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Description of *Perkinsus beihaiensis* n. sp., a new *Perkinsus* sp. Parasite in Oysters of Southern China

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ABSTRACT. Oysters were collected from coastal locations in China from 1999–2006 for parasite analyses by molecular, culture, and histological techniques. Polymerase chain reaction-based assays targeting the internal transcribed spacer (ITS) region of the ribosomal RNA gene complex were performed to detect the presence of *Perkinsus* species. Sequencing and phylogenetic analysis of amplified *Perkinsus* sp. DNAs indicated that a novel *Perkinsus* sp. infects *Crassostrea hongkongensis*, *Crassostrea ariakensis*, and other bivalve hosts from Fujian to Guangxi provinces in southern China. Prevalence of this *Perkinsus* sp. reaches as high as 60% in affected oyster populations. Analyses of nucleotide sequences of the rRNA ITS region and of large subunit rRNA and actin genes, consistently confirmed the genus affiliation of this *Perkinsus* sp., but distinguished it from currently accepted *Perkinsus* species. Parasite cell types, such as signet ring trophozoites of 2–8 μm diameter, were observed by histology, and application of both genus *Perkinsus* and *Perkinsus* species-specific in situ hybridization probes consistently labelled the same *Perkinsus* sp. cells in histological sections from infected oyster tissues. Combined phylogenetic and histological results support the identity of a new parasite species, *Perkinsus beihaiensis* n. sp.

Key Words. Actin, *Crassostrea ariakensis*, *Crassostrea hongkongensis*, ISH, parasite, PCR, ribosomal RNA.

SINCE the initial description of *Perkinsus marinus* as a significant pathogen of *Crassostrea virginica* oysters (Mackin, Owen, and Collier 1950), and especially with the recent advent of highly sensitive and specific diagnostic techniques, numerous new *Perkinsus* spp. parasites have been described worldwide, and the geographic and host ranges of many known species also have been expanded. *Perkinsus* spp. infections may be the most thoroughly studied of the molluscan disease agents, due to their notoriety for causing significant mortalities among commercially important bivalve species. *Perkinsus marinus* continues to have devastating effects on oyster populations from the Atlantic and Gulf of Mexico, USA (Burreson and Ragone Calvo 1996; Soniat 1996). Significant mortality events and depressed production also have been associated with infections of *Perkinsus olseni* among marine molluscs on the Atlantic and Mediterranean coasts of Europe (Azevedo 1989; Da Ros and Cazonier 1985; Figueras, Robledo, and Novoa 1992; Montes, Durfort, and García-Valero 2001; Santmartí et al. 1995; Villalba et al. 2005), in Australia (Goggin and Lester 1995; Lester and Davis 1981), and along the coasts of Korea (Choi and Park 1997; Park and Choi 2001) and Japan (Hamaguchi et al. 1998).

During the past decade, major research efforts have focused on the potential risks and benefits of introducing the Asian oyster, *Crassostrea ariakensis,* to the Chesapeake Bay, USA, in order to restore the severely depleted native oyster population. Field trails conducted in Virginia have documented lower mortality and faster growth in *C. ariakensis,* relative to that of the native oyster, *C. virginica* (Calvo et al. 2001). Disease resistance studies conducted as part of that investigation also suggest that *C. ariakensis* is relatively more resistant to *Haplosporidium nelsoni* and *P. marinus* infections, the two pathogens that have contributed significantly to reduction of *C. virginica* populations in Chesapeake Bay (Sindermann 1990).

In China, *C. ariakensis* is found naturally from the Bohai Sea in northern China to Beihai, Guangxi province, near China's southern border with Vietnam. A newly described oyster species, *Crassostrea hongkongensis,* occurs sympatrically with *C. ariakensis* in southern China, along the coasts of Fujian, Guangdong, and Guangxi provinces (Wang et al. 2004a). Morphological differences reported to distinguish these two oyster species are neither clear nor consistent (Lam and Morton 2003), although consistent minor anatomical differences are reported (Wang et al. 2004a). Reliable differentiation between *C. ariakensis* and *C. hongkongensis* is currently possible only by genetic techniques (Cordes and Reece 2005).

With a proposed introduction of *C. ariakensis* to the Chesapeake Bay, it is both prudent and compliant with the International Council for Exploration of the Seas (ICES) protocols (ICES, 2005) to identify the natural pathogens of this oyster species in Asia. In addition, because *C. hongkongensis* is sympatric with *C. ariakensis* in southern China waters and easily confused with *C. ariakensis* that might be imported to the USA for use as broodstock (e.g. see Zhang, Allen, and Reece 2005), it is also important to identify the pathogens and parasites of *C. hongkongensis*. Using polymerase chain reaction (PCR)-based diagnostics, we detected *P. olseni* in *C. ariakensis* in Japan, as well as in *C. ariakensis* and *C. hongkongensis* in northern China. We also detected an undescribed *Perkinsus* sp. apparently infecting several oyster host species in parts of southern China (Moss et al. 2007).

Here we provide a detailed description of *Perkinsus beihaiensis* n. sp., based on phylogenetic analyses of nucleotide sequences of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene complex, of the large subunit (LSU) rRNA gene, and of the actin gene. Histologically, this parasite shows the characteristic morphology of other described *Perkinsus* spp. and cells of this parasite enlarge after incubation for 5 days in Ray's fluid thioglycollate medium (RFTM) (Ray 1952). A PCR-based diagnostic assay has been developed and makes possible a report of the prevalence and distribution of *P. beihaiensis* n. sp. in oyster samples from southern China that were collected during 1999–2006.

MATERIALS AND METHODS

Oyster tissue samples. Wild and cultivated oysters were collected from coastal locations in China from 1999 to 2006 (Moss et al. 2007, Table 1, Fig. 1). In some cases, oysters were collected from the same locations over multiple years. Hemolymph, adductor muscle, mantle, gill tissue, or visceral mass sections were preserved in either dimethyl sulfoxide (DMSO) storage buffer (25 mM EDTA, 20% (v/v) DMSO, and saturated NaCl) or in 95% (v/v) ethanol for DNA extraction and PCR analysis. Visceral mass sections of some sample oysters were also preserved for histological examination in Davidson's solution (Shaw and Battle 1957). In addition to oyster collections reported in Moss et al. (2007), oysters were collected in April 2005 and in April and

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Table **1.** Locations sampled during this study.

Year	Sampling location	N	# PCR positive	% PCR positive	Bivalve sp. infected
2002	Tong'an, Fujian (TA)	26	1	3.8	*Crassostrea ariakensis*
2002	Shenzhen, Guangdong (SZ)	13	1	7.7	*Crassostrea hongkongensis*
2002	Chengcun, Yangxi, Guangdong (CH)	37	6	16.2	*C. hongkongensis*
2002	Guandu, Zhanjiang, Guangdong (GD) C*	35	4	11.4	*C. hongkongensis*
2002	Guandu, Zhanjiang, Guangdong (GD)	25	3	12.0	*C. hongkongensis*
2003	Zhanjiang, Guangdong (ZJ)	1	1	100.0	*C. ariakensis*
		70	32	45.7	*C. hongkongensis*
2003	Lingshui, Hainan (HN)	19	12	63.2	*Pinctada margaritifera*, *P. martensii*, unknown sp.- not distinguished
2003	Beihai, Guangxi Zhuang (BC)	59	11	18.6	*C. hongkongensis*
2005	Beihai, Guangxi Zhuang (BC05)	12	4	33.3	*C. ariakensis*
		110	36	32.7	*C. hongkongensis*
1999	Dafen River, Beihai, Guangxi Zhuang (DR)	26	9	34.6	*C. ariakensis*
		24	2	8.3	*C. hongkongensis*
2006	Dafen River, Beihai, Guangxi Zhuang (DR06)	13	2	15.4	*C. ariakensis*
		29	12	41.4	*C. hongkongensis*
2002	Podi, Beihai, Guangxi Zhuang (PO)	14	2	14.3	*C. ariakensis*
2006	Qinzhou, Guangxi Zhuang (QZ)	15	1	6.7	*C. ariakensis*
		44	3	6.8	*C. hongkongensis*

PCR results using the *Perkinsus beihaiensis*-specific assay or amplification of the ITS rRNA followed by DNA sequencing and phylogenetic analysis are noted. Bivalve species infected with *Perkinsus beihaiensis* n. sp. are indicated. C* refers to cultivated oysters.

PCR, polymerase chain reaction; ITS, internal transcribed spacer.

November 2006 for use in parasite assays and in vitro propagation efforts of *Perkinsus* sp. (Table 1). For detection of potential exotic infectious agents, oysters collected from waters in the Beihai region of southern China during April 2006 were co-habitated for up to 3 mo with native Chesapeake Bay bivalve molluscs, in quarantined disease transmission experiments. Upon termination of transmission experiments, tissue samples from Chinese oysters were also processed for parasite diagnostic assays and in vitro isolate propagation of *Perkinsus* sp. (Table 1).

Alternative Ray's fluid thioglycollate medium (ARFTM) and RFTM assays. Homogenized tissue preparations for assays using ARFTM (La Peyre et al. 2003) were inoculated into 24-well culture plates, 4–24 wells per sample. Tissues were incubated at 27 °C for 48 h to induce enlargement of *Perkinsus* sp. hypnospore cells, and unstained oyster tissue biopsies in culture plate wells were analyzed microscopically with Hoffman modulation contrast (HMC) optics, for enumeration of enlarged, refractile *Perkinsus* sp. hypnospores. Relative hypnospore densities in oyster tissues were rated as absent, light, moderate, or heavy according to the categories of Ray (1952, 1954); infection prevalences were calculated as the percentage of infected oysters detected in samples. In some cases, gill, mantle, and rectal tissues were processed for assays using RFTM (Ray 1952). Following 27 °C incubation for 5–7 days in RFTM, tissues were removed from the culture tubes, macerated on microscope slides, and stained with Lugol's iodine. Stained tissue preparations were examined by brightfield microscopy, and infection intensities of *Perkinsus* sp. were categorized on a scale from absent (0) to very heavy (5), based on the categories of Ray (1952, 1954).

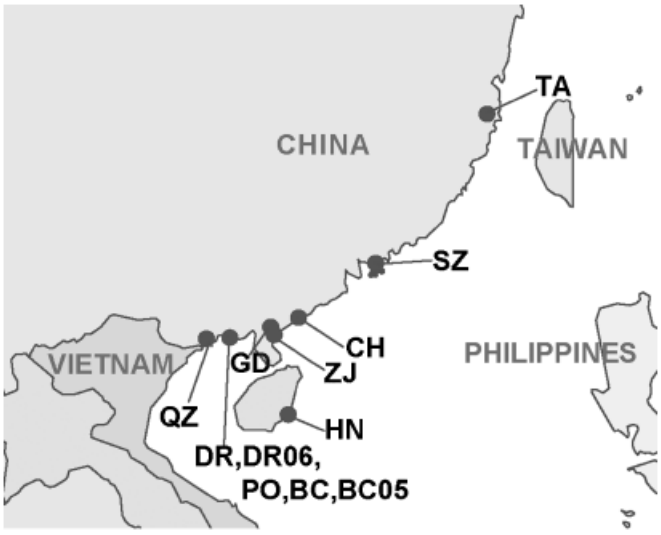

Fig. **1.** Map of sampling locations in southern People's Republic of China. *TA,* Tong'an, Fujian; *SZ*, Shenzhen; *CH*, Chengcun, Yangxi, Guangdong; *GD*, Guandu, Zhanjiang, Guangdong; *ZJ,* Zhanjiang, Guangxi Zhuang; *HN,* Lingshui, Hainan; *BC, BC05,* Beihai, Guangxi Zhuang; *PO,* Podi, Beihai, Guangxi Zhuang; *DR*, *DR06,* Dafen River, Beihai, Guangxi Zhuang; *QZ*, Qinzhou, Guangxi Zhuang.

In vitro propagation of *Perkinsus* spp. Selected ARFTM-or RFTM-incubated tissues with heaviest hypnospore densities were aseptically transferred to culture plate wells, 4–24 wells per plate, containing 2 ml of antimicrobial-supplemented Dulbecco's modified Eagle's/Ham's F12 medium with 3% (v/v) fetal bovine serum (DME/Ham's F12-3) (Burreson, Reece, and Dungan 2005). Infected tissues were disrupted and suspended in culture medium by gentle trituration with a sterile pipette. Resulting suspensions were serially diluted at 0.5 ml/well into three additional wells containing 2 ml of culture medium. Inoculated culture plates were covered, incubated at 27 °C in a humidified air atmosphere, and observed for up to 6 mo for proliferation of *Perkinsus* sp. isolates.

Nucleic acid extraction. Genomic DNA was extracted from excised oyster mantle and gill snips, or from hemocytes, using a Dneasy® Tissue Kit (Qiagen Inc., Valencia, CA), following the manufacturer's protocols, except that DNA was eluted from the column in a single 200-µl volume of elution buffer following a 10-min incubation at room temperature (approximately 20 °C).

Polymerase chain reaction-restriction fragment-length polymorphism identification of oyster host species. Species identifications of sampled oysters were carried out using a molecular diagnostic key based on PCR amplification and restriction enzyme digestion of the first ITS-1 region of the rRNA gene

complex (Cordes and Reece 2005). Polymerase chain reaction amplifications were performed with the ITS-1 primers of Hedgecock et al. (1999). The PCR reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of each primer, *Taq* polymerase at 0.25 U/μl, and template DNA at 0.2 ng/μl. Amplifications were performed with an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 52 °C for 2 min, and 72 °C for 2 min, with a final elongation at 72 °C for 5 min. Amplification products were digested with the diagnostic restriction enzymes *Hae* III or *Dde* II following manufacturer's protocols (New England Biolabs Inc., Beverly, MA). Following amplification and digestion, 4 μl of the initial PCR product and 10 μl of the digested PCR product were electrophoresed on a 3% (1.5% agarose and 1.5% low-melt agarose) agarose gel, stained with ethidium bromide, and visualized under UV light. Banding patterns were compared with those of reference oyster species for species identifications.

***Perkinsus* genus-specific PCR assay.** Screening for *Perkinsus* sp. DNA was performed using *Perkinsus* genus-specific ITS ribosomal RNA complex primers that were slightly modified from those of Casas, Villalba, and Reece (2002): forward primer PerkITS-85 (5′ CCGCTTTGTTTGGATCCC 3′) and reverse primer PerkITS-750 (5′ ACATCAGGCCTTCTAATGATG 3′). Each PCR reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, each primer at 0.1 μM, 0.025 U/μl *Taq* polymerase, 0.2 mg/ml BSA, and 0.5-μl genomic DNA (10–50 ng total). Amplifications were performed with initial denaturation at 95 °C for 4 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, 65 °C for 3 min, with a final elongation of 65 °C for 5 min. Following amplification, 4 μl of PCR product were visualized on a 2% agarose gel.

Large subunit rRNA gene amplification. A forward primer, PerkITS2 217 (5′ GTGTTCCTYGATCACGCGATT 3′) was used with a previously described reverse primer, LSU B (5′ ACGAAC GATTTGCACGTCAG 3′) (Lenaers et al. 1989) to amplify a fragment of approximately 1,170 bp from the rRNA gene complex. The targeted fragment consisted of the 3′-end of the second ITS rRNA region (ITS-2) and ended in the 5′-end of the LSU rRNA gene. Each PCR reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, each primer at 0.25 μM, 0.0625 U /μl *Taq* polymerase, 0.2 mg/ml BSA and 1.0 μl genomic DNA (10–50 ng total). Amplifications were performed with initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 1 min, 65 °C for 2 min, with a final elongation of 65 °C for 5 min. Following amplification, 8 μl of PCR product were electrophoresed on a 2% agarose gel and amplification products of the correct size were excised and gel purified using a QIAquick gel extraction kit (Qiagen Inc.).

Actin gene amplification. The forward primer, PerkActin1 130F (5′ ATGTATGTCCAGATYCAGGC 3′) and reverse primer PerkActin1 439R (5′ CTCGTACGTTTTCTCCTTCTC 3′) were used to amplify an approximately 330-bp fragment of type 1 actin gene from *Perkinsus* sp.-infected oyster tissue genomic DNA, and from genomic DNA isolated from a *Perkinsus* sp. culture sample containing abundant zoosporangia and hypnospores, oyster tissues, and contaminant microorganisms. The latter sample was obtained from an attempt to establish in vitro clonal cultures of this parasite. Each PCR reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3.0 mM $MgCl_2$, 0.1 mM of each dNTP, each primer at 0.1 μM, 0.0125 U/μl *Taq* polymerase, 0.2 mg/ml BSA, and 0.5 μl genomic DNA (10–50 ng total). Amplifications were performed with initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 58.5 °C for 45 s, 68 °C for 1 min, with a final elongation of 68 °C for 5 min. Following amplification, 10 μl of PCR product were electrophoresed on a 2% gel and amplification products of the correct size were excised and gel purified using a QIAquick gel extraction kit (Qiagen Inc.).

Cloning and sequencing. Cloning and sequencing reactions were performed as previously described (Dungan and Reece 2006; Moss, Burreson, and Reece 2006). Briefly, the ITS region, LSU rRNA gene, and actin gene PCR products were cloned into the plasmid pCR®4-TOPO® and transformed into *Escherichia coli* using a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. Transformed bacterial colonies were screened for inserts of the correct size using a boil-prep method followed by PCR amplification with the M13 vector primers. Before sequencing, PCR products from clones containing the correct insert size were treated with shrimp alkaline phosphatase (SAP) and exonuclease I (*Exo* I) (Amersham Biosciences, Piscataway, NJ). Cleaned PCR products from plasmid inserts were sequenced bi-directionally using the Big Dye Terminator Kit (Applied Biosystems, Norwalk, CT) with M13 sequencing primers, and using 5-μl reactions with 0.125 × (for ITS region and actin gene fragments) or 0.250 × (for LSU rRNA gene fragments) the concentration of Big Dye reagent specified in the manufacturer's protocols. Sequencing reaction products were precipitated using the ethanol/sodium acetate precipitation method (ABI User Bulletin, April 11, 2002). Precipitated sequencing reaction products were re-suspended in 20 μl of Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and 10 μl of each were electrophoretically separated on an ABI 3100 Prism Genetic Analyzer.

***Perkinsus beihaiensis* n. sp. diagnostic assay development.** Internal transcribed spacer region sequences of the novel *Perkinsus* sp., *P. beihaiensis* n. sp., and those of *Perkinsus* spp. previously deposited in GenBank were aligned, and regions unique to *P. beihaiensis* n. sp. were targeted for development of PCR primers. PCR reaction conditions using the *Perkinsus* genus-specific forward primer, PerkITS-85, with a unique *P. beihaiensis* n. sp. reverse primer, PerkITS-430R (5′ TCTGAGGGGCTACAATCAT 3′) were optimized and tested for specificity against known *Perkinsus* spp., closely related dinoflagellates, other oyster pathogens, and potential host organisms. Specificity of the assay was tested against *P. olseni*, *Perkinsus honshuensis*, *Perkinsus mediterraneus*, *P. marinus*, *Perkinsus chesapeaki*, *C. hongkongensis*, *C. ariakensis*, *C. virginica*, *Amphidinium carterae*, *Karlodinium micrum*, *Peridinium foliaceum*, *Cryptecodinium cohnii*, *Prorocentrum micans*, *Pseudopfiesteria shumwayae*, *Pfiesteria piscicida*, *Hematodinum* sp. from *Callinectes sapidus*, *Hematodinium* sp. from *Leocarcinus depurator*, *H. nelsoni*, and *Bonamia* sp. from *C. ariakensis*. The 460-bp targeted fragment consisted of a 3′-portion of the ITS1 region, the complete 5.8S rRNA gene, and a 5′-portion of ITS2 region of the rRNA gene complex. Each PCR reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, each primer at 0.1 μM, 0.0125 U/μl *Taq* polymerase, 0.2 mg/ml BSA, and 0.5 μl genomic DNA (10–50 ng total). Amplifications were performed with initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 57 °C for 45 s, 68 °C for 90 s, with a final elongation of 68 °C for 5 min. Following amplification, 6 μl of PCR product were electrophoresed on a 2% agarose gel and visualized as described.

Phylogenetic analysis. *Perkinsus* sp. sequences obtained from the southern China oysters were compared with those deposited in GenBank using basic local alignment search tool (BLAST) searches (Altschul et al. 1990) of the National Center for Biotechnology Information (NCBI) database, and those compiled previously by researchers at VIMS. Available ITS region, LSU rRNA gene, and actin gene sequences of known *Perkinsus* spp. were downloaded from GenBank for inclusion in phylogenetic analyses of the sequences obtained in this study. Multiple alignments of DNA sequences were performed using the ClustalW

algorithm in MacVector 8.1.2, and genetic distance (uncorrected–'p') and parsimony analyses were performed using PAUP* 4.0b10 (Swofford 2002).

GenBank sequences included in the ITS region analyses were the following: *Perkinsus qugwadi* AF15128 (outgroup taxon); *P. marinus* AY295180, AY295188, AY295189, AY295194, AY295197, AY295199; *P. chesapeaki* (= *P. andrewsi*) AF091541, AY876302, AY876304, AY876305, AY876306, AY876311; *P. olseni* (= *P. atlanticus*) AF441207, AF441209, AF441211, AF441213, AF441215, AY435092, AF473840, AY820757, AF522321, POU07701, PSU07698, PSU07699, EF204082, EF204083, EF204086; *P. mediterraneus* AY487834, AY487835, AY487837, AY487839, AY487841, AY487842; *P. honshuensis* DQ516696, DQ516697, DQ516698, DQ516699; *Perkinsus* sp. EF204015–EF204068, EF526428–EF526436, EU068080–EU068095.

GenBank sequences included in analyses of the LSU rRNA gene were the following: *P. micans* X16180 (outgroup taxon); *P. marinus* AY876319, AY876320, AY876322, AY876325, AY876328, AY876329; *P. chesapeaki* (= *P. andrewsi*) AY876344–AY876349; *P. olseni* (= *P. atlanticus*) AF509333, AY876330, AY876331, AY876332, EF204077–EF204079; *P. mediterraneus* EF204095–EF204098, EF204100; *P. honshuensis* DQ516680–DQ516682, DQ516684; *Perkinsus* sp. EF526433, EF526437–EF526441, EF526443–EF526452.

GenBank sequences included in the analyses of the actin genes were the following: *A. carterae* U84289, *Prorocentrum minimum* U84290 (outgroup taxa); Type 1 *P. marinus* AY876350, U84287, U84288; Type 1 *P. chesapeaki* (= *P. andrewsi*), AY876359–AY876361; Type 1 *P. olseni* (= *P. atlanticus*), AY876352, AY876355–AY876357, EF204109–EF204111; Type 1 *P. mediterraneus* EF204112–EF204115; Type 1 *P. honshuensis*, DQ516686–DQ516689; Type 1 *Perkinsus* sp. EF526411, EF526412, EF526414, EFF526415, EF516418, EF526420, EF526425, EF526427; Type 2 *P. marinus* TIGR4286, TIGR5138; Type 2 *P. chesapeaki* (= *P. andrewsi*) AY876358, AY876362; Type 2 *P. olseni* (= *P. atlanticus*), AY876351, AY876353, AY876354, DQ516693–DQ516695, EF204108; Type 2 *P. honshuensis*, DQ516690–DQ516692.

In situ DNA probe hybridization (ISH) assays. A genus-specific 5′-end digoxigenin-labelled *Perkinsus* spp. probe (Elston et al. 2004) was used to specifically target small subunit rRNA sequences (SSU), and a species-specific probe, PerkBehLSU (5′ GTGAGTAGGCAGCAGAAGTC 3′) was designed and used in separate hybridization reactions to target the LSU rRNA of *P. beihaiensis* n. sp. Digoxigenin-labelled oligonucleotide probes were obtained from Operon Biotechnologies Inc. (Huntsville, AL). The protocol followed for ISH was that previously published (Stokes and Burreson 1995) with the modifications of Elston et al. (2004). Pronase at a final concentration of 1.25 mg/ml was used for permeabilization during a 30-min incubation. A probe concentration of 7 ng/μl was used for hybridization with both the genus- and species-specific probes. The *P. beihaiensis* n. sp.-specific probe was tested for specificity against *Perkinsus* sp.-infected reference tissues, including *P. marinus* in *Crassostrea virginica*, *P. chesapeaki* in *Mya arenaria*, *P. mediterraneus* in *Chamelea gallina*, *P. olseni* in *Venerupis* (*Tapes*) *philippinarum*, and *P. honshuensis* in *V. philippinarum*. Negative controls included duplicate histological sections of all tested samples, which received hybridization buffer without probe during hybridization incubations.

RESULTS

Alternative Ray's fluid thioglycollate medium and RFTM assays. Thirteen of 78 (17%) live oysters collected during May 2005 from Beihai, China and analyzed by ARFTM assays, showed light *Perkinsus* sp. infections. Among DNAs from 24 of the same oysters that were tested using the genus *Perkinsus* ITS region PCR assay, nine (38%) gave positive results, and approximately half (5/9) of the PCR-positive DNAs came from oysters that also tested positive by ARFTM assays. Thus, 17–38% of *C. ariakensis* and *C. hongkongensis* from Beihai, China tested positive for the presence of *Perkinsus* sp. by one or both diagnostic assays.

In vitro propagation of *Perkinsus* sp. Low numbers of enlarged hypnospores harvested from ARFTM-incubated tissues of nine infected oysters that were processed soon after their arrival from China in 2005 were transferred into the DME/F12-3 *Perkinsus* spp. propagation medium (Burreson et al. 2005), but failed to proliferate before their overgrowth by thraustochytrid contaminants (Lyons et al. 2006). Supplementing the DME/F12-3 medium to 500 μg/ml with the fungicide metalaxyl (CAS 57837-19-1) temporarily inhibited the growth of thraustochytrid contaminants, had no inhibitory effect on in vitro proliferation of *P. olseni* isolate ATCC PRA-180; but did not promote proliferation of primary isolate cultures of *Perkinsus* sp. from the Beihai, China, oysters.

Among 76 oysters from China's Beihai region that were processed soon after their 2006 arrival, RFTM- or ARFTM-enlarged hypnospores from tissues of 17 infected oysters were sub-cultured in the DME/F12-3 *Perkinsus* spp. propagation medium. Thraustochytrid contaminants overgrew several primary cultures, and proliferation among inoculum hypnospores of *Perkinsus* sp. occurred in none.

Among six *C. hongkongensis* oysters that were collected during 2006 from Beihai, China waters and used for 95 days as Asian pathogen vectors in cohabitation experiments with Chesapeake Bay molluscs, ARFTM-enlarged *Perkinsus* sp. hypnospores from two infected oysters were sub-cultured. One oyster harbored a relatively intense *Perkinsus* sp. infection, and approximately 80% of its numerous hypnospores zoosporulated upon sub-culture in the DME/F12-3 propagation medium. Zoosporangia with diameters of 35–65 μm had single polar discharge pores and tubes, and developed motile zoospores with diameters of 3–5 μm (Fig. 2). Although zoosporulation itself is a proliferation event, motile zoospores in primary cultures of *Perkinsus* sp. from southern Chinese oysters remained contained within thick-walled zoosporangia, lost motility after several days, and failed to proliferate further. At approximately 90 days post-inoculation, non-proliferating zoospores within zoosporangia and non-proliferating culture inoculum hypnospores were harvested from selected in vitro isolate primary cultures. Harvested cells were preserved in ethanol for extraction and DNA sequencing.

In situ DNA probe hybridization assay results. In situ hybridization probes designed to target LSU rRNA of the novel southern China *Perkinsus* sp., *P. beihaiensis* n. sp., specifically hybridized to *Perkinsus* sp. cells in tissue sections from southern Chinese oysters (Fig. 4, 6). Likewise, the results observed with this *P. beihaiensis* n. sp.-specific probe complemented those obtained with the previously published *Perkinsus* spp. genus probe in oysters found to contain *Perkinsus* sp. cells. The species-specific probe did not cross-react with previously described *Perkinsus* species or host tissues in control samples, and in situ hybridization reactions conducted without probes produced no signals in sections from *Perkinsus* sp.-infected southern Chinese oysters.

Histological assays. Among Chinese oysters sampled during 2005 and 2006 that tested positive for *P. beihaiensis* n. sp. DNA by PCR assays, 16/39 (41%) showed in situ lesions when tissue sections were analyzed histopathologically. Of those host oysters, 37 (95%) were genetically identified as *C. hongkongensis*, one as *C. ariakensis*, and one as an unidentified *Crassostrea* sp. *Perkinsus* sp. lesions occurred in stomach, intestine (Fig. 3–6), and digestive gland epithelia (81%) and visceral connective tissues

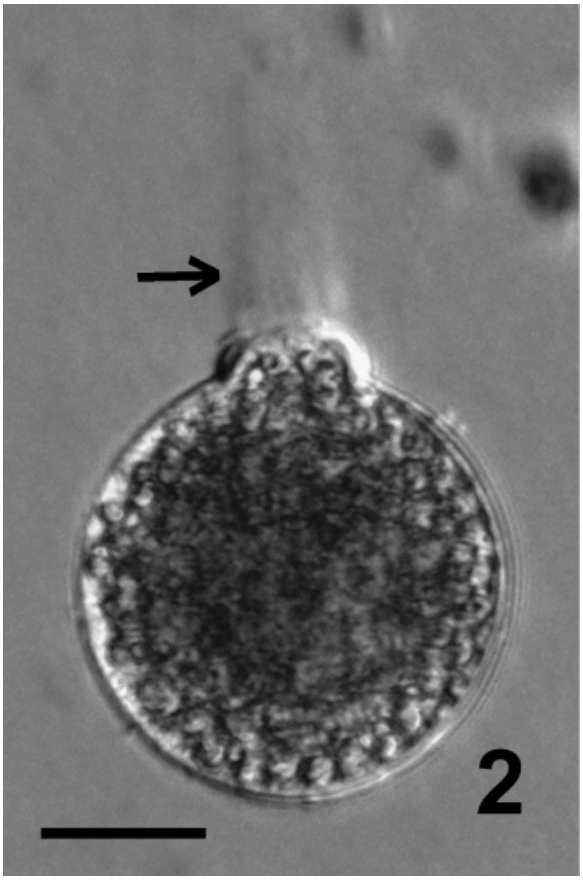

Fig. **2.** Hoffman modulation contrast image of a zoosporangium of *Perkinsus beihaiensis* n. sp. containing zoospores. Identity of zoosporangium confirmed by sequencing the ITS rRNA region (GenBank Accession numbers EU068100—EU068107). Discharge tube (arrow). Scale bar = 20 μm.

(63%), as well as among gill and mantle connective tissues (44%). In two heavy, systemic, infections numerous *Perkinsus* sp. trophozoites (Fig. 7) and proliferating schizonts (Fig. 8) were observed to have been phagocytosed, but were apparently healthy, within circulating hemocytes.

Two examples of possible *Perkinsus* sp. dispersal mechanisms were observed. In one *C. ariakensis*, numerous parasite cells were observed shedding into the stomach lumen adjacent to a necrotic epithelial lesion (Fig. 9). In a female *C. hongkongensis*, *Perkinsus* sp. cells occurred in the egg-laden lumen and epithelium of the gonoduct (Fig. 10).

Infection intensities ranged through light (31% with small focal lesions), moderate (56% with multi-focal or light systemic lesions), and heavy (13% with lethal systemic lesions) (Fig. 11, 12). *Perkinsus* sp. signet ring trophozoites of 2–8 μm diam. subdividing 4–12 μm diam. schizonts, and clusters of multiple sibling trophozoites of 2–4 μm diam. occurred among infected tissues.

Phylogenetic analyses of ITS region, LSU rRNA gene, and actin genes. The expected approximate 690-bp *P. beihaiensis* n. sp. ITS region fragment was amplified from oysters from many sampled locations (Table 1), and from cells harvested in attempts to establish primary *P. beihaiensis* n. sp. cultures. Only those samples relevant to the following species description are included in Table 1; however, a complete description of the disease survey is reported in Moss et al. (2007). The primers designed to amplify *Perkinsus* spp. LSU rRNA gene and type 1 actin gene fragments from infected oyster genomic DNA successfully amplified the expected fragments of approximately 1,170 and 330 bp, respectively, from the infected southern Chinese oysters. Sequencing of the ITS region and LSU rRNA gene fragments of the rRNA gene complex showed that in all cases the fragment amplified was the targeted DNA. When *P. beihaiensis* n. sp.-infected oyster genomic DNA was used as a template for amplification of the type 1 *Perkinsus* sp. actin gene, many sequenced fragments were found to be non-targeted DNA. However, when DNA from the enriched *P. beihaiensis* n. sp. hypnospore and zoosporangia from in vitro culture cell pellets was used as a template, the majority of the amplified fragments were found to be the targeted *P. beihaiensis* n. sp. type 1 actin gene fragment as indicated by results of BLAST searches and the phylogenetic analysis. In many instances, identical *P. beihaiensis* n. sp. ITS region sequences were found within a single oyster; however, *Perkinsus* sp. ITS region sequence variation was occasionally recorded within hosts. For example, of eight ITS fragments sequenced from *P. beihaiensis* n. sp. hypnospores harvested from a single oyster, four variants differing from each other by only a few base pairs were observed. Identical ITS region sequences were often observed in different oysters and from oysters collected from geographically separate locations.

Large subunit rRNA gene sequences were amplified from six of the *P. beihaiensis* n. sp.-positive oysters collected from Beihai in 2005. Of the total 14 PCR fragments that were sequenced, an identical sequence was found in three different oysters; however, the other 11 fragments each varied by a few base pairs with no observed pattern in the variation.

Two *P. beihaiensis* n. sp. type 1 actin gene fragments were sequenced from the genomic DNA of one oyster collected in Beihai in 2005. Fifteen additional fragments were amplified and sequenced from the *P. beihaiensis* n. sp. cell pellet DNA obtained during an unsuccessful attempt to establish a clonal culture of the parasite. Of the 17 sequences obtained, 11 PCR fragments had identical sequences; a second sequence was common to two fragments, and all other sequences were unique. In total, six unique *P. beihaiensis* n. sp. type 1 actin gene sequences were recorded.

In both distance and parsimony analyses, the nucleotide sequences of *P. beihaiensis* n. sp. from the ITS region, LSU rRNA gene, and actin gene loci consistently placed this parasite as a member of the genus *Perkinsus*; however, the sequences grouped in clades that were distinct from those of all previously described *Perkinsus* species. The topologies of the trees generated with the distance and parsimony analyses were similar in the analyses based on each of the three loci. In the ITS region parsimony analysis (Fig. 13), *P. chesapeaki* was the most distant of the *Perkinsus* spp. apart from *P. qugwadi* (outgroup). Internal transcribed spacer region nucleotide sequences of *P. beihaiensis* n. sp. formed a monophyletic clade (100% bootstrap and jackknife support) sister to a clade containing *P. olseni*, *P. marinus*, *P. honshuensis*, and *P. mediterraneus*. In that analysis, sequences of *P. mediterraneus* and *P. honshuensis* formed strongly supported (> 98% both bootstrap and jackknife support) monophyletic clades that were relatively weakly supported as sister groups with 55% bootstrap and 58% jackknife support values.

In the LSU rRNA parsimony analysis (Fig. 14), nucleotide sequences of *P. beihaiensis* n. sp. formed a highly supported clade (100% bootstrap and jackknife support). The clade containing *P. beihaiensis* n. sp. sequences fell out as sister to a clade containing *P. chesapeaki*, *P. olseni, P. marinus*, *P. honshuensis* and *P. mediterraneus*. Within that latter grouping of *Perkinsus* species, a clade with 90% bootstrap support (98% jackknife support) grouped the sequences of *P. mediterraneus* and *P. honshuensis*, and although the *P. mediterraneus* sequences formed a monophyletic

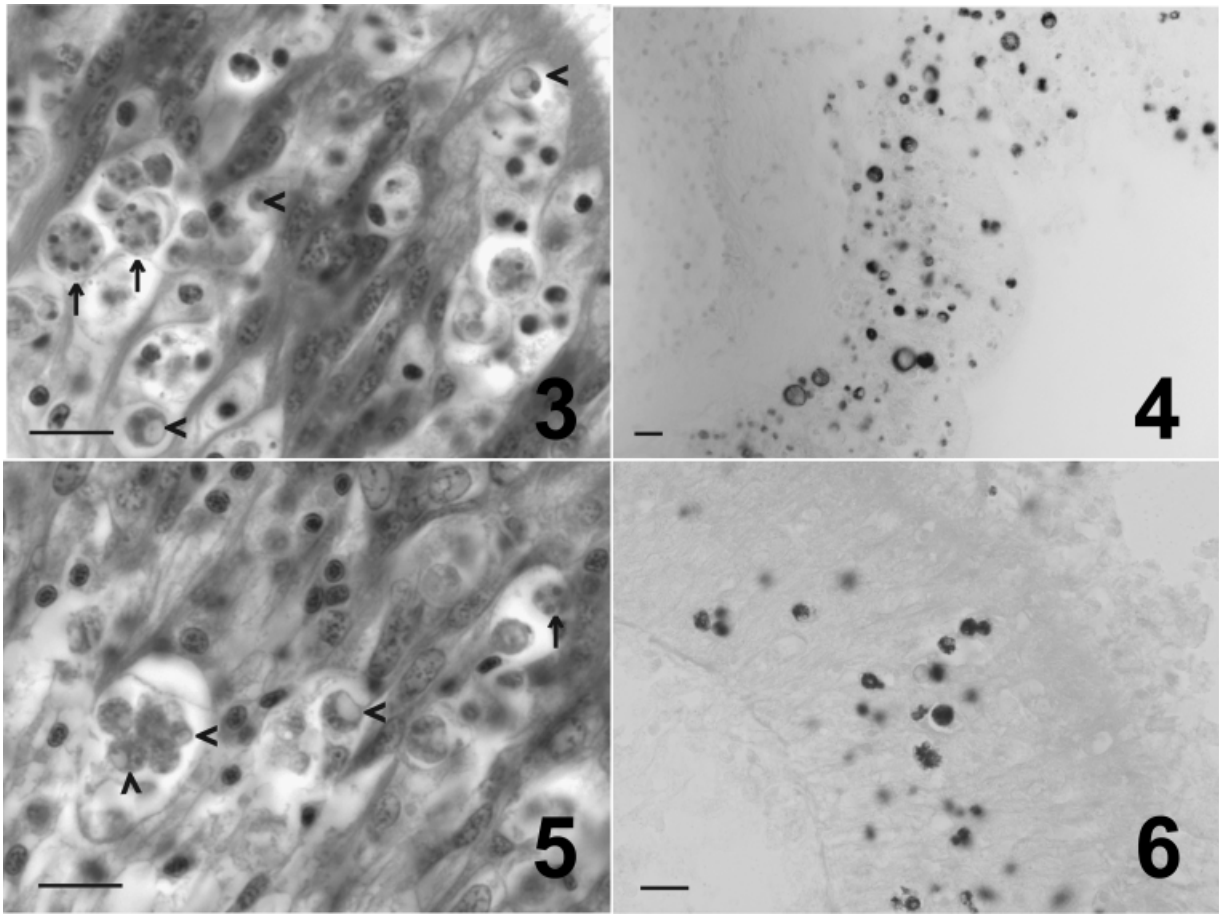

Fig. **3–6.** *Perkinsus beihaiensis* n. sp. lesions in *Crassostrea* spp. oysters. **3.** H&E-stained tissue section of *C. ariakensis* stomach epithelium showing a lesion caused by *P. beihaiensis*. Examples of trophozoites (↑) and schizonts (<) indicated. Scale bar = 10 μm. **4.** *Crassostrea ariakensis* (same individual shown in Fig. 3) showing hybridization of the *P. beihaiensis*-specific probe to cells in the stomach epithelium. Scale bar = 10 μm. **5.** H&E-stained tissue section of *C. hongkongensis* stomach epithelium showing a *P. beihaiensis*. Examples of trophozoites (↑) and schizonts (<) indicated. Scale bar = 10 μm. **6.** *Crassostrea hongkongensis* (same individual shown in Fig. 5) showing hybridization of the *P. beihaiensi*-specific probe to cells in the stomach epithelium. Scale bar = 10 μm.

group, the *P. honshuensis* sequences fell out as unresolved at the base the clade containing both species.

In actin gene sequence analyses (Fig. 15) there were two major monophyletic clades: one containing all *Perkinsus* spp. type 1 actin sequences and the other containing all type 2 *Perkinsus* spp. actin sequences (types as originally designated in Burreson et al. 2005). The clade containing *P. beihaiensis* n. sp. sequences was monophyletic and highly supported (100%) in both parsimony bootstrap and jackknife analysis. Type 1 *P. honshuensis* and *P. mediterraneus* actin nucleotide sequences formed highly supported (100% both bootstrap and jackknife) monophyletic clades within a clade that placed these taxa as sister species with 98% bootstrap (100% jackknife) support.

The genetic distances within the ITS region sequences of *P. beihaiensis* n. sp. were low (0.0%–0.2%) and in the range of the intraspecific variation observed within the currently accepted *Perkinsus* species (Table 2). Genetic distance analysis indicated that the ITS region sequences of this parasite are most closely related to those of *P. honshuensis* (88.5%–89.6% genetic similarity). The two most closely related *Perkinsus* species based on ITS region genetic distances were *P. honshuensis* and *P. mediterraneus* (96.3%–97.1% similar).

The intraspecific genetic distance observed between LSU rRNA gene sequences of *P. beihaiensis* n. sp. were low (0.0%–0.2%) and in the range expected within other *Perkinsus* species (Table 3). Because of the relatively highly conserved nature of the LSU rRNA gene sequences, observed genetic distances were also low between species. Among all *Perkinsus* spp., genetic distance analysis of LSU rRNA gene sequences placed *P. honshuensis* and *P. mediterraneus* as the most closely related (99.3%–99.9% similar).

Intraspecific variation within type 1 actin sequences of *P. beihaiensis* n. sp. was low (0.0%–0.4%) and was also within the variability observed within other *Perkinsus* species sequences (Table 4). Type 1 actin sequences of *P. beihaiensis* n. sp. were most closely related to *P. olseni* (83.4%–86.2% similar). *Perkinsus honshuensis* and *P. mediterraneus* were suggested to be the two most closely related of the other *Perkinsus* species (93.0%–93.8% genetic similarity).

Host and geographic distribution. Based on results from sequencing products of the genus *Perkinsus*-specific assay, as well as results from the *P. beihaiensis* n. sp.-specific PCR assay described here, the geographic distribution of *P. beihaiensis* n. sp. in sampled oyster populations extends at least from Tong'an in Fujian province to Qinzhou and locations surrounding Beihai, Guangxi province, in southern China (Table 1). DNA of this parasite was detected in *C. ariakensis* and *C. hongkongensis* oysters, as well as in *Pinctada margaritifera* and *P. martensii* pearl oysters, and several oyster specimens that were not identified genetically

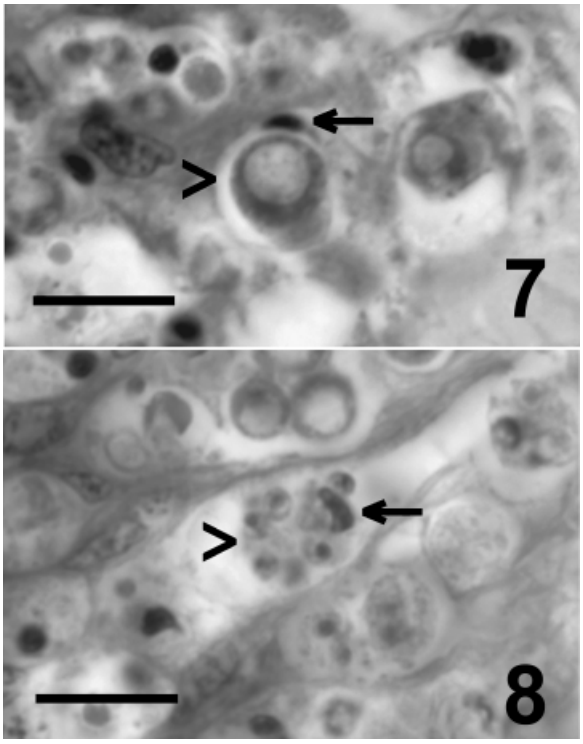

Fig. **7–8.** Oyster hemocyte defensive responses to *Perkinsus beihaiensis* n. sp. parasites. **7.** H&E-stained tissue section showing a *P. beihaiensis* trophozoite (<) encapsulated within an oyster hemocyte. Hemocyte nucleus (↑) visible. Scale bar = 10 μm. **8.** H&E stained tissue section of a *P. beihaiensis* schizont (<) encapsulated within an oyster hemocyte. Hemocyte nucleus (↑) visible. Scale bar = 10 μm

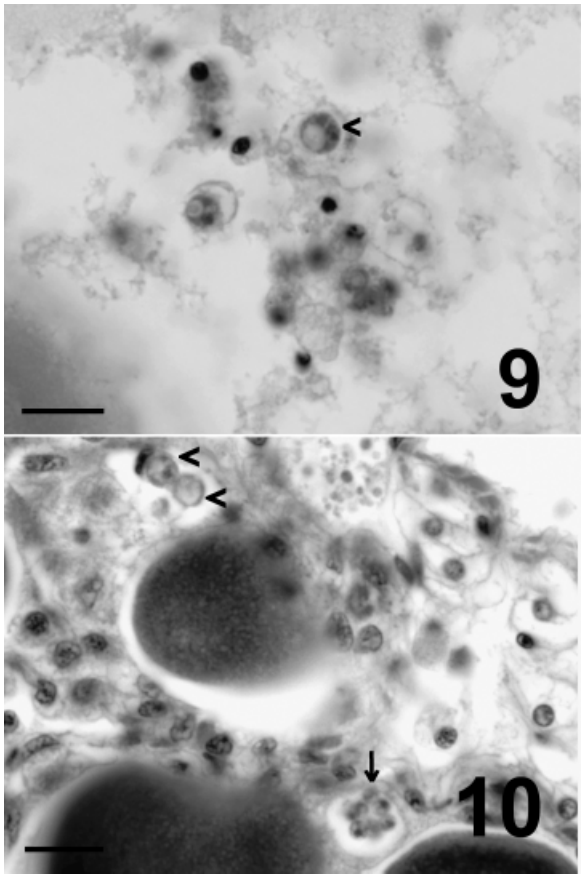

Fig. **9–10.** *Perkinsus beihaiensis* n. sp. trophozoites in tissues of *Crassostrea* spp. oysters. **9.** H&E-stained tissue section of a *Perkinsus beihaiensis* n. sp. trophozoite (<) visible within the stomach lumen of a *Crassostrea ariakensis* oyster. Scale bar = 10 μm. **10.** H&E stained tissue section of a *Perkinsus beihaiensis* trophozoites (<) and schizonts (↑) visible in the epithelia surrounding the gonoducts of a *Crasssostrea hongkongensis* oyster. Scale bar = 10 μm.

using available tools (Table 1). The abundance of *C. hongkongensis* was higher than that of *C. ariakensis* (470 *C. hongkongensis* vs. 107 *C. ariakensis*) in our samples from southern China locations, and DNA from *P. beihaiensis* n. sp. was more frequently found in *C. hongkongensis* than in *C. ariakensis* (23.8% of *C. hongkongensis* vs. 18.7% of *C. ariakensis*). DNA of *P. beihaiensis* n. sp. was detected in as little as 3.8% (1/26) of the *C. ariakensis* sampled from Tong'an, Fujian, in 2002. Results from a 2003 oyster sample from Zhanjiang, Guangdong, suggest, however, that the prevalence of *P. beihaiensis* n. sp. infections in *Crassostrea* spp. oysters can be as high as 46.5% (Table 1).

DISCUSSION

We conducted a comprehensive phylogenetic analysis of a new *Perkinsus* sp. parasite infecting oysters from coastal southern China. Phylogenetic analyses based on nucleotide sequences of the ITS region, the LSU rRNA gene, and the type 1 actin gene of this parasite consistently placed it within the genus *Perkinsus*. However, sequences at each locus form well-supported, distinct, monophyletic clades, when compared with those from other *Perkinsus* spp. described to date. With these consistent genetic similarities and differences, in addition to observations of parasite cell morphology typical of *Perkinsus* spp., and enlargement in RFTM media, we conclude that this parasite represents a new *Perkinsus* sp. for which we propose the name *P. beihaiensis* n. sp.

Perkinsus beihaiensis n. sp.

Diagnosis. Infections occur among oyster digestive epithelia and various connective tissues. Histologically, trophozoites in oyster tissues are spherical, 2–6 μm in diameter, with a single, eccentric nucleus that typically contains a prominent nucleolus, and a large, eccentric vacuole that occupies much of the cell volume. In situ proliferation is by schizogony of 4–12 μm mother cells to yield clusters of 4–20 sibling daughter cells. Lesions occur with decreasing frequency among visceral connective tissues, stomach and intestinal epithelia, mantle and gill connective tissues, and digestive gland epithelia.

DNA nucleotide sequences. In phylogenetic analyses, nucleotide sequences from the ITS region and LSU rRNA gene of the rRNA gene complex and type 1 actin gene will form distinct monophyletic clades, separate from those of the other known *Perkinsus* species, including *P. marinus*, *P. chesapeaki*, *P. mediterraneus*, *P. olseni*, *P. honshuensis*, and *P. qugwadi*.

Reference material deposited. Replicate H&E-stained histological sections from infected *C. hongkongensis* and *C. ariakensis* oysters that were confirmed by both PCR and ISH assays to be infected by *P. beihaiensis* n. sp., were deposited as respective hapantotype and paratype reference materials, with both the United States Department of Agriculture National Parasite Collection (USNPC) (http://www.lpsi.barc.usda.gov/bnpcu) (USNPC 100051 and USNPC 100052), and with the Office International des Epizooties (OIE) genus *Perkinsus* reference collection at the Virginia Institute of Marine Science (http://www.vims.edu/env/research/shellfish/oie). Nucleotide sequences of the ITS region and LSU rRNA genes of the rRNA gene complex and actin genes

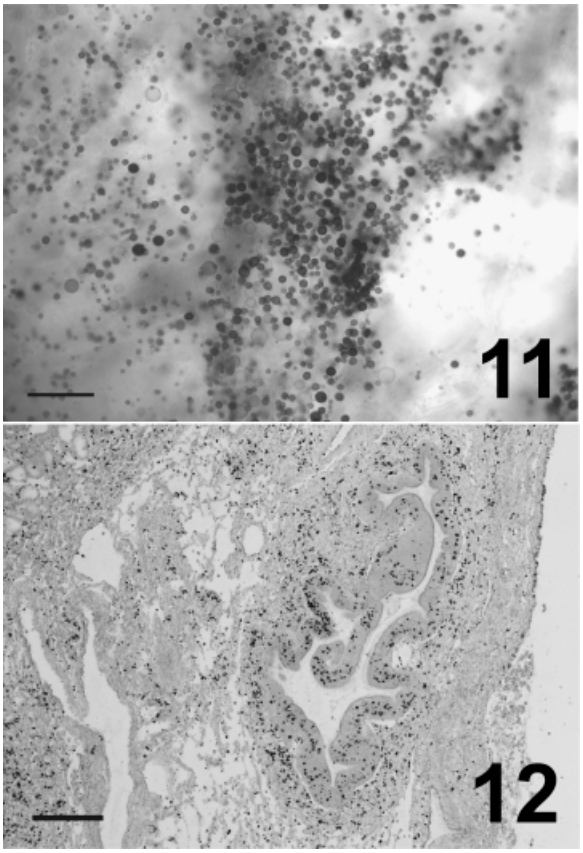

Fig. **11–12.** *Perkinsus beihaiensis* n. sp. cells in tissues of heavily infected *Crassostrea hongkongensis* oysters. **11.** Low-magnification image of numerous *Perkinsus beihaiensis* hypnospores within gill and mantle tissues of a heavily infected oyster after incubation in RFTM and staining with Lugol's iodine. The number and density of cells observed represent a very heavy infection according to Ray (1952, 1954). Scale bar = 200 μm. **12.** Low-magnification image showing widespread hybridization of the *Perkinsus beihaiensis*-specific probe abundant pathogen cells in a histological section of visceral tissues of an oyster. Scale bar = 200 μm.

are deposited with GenBank (http://www.ncbi.nlm.nih.gov/Genbank) under the accession numbers listed in Table 5.

Type host. *Crassostrea hongkongensis*.

Other hosts. *Crassostrea ariakensis, P. martensii* (by PCR only), *P. margaritifera* (by PCR only).

Type locality. Beihai region, Guangxi Zhuang, People's Republic of China (21°32′57.70″N, 109°10′10.03″E).

Etymology. The species name refers to Beihai, the city in China near waters where this parasite has frequently been detected in oysters.

Higher classification. Chromalveolata (super-group), Alveolata (first rank), Dinozoa (second rank), Perkinsidae (third rank) (Adl et al. 2005).

The ITS region of the rRNA complex has previously been used to examine relationships within the genus, and discriminate between *Perkinsus* spp. (Brown, Hudson, and Reece 2004; Casas et al. 2002; Dungan et al. 2002; Dungan and Reece 2006; Goggin 1994; Park et al. 2006). In addition, the LSU rRNA and actin genes have been used to further clarify taxonomic placements because analyses of these regions offer resolution at multiple levels. The ITS region of the rRNA gene complex is transcribed, though it is not translated into a functional protein with potential fitness effects; therefore its sequences are often found to be more variable than those of adjacent rRNA gene loci. ITS region, LSU rRNA gene, and the type 1 actin gene sequences of *P. beihaiensis* consistently place it within the genus *Perkinsus*, though distinct from all other described *Perkinsus* species. For each analyzed genetic locus, observed intraspecific variation of *P. beihaiensis* sequences were within ranges seen for other described *Perkinsus* spp. Interspecific distances within the genus were typical of what has been observed in previous studies between accepted *Perkinsus* species, and were less than those found between *Perkinsus* species and outgroup taxa (Burreson et al. 2005; Dungan and Reece 2006; Dungan et al. 2007).

Although we were only able to successfully amplify and sequence type 1 actin gene fragments from *P. beihainesis*, we cannot discount the existence of a type 2 actin gene(s). Until recently, only type 1 actin gene sequences from *P. marinus* existed in GenBank, but as part of the genome-wide sequencing effort, type 2 actin gene sequences have been deposited in the *P. marinus* TIGR database. The conserved nature of actin gene DNA sequences across very different taxa, combined in this case with the absence of a pure culture of *P. beihaiensis*, make it difficult to amplify pathogen DNA selectively from the milieu of host and symbiont DNAs. The type 1 *Perkinsus* spp. actin gene primers reported in this study successfully amplified targeted DNA from a semi-enriched *P. beihaiensis* genomic DNA source; however, these primers had limited success in selectively amplifying targeted actin genes from genomic DNAs of *P. beihaiensis*-infected oysters.

The *P. beihaiensis*-specific PCR primer set specifically amplifies this species' ITS region, and the in situ hybridization probe specifically hybridizes to the nucleic acids of *P. beihaiensis* cells in histological tissue sections. Polymerase chain reaction assay specificity was tested on DNA samples from infected and uninfected host oysters, and multiple other closely related organisms. Specificity of the ISH assay was confirmed, and we observed no probe binding to non-target *Perkinsus* sp. cells in tested sections. Additionally, *P. beihaiensis*-specific probe assay results mirrored results obtained with the genus *Perkinsus*-specific probe in tissue sections from oysters in which only *P. beihaiensis* was detected by PCR assays. The successful PCR amplification and ISH labelling of *P. beihaiensis* cells in infected *C. ariakensis* and *C. hongkongensis* oyster tissues demonstrate that these assays may be used to screen potential hosts for *P. beihaiensis* infections and to localize parasite cells in situ.

In histological analyses, cells of *P. beihaiensis* were most commonly found in visceral mass connective tissues and in stomach and intestinal epithelia of infected oysters. Early infections of *P. olseni* in *V.* (*Tapes) decussatus*, *P. chesapeaki* in *M. arenaria* or *Tagelus plebeius*, and *P. marinus* in *C. virginica* are often limited to the gill and mantle tissues (Casas et al. 2002; Dungan et al. 2002; Mackin 1951); tissues that are generally considered to be points of entry for those parasites. *Perkinsus marinus* infections in *C. virginica* often become systemic, though *P. marinus* cells may be localized in digestive epithelia, and these organs have often been found to have the highest densities of parasite cells (Oliver et al. 1998). Infections by *P. olseni* occur almost exclusively among connective tissues, and RFTM analysis of gill tissues alone is commonly used for diagnosis of *P. olseni* in clams (Villalba et al. 2005).

For the current study, gill and mantle, and occasionally rectal tissues, were used for PCR and RFTM assays. With the apparent rarity of *P. beihaiensis* lesions in gill and mantle tissues that we observed histologically with both ISH and H&E analyses (38% of lesions seen in gill), our PCR and RFTM assays of gill tissues may have underestimated prevalences and intensities of *P. beihaiensis* infections among tested Chinese oysters.

Visceral mass tissues are not commonly targeted as sources of PCR-template samples because DNA extracted from these tissues often carries high levels of PCR-inhibitory substances associated with digestive organs. Extraction methods exist that may facilitate

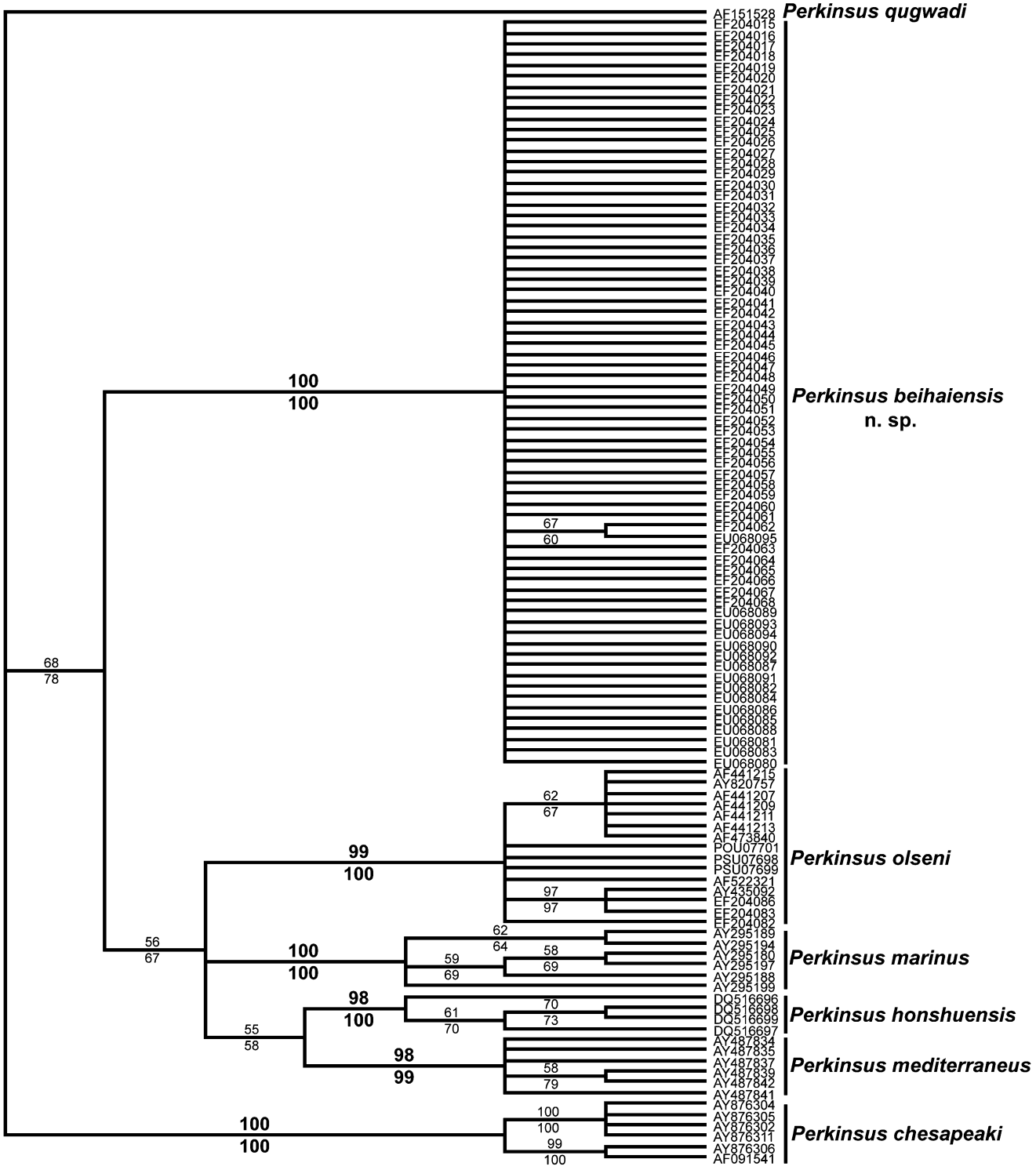

Fig. **13.** Relationships among *Perkinsus* spp. based on ITS region gene sequences. Maximum parsimony bootstrap support values for each clade are given above the lines and jackknife support values are given below the lines. Bold support values indicate species clades.

amplification of DNA isolated from digestive gland tissues; however, the complexity of those methods renders them impractical for use in routine diagnostics. Future research goals will be to determine the optimal diagnostic method for *P. beihaiensis*. Until optimal diagnostic tissues are identified, we suggest that a complete histological analysis in concert with PCR analysis may be necessary in order to adequately assess prevalences and intensities of *P. beihaiensis* infections.

Despite the promising and extensive proliferative zoosporulation that occurred among several *P. behaiensis* primary cultures, our efforts to establish continuous in vitro isolate cultures of *P. beihaiensis* have so far failed. Constituents of *Perkinsus* spp.

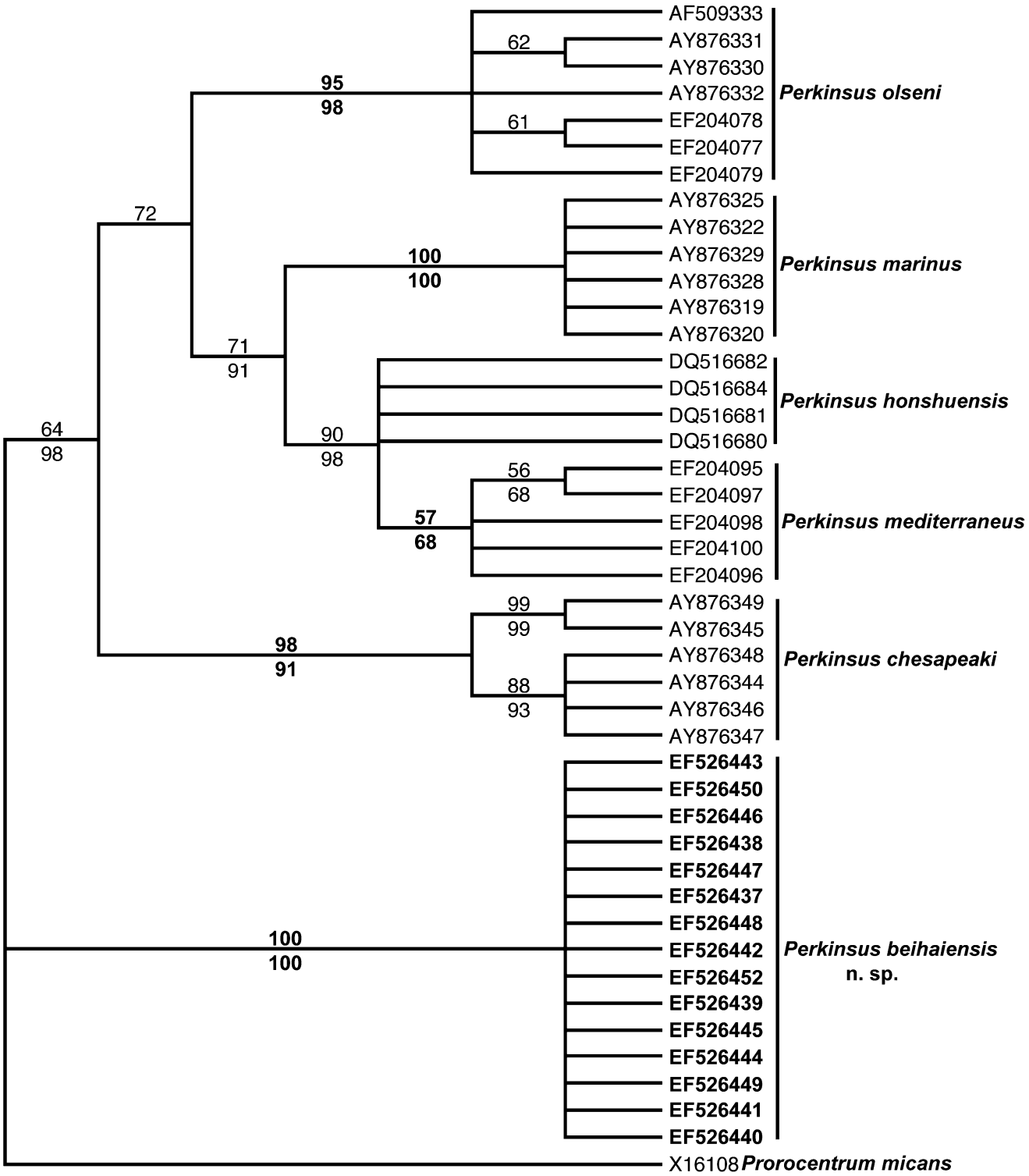

Fig. **14.** Relationships among *Perkinsus* spp. based on large subunit (LSU) rRNA gene sequences. Maximum parsimony bootstrap support values for each clade are given above the lines and jackknife support values are given below the lines. Bold support values indicate species clades.

culture media, including compositions and concentrations of mineral salts, trace elements, metabolites, nucleotide precursors, and vitamins were originally developed for *P. marinus*, and were based on compounds found normally in *C. virginica* oyster tissues (Gauthier and Vasta 1993; La Peyre, Faisal, and Burreson 1993). Although those media and their modifications have been subsequently used to propagate diverse isolates of *Perkinsus* spp. from a wide variety of mollusc hosts, they appear to be deficient in the several modifications that we used for in vitro propagation of *P. beihaiensis* infecting *C. ariakensis* and *C. hongkongensis*

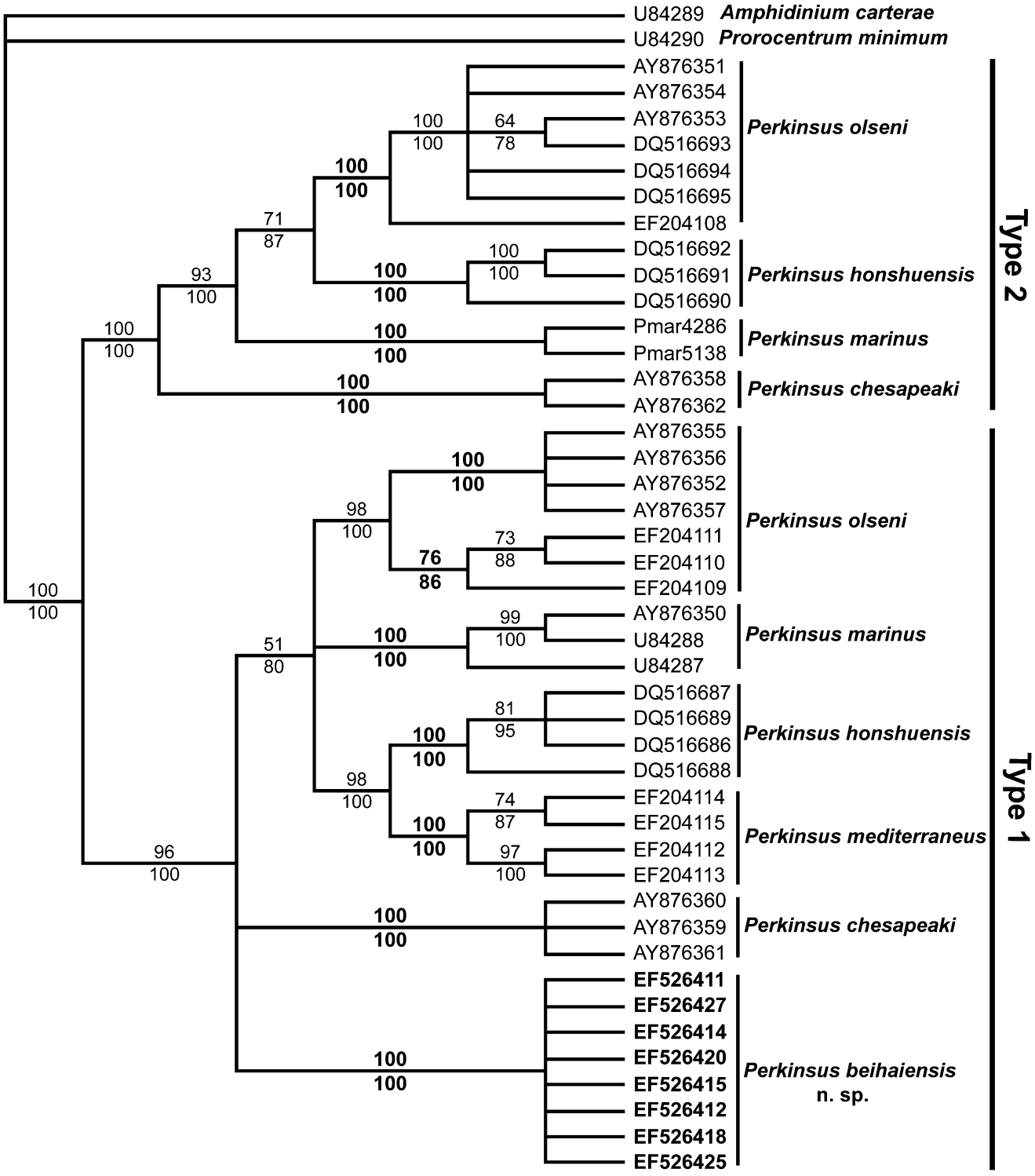

Fig. **15.** Relationships among *Perkinsus* spp. based on actin gene sequences. Maximum parsimony bootstrap support values for each clade are given above the lines and jackknife support values are given below the lines. Bold support values indicate species clades.

oysters from southern China. *Perkinsus* spp., such as *P. marinus* and *P. olseni*, have been shown to proliferate in several media, and within ranges of temperature and salinity conditions (Chu, Volety, and Constantin 1994; Ordás, Novoa, and Figueras 1999); while *P. mediterraneus* has proven to be extremely fastidious and slow-growing in vitro (Casas et al. 2008). We anticipate that critical, minor modifications to existing medium compositions and/or incubation parameters will yield continuous in vitro isolate cultures and archival-type strains of *P. beihaiensis*.

The distribution and prevalence of *P. beihaiensis* suggests that it may be widespread in Chinese oysters, particularly *C. hongkongensis*, in coastal localities from Tong'an, Fujian to

Table **2.** Range of pairwise distance (uncorrected-'p') values observed among ITS rDNA region sequences of currently accepted *Perkinsus* spp. (except *P. qugwadi*) from GenBank and those of the *Perkinsus beihaiensis* n. sp. obtained in this study.

Species	*Perkinsus marinus*	*Perkinsus chesapeaki*	*Perkinsus mediterraneus*	*Perkinsus olseni*	*Perkinsus honshuensis*	*Perkinsus beihaiensis* n. sp.
Perkinsus marinus	**0.000–0.004 0.4%**	0.123–0.140	0.048–0.061	0.048–0.061	0.046–0.055	0.110–0.124
Perkinsus chesapeaki	12.3–14.0%	**0.000–0.017 1.7%**	0.122–0.136	0.124–0.135	0.115–0.122	0.163–0.175
Perkinsus mediterraneus	4.8–6.1%	12.2–13.6%	**0.000–0.006 0.6%**	0.042–0.055	0.029–0.037	0.110–0.120
Perkinsus olseni	4.8–6.1%	12.4–13.5%	4.2–5.5%	**0.000–0.005 0.5%**	0.043–0.055	0.105–0.120
Perkinsus honshuensis	4.6–5.5%	11.5–12.2%	2.9–3.7%	4.3–5.5%	**0.000–0.004 0.4%**	0.104–0.115
Perkinsus beihaiensis n. sp.	11.0–12.4%	16.3–17.5%	11.0–12.0%	10.5–12.0%	10.4–11.5%	**0.000–0.002 0.2%**

The range of within species sequence distances are given across the diagonal. Raw distance value ranges between species are given above the diagonal and ranges for percent distance values are given below.

Beihai, Guangxi, near the southern border of China with Vietnam. We have found DNA of this parasite additionally in high prevalence in *C. ariakensis*, and in *P. margeratifera*, *P. martensii*, and in some unidentified bivalve mollusc species. Infection prevalences averaged over our southernmost sampling sites indicate that approximately 20% of regional mollusc populations may be infected by *P. behaiensis* during some seasons. More extensive host and seasonal sampling will clarify patterns and natural levels of parasite prevalence in affected populations.

Histopathological data collected thus far indicate that infection by *P. beihaiensis* is detrimental to host oysters. In this study, defensive hemocyte infiltration occurred in *P. beihaiensis*-infected oyster tissues. In low-intensity infections, *P. beihaiensis* cells were detected in epithelia of the stomach and intestine and in the digestive tubules and ducts, potentially leading to interference with nutrient uptake and absorption by the oyster. In moderate to severe infections, *P. beihaiensis* cells were systemically distributed and abundant in virtually all tissues, including the visceral mass connective tissues, stomach and intestinal epithelia, gills, mantle, and gonoducts, with necrotic loss of normal tissue architecture readily apparent. Although we cannot attribute parasite body burden to a loss of condition or death, a few moribund or dead oysters were found to have very heavy infection intensities.

It has been suggested that heavy infections of *Perkinsus* spp. may have significant negative physiological effects on oysters. Studies have shown that *P. marinus* infections reduce oyster growth, and are especially pathogenic during summer, when the abundance of parasites in tissues is high. Oyster condition index is reduced during the summer, which is the post-spawning period, and therefore, it is not surprising that oyster condition index is negatively associated with *P. marinus* infection intensity (Albright et al. 2007; Andrews 1961). In addition, reproductive output may be affected (Dittman, Ford, and Padilla 2001) and metabolic costs of *Perkinsus* sp. parasitism may be greater than those that could be met by the normal feeding activity of the infected bivalve (Casas et al. 2002; Choi et al. 1989).

Although there exists a long history of oyster culture in the southern provinces of China, there are few previous reports of apparent disease-or pathogen-induced mortality in shellfish populations from that region. Past accounts of mollusc disease in that region include a mortality event in *Ostrea edulis* caused by a bloom of a toxic *Prorocentrum* sp. dinoflagellate (Zhang, Munday, and Handlinger 1995), reports of rickettsia-like organisms in pearl oysters, *P. maxima* and *P. fucata* (Wu and Pan 1999) and in *C. ariakensis* (Wu and Pan 2000), and mass mortality of abalone, *Haliotis diversicolor*, attributed to the outbreak of a viral infection (Wang et al. 2004b). Here we report on the existence of a previously unknown and pathogenic oyster parasite in southern China. *Perkinsus beihaiensis* n. sp. occurs throughout an extensive geographic range in that region and, based on the prevalence and pathology of *P. beihaiensis* infections, there may exist a disease risk to wild and cultured oyster populations in the region.

Table **3.** Range of pairwise distance (uncorrected-'p') values observed among LSU rDNA region sequences of currently accepted *Perkinsus* spp. (except *P. qugwadi*) from GenBank and those of the *Perkinsus beihaiensis* n. sp. obtained in this study.

Species	*Perkinsus marinus*	*Perkinsus chesapeaki*	*Perkinsus mediterraneus*	*Perkinsus olseni*	*Perkinsus honshuensis*	*Perkinsus beihaiensis* n. sp.
Perkinsus marinus	**0.000–0.002 0.2%**	0.035–0.040	0.022–0.026	0.001–0.029	0.021–0.025	0.044–0.049
Perkinsus chesapeaki	3.5–4.0%	**0.000–0.005 0.5%**	0.023–0.032	0.022–0.040	0.025–0.032	0.036–0.044
Perkinsus mediterraneus	2.2–2.6%	2.3–3.2%	**0.000–0.003 0.3%**	0.017–0.026	0.001–0.007	0.044–0.051
Perkinsus olseni	0.1–2.9%	2.2–4.0%	1.7–2.6%	**0.000–0.003 0.3%**	0.019–0.025	0.035–0.049
Perkinsus honshuensis	2.1–2.5%	2.5–3.2%	0.1–0.7%	1.9–2.5%	**0.000–0.004 0.4%**	0.044–0.052
Perkinsus beihaiensis n. sp.	4.4–4.9%	3.6–4.4%	4.4–5.1%	3.5–4.9%	4.4–5.2%	**0.000–0.002 0.2%**

The range of within species sequence distances are given across the diagonal. Raw distance value ranges between species are given above the diagonal and ranges for percent distance values are given below.

Table **4.** Range of pairwise distance (uncorrected-'p') values observed among type 1 actin gene region sequences of currently accepted *Perkinsus* spp. (except *P. qugwadi*) from GenBank and those of the *Perkinsus beihaiensis* n. sp. obtained in this study.

Species	*Perkinsus marinus*	*Perkinsus chesapeaki*	*Perkinsus mediterraneus*	*Perkinsus olseni*	*Perkinsus honshuensis*	*Perkinsus beihaiensis* n. sp.
Perkinsus marinus	**0.000–0.018 1.8%**	0.152–0.170	0.125–0.131	0.114–0.131	0.142–0.145	0.173–0.190
Perkinsus chesapeaki	15.2–17.0%	**0.000–0.009 0.9%**	0.163–0.176	0.145–0.159	0.173–0.183	0.163–0.180
Perkinsus mediterraneus	12.5–13.1%	16.3–17.6%	**0.000–0.007 0.7%**	0.104–0.118	0.062–0.070	0.163–0.176
Perkinsus olseni	11.4–13.1%	14.5–15.9%	10.4–11.8%	**0.000–0.018 1.8%**	0.125–0.131	0.138–0.166
Perkinsus honshuensis	14.2–14.5%	17.3–18.3%	6.2–7.0%	12.5–13.1%	**0.000–0.000 0.0%**	0.163–0.170
Perkinsus beihaiensis n. sp.	17.3–19.0%	16.3–18.0%	16.3–17.6%	13.8–16.6%	16.3–17.0%	**0.000–0.004 0.4%**

The range of within species sequence distances are given across the diagonal. Raw distance value ranges between species are given above the diagonal and ranges for percent distance values are given below.

Table **5.** GenBank Accession Numbers for *Perkinsus beihaiensis* n. sp. sequences associated with this study.

Locus	GenBank accession numbers
rRNA internal transcribed spacer region	EF204015–EF204069, EF526428–EF526436, EU068080–EU068095, EU068100–EU068107
LSU rRNA gene	EF526437–EF526452
Actin gene	EF526411–EF526427

With a pending Environmental Impact Statement (EIS) and ruling regarding the introduction of *C. ariakensis* to Chesapeake Bay, we are now aware of a new and pathogenic parasite, *P. beihaiensis*, that infects *C. ariakensis*, *C. hongkongensis,* and other oyster species, and occurs in potential broodstock sites in southern China. The natural pathogenicity of this parasite to its host is not yet fully known, although it is the subject of ongoing research. A fear is that non-native pathogens, either introduced directly with rogue introductions of *C. ariakensis* or indirectly such as through ballast water may impact the oyster restoration effort (using *C. ariakensis*), or harm other Chesapeake Bay bivalves such as *C. virginica* or the hard clam *M. mercenaria*. Therefore, our ongoing research should seek to understand the pathogenicity and transmissibility of *P. beihaiensis* to its native hosts, as well as to native Chesapeake Bay bivalve species.

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