. The tree was rooted with sequences from the dinoflagellates *P. minimum* and *A. carterae*. Bootstrap support values above 50% are indicated at nodes. Sequences from the individual species are labeled, and GenBank accession numbers are given as the taxon labels for each branch.
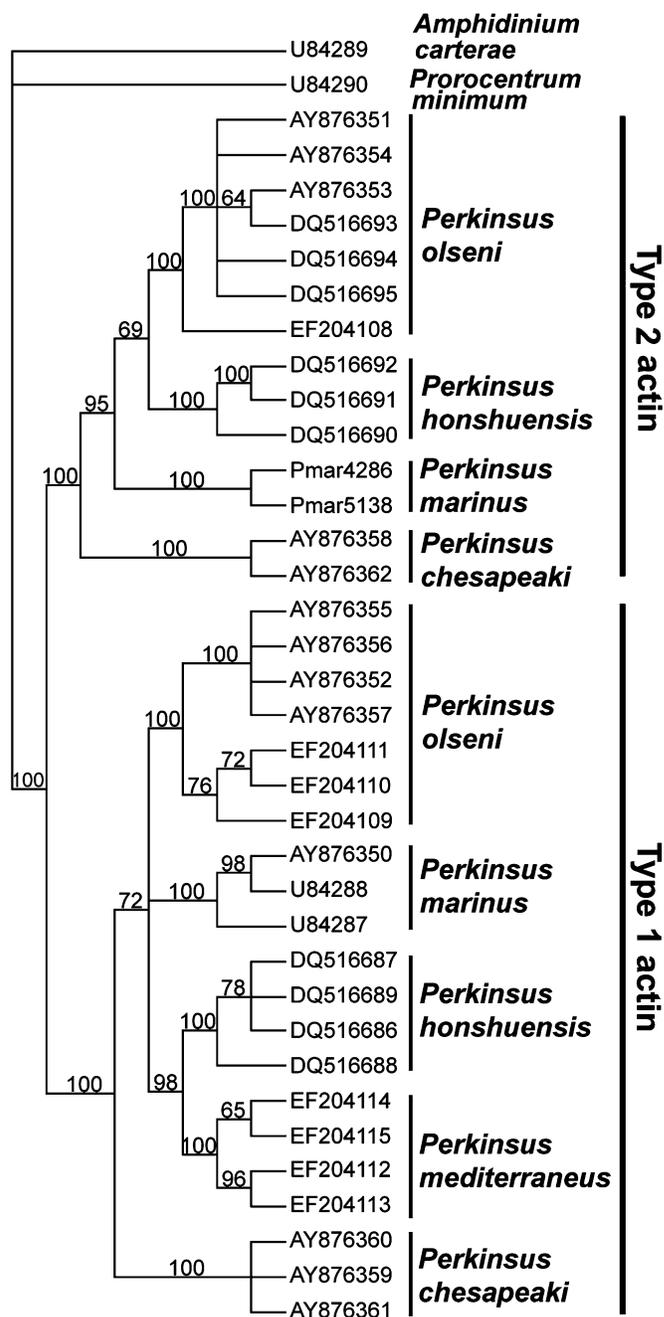
**Enzymatic activities of culture supernatants.** Significant enzyme activities were detected in the culture supernatants of the *P. mediterraneus* isolates. Activities were detected for 11 of 19 enzymes examined. In unconcentrated (1x) culture supernatants, moderate enzymatic activities ( ∼ 20 nmol of hydrolyzed substrate) were detected for acid phosphatase, β-glucosidase, and naphtol-AS-BI-phosphohydrolase; low enzymatic activities (5 to < 20 nmol of hydrolyzed substrate) were detected for esterase, esterase lipase, and α-mannosidase; and very low enzymatic
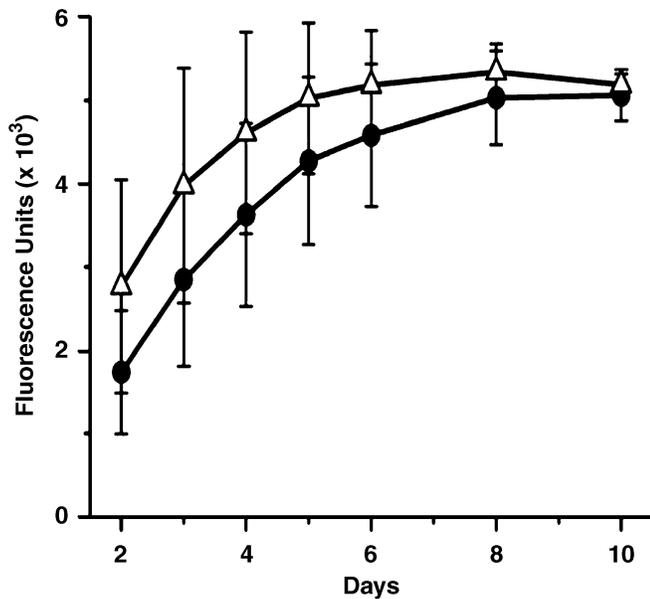
**Fig. 8.** Cumulative metabolic activity (mean ± SD) of *Perkinsus mediterraneus* isolates. Cumulative metabolic activities of isolate Pmed Ht2 (open triangles) and isolate Pmed-G2 (filled circles) were measured over 10 d using the AlamarBlue assay. Values are expressed in fluorescence units (fu).
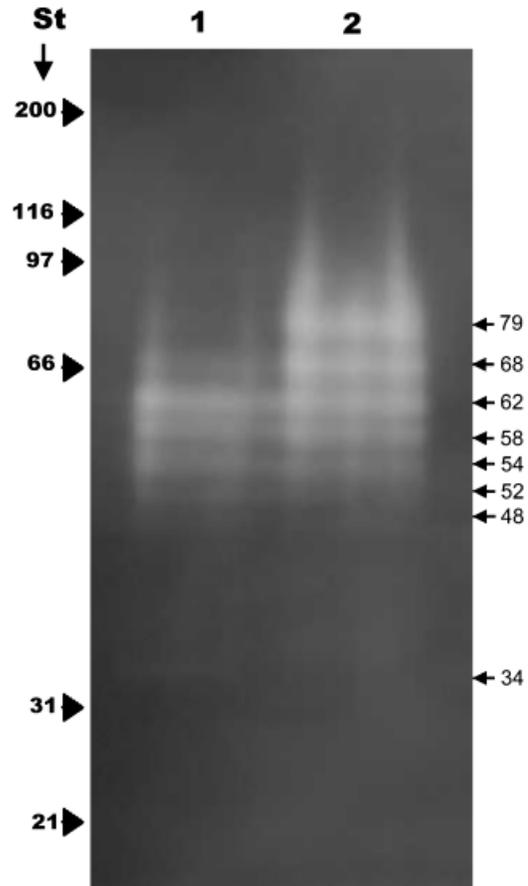


**Fig. 9.** Gelatin sodium dodecylsulfate-polyacrylamide gel electrophoresis of cell-free culture supernatants of *Perkinsus mediterraneus*. Supernatants from isolates Pmed-Ht2 (lane 1) and Pmed-G3 (lane 2) were 6-wk-old, and both isolates were seeded with $5 \times 10^5$ cells/ml. Following electrophoresis of supernatants, the gel was incubated for 4 h at 37 °C to allow proteolysis of the embedded gelatin substrate. Both lanes contain 1 μg of supernatant protein. Positions of the approximate molecular weights (kDa) of *P. mediterraneus* proteolytic bands are indicated on the right based on molecular mass standards (kDa) indicated on the left.

activities (<5 nmol of hydrolyzed substrate) were detected for alkaline phosphatase and lipase. Low activity (<5 nmol of hydrolyzed substrate) of three additional enzymes, α-chymotrypsin, α-glucosidase, and *N*-acetyl-β-glucosaminidase, was also detected in 5x-concentrated culture supernatants. No differences in enzymatic activities were detected between the isolates Pmed-Ht2 and Pmed-G3 (data not shown).

**Detection and characterization of proteases in culture supernatants.** Electrophoretic separation of the culture supernatant proteins by gelatin SDS-PAGE revealed multiple proteolytic bands with approximate molecular masses ranging from 34 to 79 kDa (Fig. 9). Eight proteolytic bands with approximate molecular weights of 34, 48, 52, 54, 58, 62, 68, and 79 kDa were visible after gelatin SDS-PAGE of isolate Pmed-Ht2 culture supernatants (Fig. 9). The major extracellular proteolytic band of isolate Pmed-Ht2 was approximately 62 kDa (36.8%), and 87.7% of the proteolytic activity was concentrated in bands within the 54–68 kDa range. Seven out of eight of the proteolytic bands of isolate Pmed-Ht2 were also observed in the culture supernatants of isolate Pmed-G3, except where a 37-kDa proteolytic band from isolate Pmed-G3 replaced a 34-kDa band from isolate Pmed-Ht2 (data not shown); the 37-kDa proteolytic band in Pmed-G3 appeared very faint in the gelatin gel and could not be captured by digital photography. Ninety percent of Pmed-G3 proteolytic activity was in bands ranging from 58 to 79 kDa, and the 79-kDa proteolytic band accounted for approximately 43.9% of the total proteolytic activity (Fig. 9).

Pre-incubation of the culture supernatant of *P. mediterraneus* with the serine protease inhibitor AEBSF resulted in a marked decrease in protease activity (28.1% ± 12.2% of control). No decrease in protease activity was measured when inhibitors for cysteine, aspartic, or metalloprotease classes were incubated with *P. mediterraneus* culture supernatant (E64: 111.9% ± 2.2%; pepstatin A: 120.5% ± 2.2%; *o*-phenantroline: 116.9% ± 18.1%) suggesting that *P. mediterraneus* proteases belong to the serine class.

## DISCUSSION

In this study, continuous cultures of *P. mediterraneus* were established for the first time. Morphological observations using light and transmission electron microscopy, enlargement in ARFTM, and staining of hypnospores with Lugol's iodine solution indicated that the cultured parasite belonged to the genus *Perkinsus*. Phylogenetic analysis of ITS region, LSU rRNA gene, and type 1 actin gene sequences of this parasite also confirmed that the cultured parasite was in the *Perkinsus* genus, and the ITS region sequences matched those determined previously for *P. mediterraneus* (Casas et al. 2004). Preliminary analysis of *P. mediterraneus* culture supernatants revealed the presence of several enzymes with esterase-, lipase-, phosphatase-, glycosidase-, and protease-like activities. *Perkinsus mediterraneus* proteases were further characterized as belonging to the serine class, which is the same class of proteases secreted by the eastern oyster parasite *P. marinus* (La Peyre et al. 1995).

*Perkinsus mediterraneus* cultures were successfully established from gill and heart tissues of infected European flat oysters, using the same procedures described previously for obtaining *P. marinus* and *P. olseni* cultures (Casas et al. 2002; La Peyre, Faisal, and

Burreson 1993). Cultured *P. mediterraneus* cells had the morphological characteristics typical of the trophozoites and schizonts of *Perkinsus* spp. (Burreson et al. 2005; Casas et al. 2002; Coss, Robledo, and Vasta 2001; Dungan and Reece 2006; Dungan et al. 2007b; La Peyre et al. 1993; Perkins 1996; Robledo et al. 2002; Sunila, Hamilton, and Dungan 2001). In vitro *P. mediterraneus* trophozoites (19.6 ± 4.3 μm), however, were larger than reported for *P. mediterraneus* trophozoites in infected *O. edulis* tissues (7.9 ± 0.3 μm, Casas et al. 2004), and larger than trophozoites of *P. marinus* (4.9 ± 0.9 μm), *P. olseni* (8.5 ± 1.9 μm), or *P. chesapeaki* (10.0 ± 2.4 μm) that were also cultured in JL-ODRP-2F medium (La Peyre et al. 2006), or *P. honshuensis* (10.2 ± 3.7 μm) cultured in DME/F12-3 medium supplemented with 3% (v/v) fetal bovine serum and various other nutrients (Dungan and Reece 2006). Similarly, in vitro *P. mediterraneus* schizonts were larger (32.7 ± 7.1 μm) than in vitro schizonts of *P. marinus* (15.1 ± 2.2 μm), *P. olseni* (15.3 ± 3.3 μm), *P. chesapeaki* (15.3 ± 2.3 μm), or *P. honshuensis* (20.3 ± 5.4 μm) (Dungan and Reece 2006; La Peyre et al. 2006). The large size of *P. mediterraneus* cells in vitro may indicate a stress response, similar to previous observations of enlargement of *Perkinsus* spp. trophozoites when cultured in medium with low salt concentrations (La Peyre et al. 2006). It may be a revealing anecdotal observation that when infected *O. edulis* tissues were placed in culture medium and *P. mediterraneus* started to proliferate at the beginning of our study, the trophozoites were smaller (< 10 μm) and their density increased more rapidly than after several subcultures, indicating that tissue-derived factors may have improved *P. mediterraneus* proliferation in JL-ODRP-2F culture medium.

The low growth rate of *P. mediterraneus* in vitro compared with other *Perkinsus* spp. cultured in the same medium (La Peyre et al. 2006) does suggest suboptimal culture conditions for *P. mediterraneus*; *P. mediterraneus* density typically increased by 2–6-fold following subculture in fresh medium or in mixtures of fresh and conditioned media while the densities of other *Perkinsus* spp. increased by 10-to-30-fold following subculture in fresh JL-ODRP-2F medium (La Peyre et al. 2006). Our culture medium JL-ODRP-2F (1,025 mOsm/kg, 35 ppt seawater salinity equivalence) was formulated with high salt concentrations because infected *O. edulis* were collected in waters off the Mediterranean Island of Menorca, where salinity is typically 38 ppt. It is possible that the high salt concentrations of JL-ODRP-2F are not optimal for *P. mediterraneus*, because proliferation rates of three other *Perkinsus* species were reduced in JL-ODRP-2F medium when compared with their growths to JL-ODRP-2A medium (La Peyre et al. 2006), a culture medium similar in composition, but with lower salt concentrations (775 mOsm/kg, 25 ppt seawater salinity equivalence). Clearly, culture conditions including salinity, temperature, nutrient concentrations, and seeding density require optimization in order to maximize the proliferation rate of *P. mediterraneus* in vitro, and to ensure trophozoite and schizont sizes in vitro are comparable to the sizes of those cell types observed in vivo.

*Perkinsus mediterraneus* released a number of hydrolytic enzymes into the culture medium. Most of these enzymes are also produced by *P. marinus*, *P. olseni*, and *P. chesapeaki* cultured under the same conditions, but their relative concentrations appear to be unique to each *Perkinsus* species (this study; Casas 2002; SC. and JL. unpubl. data). Typically, culture supernatants of *P. mediterraneus* have high naphtol-AS-BI-phosphohydrolase and acid phosphatase activities, while supernatants of *P. marinus* cultures have high α-chymotrypsin activity, while those of *P. chesapeaki* have high alkaline phosphatase activity and those of *P. olseni* have high esterase, esterase lipase, and α-mannosidase activities, relative to the activities of other enzymes. One exception is α-glucosidase activity, which is high in all *Perkinsus* species

(this study; Casas 2002; SC. and JL. unpubl. data). The API ZYM enzymatic profiles of *Perkinsus* species may therefore be useful for a rapid preliminary identification of *Perkinsus* species cultured under the conditions reported. However, DNA sequence analysis should always be used to confirm identification of *Perkinsus* spp., until more is known about the variations in enzymatic profiles of these parasites under different culture conditions, and between isolates of the same species. The significance of the differences in enzymatic profiles between *Perkinsus* species in the progression of their diseases in their respective hosts remains to be determined. The ECP of *P. mediterraneus* and other *Perkinsus* species must first be characterized at the biochemical and molecular levels before their roles in parasite metabolism or as virulence factors can be evaluated.

Analysis of the culture supernatants of *P. mediterraneus* by gelatin SDS-PAGE revealed the presence of proteolytic bands, and showed mostly quantitative differences between protease profiles of the two isolates. Differences in proteolytic band profiles of culture supernatants between isolates of *P. marinus* have also been reported (La Peyre and Faisal 1997), the only other *Perkinsus* species that has been shown to secrete proteases (La Peyre et al. 1995). *Perkinsus marinus* appears to have higher protease activity than *P. mediterraneus* in vitro. The protease activities of 6-wk-old culture supernatants of *P. mediterraneus* isolate Pmed-Ht2 seeded $1 \times 10^6$ cells/ml (0.26 ± 0.01 U/ml) and Pmed-G3 seeded at $5 \times 10^5$ cells/ml (0.26 ± 0.01 U/ml) were lower than that of *P. marinus* isolate P-1 seeded at $2 \times 10^5$ cells/ml (1.29 ± 0.07 U/ml; SC. and JL., unpubl. data). This observation will need to be verified once *P. mediterraneus* culture conditions are optimized. At this time, only one protease of *P. marinus* has been isolated and characterized, a 41.7 kDa monomeric, *N*-glycosylated serine protease, designated as perkinsin, the major extracellular protease produced by this species in vitro (Faisal et al. 1999; La Peyre et al. 1995). Proteases generating the other proteolytic bands of *P. marinus* have yet to be chemically characterized and identified; it is still unknown if these bands represent distinct proteases or are products of perkinsin autolysis or aggregation (Faisal et al. 1999). Similar work will need to be undertaken to further characterize the proteases of *P. mediterraneus*. Extensive lysis of *O. edulis* tissues infected by *P. mediterraneus* has not been observed, in contrast to observations of *C. virginica* tissues heavily infected with *P. marinus*, but that may simply be because no heavily infected *O. edulis* have been examined to date (Casas et al. 2004). Additional histopathological studies using *O. edulis* with heavy infections of *P. mediterraneus* need to be undertaken, to better characterize the damage caused by this parasite in its host.

A close phylogenetic relationship between *P. mediterraneus* and *P. honshuensis* was suggested by results of previous phylogenetic analyses of their ITS region sequences (Dungan and Reece 2006). At that time, however, sequences from additional loci were unavailable to substantiate a potential for such a relationship. Large subunit rRNA and type 1 actin gene sequences for *P. mediterraneus* were included here in a more comprehensive phylogenetic analysis of *P. mediterraneus*. Additional information obtained from the LSU rRNA gene and type 1 actin gene sequences of *P. mediterraneus* supports the hypothesized close sister relationship between these two *Perkinsus* species. It is possible that *P. honshuensis* described in *Venerupis* ( = *Tapes*) *philippinarum* from Japan (Dungan and Reece 2006) and *P. mediterraneus* described in *O. edulis* from the Mediterranean Sea (Casas et al. 2004) at one point existed as sympatric species, with divergence as indicated by the monophyletic species clades in both the ITS region and actin gene analyses, occurring through time with geographic separation.

Although *Perkinsus*-like organisms have been reported in bivalve mollusks along the Mediterranean coasts of Spain

(Sagristà, Durfort, and Azevedo 1996), France (Goggin 1992), and Italy (Canestri-Trotti et al. 2000; Da Ros and Canzonier 1985; Rubini et al. 1996) since the 1980s, it is only recently that molecular diagnostic techniques have been applied to identify these organisms at the species level in the Mediterranean Sea (Abollo et al. 2006; Casas et al. 2004). The areas and host species sampled remain limited: *P. mediterraneus* infections have been confirmed among *O. eduli*s oysters from the Balearic Island of Menorca (Spain) (Casas et al. 2004), and *P. olseni* infections confirmed among *Tapes philippinarum* clams from the Marano Lagoon (North Adriatic Sea, Italy) (Abollo et al. 2006). The geographic range of both *Perkinsus* species in the Mediterranean Sea needs to be determined, in addition to examining the possibility that other *Perkinsus* spp. may be found in the region. This is timely considering that shellfish aquaculture production is rapidly expanding in the Mediterranean Sea and newly introduced species of bivalve mollusks, such as *T. philippinarum* that are cultured there, may introduce exotic *Perkinsus* spp. or other molluskan pathogens to both wild and cultured shellfish. Further expansion of shellfish aquaculture in the Mediterranean Sea should be accompanied by examination of the distribution and pathogenicity of *Perkinsus* species to limit their impact and spread. In this regard, the ability to propagate *P. olseni* and now *P. mediterraneus* will greatly facilitate studies on the pathogenicity of these parasites to native molluscan species.

## ACKNOWLEDGMENTS

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