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Characterization of exotic pathogens associated with the suminoe oyster, Crassostrea ariakensis

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CHARACTERIZATION OF EXOTIC PATHOGENS ASSOCIATED WITH THE SUMINOE OYSTER, *CRASSOSTREA ARIAKENSIS*

A Dissertation

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by

Jessica A. Moss

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

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DEDICATION

To my parents, whose love and support continue to guide me through all of my endeavors and to Hamish for his love, generosity, and laughter.

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ABSTRACT

The eastern oyster, *Crassostrea virginica*, once an integral part of the ecology and economy of Chesapeake Bay, has been severely depleted. Factors leading to the decline of the eastern oyster include over-harvesting, environmental degradation and disease pressure caused by the protozoans *Haplosporidium nelsoni* and *Perkinsus marinus*, known commonly as MSX and Dermo, respectively. Studies regarding the feasibility of introducing a non-native oyster to the Bay were initiated, and field-based research on an Asian oyster, *Crassostrea ariakensis*, suggested that it might be a viable species for introduction.

Research surrounding the proposed introduction has focused on recommendations such as those from the International Council for Exploration of the Seas, suggesting that the "ecological, genetic and disease relationships of the species in its natural range and environment" be examined. In order to assess the disease risks associated with *C. ariakensis,* a parasite survey of oysters collected from China, Japan and Korea was undertaken to examine the pathogens associated with *C. ariakensis* in its natural range. The protozoan parasites, *Perkinsus olseni* and a new *Perkinsus* sp., as well as multiple genetic strains of molluscan herpesvirus, were discovered using molecular diagnostic methods. Molluscan herpesvirus and *Perkinsus* spp. protozoans are known to cause mortality of many commercially important bivalve species.

Characterization of the new *Perkinsus* sp. included a comprehensive analysis of three DNA loci along with histological examination of the *Perkinsus* sp. cells in preserved tissue sections. Challenge experiments were undertaken using *P. olseni* and the new *Perkinsus* sp. in order to assess the transmission risk of these exotic microbes to the eastern oyster and the hard clam, *Mercenaria mercenaria.* The laboratory experiments suggest that bivalve shellfish native to Chesapeake Bay may be susceptible to the alien *Perkinsus* spp. associated with *C. ariakensis.* In addition, *C. ariakensis* may acquire moderate to lethal infections of *P. marinus* under stressful conditions. In light of the proposed introduction of *C. ariakensis*, it appears that there is a great disease risk associated with this Asian oyster species with the potential to have a negative impact on the naïve shellfish populations of Chesapeake Bay.

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INTRODUCTION

Decline of the Oyster industry in Chesapeake Bay

The eastern oyster, *Crassostrea virginica*, was once an integral part of the economy of Chesapeake Bay. In colonial times, oysters provided not only an abundant food source, but oyster reefs additionally created unique habitats that played an ecologically important role in food webs supporting other commercially important fish and crab species. Oysters also acted as key regulators of the water quality of the Bay, whereby it was estimated that prior to 1870, Bay oyster populations had the capacity to filter the waters of the Bay in approximately 30 days (Pomeroy et al. 2006). Natural populations of *C. virginica* have been severely depleted. Peak oyster harvests observed in the late 1800s were reduced by 60% between 1880 and 1930 due to over-dredging on deep channel reefs (National Research Council, 2003). Further decline in the late 1950s has been attributed to continued over-harvesting and the impact of two protozoan pathogens, *Haplosporidium nelsoni* and *Perkinsus marinus,* the parasites responsible for the diseases known as MSX and Dermo, respectively (Andrews 1988, Burreson and Ragone Calvo 1996). Use of agricultural pesticides, elevated levels of tributyl tin and other environmental contaminants have also been implicated as factors in the decline of oyster harvests (National Research Council, 2003). In the state of Virginia, landings once in excess of 6 million bushels in the 1930s have declined to less than 20,000 bushels annually since the 1990s.

Non-native oyster introduction proposed

The decline in oyster production in Virginia and Maryland led to the formation of a number of panels in the early 1990s to make recommendations on restoring oyster populations to Chesapeake Bay. One option being considered is the introduction of a non-native oyster species for ecological and fishery enhancement. A Virginia Institute of Marine Science (VIMS) study conducted in 1996 focusing on the Pacific oyster, *Crassostrea gigas,* documented lower disease susceptibility in *C. gigas* than in the native eastern oyster, however growth rates of the Pacific oyster were equal or inferior to the native oyster in Chesapeake Bay (Calvo et al. 1999). A 1998 field-based study on another Asian oyster, *Crassostrea ariakensis*, documented rapid growth and survival in that species, as compared to *C. virginica,* even when endemic diseases were present (Calvo et al. 2001). In addition, marketability testing of *C. ariakensis* has shown that it has an acceptable taste for the local consumer (Grabowski et al. 2003).

In China, *Crassostrea ariakensis* is found naturally from the Bohai Sea in northern China to Beihai, Guangxi, in southern China near the border with Vietnam. A newly described species, *Crassostrea hongkongensis,* has been found to coexist with *C. ariakensis* in southern China, along the coast of Fujian, Guangdong, and Guangxi provinces (Wang et al. 2004). *Crassostrea hongkongensis* is preferred by local fishermen for oyster aquaculture in southern China due to higher meat quality and productivity (Zhou and Allen 2003). Morphological differences between these two oyster species are not very clear (Lam and Morton 2003), although slight anatomical differences have been reported (Wang et al. 2004). Past morphological and mitochondrial DNA phylogenetic studies have suggested that *C. ariakensis* is the "red meat" form (color of the soft body) of *C. rivularis,* an oyster species that is widely cultured in China while the "white meat"

form of *C. rivularis* is the same species as the newly described oyster species, *C. hongkongensis* (Lam and Morton 2003,Wang et al. 2004). Through phylogenetic analysis of many Asian oyster species, individuals identified as "red" or "white" based on morphology by researchers in the field did not segregate into monophyletic clades in an analysis based on the first internal transcribed spacer ribosomal RNA region (ITS-1) and cytochrome oxidase I (COI) gene regions (Cordes and Reece 2005), suggesting that morphological characteristics may not be appropriate for distinguishing between these two species. The fact that genetic data conflict with species designations based on morphological characters has therefore highlighted the need for confirmatory genetic identification of Asian *Crassostrea* species. For example, an intentional introduction of *C. ariakensis* from Beihai, China to the west coast, USA occurred in 1999. Genotypic analysis revealed that 48% (24 of 50) of those brood stock oysters, imported as *C. ariakensis,* were, in fact, *C. hongkongensis* (Zhang et al. 2005).

Additionally, because of the confusion associated with taxonomic identification of *C. ariakensis,* existing records of biology and ecology of *C. ariakensis* must be read with caution. Current trials using triploid *C. ariakensis* suggest that this species can tolerate a wide range of temperature and salinity conditions (NOAA Quarterly Review, Spring 2005).

Assessing the risk of *Crassostrea ariakensis* introduction

In 2003, the U.S. Congress authorized the Army Corps of Engineers to prepare an Environmental Impact Statement (EIS) examining the possible risks and benefits of introducing a non-native oyster to Chesapeake Bay. The EIS is being prepared with the Army Corps of Engineers as the lead federal agency, in conjuction with two state agencies, the Maryland Department of Natural Resources and the Virginia Marine

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molluscan herpesviruses, two species of *Perkinsus*, *Perkinsus olseni* and a new *Perkinsus* species, *Chlamydia*-like organisms, ciliates, *Nematopsis* sp., cestodes, viral gametocytic hypertrophy and *Steinhausia*-like microsporidians in oysters from potential *C. ariakensis* broodstock sites in Asia.

Steinhausia-like microsporidians were observed in oyster ova, indicating a real possibility for vertical transmission. Vertical transmission of microsporidians has been demonstrated previously in invertebrates such as amphipod crustaceans and *Daphnia* (Kelly et al. 2003, Galbreath et al. 2004, Haine et al. 2004, Vizoso and Ebert 2004). Viral gametocytic hypertrophy has been reported in *Crassostrea virginica* from Chesapeake Bay (Farley 1978), and a *Steinhausia*-like microsporidian has been reported in the clam *Macoma balthica* in Chesapeake Bay (Farley 1977). The parasites observed in Asian oysters, however, may be different strains or species than those in Chesapeake Bay, and could pose problems if introduced.

Although current quarantine measures should minimize the risk of introduction of exotic diseases with Asian *C. ariakensis*, there is obvious concern regarding accidental introduction of exotic pathogens associated with failed quarantine measures, rogue introductions of Asian oysters directly to Chesapeake Bay, or ballast water introductions. Failure of adequate water treatment in those facilities importing oysters from Asia could result in contamination of Bay waters with pathogens associated with the exotic oysters. Furthermore, the mid-Atlantic coast is frequently at risk for experiencing hurricanes such as hurricane Floyd (September 15--22, 1999) and Isabelle (September 2003) that severely impacted the Bay, having the potential to damage the infrastructure and function of buildings designated as quarantine facilities and lead to accidental release of exotic pathogens.

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Virginia, in Chesapeake Bay, ranked fifth and second, respectively, in the amount of foreign ballast water received (Smith et al. 1999). Diverse assemblages of ballast water organisms have been reported in ships arriving in Chesapeake Bay including crustaceans, molluscs, ctenophores, echinoderms, rotifers, nematodes, ciliates, diatoms, copepods and cyanobacteria (Smith et al.1999), as well as viruses and pathogens such as *Vibrio cholerae* and *Pfiesteria piscidica* (Dobbs et al. 2003). One thing that limits the establishemen of ballast-introduced organisms is their ability to survive in the environment into which they are discharged (Smith et al. 1999). *Perkinsus* spp. have been shown to tolerate a wide range of salinities and temperatures (see below), therefore it is possible that Chesapeake Bay may be a suitable habitat for exotic *Perkinsus* spp. arriving here through Asian shipping traffic. The impacts that these *Perkinsus* spp. would have on the native Chesapeake Bay bivalves such as *C. virginica* and *M. mercenaria,* or even on introduced *C. ariakensis,* are not known.

Mollusc herpesviruses

The detection of multiple genetic strains of mollusc herpesviruses in Asia (Chapter 1) also poses a real risk to Chesapeake Bay. Mollusc herpesviruses are found in a growing number of bivalve species including the eastern oyster *Crassostrea virginica* (Farley, 1972), the Pacific oyster *Crassostrea gigas* in Australia and France (Hine et. al. 1992, Nicolas et. al. 1992, Renault et al. 1994), the European flat oyster *Ostrea edulis* (Comps and Cochennech 1993, Renault et al. 2000a), the southern flat oyster *Ostrea angasi* in Australia (Hine 1997), the Chilean oyster *Tiostrea chilensis* larvae in New Zealand (Hine et al. 1998), the European clam *Ruditapes decussatus* (Renault and Arzul, 2001), the Manila clam *R. philippinarum* (Renault 1998, Renault et al. 2001), and the

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Perkinsus species have been described in a variety of molluscs worldwide. *Perkinsus marinus* was first described as a pathogen of *Crassostrea virginica* (Mackin et al. 1950, Andrews and Hewatt 1957) and is known for its devastating effects on eastern and Gulf Coast USA oyster populations (Burreson and Ragone Calvo 1996). *Perkinsus olseni* and *P. atlanticus* have recently been synonymized based on molecular evidence (Murell et al. 2002), further expanding an already wide host and geographic range for *P. olseni.* *Perkinsus olseni* has been reported in abalone, *Haliotis ruber* and *Haliotis laevigata* in Australia (Lester and Davis 1981, Goggin and Lester 1995), in the pearl oyster *Pinctada maxima* (Norton et al. 1993) in Australia and in clams, *Austrovenus stutchburyi* in New Zealand (Dungan et al. 2007). *Perkinsus olseni* has been blamed for the death of carpet shell clams, *Ruditapes decussatus,* in Portugal (Azevedo 1989) and Spain (Villalba et al. 2005) and manila clams, *R. philippinarum,* in Spain (Santmartí et al. 1995), and has additionally been reported in Italy (Da Ros and Cazonier 1985). *Perkinsus olseni* also infects the manila clam in South Korea (Choi and Park 1997, Park et al. 1999, Park and Choi 2001) and Japan (Hamaguchi et al. 1998, Dungan and Reece 2006). *Perkinsus olseni* was also recently detected in the undulated surf clam, *Paphia undulata*, from the Gulf of Thailand (Leethochavalit et al. 2004), in cockles, *Austrovenus stutchburyi*, in New Zealand (Dungan et al. 2007), and in the venus clam, *Protothaca jedoensis*, in Korea (Park et al. 2006). *Perkinsus chesapeaki* and *P. andrewsi* have also been recently synonymized (Dungan et al. 2002). *Perkinsus chesapeaki* has been described in the soft clam, *Mya arenaria* (McLaughlin et al. 2000) and in *Tagelus plebius* in Chesapeake Bay (Dungan et al. 2002). *Perkinsus chesapeaki* has also been reported in *Macoma baltica* (Coss et al. 2001) in Delaware Bay. *Perkinsus qugwadi* was described in the Japanese scallop, *Patinopecten yessoensi* (Blackbourn et al. 1998), although

hypnospores of this parasite do not enlarge in thioglycollate fluid media (Blackbourn et al. 1988), a characteristic of all other *Perkinsus* spp., and molecular evidence further suggests that *P. qugwadi* may be only a distant relative of other *Perkinsus* species. *Perkinsus mediterraneus* has been described in the European flat oyster, *Ostrea edulis* (Casas et al. 2004) and *P. honshuensis* was recently reported in *R. philippinarum* in southern Japan (Dungan and Reece 2006) where it co-exists with *P. olseni.*

The life cycle of all *Perkinsus* spp. consists of three stages, though the cell morphology may vary slightly between species. Intraspecific morphological variation may be dependent on host (Goggin and Lester 1995). The smallest *Perkinsus* spp. cells are uninucleate, round immature trophozoites usually found within the hemocytes of infected invertebrate hosts. When immature trophozoites become mature they develop a vacuoplast, grow in overall size and gain a large, eccentric vacuole. This morphology is classically called a "signet ring." In addition, the nucleolus enlarges and becomes visible under a light microscope (Perkins 1996). In vivo, trophozoites divide by palintomy forming a meront containing 2--64 cells, however *P. marinus* grown in ODRP-3 or DME:Ham's F12 medium may divide solely by binary fission (Perkins 1996, JAM personal observation). The cell wall of mature meronts ruptures to release immature trophozoites. In seawater, mature trophozoites enlarge, the cell wall thickens, the vacuoplast disappears and the eccentric vacuole enlarges, forming hypnospores or prezoosporangia. In seawater, it has been shown experimentally that *Perkinsus* spp. may undergo zoosporulation inside the enlarged cell wall often creating 4--32 or more immature zoospores within a zoosporangium. Zoosporulation of *P. chesapeaki* and *P. olseni* is commonly observed when cultured in DME:Ham's media (Burreson et al. 2005, Dungan et al. 2007). Zoospores develop biflagellated flagella and are released through a

discharge tube on the surface of the zoosporangia. It is assumed that the zoospores eventually lose their flagella and become rounded cells (Perkins 1996) because flagellated *Perkinsus* cells have never been seen infecting a host. Trophozoites of all *Perkinsus* species except for *P. qugwadi* may be induced to form zoosporangia in vitro when incubated in thioglycollate medium. In thioglycollate medium, trophozoites enlarge and changes in the cell wall enable staining with Lugol's iodine. This is the basis for the Ray's fluid thioglycollate media (RFTM) assay routinely used for *Perkinsus* diagnosis. Under laboratory conditions, all life stages, trophozoites, hypnospores and zoospores of *P. marinus* have been shown to cause infection in oysters (Chu 1996).

The links between temperature and salinity associated with *P. marinus* infection dynamics have been well studied; however, it is also thought that the same environmental parameters similarly influence *P. olseni.* Warm water temperatures, 20--25 °C, and high salinity >15 ppt, have been shown to cause maximal proliferation of *P. marinus* parasite cells, as well as highest incidences of infection in oysters, as the rate of infection in nature is believed to be proportional to the number of waterborne infective cells (Andrews and Hewatt 1957, Chu et al. 1994). *Perkinsus marinus* cells can survive in over winter conditions of temperatures as low as 4 °C and a salinity of 4 ppt (Chu and Greene 1989, Ragone Calvo and Burreson 1994, Chu 1996). Due to the effects of salinity and temperature on the parasite, the infections caused by *P. marinus* are generally seasonal in the Chesapeake Bay. Oyster mortality rates in the parts of the bay with >12 ppt salinity begin to increase in early August, with *P. marinus* maximum prevalence peaking in September and a minimum prevalence observed during the winter with little to no observed mortality during the winter months. Mortality of hosts depends on the level of infection, with an apparent dose of 10--10^2 cells being enough to establish infection

(Chu 1996). Infection intensity has also been linked to oyster size. Smaller oysters usually will not acquire infections during their first summer of *P. marinus* exposure, presumably because of their inherent reduced filtering ability when compared to more mature oysters, resulting in the smaller oysters failing to accumulate enough cells to cause infection (Burreson and Ragone Calvo 1996).

Maximal *Perkinsus olseni* proliferation, in vitro, occurs at temperatures between 15--32 °C and salinities between 25--35ppt though cells may tolerate more extreme conditions in vivo (Ordas and Figueras, 1998, Casas et al. 2002). In a 5 yr study conducted in Spain, there was an annual pattern of *P. olseni* infection of *Ruditapes decussatus.* Lower mean infection intensity and prevalence was observed in winter and higher infection intensity and prevalence was observed from spring to autumn. The temporal pattern of parasite development was significantly associated with the seawater temperature; maximum infection intensity occurred when seawater temperature was >15 °C. Mortality of *R. decussatus* peaked in spring and summer, after peaks of high parasite infection intensity and coinciding with high seawater temperature. It has been suggested that, like *P. marinus* infections in *C. virginica*, *P. olseni* infection in clams is related to age or size, with parasite cells only detectable in clams >20 cm (>1yr old) (Villalba et al. 2005).

Dissertation research summary

The initial parasite survey of *C. ariakensis* and sympatric bivalve shellfish species highlighted the existence of both viral and protozoan pathogens that could pose a severe risk to native Bay bivalve species. Molecular and histological characterization of a new Chinese *Perkinsus* species was followed by laboratory experiments undertaken to examine the potential for transmission of this exotic *Perkinsus* species to the native

eastern oyster and the hard clam, *Mercenaria mercenaria.* Pure cultures of *P. olseni* as well as cells harvested from naturally-infected clam tissues were used in direct inoculation experiments and a bath challenge experiment to additionally look at the potential for transmission and pathogenicity of this *Perkinsus* species to local Bay bivalves. Results of *Perkinsus* spp. transmission experiments suggested that *C. virginica* and *M. mercenaria* may be susceptible to both of these exotic *Perkinsus* species.

Chapter 1. Survey of *Crassostrea ariakensis* and other oyster species in Asia for *Perkinsus* spp., molluscan herpesviruses, and other parasites: implications for non-native oyster introduction to Chesapeake Bay

ABSTRACT

With the drastic decline of eastern oyster, *Crassostrea virginica*, populations in the Chesapeake Bay due to over-fishing, diseases and habitat destruction, there is interest in Maryland and Virginia in utilizing the non-native oyster species, *Crassostrea ariakensis,* for aquaculture, fishery resource enhancement, and ecological restoration. The International Council for the Exploration of the Sea (ICES) recommends that non-native species be examined for ecological, genetic and disease relationships in the native range prior to a deliberate introduction to a new region. Therefore, a pathogen survey of *C. ariakensis* and other sympatric oyster species was conducted on samples collected in the People's Republic of China, Japan and Korea, using molecular diagnostics and histopathology. Molecular assays focused on two types of pathogens: protistan parasites in the genus *Perkinsus* and herpesviruses, both with known impacts on commercially important molluscan species around the world, including in Asia. PCR amplification and DNA sequence data from the internal transcribed spacer region of the ribosomal RNA gene complex revealed the presence of two *Perkinsus* species not currently found in USA waters: *Perkinsus olseni* and an undescribed *Perkinsus* species. In addition, molecular analyses revealed three different strains of molluscan herpesviruses in oysters from

several potential *C. ariakensis* broodstock acquisition sites in Asia. Viral gametocytic hypertrophy, *Chlamydia*-like organisms, a *Steinhausia*-like microsporidian, *Perkinsus* sp., *Nematopsis* sp., ciliates, and cestodes were also detected by histopathology.

INTRODUCTION

The eastern oyster, *Crassostrea virginica,* is important both economically and ecologically in the Chesapeake Bay. Oyster populations have been in a severe state of decline in recent decades due to combined effects of over-harvesting, habitat loss and disease pressures from marine pathogens (Mann et al. 1991). In the State of Virginia, oyster landings exceeding 6 million bushels in the 1930s have declined to less than 20,000 bushels since the 1990s (National Research Council 2003, Allen 2005). In 1995, a Virginia General Assembly resolution requested that the Virginia Institute of Marine Science determine the appropriate legal process for, and examine the feasibility of, introducing a non-native oyster species to enhance ecological benefits and revitalize the oyster industry in the Chesapeake Bay region. Initial results of research using the Pacific oyster, *Crassostrea gigas*, which has been successfully introduced at several locations around the world, suggested that this oyster species would not perform well in the Chesapeake Bay (Calvo et al. 1999). The search for another non-native oyster species that might be more suitable for the regional conditions was therefore initiated.

Considerable interest has recently focused on the Suminoe oyster, *Crassostrea ariakensis*, which resembles the native oyster, *Crassostrea virginica,* in taste (Grabowski et al. 2003), and is tolerant of temperate to sub-tropical water temperatures, and variable salinities. Field trials conducted in Virginia waters have documented lower mortality and faster growth by the Suminoe oyster, as compared with the native oyster. Disease surveys of triploid Suminoe oysters deployed in these side-by-side trials also suggest that,

in comparison to the native eastern oyster, *C. ariakensis* is relatively resistant to infections of *Haplosporidium nelsoni* and *Perkinsus marinus* (Calvo et al. 2001, Orner 2005), the two major parasites that have decimated *C. virginica* oyster populations in Chesapeake Bay since the 1950s (Sindermann 1990).

Based on significant impacts that non-native introductions of various aquatic species have had around the world, the International Council for the Exploration of the Seas (ICES) developed a Code of Practice on Introductions and Transfers of Marine Organisms (ICES 2005). The code describes a series of protocols to be followed prior to introductions of exotic animals. A thorough review of the ecological, genetic and disease impacts on native bivalves of the proposed introduced species in its natural range and in donor locations is recommended. These, and similar recommendations from other organizations, were the impetus behind a survey that we conducted on the parasites of *Crassostrea ariakensis* and other sympatric oysters in the *C. ariakensis* native range of China, Japan and Korea.

Previous research has documented the harmful impact of both *Perkinsus* spp. and herpes-like viruses on molluscan species in Asia (Choi and Park 1997, Park and Choi 2001, Chang et al. 2005). This fact, along with the ready availability of molecular assays for these organisms (Casas et al. 2002, Renault et al. 2000a), prompted the specific screening for these pathogens in Asian oyster populations. Since the discovery of *P. marinus* in *Crassostrea virginica* oysters along the Gulf of Mexico and Atlantic coasts of the USA in the late 1940s and early 1950s (Ray 1952, Mackin et al. 1950), *Perkinsus* spp. parasites have been found worldwide, and many are reported to cause disease in commercially important mollusc species. *Perkinsus marinus* has garnered recognition for its devastating effects on Atlantic and Gulf of Mexico USA oyster populations (Andrews

and Hewatt 1957, Burreson and Ragone Calvo 1996), and in the Chesapeake Bay region, *P. chesapeaki* (= *P. andrewsi*) (Burreson et al. 2005) has been associated with disease outbreaks in *Mya arenaria* and *Tagelus plebeius* (Dungan et al. 2002). Clam and oyster mortalities have also occurred in conjunction with *Perkinsus* spp. infections on the Atlantic and Mediterranean coasts of Europe (Da Ros and Cazonier 1985, Azevedo 1989, Figueras et al. 1992, Santmartí et al. 1995, Montes et al. 2001, Villalba et al. 2005), in Australia (Lester and Davis 1981, Goggin and Lester 1995) and in Korea (Choi and Park 1997, Park and Choi 2001).

In addition to *Perkinsus* spp. parasites, herpes-like viruses and herpesviruses can be a devastating problem leading to severe economic losses, particularly in hatcheries where they can cause massive mortality in larvae and juvenile oysters (Hine et al.1992, Le Deuff et al. 1994, Arzul et al. 2001, Friedman et al. 2005). Numerous cases of herpes-like viruses affecting commercial marine molluscs have been reported around the world, the earliest in 1972 in *Crassostrea virginica* in Maine, USA (Farley et al. 1972). The herpes-like viruses have been reported in the Pacific oyster, *C. gigas* (Hine et al. 1992, Nicolas et al. 1992, Renault et al. 1994, Friedman et al. 2005), the European flat oyster *Ostrea edulis* (Comps and Cochennec 1993, Renault et al. 2000a), the Australian flat oyster, *Ostrea angasi* (Hine and Thorn 1997), larvae of the Chilean oyster, *Tiostrea chilensis,* in New Zealand (Hine et al. 1998), the European carpet shell clam, *Ruditapes decussatus* (Renault et al. 2001), the Manila clam, *Ruditapes philippinarum*, (Renault 1998) and the scallop, *Pecten maximus,* in France (Arzul et al. 2001). A similar herpes-like virus may be responsible for mortality events in abalone, *Haliotis diversicolor supertexta,* in Taiwan (Chang et al. 2005).

Unlike the larvae and some juvenile oysters, adult *Crassostrea gigas* oysters appear capable of surviving asymptomatically with OsHV-1 infections (the original strain of mollusc herpesvirus sequenced from French *C. gigas*) (Arzul et al. 2002) and prior to death, no gross physiological signs are detectable in the infected individuals. Histopathological signs indicative of infection include enlarged and abnormally shaped nuclei and abnormal chromatin patterns throughout the connective tissue (Renault et al. 1994). The experience in French hatcheries has shown that this pathogen is likely to be vertically transmitted from broodstock to progeny, and can be very difficult to eradicate from facilities (Arzul et al. 2001). Consequently, if introduced along with *C. ariakensis*, molluscan herpesviruses could have a devastating impact on the remaining *C. virginica* populations in Chesapeake Bay, and on the growing aquaculture industry in the USA mid-Atlantic region.

In accordance with the ICES recommendation that non-native species be examined for disease in their native range prior to a deliberate introduction into a new region, a pathogen survey was conducted of *C. ariakensis* and other sympatric oyster species on samples collected in China, Japan and Korea. *Perkinsus* sp. and other metazoan parasites were observed by histology peformed by the VIMS pathology laboratory. Molecular diagnostics developed to target *Perkinsus* spp. and OsHV-1 identified two *Perkinsus* species not currently found in USA waters (*Perkinsus olseni* and an undescribed *Perkinsus* species), as well as three genetic variants of molluscan herpesviruses that are highly similar in DNA sequence to OsHV-1 in the genomic region analyzed. It should be noted that recent studies using these same molecular diagnostic assays have detected the two endemic *Perkinsus* species, *P. marinus* and *P. chesapeaki* (Burreson et al. 2005, Audemard et al. 2006), however no evidence of molluscan

herpesviruses has been detected previously in bivalve hosts along the US east and Gulf coasts (Friedman et al. 2005).

MATERIALS AND METHODS

Sample collection and preparation. *Crassostrea ariakensis* and, inadvertently, several other oyster species samples (see below), were collected from 23 coastal sites in China, Japan and Korea between 1999 and 2005 (Fig. 1 and Table 1). Additional oyster hemolymph samples were taken from hatchery-reared *C. ariakensis* including F1 northern China *C. ariakensis* (NCA) spawned from broodstock collected from the Yellow River in China in 1999, F1 southern China *C. ariakensis* (SCA) spawned from broodstock collected from the Dafen River in China in 1999, and west coast *C. ariakensis* (WCA), spawned from broodstock imported to VIMS from Washington, USA in 1999. Adductor muscle, mantle, gill tissue and/or hemolymph from each individual were preserved in either DMSO (25 mM EDTA, 20% DMSO and saturated NaCl) or 95% ethanol for DNA extraction and PCR analysis. When samples were additionally preserved and processed for histological analysis (Table 1), a sterile blade was used to excise a transverse tissue section through the visceral mass, and histological samples were fixed in Davidson's solution (Shaw and Battle 1957). Because of the large size of the oysters in the 2002 Chinese samples, two tissue sections were preserved for each oyster, one that included digestive gland, gill and mantle, and one that included adductor muscle, heart and kidney. Paraffin-infiltrated histological tissues were embedded, sectioned at 5--6 μm thickness, and sections were stained with Mayer's hematoxylin and eosin for microscopic analyses. Examinatin of hematoxylin and eosin stained tissue

sections from those oysters collected in 2002 were performed by members of the VIMS oyster pathology laboratory.

Nucleic acid extraction. Genomic DNA was extracted individually for each oyster, from excised mantle and gill snips, using the DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA) following manufacturer's protocols. Hemolymph samples were centrifuged at 16,000 x g for 5 min and precipitated floccules were then subjected to DNA extraction with the DNeasy® Tissue Kit. DNA was eluted in 50--200 µl of elution buffer.

Test for amplifiable DNA. In order to assure that amplifiable DNA was present in all extracted samples, genomic DNAs were tested using universal small subunit ribosomal RNA (SSU-rRNA) gene primers 16S-A (5′-CCGAATTCGTCGACAACCTGGTTGATCCTGCCAGT-3′) and 16S-B (5′-GGATCCAAGCTTGATCCTTCTGCAGGTTCCCTAC-3′) (modified from Medlin et al. 1988) with an expected amplification product of approximately 1,800 bp. Each PCR reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.75 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5 µM of each primer, 0.0125 U μl^{-1} *Taq* polymerase, 0.2 mg ml^{-1} bovine serum albumin (BSA), and 0.5 µl genomic DNA (10--50 ng total). Amplifications were performed with an initial denaturation of 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 45 °C for 30 s, 65 °C for 2 min, with a final elongation at 65 °C for 2 min. Following amplification, 3 µl of PCR product was analyzed by agarose gel electrophoresis (2%), stained with ethidium bromide and visualized under UV light. Images were recorded with an Alpha Innotech FlourChem® (San Leandro, CA) imaging system.

Genus-specific *Perkinsus* spp. PCR assay. Screening for *Perkinsus* spp. DNA was performed using *Perkinsus* genus-specific primers that target the internal transcribed spacer (ITS) region of the rRNA gene complex, modified slightly from Casas et al. (2002); PerkITS-85 (5′-CCGCTTTGTTTGGATCCC-3′) and 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 1.0 μM, 0.025 U μl^{-1} *Taq* polymerase, 0.05 mg ml^{-1} BSA, and 0.5 μl genomic DNA (10--50 ng total). Amplifications were performed with an initial denaturation of 95 °C for 4 min followed by 40 cycles of: 95 °C for 1 min, 53 °C for 1 min, 65 °C for 3 min, with final elongation at 65 °C for 5 min. Following amplification, 4 μl of PCR product was analyzed as described above.

Molluscan herpesvirus PCR assay. The 'A' region of the molluscan herpesvirus genome encoding a gene of unknown function (Batista et al. 2007) was amplified using nested 'A' region primer pairs (Renault et al. 2001). First a product of approximately 1,000 bp was amplified, followed by an approximate 900 bp product in the nested reaction. For the A3 (5′-GCCAACCGTTGGAACCATAACAAGCG-3′) / A4 (5′-GGGAATGAGGTGAACGAAACTATAGACC 3′) primer pair (external primers), the PCR reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.0 mM $MgCl_2$, BSA 0.4 mg ml^{-1}, 0.8 mM of each dNTP, 0.24 uM of each primer, *Taq* polymerase at 0.24 U μl^{-1}, and template DNA at 20 ng μl^{-1}. Amplifications were performed with an initial denaturation at 94 °C for 4 min, followed by 35 cycles of: 94 °C for 4 min, 50 °C for 30 sec, and 72 °C for 30 sec, with final elongation at 72 °C for 5 min. Reaction conditions and reagent concentrations were the same for the internal amplification reaction using the A5 (5′-CGCCCCAACCACGATTTTTCACTGACCC-

3′)/A6 (5′-CCCGTCAGATATAGGATGAGATTTG-3′) primer pair; however, 0.5 μl of the initial PCR reaction after the A3/A4 amplification was used in the A5/A6 reaction in place of genomic DNA. Following amplification using the A5/A6 primers, 5 μl of PCR product was analyzed by agarose gel electrophoresis as described above.

In situ DNA probe hybridization (ISH) assays. Paraffin-infiltrated tissues from two different animals, one collected from Podi, Beihai, China, in 1999, and another from Beihai, China, collected in 2005, were cut into 5 μm tissue sections for in situ hybridization (ISH) assays. A genus-specific, 5′ digoxigenin-labeled genus-*Perkinsus* probe (Elston et al. 2004) was used to specifically target *Perkinsus* spp. SSU-rRNA sequences. Digoxigenin-labeled oligonucleotides were obtained from Operon Biotechnologies, Inc. (Huntsville AL). The ISH protocol of Stokes and Burreson (1995) was followed, with the modifications of Elston et al. (2004). Pronase at a final concentration of 0.125 mg mL^{-1} was used for permeabilization during a 30 min incubation. A probe concentration of 7 ng μl^{-1} was used for hybridization. An anti-digoxigenin antibody linked to alkaline phosphatase was used in conjunction with NBT/BCIP for colorimetric detection of bound probe. Negative controls included duplicate histological sections of all tested samples, which received hybridization buffer without probe during hybridization incubations.

PCR-RFLP identification of oyster host species. Species identification of host oyster samples was carried out using a molecular diagnostic key based on the PCR amplification and restriction enzyme digestion of the ITS-1 gene region (Cordes and Reece 2005). PCR amplifications was performed using the 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^{-1} and template DNA at 0.2 ng μl^{-1}. 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 and *Dde* II following manufacturer protocols (New England Biolabs, Inc., Beverly, MA, USA). Following amplification and digestion, 4 µl of the initial PCR product and 10 µl of the digested PCR product, respectively, 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 to those of reference oyster species for species identifications.

Cloning and Sequencing. PCR products of the *Perkinsus* spp. ITS region and those amplified by primers designed to target OsHV-1 sequences 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 using a boil-prep method followed by a PCR-based screening reaction using the M13 forward and reverse primer pairs supplied in the cloning kit, or by *Eco* RI digest following plasmid DNA isolation.

When using the *Eco* RI digest method, 4 ml of 2YT media was inoculated with transformed bacterial colonies and incubated for 12--15 h in a 37°C-water bath while shaking at 200 rpm. Plasmid DNA was purified from bacterial cultures using a Qiaprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and electrophoresed on a 2% agarose gel. *Eco* RI restriction digestions were performed in 15 µl reactions containing 3.0 µl of purified plasmid DNA, 10.2 µl of sterile distilled water, 1.5 µl of 10x reaction buffer, and 0.3 µl of *Eco* RI restriction endonuclease. Plasmid DNA was digested at 37 °C for 3 h,

and 10 µl of the digested plasmid DNA was electrophoresed on a 3% (1.5% agarose and 1.5% low melt agarose) agarose gel, stained with ethidium bromide and visualized under UV light.

For the PCR-based screening method, bacterial colonies were picked from agar plates using a sterile wooden toothpick, and inoculated into 10 µl of sterile water in 200 µl plastic strip tubes. Inoculated water samples were boiled for 4 min at 94 °C and 0.5 µl of the boiled preparation was used in a PCR reaction using the M13 forward and reverse primers as described previously (Moss et al. 2006). Following amplification with the M13 primer pair, 3 µl of PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized under UV light as described above.

Prior to 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) in order to degrade nucleotides and single-stranded DNA (primers) remaining after PCR. Five microliters of the M13 PCR product was combined with 0.5 units of SAP and 5.0 units of *Exo* I and incubated at 37 °C for 30 min, 80 °C for 15 min and 15 °C for 5 s.

Plasmid inserts or PCR products of plasmid inserts were sequenced bi-directionally, using the ThermoSequenase labeled primer cycle sequencing kit (Amersham Pharmacia, Cleveland, OH) according to methods as described previously (Reece and Stokes 2003), or the Big Dye Terminator kit (Applied Biosystems, Norwalk, CT) with M13 sequencing primers as described previously (Moss et al. 2006).

Analysis of *Perkinsus* spp. and molluscan herpesvirus sequences. *Perkinsus* spp. and molluscan herpesvirus sequences were compared to those deposited in GenBank, and those compiled previously by researchers at VIMS, using BLAST (basic

local alignment search tool) searches (Altschul et al. 1990) of the National Center for Biotechnology Information (NCBI) database.

Available ITS region sequences from *Perkinsus* spp., and 'A' fragment sequences of molluscan herpesviruses, were downloaded from GenBank and included in phylogenetic analyses of the sequences obtained in this study. Representative *Perkinsus* spp. ITS region sequences included the following: *P. marinus* AY295177--AY295186; *P. chesapeaki* AF091541, AF440466, AF440468, AY876302, AY876304, AY876306, AY876308, AY876312, AY876314; *P. olseni* AF441207--AF441211, AF441213--AF441217; *P. mediterraneus* AY487834--AY487843; *P. honshuensis* DQ516696--DQ516702 and *P. qugwadi* AF15128. Representative molluscan herpesvirus sequences included the genome sequence of OsHV-1, AY509253 and sequences AY459364 and AY459362.

Perkinsus spp. ITS region and molluscan herpesvirus sequences were aligned separately using the CLUSTAL-W algorithm (Thompson et al. 1994) in MacVector 8.0.1, with open and extend gap penalties of seven and three, respectively. Neighbor-joining and parsimony analyses of *Perkinsus* spp. ITS region sequences were conducted using PAUP*4b10.0 (Swofford 2002). Bootstrap analyses were done with 10 random additions of 100 bootstrap replicates with gaps treated as missing data. For jackknife analyses, 30% deletion was done with 10 random additions and 100 replicates with gaps treated as missing data.

RESULTS

Host identifications. The species identification of each individual was determined using the molecular genetic PCR-RFLP key developed by Cordes and Reece

(2005) (Table 1). Although only the host species *Crassostrea ariakensis* was targeted for this study, and the animals were identified by fishermen and scientists in Asia as *C. ariakensis*, many other *Crassostrea* species, and even a *Saccostrea* sp., were found among the samples (Zhang et al. 2005, Cordes and Reece 2005).

PCR-based screening results. PCR-based screening results from locations in Japan, China and Korea (Fig. 1) revealed that molluscan herpesviruses and *Perkinsus* spp. parasites are widespread in Asian populations of *Crassostrea ariakensis*, *Crassostrea hongkongensis* and other oyster species found at the same sites (Table 2). The Itoki River, Japan; Souchang River and Chengcun, China; and Kahwa and Sumjin River, South Korea sites had the highest prevalence of molluscan herpesviruses of the locations sampled (Table 2). DNA evidence of *Perkinsus* spp. infections in *C. ariakensis* and other bivalve species was seen at several Japanese and Chinese sampling sites. DNA from the undescribed *Perkinsus* species was detected in all samples collected between 1999 and 2005 from sites in southern China indicating that the parasite is endemic to that region (Table 2).

Perkinsus marinus DNA was detected in Virginia Institute of Marine Science (VIMS), USA hatchery stocks of *Crassostrea ariakensis*, however, none of the oysters screened from the VIMS hatchery were PCR-positive for molluscan herpesviruses (Table 2).

Histological screening results. The viral, bacterial, protistan and metazoan parasites detected in oysters by histopathology at eight sites sampled in 2002 are listed in Table 3 and illustrated in Fig. 2 and Fig. 3. Most parasites were uncommon. Although molluscan herpesvirus DNA was detected by PCR in 4 of 37 animals screened from one of the samples also examined by histology, no histological evidence of herpes viral

Table 1. Sampling sites, abbreviations used, sample sizes, bivalve species present, analyses performed and general results (+ or -) of assays. Sampling sites in China are ordered from northern to southern locations, and are listed chronologically for multiple collections from a single site. Positive (+) assay results in the molecular assays indicate that either molluscan herpesvirus or *Perkinsus* sp. DNA was detected (Table 2) and for the histological analysis that a potential pathogen was detected (Table 3).

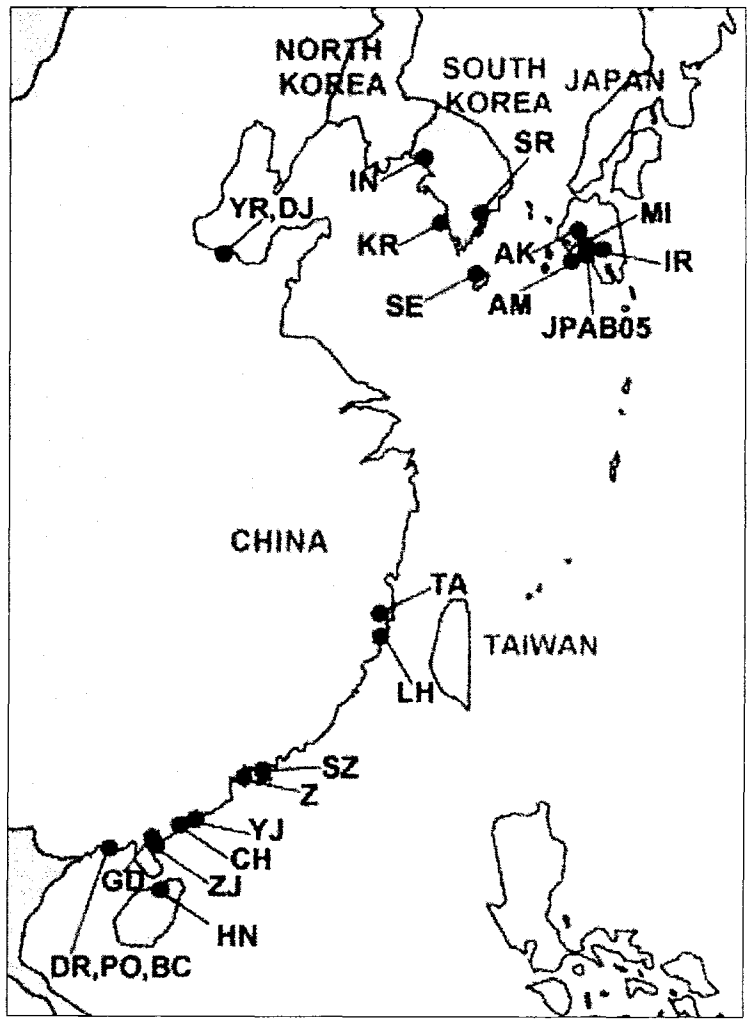
Year	Sampling Location (sample abbreviation)	Natural (N) Cultivated (C)	Sample Size (N)	Oyster Species	Assays Performed	Assay Results (+ or -)
	Japan					
1999	Itoki River, Kyushu (IR)	N	50	*C. ariakensis*	Molecular	+
2003	Ariake Sea (AK)	N	24	*C. gigas, C. sikamea*	Molecular	-
2004	Amakusa (AM)	N	60	*C. gigas*	Molecular	-
2004	Midori (MI)	N	68	*C. gigas*	Molecular	-
2005	Mie Prefecture (JPAB05)	C	217	*C. ariakensis, C. sikamea, C. gigas*	Molecular	+
	South Korea					
2004	Seogwipo (SE)	unknown	161	*Saccostrea* sp., *C. gigas*, unknown sp.	Molecular	-
2004	Kahwa River (KR)	unknown	35	*C. ariakensis, C. gigas*	Molecular	+
2004	Sumjin River (SR)	unknown	20	*C. ariakensis*	Molecular	+
2004	Kanghwa Island, Inchon (IN)	unknown	20	*C. ariakensis*	Molecular	+
	People's Republic of China (PRC)					
1999	Yellow River, Bohai Sea, Shandong (YR)	N	43	*C. ariakensis*	Molecular	+
2002	Dajiawa, Shandong (DJ)	N	26	*C. ariakensis*, *C. hongkongensis*	Molecular Histological	- -

2002	Chengcun, Yanxi, Fujian (CH)	C	37	*C. ariakensis, C. hongkongensis*	Molecular Histological	+ +
2002	Longhai, Fujian (LH)	C	9	*C. hongkongensis*	Molecular Histological	- +
2002	Tong'an, Fujian (TA)	N	28	*C. ariakensis, C. hongkongensis*	Molecular Histological	- +
1999	Yamen River, Zhuhai, Guangdong (Z)	N	50	*C. hongkongensis*	Molecular	+
1999	Souchang River, Yangjiang, Guangdong (YJ)	N	50	*C. hongkongensis*	Molecular	+
2002	Shenzhen, Guangdong (SZ)	N	13	*C. ariakensis, C. hongkongensis*	Molecular Histological	+ +
2002	Guandu, Zhanjiang, Guangdong (GD_C)	C	35	*C. hongkongensis*	Molecular Histological	+ +
2002	Guandu, Zhanjiang, Guangdong (GD_N)	N	25	*C. hongkongensis*	Molecular Histological	+ +
2003	Zhanjiang, Guangdong (ZJ)	C	71	*C. ariakensis, C. hongkongensis*	Molecular	+
1999	Dafen River, Beihai, Guangxi Zhuang (DR)	N	50	*C. ariakensis, C. hongkongensis*	Molecular	+
2002	Dafen River, Beihai, Guangxi Zhuang (DR_02)	N	17	*C. ariakensis, C. hongkongensis*	Molecular Histological	- +
2002	Podi, Beihai, Guangxi Zhuang (PO)	C	39	*C. ariakensis, C. hongkongensis*	Molecular Histological	+ +
2003	Beihai, Guangxi Zhuang (BC)	N	64	*C. ariakensis, C. hongkongensis*	Molecular	+ +
2005	Beihai, Guangxi Zhuang (BC_05)	N	113	*C. ariakensis, C. hongkongensis*	Molecular Histological	+ -
2003	Lingshui, Hainan(HN)	C	19	*Pinctada margaritifera, P. martensii,* unknown sp.	Molecular	+

	USA hatchery stocks					
2000	SCA hatchery (SCA) (F1 of DR, PRC)	C	52	*C. ariakensis, C. hongkongensis*	Molecular	-
2002	NCA hatchery (NCA) (F1 of YR, PRC)	C	50	*C. ariakensis*	Molecular	+
2003	WCA hatchery (WCA)	C	49	*C. ariakensis*	Molecular	+

Figure 1. Map of sampling locations. *IR* indicates Itoki River, Kyushu, Japan; *YR*, Yellow River, Bohai Sea, China; *Z*, Yamen River, Zhuhai, Guangdong Province, China; *YJ*, Souchang River, Yangjiang, Guangdong Province, China; *DR*, Dafen River, Beihai, Guangxi Zhuang Province, China; *CH*, Chengcun, Yanxi, Fujian Province, China; *GD*, Guandu, Zhanjiang, Guangdong Province, China; *SZ*, Shenzhen, Guangdong Province, China; *PO*, Podi, Beihai, Guangxi Zhuang Province, China; *DJ*, Dajiawa, Shandong Province, China; *LH* Longhai, Fujian Province, China; *TA* Tong'an, Fujian Province, China; *SE*, Seogwipo, South Korea; *KR*, Kahwa River, South Korea; *SR*, Sumjin River, South Korea; *IN*, Kanghwa Island Inchon, South Korea; *HN* Lingshui, Hainan, China; *ZJ* Zhanjiang, Guangxi Zhuang Province, China; *BC, BC05*, Beihai, Guangxi Zhuang, China; *AK, JPAB05*, Ariake Sea, Japan; *AM* Amakusa, Japan; *MI* Midori, Japan.

Figure 1.



infection was observed. In addition to the parasites listed in Table 3, a sample of 33 *Crassostrea hongkongensis* from southern China collected in 2003 had 18 of 26 female oysters (69%) infected with a *Steinhausia*-like microsporidian in the ova (Fig. 2D). Unfortunately, the exact source location for this sample is unknown.

Among the 17 oysters sampled from Beihai, China, in 2005 that tested positive for *Perkinsus* sp. infections by PCR, only one showed lesions typical of *Perkinsus* sp. when examined histologically. In oyster BC05Ca-20, numerous mature *Perkinsus* sp. signet ring trophozoites of 5--10 μm diameters, subdividing 5--10 μm diameter schizonts and clusters of multiple immature sibling trophozoites of 3--4 μm diameters occurred (Fig. 3). *Perkinsus* sp. parasite cells (552) enumerated in one section occurred in multi-focal stomach epithelium lesions (43%), rare lesions in digestive gland epithelia, among connective tissues of the mantle (27%), visceral mass (15%), and gills (10%) and systemically circulating both free and phagocytosed within host hemocytes, in the oyster vasculature (3%).

ISH results. In situ hybridization probes designed to target *Perkinsus* species cells hybridized only to *Perkinsus* sp. cells in oyster samples positive for *Perkinsus* sp. DNA by PCR analysis. Figure 3A and B shows two consecutive sections from the *Crassostrea hongkongensis* from Beihai, China, BC05Ca-20, described above, infected with the undescribed *Perkinsus* species. Figure 3A is an H&E-stained section showing *Perkinsus* sp. cells in the stomach epithelium of the oyster. Figure 3B shows positive *in situ* hybridization with the genus-specific probe to *Perkinsus* sp. cells. The *Perkinsus* genus-specific probe labeled cells in connective tissues of the gills and mantle, as well as in the epithelia of intestine and stomach. In situ hybridization reactions conducted without probe produced no signal in tissues of the same host oyster.

Table 2. Prevalence data for *Perkinsus* spp. and molluscan herpesvirus (HV) pathogens based on molecular assays. Samples where either one or both of these pathogens were detected are listed, all other samples were negative in both assays. The bivalve species infected with either pathogen at a sampling site are indicated. The symbol "n/d" indicates that identification of the *Perkinsus* sp. was not done. *No DNA remained for species identification of one oyster with *Perkinsus* sp. DNA in this sample.

Year	Sample	N	# HV positive	HV prevalence (%)	# *Perkinsus* sp. positive	*Perkinsus* sp. prevalence (%)	*Perkinsus* sp.	Bivalve spp.
	Japan							
1999	Itoki River, Kyushu (IR)	50	13	26.0	9	18.0	*P. olseni*	*C. ariakensis*
2005	Mie Prefecture (JPAB05)	170	5	2.9	0	-		*C. ariakensis*
		29	2	6.9	0	-		*C. gigas*
		45	2	4.4	0	-		*C. sikamea*
	South Korea							
2004	Kahwa River (KR)	35	10	28.6	0	-		*C. ariakensis*
2004	Sumjin (SR)	20	8	40.0	0	-		*C. ariakensis*
2004	Kanghwa Island, Inchon (IN)	20	1	5.0	0	-		*C. ariakensis*
	China							
1999	Yellow River, Bohai Sea, Shandong (YR)	43	0	-	2	4.7	*P. olseni*	*C. ariakensis*
2002	Tong'an, Fujian (TA)	28	0	-	1	3.6	undescribed	*C. ariakensis*
2002	Chengcun, Yanxi, Fujian (CH)	37	4	10.8	6	16.2	undescribed	*C. hongkongensis*

1999	Yamen River, Zhuhai, Guangdong (Z)	50	5	10.0	5	10.0	*P. olseni*	*C. hongkongensis*
1999	Souchang River, Yangjiang, Guangdong (YJ)	50	10	20.0	1	2.0	*P. olseni*	*C. hongkongensis*
2002	Shenzhen, Guangdong (SZ)	13	0	-	1	7.6	undescribed	*C. hongkongensis*
2002	Guandu, Zhanjiang, Guangdong, (GD_C)	35	0	-	4	11.4	undescribed	*C. hongkongensis*
2002	Guandu, Zhanjiang, Guangdong (GD_N)	25	0	-	3	12.0	undescribed	*C. hongkongensis*
2003	Zhanjiang, Guangdong, (ZJ)	1	0	-	1	100.0	undescribed	*C. ariakensis*
		70	0	-	32	45.7	undescribed	*C. hongkongensis*
1999	Dafen River, Beihai, Guangxi Zhuang (DR)	26	0	-	9	34.6	undescribed	*C. ariakensis*
		24	1	4.1	2	8.3	undescribed	*C. hongkongensis*
2002	Podi, Beihai, Guangxi Zhuang (PO)	14	0	-	2*	14.3	undescribed	*C. ariakensis*
		24	0	-	2*	8.3	undescribed	*C. hongkongensis*
2003	Beihai, Guangxi Zhuang (BC)	59	0	-	11	18.6	undescribed	*C. hongkongensis*
2005	Beihai, Guangxi Zhuang (BC_05)	12	0	-	4	33.3	undescribed	*C. ariakensis*
		101	0	-	36	35.6	undescribed	*C. hongkongensis*
2003	Lingshui, Hainan (HN)	19	0	-	12	63.2	undescribed	*Pinctada margaritifera, P. martensii,* unknown

								sp.- not distinguished
2002	VIMS NCA	49	0	-	10	20.4	*P. marinus*	*C. ariakensis*
2003	VIMS WCA	50	0	-	1	2.0	*P. marinus*	*C. ariakensis*

Table 3. Histological analysis of oysters collected at seven locations in China in 2002.

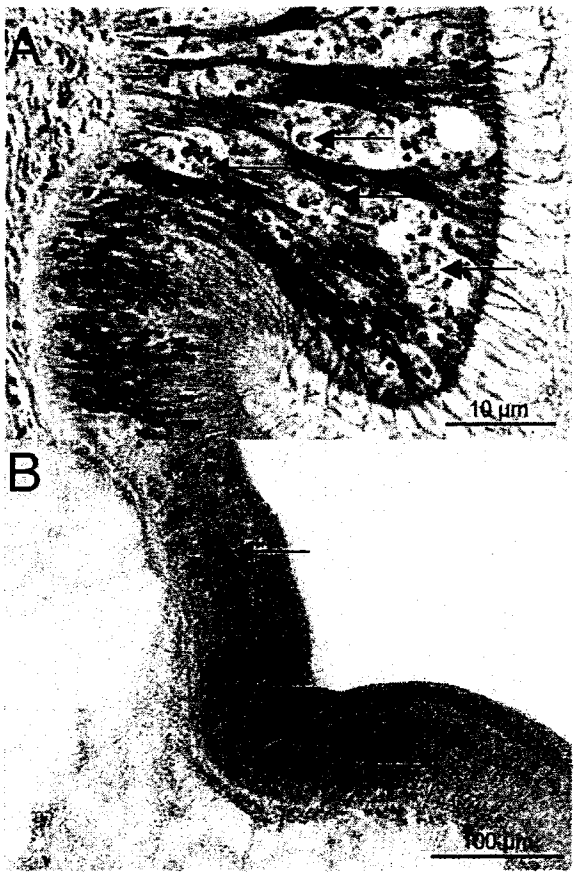
Sampling Location	N	Natural (N), Cultured (C)	Viral gametocytic hypertrophy	*Chlamydia*-like organisms	*Perkinsus* sp.	Ciliates	*Nematopsis* sp.	Cestodes
Dajiawa, Shandong	29	N	0	0	0	4	0	0
Chengcun, Yangxi, Fujian	31	C	0	1	1	2	0	1
Longhai, Fujian	28	C	0	3	0	2	0	0
Tong'an, Fujian	29	N	0	1	0	2	11	1
Shenzhen, Guangdong	26	N	0	1	0	1	0	0
Guandu, Zhanjiang, Guangdong	30	N	1	1	0	0	9	0
Guandu, Zhanjian, Guangdong	60	C	1	6	3	0	14	0
Dafen River, Beihai, Guangxi Zhuang	30	N	0	1	0	2	0	6
Podi, Beihai, Guangxi Zhuang	30	C	0	2	2	2	0	5

Fig. 2. Parasites observed by histopathology in *Crassostrea* sp. in China. (A) Viral gametocytic hypertrophy (arrow) in gonad of wild *C. hongkongensis* from Guandu, Zhanjiang, China (B) *Chlamydia*-like inclusions (arrows) in digestive tubules of wild *C. hongkongensis* from Guandu, Zhanjiang, China (C) Ciliates (arrows) attached to gill epithelium in cultured *C. hongkongensis* from Chengcun, Yanxi, Fujian, China (D). *Steinhausia*-like microsporidian in ovum of wild *C. hongkongensis* from southern China. (E) Spores of *Nematopsis*-like gregarines (arrows) in cultured *C. hongkongensis* from Guandu, Zhanjiang, China. (F) Encapsulated metacestode (arrow) in gill tissue of wild *C. hongkongensis* or *C. ariakensis* from Dafen River, Beihai, Guangxi Zhuang, China.

Figure 2.

Figure 3. (A) H&E-stained histological section of a *Crassostrea hongkongensis* oyster showing *Perkinsus* sp. cells in the stomach epithelium (arrows). (B) *In situ* hybridization with the *Perkinsus* sp. genus-specific probe to *Perkinsus* sp. cells (arrows) in the stomach epithelium.

Figure 3.



***Perkinsus* spp. ITS region sequences.** ITS region PCR amplification products were sequenced from a select number of positive oyster samples. All sequences were deposited in GenBank (Table 4). Sequencing of the ITS region amplicons from the *Perkinsus* spp. assay indicated that the Chesapeake Bay-native *P. marinus* was found in VIMS hatchery stocks of *Crassostrea ariakensis.* This is not surprising, since hatchery-reared oysters are exposed to water coming from the adjacent York River, which is endemic for *P. marinus*. ITS region sequences from oysters from the NCA and WCA hatchery stocks formed a monophyletic clade with known *P. marinus* sequences, with 100% bootstrap support in both neighbor joining distance analysis and in maximum parsimony analysis (Fig. 4, Fig. 5). *Perkinsus* sp. ITS region sequences amplified from DNAs extracted from *C. ariakensis* and *Crassostrea hongkongensis* oysters collected from several Asian sites; including the Yamen and Yellow rivers, China and the Itoki River, Kyushu, Japan, formed a monophyletic clade with known *P. olseni* ITS region sequences in both neighbor joining distance analysis, as well as maximum parsimony analysis (100% bootstrap support) (Fig. 4, Fig. 5).

Crassostrea ariakensis and *C. hongkongensis* oysters collected from locations in China along the southern coast from Tong'an, Fujian to the Dafen River, Beihai, appeared to be infected with an undescribed *Perkinsus* species. The genus-specific *Perkinsus* spp. primers amplified unique nucleotide sequence fragments of approximately 689 bp from numerous oysters. Forty-two clones were sequenced from 16 individual oysters selected from six different samples, and were 99.3% similar (uncorrected-p) to each other. Overall, 28 of the 42 clones (67%) shared a common ITS rRNA sequence, while the other 14 clones all were all unique. BLAST analyses of GenBank suggested that these were *Perkinsus* spp. sequences. Pairwise distances and molecular phylogenetic

Table 4. GenBank accession numbers associated with *Perkinsus* spp. ITS rRNA and molluscan herpesvirus (HV) sequences generated in this study.

Pathogen	Source	GenBank accession numbers
Perkinsus olseni	Itoki River, Kyushu, Japan (IR)	EF204075, EF204076
Perkinsus olseni	Yellow River, Bohai Sea, Shandong, China (YR)	EF204073, EF204074
Perkinsus olseni	Yamen River, Zhuhai, Guangdong, China (Z)	EF204070-EF204072
undescribed *Perkinsus* sp.	Tong'an Fujian, China (TA)	EF204046-EF204050
undescribed *Perkinsus* sp.	Chengcun, Yanxi, Fujian, China (CH)	EF204034, EF204035, EF204043
undescribed *Perkinsus* sp.	Shenzhen, Guangdong, China (SZ)	EF204015-EF204018, EF204029-EF204031, EF204036, EF204038, EF204039, EF204041
undescribed *Perkinsus* sp.	Guandu, Zhanjiang, Guangdong, China (GD)	EF204022, EF204051-EF204056
undescribed *Perkinsus* sp.	Dafen River, Beihai, Guangxi Zhuang, China (DR)	EF204021, EF204024-EF204028, EF204040, EF204044, EF204045
undescribed *Perkinsus* sp.	Podi, Beihai, Guangxi Zhuang, China (PO)	EF204019, EF204020, EF204023, EF204032, EF204033, EF204042
Perkinsus marinus	VIMS hatchery (WCA)	EF204008-EF204011
Perkinsus marinus	VIMS hatchery (NCA)	EF204012-EF204014
HV	Ariake Sea, Mie, Japan (JPAB05)	EF221836-EF221839
HV	Kahwa River, South Korea (KR)	EF221840
HV	Sumjin River, South Korea (SR)	EF221841
HV	Yamen River, Zhuhai, Guangdong, China (Z)	EF221843
HV	Chengcun, Yanxi, Fujian, China (CH)	EF221842
HV	Souchang River, Yangjiang, Guangdong, China (YJ)	EF221844

analyses confirmed that these sequences were closely related to those of known *Perkinsus* species. In both neighbor joining and maximum parsimony analyses, these new parasite ITS region sequences grouped with those of known *Perkinsus* spp. (Fig. 4, Fig. 5). Mean pairwise distances (uncorrected-p) of ITS region nucleotide sequences within known *Perkinsus* spp. ranged from 0.2% in the undescribed *Perkinsus* sp. to 1.6% variation in *Perkinsus chesapeaki*.

Only one ITS region nucleotide sequence was available for *Perkinsus qugwadi*, therefore mean intraspecific ITS variation could not be calculated. ITS rRNA of this new *Perkinsus* sp. was most distantly related to *P. qugwadi* (63.6%--63.7% similarity), and most closely related to the recently described species, *Perkinsus honshuensis* (89.0%--89.8% similarity) (Table 5). Nucleotide sequences from this apparent new parasite form a well-supported sister group (100% bootstrap support within this species) to the larger *Perkinsus* spp. clade that includes *P. marinus*, *P. mediterraneus* and *P. olseni* (Fig. 4, Fig. 5).

Molluscan herpesvirus sequences. Molluscan herpesvirus DNA was found in *Crassostrea ariakensis* populations at a site in the Itoki River, Ariake Bay, Japan sampled in 1999, and was also found in the 2005 samples taken from Mie Prefecture, Japan near the first sampling site. A portion of oysters collected at the Kahwa River and Sumjin River, South Korea sampling sites, as well as in the Yamen River, Souchang River, Dafen River, and Chengcun, China sites, were also positive for molluscan herpesvirus DNA (Table 3).

A subset of oysters positive for molluscan herpesvirus DNA from the Yamen River, Souchang River, Chengcun, China, and Mie, Japan, were chosen as representatives from those populations, and their herpesvirus DNA was cloned and sequenced. The

Figure 4. *Perkinsus* spp. ITS rRNA gene sequences. Neighbor joining tree determined by analysis of the ITS gene sequences of known *Perkinsus* spp. sequences and the ITS gene sequences of those *Perkinsus* spp. found in oysters collected in Japan and China. GenBank accession numbers associated with each sample from this study **(bold)** are listed in Table 4.

Figure 4.

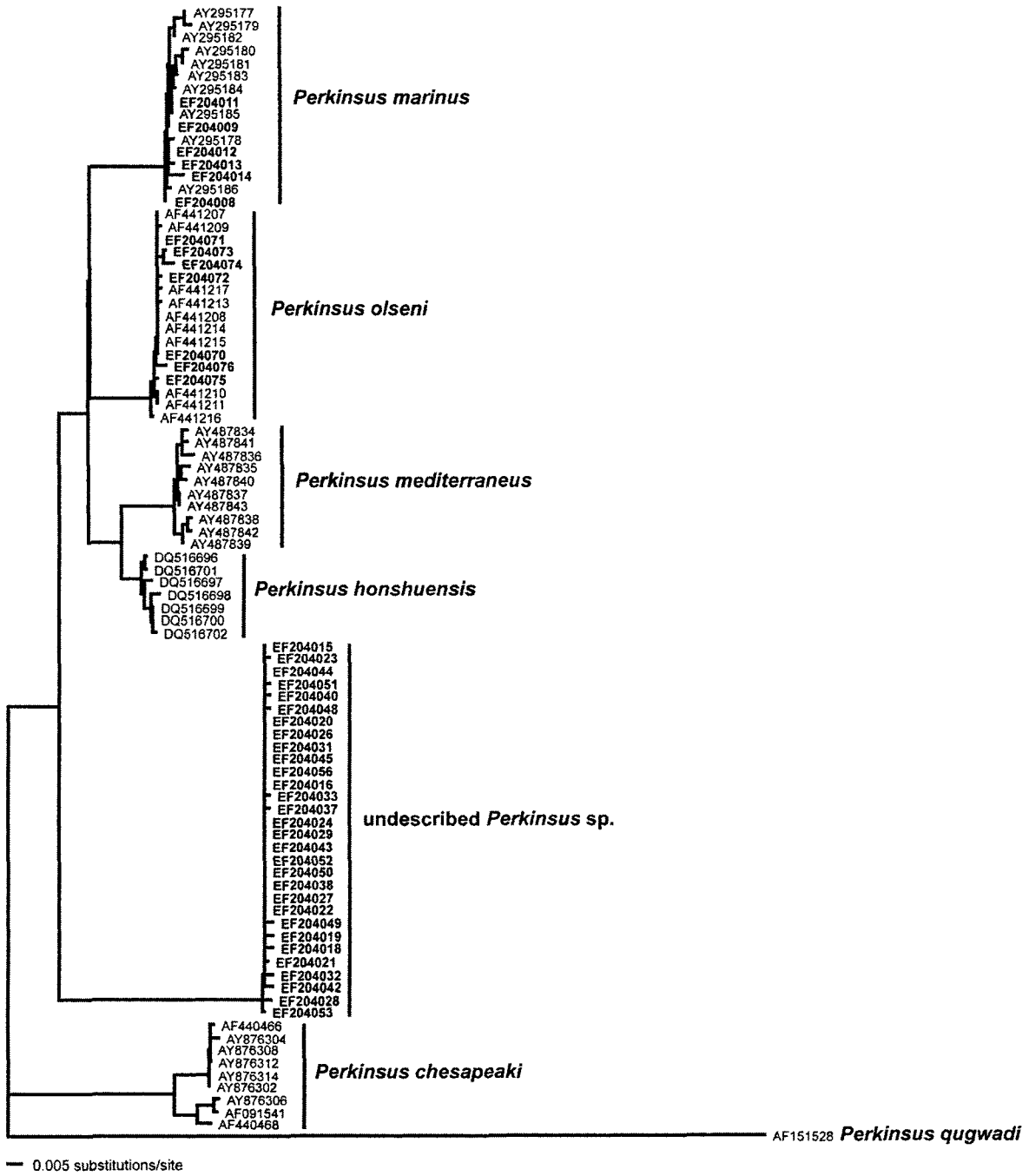


Figure 5. *Perkinsus* spp. ITS rRNA gene sequences. Maximum parsimony tree determined by analysis with 100 replicates of 10 random additions of the ITS gene sequences of the ITS gene sequences of known *Perkinsus* spp. sequences and the ITS gene sequences of those *Perkinsus* spp. found in oysters collected in Japan and China. 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. GenBank accession numbers associated with each sample from this study **(bold)** are listed in Table 4.

Figure 5.

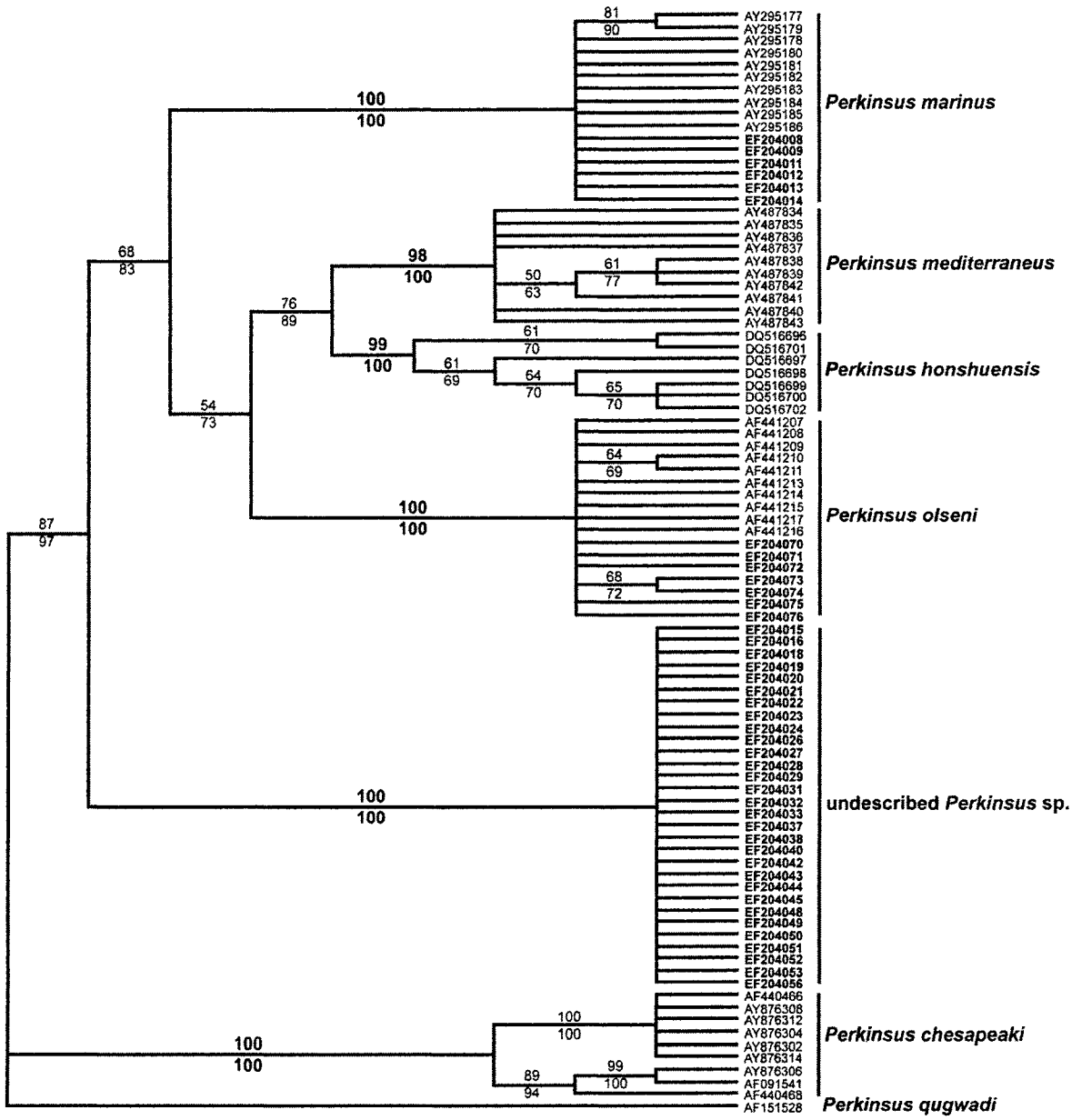


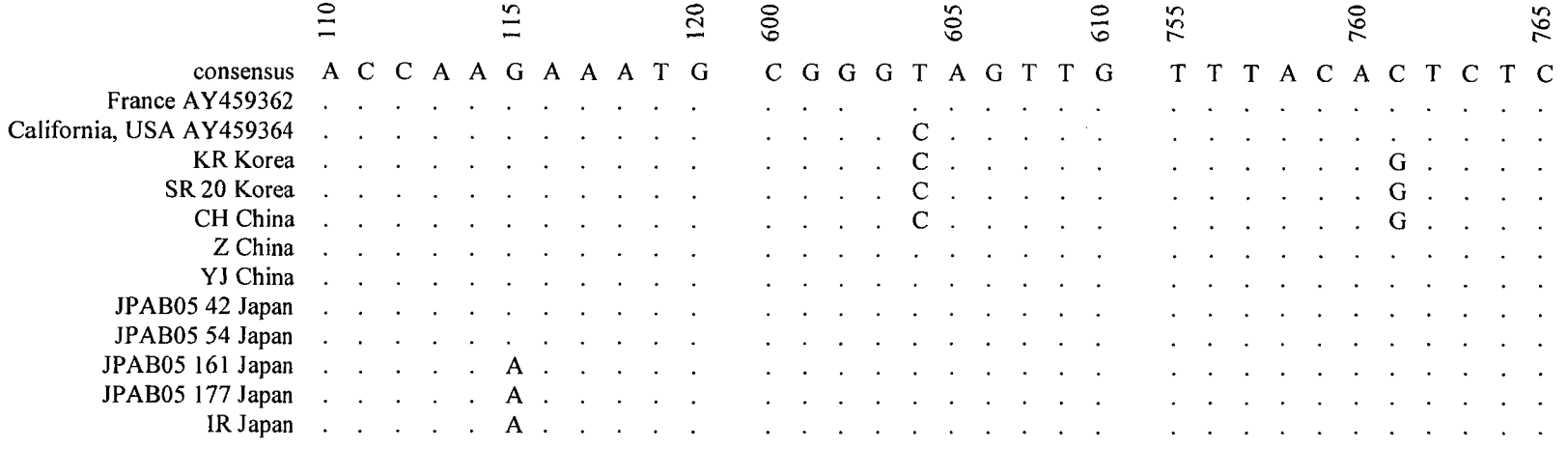
Table 5. Range of sequence similarities and pairwise distances (uncorrected '*p*' values) observed among rDNA ITS region sequences of currently accepted *Perkinsus* spp. from GenBank, and those of the undescribed *Perkinsus* sp. obtained in this study. The range of observed within species sequence distance is given across the diagonal. Raw distance value ranges between species are given above the diagonal, and ranges for percent distance values are given below. "---" Only one sequence for *P. qugwadi* was available.

Species	*P. marinus*	*P. chesapeaki*	*P. mediterraneus*	*P. olseni*	*P. honshuensis*	undescribed *Perkinsus* sp.	*P. qugwadi*
P. marinus	**0.000-0.004 0.4%**	0.119-0.139	0.053-0.069	0.049-0.063	0.052-0.062	0.112-0.126	0.323-0.330
P. chesapeaki	11.9-13.9%	**0.000-0.016 1.6%**	0.134-0.148	0.126-0.138	0.117-0.126	0.156-0.175	0.342-0.350
P. mediterraneus	5.3-6.9%	13.4-14.8%	**0.000-0.007 0.7%**	0.050-0.065	0.029-0.040	0.119-0.129	0.331-0.335
P. olseni	4.9-6.3%	12.6-13.8%	5.0-6.5%	**0.000-0.003 0.3%**	0.049-0.060	0.107-0.119	0.333-0.336
P. honshuensis	5.2-6.2%	11.7-12.6%	2.9-4.0%	4.9-6.0%	**0.000-0.006 0.6%**	0.102-0.110	0.327-0.331
undescribed *Perkinsus* sp.	11.2-12.6%	15.6-17.5%	11.9-12.9%	10.7-11.9%	10.2-11.0%	**0.000-0.002 0.2%**	0.363-0.364
P. qugwadi	32.3-33.0%	34.2-35.0%	33.1-33.5%	33.3-33.6%	32.7-33.1%	36.3-36.4%	---

molluscan herpesvirus DNA amplified from *Crassostrea ariakensis* oysters collected from the Chinese sites and from the two South Korean sites had variations in observed molluscan herpesvirus DNA sequences and polymorphic sequences were found within and between oysters from different locations. The level of polymorphism between the viral sequences was low (3.7%) with a combined total of 34 individual randomly distributed single nucleotide differences over the entire 917 bp sequence from all of the clones analyzed. Three polymorphic nucleotide sites were observed that generally had consistent polymorphisms across all individuals within a collection site, except for individuals from the 2005 Ariake Sea, Japan sampling (Fig. 6). The molluscan herpesvirus DNA amplified from the Yamen River and Souchang River, China, *C. ariakensis* were similar to those viral sequences found in French *C. gigas* (LeDeuff & Renault 1999, Renault et al. 2000b), sharing a thymine at a polymorphic site 604 bp from the 5′ end of the fragment (primers removed). The molluscan herpesvirus sequences amplified from the Chengcun, China, *C. ariakensis*, and those from both the Kahwa and Sumjin rivers, Korea, are similar to that found in *C. gigas* from Tomales Bay, California, USA (Friedman et al. 2005) and in the Itoki River, Japan *C. ariakensis*, sharing a cytosine at that particular polymorphic site. The viral DNA amplified from the Itoki River samples and from some of the 2005 Ariake Sea, Japan samples appears to have a unique polymorphism at a site 115 bp from the 5' end of the fragment, sharing an adenine residue where all other viral sequences share a guanine. From the 2005 Ariake Sea, Japan, molluscan herpesvirus PCR fragments were cloned and sequenced from three molluscan herpesvirus-positive *C. ariakensis* oysters and one positive *Crassostrea sikamea*. The DNA sequences from these oysters suggest that two of the oysters, one *C. ariakensis* and the *C. sikamea,* were infected with the same strain of molluscan

Figure 6. Alignment of molluscan herpesvirus 'A' region sequences isolated from oysters showing the polymorphic sites unique to viral sequences at each location. Viral sequences found in France and in Tomales Bay, California, USA have been deposited previously in GenBank, and accession numbers are listed accordingly.

Figure 6.



herpesvirus found in the Japanese *C. ariakensis* collected in 1999 from the Itoki River, and the other two *C. ariakensis* were infected with the strain commonly seen in France and parts of China. Viral DNAs amplified from Chengcun, China, and Korean *C. ariakensis* shared an additional polymorphic site 761 bp from the 5′ end of the A5/A6 fragment. These viral sequences share a guanine at this site, where all other viral sequences share a cytosine.

DISCUSSION

Several potential disease agents, including two different *Perkinsus* species, molluscan herpesviruses and a *Steinhausia*-like microsporidian, were detected in oysters collected from sites in Asia where potential *Crassostrea ariakensis* broodstocks for Chesapeake Bay might originate. Standard ICES protocols should minimize the risk of introducing horizontally transmitted pathogens with the host, since broodstock would be held under strict quarantine conditions, and only progeny of oysters brought to the USA would be introduced into the environment. However, neither the ICES protocols nor a mechanical procedure such as cleansing gametes to remove superficial parasites would prevent transmission of pathogens if they infect the gametes themselves. If vertical transmission of any pathogen identified in this survey occurred, they could be introduced to the Chesapeake Bay by vertical transmission from infected broodstock to F1 or F2 *C. ariakensis* progeny oysters, with the potential for serious negative impacts to already depleted native oyster populations. In addition, *C. ariakensis* could act as a reservoir host for exotic pathogens that may be introduced by other means. For example, ballast water may have been the source of a possible exotic *Bonamia* sp. that caused a severe mortality event during 2003 in *C. ariakensis* deployed in North Carolina waters (Burreson et al.

2004), but has not been documented to infect local native bivalves. The possibility of vertical transmission of viruses among aquatic organisms is recognized (Bootland et al. 1991, Lo et al. 1997, Tsai et al. 1999), and recent transmission studies in France with *Crassostrea gigas* have detected OsHV-1 in three successive generations of oysters (Barbosa-Solomieu et al. 2005). In this study, three genetic variants of molluscan herpesviruses were detected by PCR.

Two of the pathogens observed histologically pose a threat of introduction to Chesapeake Bay via infected broodstock. Viral gametocytic hypertrophy and the *Steinhausia*-like microsporidian were both observed in oyster ova. Viral gametocytic hypertrophy has been reported in *Crassostrea virginica* from Chesapeake Bay (Farley 1978), and a *Steinhausia*-like microsporidian has been reported in the clam *Macoma balthica* in Chesapeake Bay (Farley 1977). The parasites observed in Asian oysters, however, may be different strains or species than those in Chesapeake Bay, and could pose problems if introduced. There is substantial evidence for vertical transmission of some microsporidian parasites of invertebrate hosts (Kelly et al. 2003, Galbreath et al. 2004, Vizoso & Ebert 2004), therefore indicating a real possibility for vertical transmission of these Asian pathogens.

Although there is no current evidence to suggest that *Perkinsus* sp. parasites may be vertically transmitted, and therefore might not be introduced to a new area through importation of small numbers of infected broodstock that are held in quarantine, past studies have found protozoan cells that were described as "*Perkinsus*-like", but were not a true *Perkinsus* sp., in male and female scallop gonads. Subsequent infection of the larvae post-spawning was observed, suggesting the possibility of vertical transmission (Karlsson 1991, Whyte et al. 1993). ICES protocols are being followed, however, the

potential for vertical transmission of the pathogens observed in this study, and the associated risks of introducing the non-native host, must be recognized. Consistent and careful disease testing of deployed oysters may need to be conducted if an introduction occurs. It should be noted that there was no evidence of molluscan herpesviruses in the current VIMS hatchery stocks of *C. ariakensis* that were screened, and although *Perkinsus* sp. DNA was detected in these samples, it was *P. marinus* DNA, an endemic species. There is currently no evidence of *Perkinsus olseni* or the new undescribed *Perkinsus* species in the VIMS stocks.

Most parasites observed in the histological analyses conducted here (Table 3) were generally uncommon, and are unlikely to be introduced to a new area via infected broodstocks. Histological observations indicated tissue tropisms by *Perkinsus* sp. pathogens among both connective tissues and digestive system epithelia of oysters from Beihai, China. These observations are consistent with the detection of *Perkinsus* sp. DNAs in this sample, using molecular tools (see below).

Molecular detection assays identified *Perkinsus* spp. and molluscan herpesvirus DNA in many of the samples surveyed. *Perkinsus olseni* DNA was detected in several samples of Asian *Crassostrea ariakensis* and *C. hongkongensis*. *Perkinsus olseni* is known to be widely distributed among molluscs in the Pacific and eastern Atlantic oceans (Lester & Davis 1981). With the synonymization with *P. atlanticus* (Murrell et al. 2002), *P. olseni* has been reported to infect a variety of hosts from around the world (Villalba et al. 2004), including the blacklipped and the green-lipped abalone in Australia (Lester & Davis 1981), the carpet shell clam in Portugal (Azevedo 1989), and the Manila clam, in Spain, Portugal, northern China, Korea, and Japan (Dungan & Reece 2006). The discovery of *P. olseni* in *C. ariakensis, C. hongkongensis*, and *C. gigas* in Japan and

southern China expands the currently known geographic and host distribution of that parasite.

In addition to *P. olseni*, a previously undescribed *Perkinsus* species was found in populations of *C. ariakensis* and *C. hongkongensis* in southern China, as well as in some pearl oysters and unidentified bivalves. As discussed in several recent publications (Burreson et al. 2005, Reece and Dungan 2006), molecular methods are the only reliable way to distinguish *Perkinsus* spp., because host and environmental elements may influence host morphological characteristics. ITS region *Perkinsus* sp. sequences amplified from southern China host sample DNAs grouped with those sequences from other *Perkinsus* spp. However, as with each of the other species, they form a unique monophyletic clade within the genus indicating that this is a unique species. The similarity among the sequences within this clade, and the genetic distance between these sequences and those of other *Perkinsus* spp., are consistent with the distances observed in previous studies that have used the ITS region sequences to discriminate species or strains (Brown et al. 2004, Burreson et al. 2005, Dungan and Reece 2006). Sequencing of multiple gene regions is recommended to provide additional support for *Perkinsus* spp. phylogenies based on the ITS rRNA region. To date, phylogenies that were based on other regions including the large subunit ribosomal RNA and actin gene sequences have confirmed results obtained using ITS rRNA (Dungan and Reece 2006, Dungan et al. 2007).

More than 30 years ago Farley (1972) reported, based on transmission electron microscopy, a herpes-like virus infecting *Crassostrea virginica* oysters from Maine. A recent survey of oysters from the Atlantic, Gulf of Mexico, and Pacific coasts of the USA, which used molecular diagnostic tools designed originally to detect OsHV-1,

indicated that a herpes-like virus is currently found only on the Pacific coast of the United States in Tomales Bay, California and not along the US East or Gulf coasts (Friedman et al. 2005). Molluscan herpes-like viruses also occur in France (Nicolas et al. 1992, Comps and Cochennec 1993, Renault et al. 1994, Renault et al. 2000a, Renault et al. 2001, Arzul et al. 2002), Australia (Hine and Thorn 1997), New Zealand (Hine and Thorn 1997, Hine et al. 1998), and Taiwan (Chang et al., 2005). Previously, at least two genetic strains of molluscan herpesviruses were described; with DNA sequence polymorphisms in the 'A' region distinguishing between the original strain (OsHV-1) found in France, another French variant was described later (Arzul et al. 2001) and a recently described variant strain found in California (Friedman et al. 2005). Based on the observation of three total polymorphic sites within the 'A' region, this study suggests that there are at least two different genetic strains of molluscan herpesvirus in Japan, one strain in Korea and two strains in China.

Initial sequencing of molluscan herpesvirus DNA detected in *Crassostrea ariakensis* from sites in Korea and in *C. hongkongensis* in Chengcun, China, suggested, based on the site at 604 bp, that these oysters could be infected with the same genetic strain that was detected in *C. gigas* from Tomales Bay, California (Friedman et al. 2005). However, additional DNA sequencing revealed that the viral DNAs amplified from oysters in Chengcun, China and from Korean *C. ariakensis* share an additional polymorphic site, making them unique from the California molluscan herpesvirus strain. Interestingly, molluscan herpesvirus sequences found in oysters from Souchang River and Yamen River, China, had the same 'A' region sequence as OsHV-1 that was first detected in *C. gigas* from France (Le Deuff and Renault 1999, Renault et al. 2000b). However, molluscan herpesvirus sequences from Japanese oysters have a unique pattern

of differences at the three polymorphic sites, suggesting that the two Japanese molluscan herpesvirus strains could be unique to that geographic region. Sequencing of additional gene regions of the molluscan herpesvirus found in Asia may further discriminate strains suggested by the polymorphisms observed here in the 'A' fragment. Overall, this study expands the current known host geographic range of molluscan herpesviruses to include Japan, China and Korea, where it infects *C. ariakensis, C. hongkongensis, C. gigas* and *C. sikamea.*

DISCLAIMER

According to the International Code of Zoological nomenclature (Article 9), the use of a new species name in an unpublished document such as this dissertation does not meet the criteria of the Code. Following the criteria outlined by the code, the new *Perkinsus* species name introduced in the following chapter would be invalid, however this chapter will be submitted for publication, therefore I request that the use of the new species name be allowed in this chapter and in all following references in this document solely for the purposes of this dissertation.

Chapter 2. A Novel *Perkinsus* sp. Parasite of Oysters in Southern China; Description of *Perkinsus beihaiensis* n. sp.

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* spp. parasites. Sequencing and phylogenetic analysis of amplified *Perkinsus* spp. DNAs indicated that a novel *Perkinsus* sp. infects *Crassostrea hongkongensis, Crassostrea ariakensis* and other oyster hosts from the Fujian to Guangxi provinces in southern China. Prevalence of this *Perkinsus* sp. reaches as high as 60% in affected oyster populations. Analyses of ITS region nucleotide sequences and of large subunit ribosomal RNA 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 histologically, and application of both genus *Perkinsus* and *Perkinsus* species-specific in situ hybridization probes consistently labeled the same *Perkinsus* sp. cells in histological sections from infected oyster tissues. Combined phylogenetic and histological results support the identity of the new parasite species, *Perkinsus beihaiensis* n. sp., in oysters from southern China.

INTRODUCTION

Since the initial description of *Perkinsus marinus* as a significant pathogen of *Crassostrea virginica* oysters (Mackin et al. 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 Atlantic and Gulf of Mexico USA oyster populations (Burreson and Ragone Calvo 1996, Soniat 1996). Significant mortality events and depressed production have also been associated with *P. olseni* infections among marine molluscs on the Atlantic and Mediterranean coasts of Europe (Azevedo 1989, da Ros and Cazonier 1985, Figueras et al. 1992, Montes et al. 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 *Perkinsus marinus* infections, the

two pathogens that have contributed significantly to reduction of *C. virginica* populations in Chesapeake Bay (Sindermann 1990).

In China, *Crassostrea 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. 2004). 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. 2004). Reliable differentiation between *C. ariakensis* and *C. hongkongensis* are currently possible only by genetic techniques (Cordes and Reece 2005).

With an introduction of *C. ariakensis* to the Chesapeake Bay proposed, 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, since *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 (for example see Zhang et al. 2005), it is also important to identify the pathogens and parasites of *C. hongkongensis*. Using PCR-based diagnostics, *P. olseni* was detected in *C. ariakensis* in Japan, as well as in *C. ariakensis* and *C. hongkongensis* in northern China. An undescribed *Perkinsus* sp. was also detected apparently infecting several oyster host species in parts of southern China (Moss et al. 2007).

Herein is provided a detailed description of *Perkinsus beihaiensis* n. sp., based on phylogenetic analyses of nucleotide sequences from the internal transcribed spacer region of the ribosomal RNA (rRNA) gene complex, of large subunit 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 five 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--2006 (Chapter 1, Moss et al. 2007, Figure 1, Table 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% DMSO, and saturated NaCl) or in 95% ethanol, for DNA extraction and PCR analysis. Visceral mass sections of some sample oysters were also preserved in Davidson's solution (Shaw and Battle 1957) for histological examination. In addition to oyster collections reported in Moss et al. (2007), oysters were collected in April 2005, and in April and November 2006, for use in parasite assays and *Perkinsus* sp. in vitro propagation efforts (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 *Perkinsus* sp. in vitro isolate propagation (Table 1).

Figure 1. Map of sampling locations. *BC, BC05,* Beihai, Guangxi Zhuang, China; *CH,* Chengcun, Yanxi, Fujian Province, China; *DR*, *DR06,* Dafen River, Beihai, Guangxi Zhuang Province, China; *GD*, Guandu, Zhanjiang, Guangdong Province, China; *HN,* Lingshui, Hainan, China; *PO,* Podi, Beihai, Guangxi Zhuang Province, China; *QZ06*, Qinzhou, Guangxi Zhuang, China; *SZ*, Shenzhen, China; *TA,* Tong'an, Fujian Province, China; *ZJ,* Zhanjiang, Guangxi Zhuang Province, China.

Figure 1.

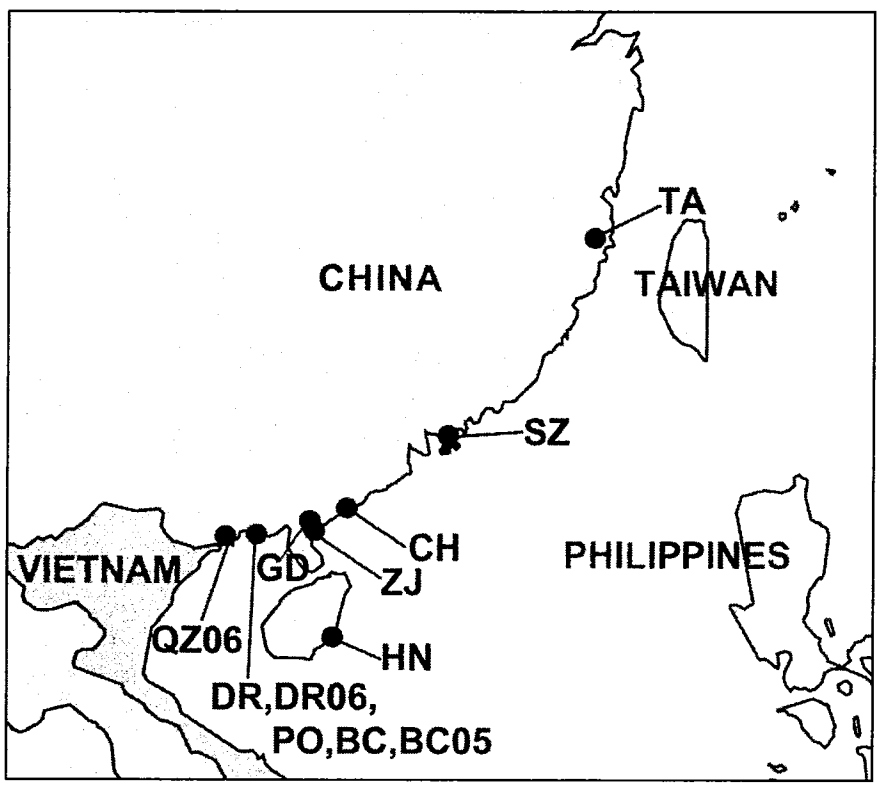


Table 1. Locations sampled during this study. PCR results using the *P. beihaiensis*-specific assay or amplification of the ITS rRNA followed by DNA sequencing and phylogenetic analysis are noted. Bivalve species infected with *Perkinsus beihaiensis* n. sp. are indicated. C* refers to cultivated oysters.

Year	Sampling Location	N	# PCR positive	% PCR positive	Bivalve sp. infected
2002	Tong'An, Fujian (TA)	28	1	3.6	*C. ariakensis*
2002	Chengcun, Yanxi, Fujian (CH)	37	6	16.2	*C. hongkongensis*
2002	Shenzhen (SZ)	13	1	7.7	*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*
1999	Dafen River, Beihai, Guanxi 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*
		24	2	8.3	*C. hongkongensis*
2003	Beihai, Guangxi Zhuang (BC)	59	11	18.6	*C. hongkongensis*
2003	Lingshui, Hainan (HN)	19	12	63.2	*Pinctada margaritifera, P. martensii,* unknown sp.- not distinguished
2005	Beihai, Guanxi Zhuang (BC05)	12	4	33.3	*C. ariakensis*
		101	36	35.6	*C. hongkongensis*
2006	Qinzhou, Guangxi Zhuang (QZ06)	15	1	6.7	*C. ariakensis*
		44	3	6.8	*C. hongkongensis*

ARFTM and RFTM assays. Homogenized tissue preparations for alternative Ray's Fluid Thioglycollate Medium (ARFTM) assays (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); and infection prevalences were calculated as the percentage of infected oysters detected in samples. In some cases, gill, mantle, and rectal tissues were processed for Ray's fluid thioglycollate medium (RFTM) assays (Ray 1952). Following 27 °C incubation for 5--7 d 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 *Perkinsus* sp. infection intensities were categorized on a scale from absent (0) to very heavy (5), based on the categories of Ray (1952, 1954).

In vitro propagation of *Perkinsus* spp. Selected ARFTM- or RFTM-incubated tissues with heaviest *Perkinsus* sp. hypnospore densities were aseptically transferred to culture plate wells, 4--24 wells per plate, containing 2 ml of antimicrobial-supplemented DME/F12-3 culture medium (Burreson et al. 2005). Infected tissues were disrupted and suspended in culture medium by gentle trituration with a sterile pipet. Resulting suspensions were serially diluted at 0.5 ml $well^{-1}$ 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 *Perkinsus* sp. isolate proliferation.

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), as described in Chapter 1.

PCR-RFLP 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 internal transcribed spacer (ITS-1) region of the rRNA gene complex (Cordes and Reece 2005) as described in Chapter 1.

***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 et al. (2002) as described in Chapter 1.

LSU rRNA gene amplification. A forward primer, PerkITS2 217 (5' GTGTTCCTYGATCACGCGATT 3') was used with a previously described reverse primer, LSU B (5' ACGAACGATTTGCACGTCAG 3') (Lenaers et al. 1989), to amplify a fragment of approximately 1170 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 large subunit (LSU) rRNA gene. Each PCR reaction contained the following: 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, each primer at 0.25 μM, 0.0625 U μl^{-1} *Taq* polymerase, 0.2 mg ml^{-1} 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 was 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., Valencia, CA).

Actin gene amplification. The forward primer, PerkActin1 130F (5' ATGTATGTCCAGATYCAGGC 3') and reverse primer PerkActin1 439R (5' CTCGTACGTTTTCTCCTTCTC 3') were used to amplify an approximate 330-bp fragment of type 1 *Perkinsus* sp. actin gene DNA from *Perkinsus* sp.-infected oyster tissue genomic DNA, and from genomic DNA isolated from a *Perkinsus* sp. culture sample containing abundant *Perkinsus* sp. 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 (see above). Each PCR reaction contained the following: 20 mM Tris-HCl (pH8.4), 50 mM KCl, 3.0 mM $MgCl_2$, 0.1 mM of each dNTP, each primer at 0.1 μM, 0.0125 U μl^{-1} *Taq* polymerase, 0.2 mg ml^{-1} 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 was 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., Valencia, CA).

Cloning and Sequencing. Cloning of the ITS region, LSU rRNA gene and actin gene PCR products as well as sequencing reactions were performed as previously described (Chapter 1, Moss et al. 2006, Moss et al. 2007, Dungan and Reece 2006).

***Perkinsus beihaiensis* n. sp. diagnostic assay development.** ITS region sequences of the novel *Perkinsus* sp., *Perkinsus beihaiensis* n. sp., and those of *Perkinsus* spp. previously deposited in GenBank were aligned, and regions unique to *Perkinsus*

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, P. honshuensis, P. mediterraneus, P. marinus, P. chesapeaki, Crassostrea hongkongensis, C. ariakensis, C. virginica, Amphidinium carterae, Karlodinium micrum, Peridinium foliaceum, Cryptecodinium cohnii, Prorocentrum micans, Pseudopfiesteria shumwayae, Pfiesteria piscida, Hematodinum* sp. from *Callinectes sapidus, Hematodinium* sp. from *Leocarcinus depurator, Haplosporidium 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^{-1} *Taq* polymerase, 0.2 mg ml^{-1} 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 was electrophoresed on a 2% agarose gel and visualized as described.

Phylogenetic analysis. *Perkinsus* sp. sequences obtained from the southern China oysters were compared to those deposited in GenBank, using BLAST (basic local alignment search tool) 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); *Perkinsus marinus* AY295180, AY295188, AY295189, AY295194, AY295197, AY295199; *Perkinsus chesapeaki* (= *P. andrewsi*) AF091541, AY876302, AY876304, AY876305, AY876306, AY876311; *Perkinsus olseni* (= *P. atlanticus*) AF441207, AF441209, AF441211, AF441213, AF441215, AY435092, AF473840, AY820757, AF522321, POU07701, PSU07698, PSU07699, EF204082, EF204083, EF204086; *Perkinsus mediterraneus* AY487834, AY487835, AY487837, AY487839, AY487841, AY487842; *Perkinsus 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: *Prorocentrum micans* X16180 (outgroup taxon); *Perkinsus 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: *Amphidinium carterae* U84289, *Prorocentrum minimum* U84290 (outgroup taxa); Type 1 *Perkinsus 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 assays. A genus-specific 5′ end digoxigenin-labeled *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 *Perkinsus beihaiensis* n. sp. Digoxigenin-labeled 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 125 μg mL^{-1} was used for permeabilization during a 30-min incubation. A probe concentration of 7 ng μl^{-1} 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

ARFTM 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 Beihai, China *C. ariakensis* and *C. hongkongensis* oysters 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 2005 arrival from China, 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 $\mu g\ ml^{-1}$ 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 *Perkinsus* sp. primary isolate cultures 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 *Perkinsus* sp. inoculum hypnospores occurred in none.

Among six *C. hongkongensis* oysters that were collected during 2006 from Beihai, China waters and used for 95 d 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 d 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.

ISH 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 (Figs. 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.

Figure 2. Hoffman modulation contrast image of *Perkinsus beihaiensis* n. sp. zoosporangia containing zoospores. Identity of zoosporangia confirmed by sequencing the ITS rRNA region (GenBank accession numbers EU068100--068107). Discharge tube labeled with arrow. Scale bar = 20 μm.

Figure 2.

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 (63%), as well as among gill and mantle connective tissues (44%). In two heavy, systemic, infections that were observed, numerous *Perkinsus* sp. trophozoites (Fig. 7) and proliferating schizonts (Fig. 8) were observed phagocytosed, but apparently healthy, within circulating hemocytes. Two examples of possible *Perkinsus* sp. dispersal mechanisms were observed. In one oyster, a *C. ariakensis*, numerous parasite cells were observed shedding into the stomach lumen adjacent to a necrotic epithelial lesion (Fig. 9). In another oyster, a female *C. honkongensis*, *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, Fig. 12). *Perkinsus* sp. signet ring trophozoites of 2--8 μm diameters, subdividing 4--12 μm diameter schizonts, and clusters of multiple sibling trophozoites of 2--4 μm diameters occurred among infected tissues.

Figures 3—6. 3. H&E stained tissue section of *Crassostrea ariakensis* stomach epithelia showing a *Perkinsus beihaiensis* n. sp. lesion. Examples of trophozoites (↑) and schizonts (<) indicated. Scale bar = 10 μm. 4. *Crassostrea ariakensis* (same individual as shown in Fig. 3) showing hybridization of the *Perkinsus beihaiensis* n. sp.-specific probe to cells in the stomach epithelia. Scale bar = 10 μm. 5. H&E stained tissue section of *Crassostrea hongkongensis* stomach epithelia showing a *Perkinsus beihaiensis* n. sp. lesion. Examples of trophozoites (↑) and schizonts (<) indicated. Scale bar = 10 μm. 6. *Crassostrea hongkongensis* (same individual as shown in Fig. 5) showing hybridization of the *Perkinsus beihaiensis* n. sp. specific probe to cells in the stomach epithelia. Scale bar = 10 μm.

Figure 3--6.

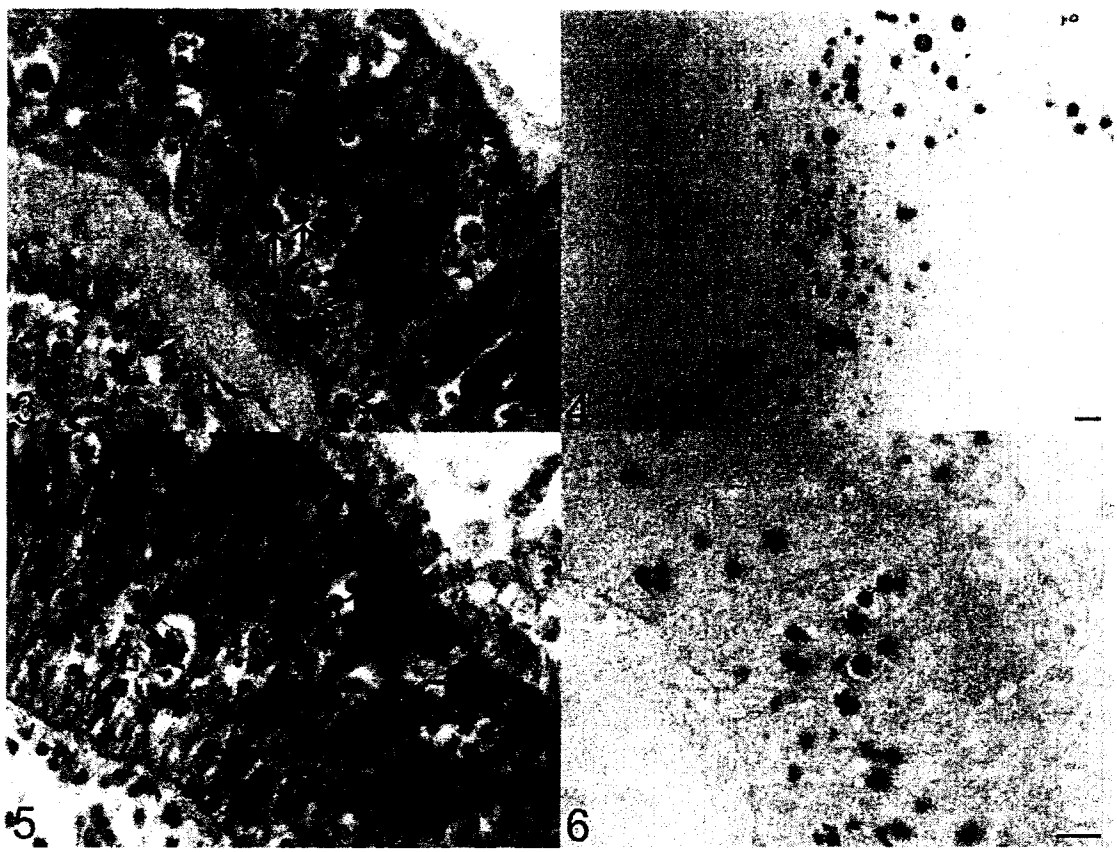


Figure 7—8. 7. H&E stained tissue section showing a *Perkinsus beihaiensis* n. sp. trophozoite (<) encapsulated within an oyster hemocyte. Hemocyte nucleus (↑) visible. Scale bar = 10 μm. 8. H&E stained tissue section of a *Perkinsus beihaiensis* n. sp. schizont (<) encapsulated within an oyster hemocyte. Hemocyte nucleus (↑) visible. Scale bar = 10 μm.

Figure 7—8.

Figure 9--10. 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* n. sp. trophozoites (<) and schizonts (↑) visible in the epithelia surrounding the gonoducts of a *Crasssostrea hongkongensis* oyster. Scale bar = 10 μm.

Figure 9—10.

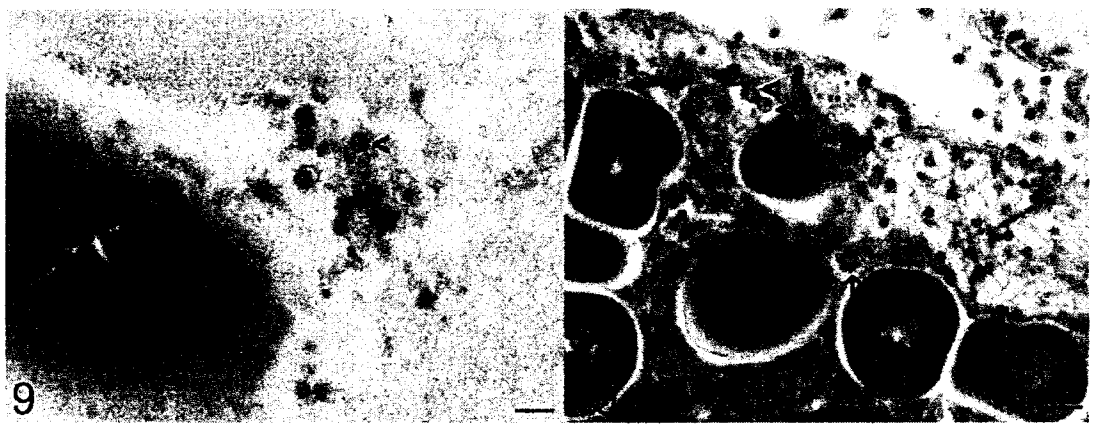
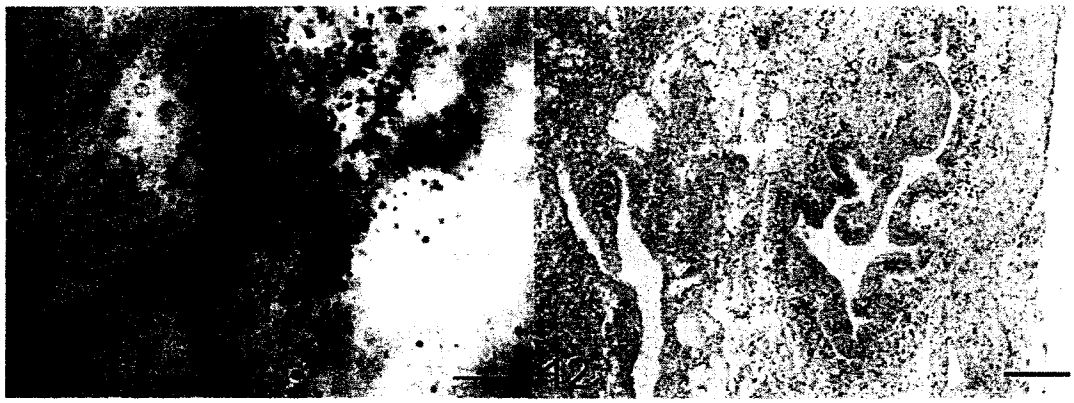


Figure 11--12. 11. Low magnification photograph of Lugol's iodine-stained *Perkinsus beihaiensis* hypnospores within *Crassostrea hongkongensis* gill and mantle tissues after incubation in RFTM. The number and density of cells observed represents a very heavy infection according to Ray (1952, 1954). Scale bar = 200 μm. 12. Low magnification photograph showing hybridization of the *Perkinsus beihaiensis* n. sp.-specific probe to cells in all tissues of a *C. hongkongensis* oyster. Scale bar = 200 μm.

Figure 11—12.



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 listed in 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 1170 bp 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, within-host *Perkinsus* sp. ITS region sequence variation was occasionally recorded. 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.

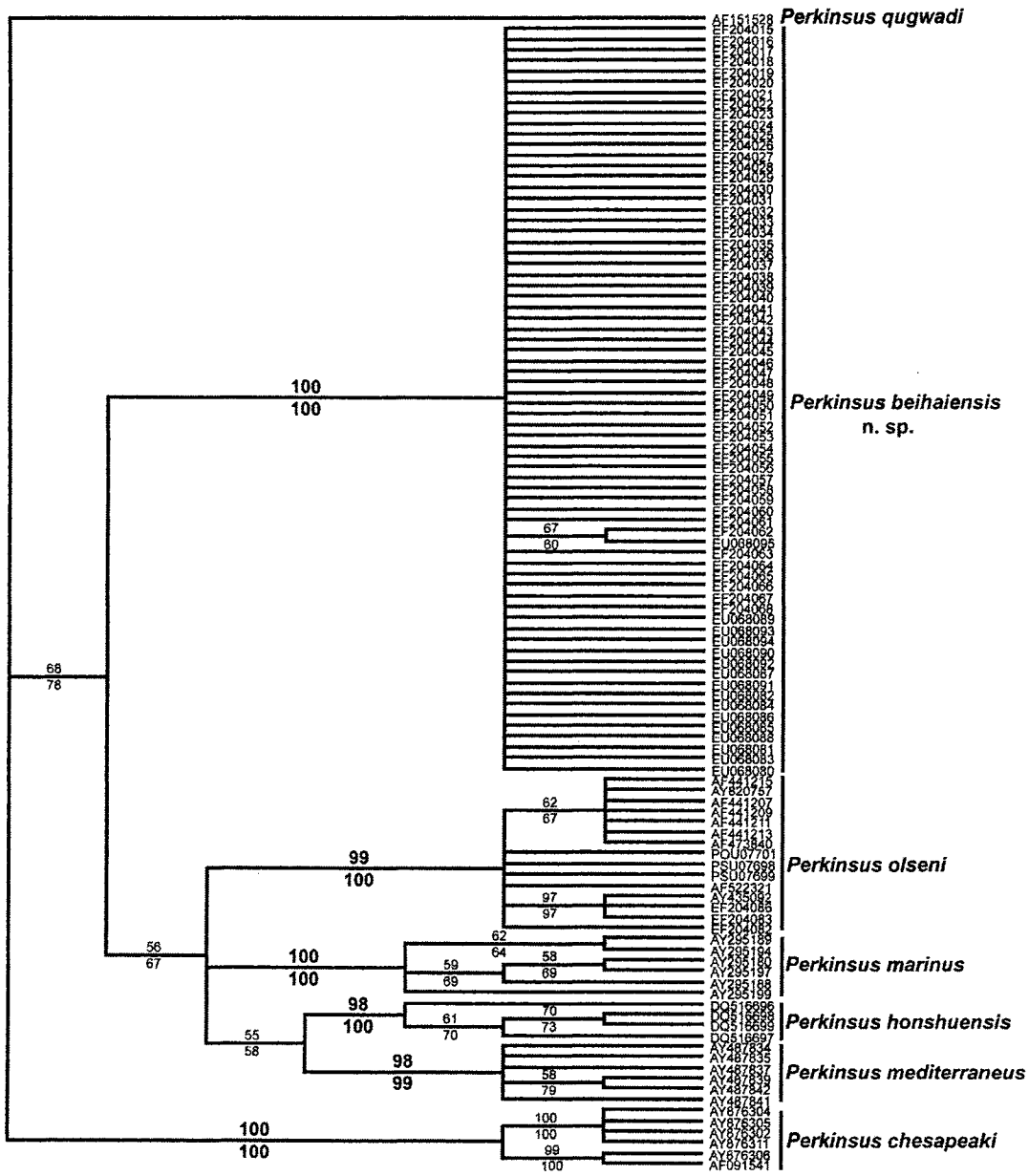
LSU rRNA gene sequences were amplified from six of the *P. beihaiensis* n. sp.-positive oysters collected from Beihai in 2005. Of the total 14 PCR fragments that were sequenced, an identical sequence was found in three different oysters; however, the other 11 fragments each varied by a few base pairs 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). ITS region nucleotide sequences of *P. beihaiensis* n. sp. formed a monophyletic clade (100%) bootstrap support 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.

Figure 13. *Perkinsus* spp. 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.

Figure 13.



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 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 distance within the ITS region sequences of *P. beihaiensis* n. sp. was low (0.0--0.2%), and is within 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).

Figure 14. *Perkinsus* spp. 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.

Figure 14.

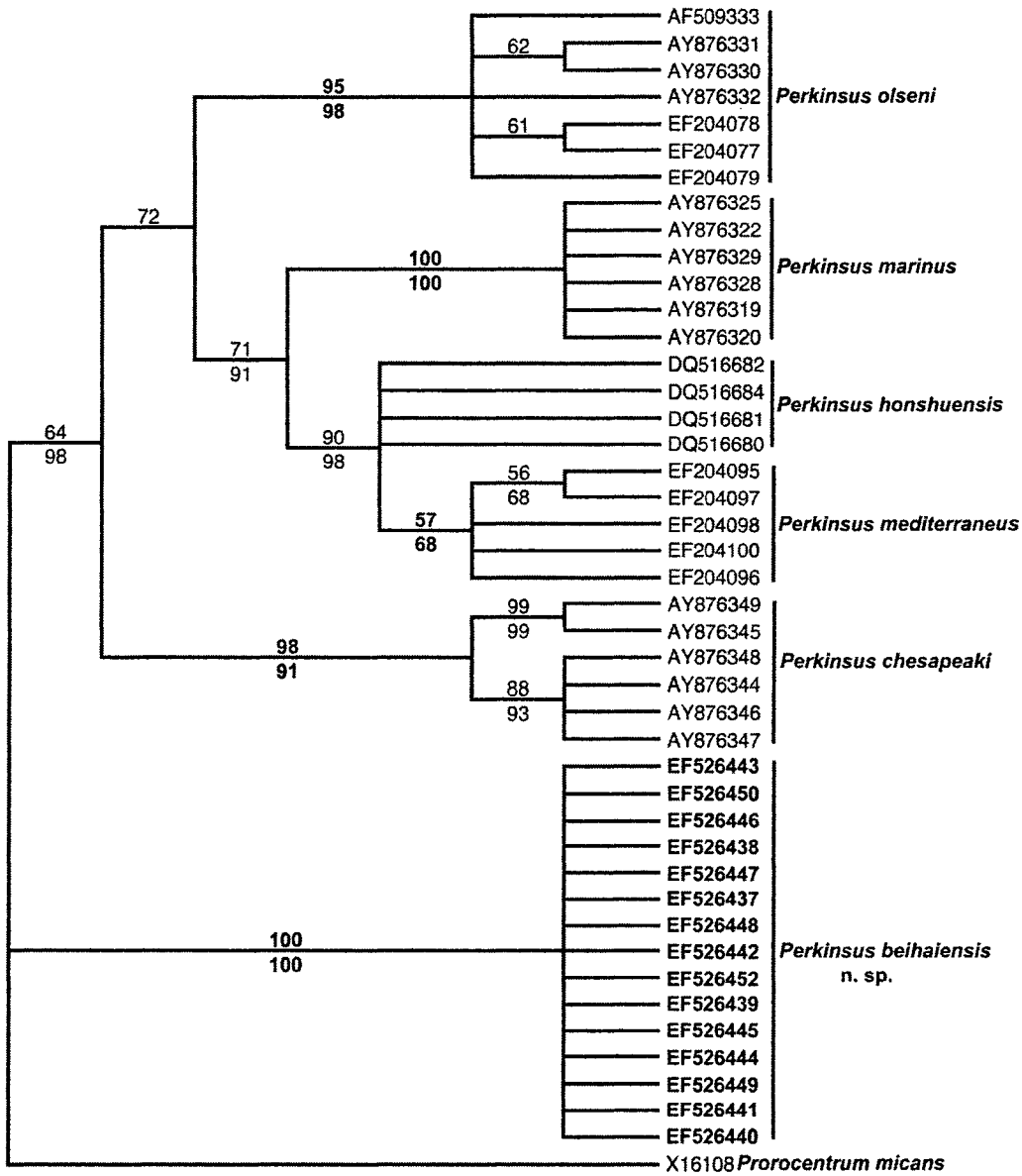
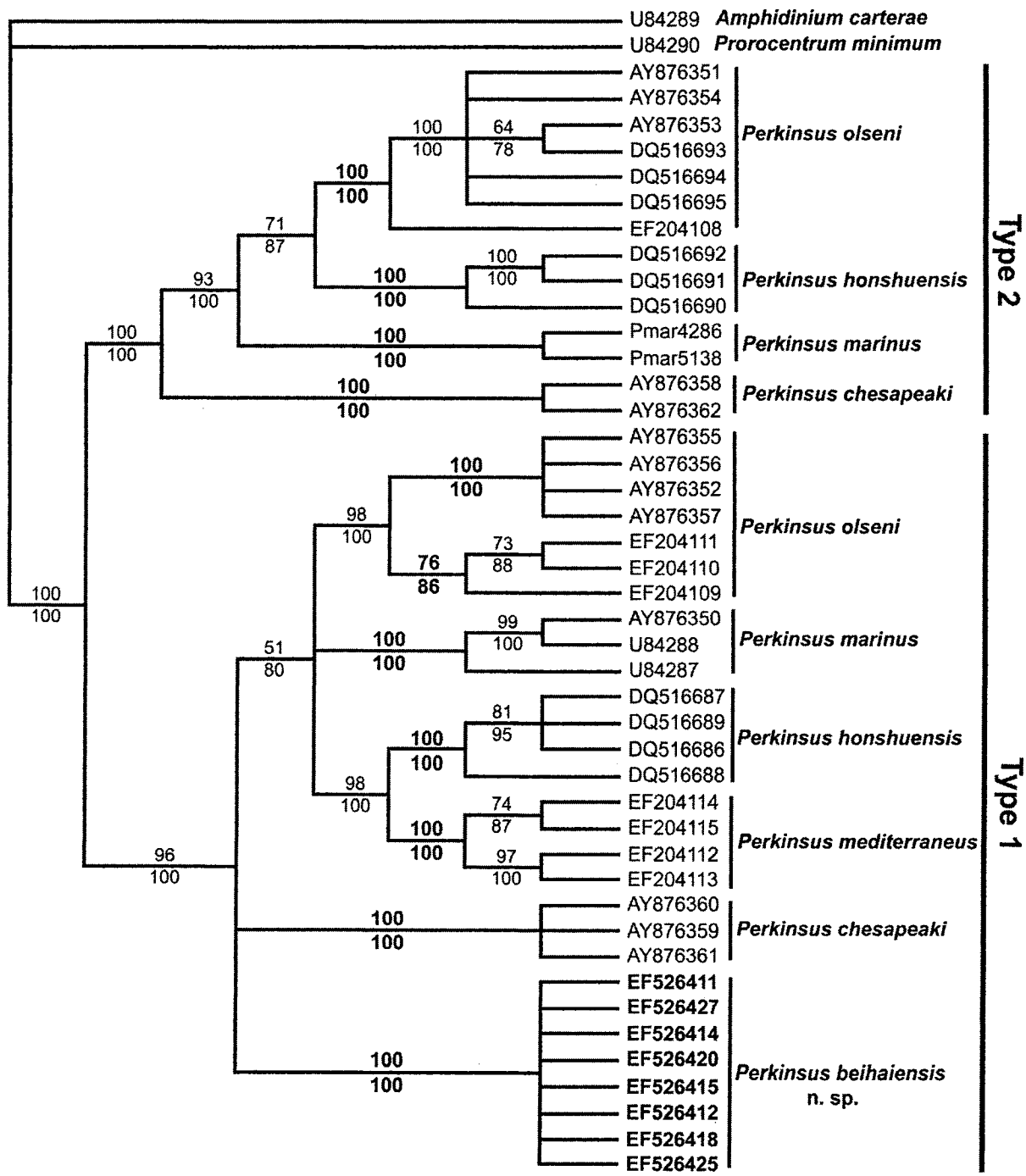


Figure 15. *Perkinsus* spp. actin gene sequences. Maximum parsimony bootstrap support values for each clade are given above the lines and jackknife support values are given below the lines. Bold support values indicate species clades.
Perkinsus species based on ITS region genetic distances were *P. honshuensis* and *P. mediterraneus* (96.3--97.1% similar).

Figure 15.



The intraspecific genetic distance observed between LSU rRNA gene sequences of *P. beihaiensis* n. sp. was low (0.0--0.2%), and within the range expected between other *Perkinsus* species (Table 3). Due to the relatively highly conserved nature of the LSU rRNA gene sequences, observed genetic distances were 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 *Crassostrea 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 using available tools (Table 1). The abundance of *C. hongkongensis* was higher than that of *C. ariakensis* (470 *C. hongkongensis* was higher than that of *C. ariakensis* (470 *C. hongkongensis* versus 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*

Table 2. Range of pairwise distances (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. 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.

Species	*P. marinus*	*P. chesapeaki*	*P. mediterraneus*	*P. olseni*	*P. honshuensis*	***P. beihaiensis* n. sp.**
P. 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
P. 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
P. 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
P. 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
P. 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
***P. 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%**

Table 3. Range of pairwise distances (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. The range of within species sequence distance 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.

Species	*P. marinus*	*P. chesapeaki*	*P. mediterraneus*	*P. olseni*	*P. honshuensis*	***P. beihaiensis* n. sp.**
P. 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
P. 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
P. 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
P. 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
P. 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
***P. 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%**

Table 4. Range of pairwise distances (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. 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.

Species	*P. marinus*	*P. chesapeaki*	*P. mediterraneus*	*P. olseni*	*P. honshuensis*	***P. beihaiensis* n. sp.**
P. 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
P. 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
P. 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
P. 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
P. 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
***P. 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%**

(23.8% of *C. hongkongensis* versus 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, however, results from a 2003 oyster sample from Zhanjiang, Guangdong, suggest that the prevalence of *P. beihaiensis* n. sp. infections in *Crassostrea* spp. oysters can be as high as 46.5% (Table 1).

DISCUSSION

A comprehensive phylogenetic analysis was conducted of a unique, new *Perkinsius* 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 to those from other *Perkinsus* spp. described to date. With these consistent genetic similarities and differences, in addition to observations of typical *Perkinsus* spp. parasite cell morphology and enlargement in RFTM media, I conclude that this parasite represents a new *Perkinsus* sp. for which I propose the name *Perkinsus beihaiensis* n. sp. as follows.

***Perkinsus beihaienesis* 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 schizogany 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 intestine 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 holotype and paratype reference materials, with both the USDA National Parasite Collection (http://www.lpsi.barc.usda.gov/bnpcu) (USNPC 100051 and USNPC 100052), and with the 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 are deposited with GenBank (http://www.ncbi.n1m.nih.gov/Genbank) under the accession numbers listed in Table 5.

Table 5. GenBank accession numbers for *Perkinsus beihaiensis* n. sp. sequences associated with this study.

Locus	GenBank Accession Numbers
Internal transcribed spacer rRNA	EF204015-EF204068, EF52642--EF526436, EU06808--EU068095, EU068100--EU068107
LSU rRNA	EF526437--EF526452
Actin gene	EF52641--EF526427

Type hosts. *Crassostrea hongkongensis*

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

Type locality. Beihai region, Guangxi Zhuang, People's Republic of China

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

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

The internal transcribed spacer region of the ribosomal RNA complex has previously been used to examine relationships within the genus, and discriminate between *Perkinsus* spp. (Brown et al. 2004, Casas et al. 2002, Dungan et al. 2002, Dungan and Reece 2006, Goggin 1994, Park et al. 2006). In addition, the large subunit 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 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. Internal transcribed spacer 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 I was only able to successfully amplify and sequence type 1 actin gene fragments from *P. beihainesis*, I 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 *Perkinsus beihaiensis*-specific PCR primer set specifically amplifies this species' ITS region DNA, and the in situ hybridization probe specifically hybridizes to the nucleic acids of *P. beihaiensis* cells in histological tissue sections. PCR 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 I 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 labeling 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, *P. beihaiensis* cells were most commonly found in visceral mass connective tissues of infected oysters, and in stomach and intestinal epithelia. Early infections of *P. olseni* in *Venerupis (Tapes) decussatus, P. chesapeaki* in *Mya arenaria* or *Tagelus plebeius,* and *P. marinus* in *Crassostrea virginica* are often limited to the gill or mantle (Casas et al. 2002, Dungan et al. 2002, Mackin 1951), and these organs 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), the 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 carry high levels of PCR-inhibitory substances associated with digestive organs (Abolomaaty et al. 2007). Extraction methods exist that may facilitate amplification of DNA isolated from digestive gland tissues; however, the complexity of those methods make them currently 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, I 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, efforts to establish continuous in vitro isolate cultures of *P. beihaiensis* have failed to date. Constituents of *Perkinsus* spp. 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 2002, La Peyre et al. 1993). Although those media and their modifications have been subsequently used to propagate diverse *Perkinsus* spp. isolates 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* 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 et al. 1994, Ordas and Figueras 1998); while *P. mediterraneus* has proven to be extremely fastidious and slow-growing in vitro (Casas et al., submitted). Critical, minor modifications to existing medium compositions and/or incubation parameters may yield continuous *P. beihaiensis* in vitro isolate cultures and archival type-strains in the future.

The distribution and prevalence of *P. beihaiensis* suggests that it may be widespread in Chinese oysters, particularly *Crassostrea hongkongensis*, in coastal localities from Tong'an, Fujian to Beihai, Guangxi, near the southern border of China

with Vietnam. DNA of this parasite has been found additionally in high prevalence in *C. ariakensis,* and in *Pinctada margeratifera, Pinctada martensii,* and in some unidentified bivalve mollusc species. Infection prevalences averaged over the 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, 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 observed parasite body burden cannot be directly attributed 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. Early studies showed that *P. marinus* infections reduce oyster growth, and are especially pathogenic during summer, when the parasite tissue abundance is high. It has been postulated that condition index may be reduced as well, although often the highest infection intensities occur during the summer post-spawning period (Andrews 1961). In addition, reproductive output may be affected (Dittman et al. 2001) and metabolic costs of *Perkinsus* spp. 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 et al. 1995), reports of rickettsia-like organisms in pearl oysters, *Pinctada maxima* and *Pinctada fucata* (Wu and Pan 1999) and *C. ariakensis* (Wu and Pan, 2000), and mass mortality of abalone, *Haliotis diversicolor*, attributed to the outbreak of a viral infection (Wang et al. 2004). Here I report on the existence of a previously unknown and pathogenic oyster parasite in southern China. *Perkinsus beihaiensis* 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.

With a pending Environmental Impact Statement (EIS) and ruling regarding the introduction of *C. ariakensis* to Chesapeake Bay, we are now aware of the new and pathogenic *Perkinsus beihaiensis* parasite 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 *Mercenaria mercenaria.* Therefore, future 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.

Chapter 3. *Perkinsus olseni* transmission studies

ABSTRACT

The Suminoe oyster, *Crassostrea ariakensis,* has been being considered for introduction into the Chesapeake Bay, USA, since the early 1990s for the purpose of restoring the commercial harvest of oysters and/or for developing a non-native oyster aquaculture industry. During a survey of *Crassostrea* spp. oysters conducted in Asia (Chapter 1), the protozoan parasite, *Perkinsus olseni*, was found in *C. ariakensis* and aother sympatric oyster species including *C. hongkonensis.* In light of the proposed introduction of *C. ariakensis,* challenge studies were performed in order to ascertain the pathogenicity of *P. olseni* both to *C. ariakensis*, as well as to Bay bivalves, the eastern oyster, *Crassostrea virginica*, and the hard clam, *Mercenaria mercenaria.* Two direct inoculation experiments were performed in which cultured *P. olseni* cells were inoculated into the pallial cavity of naïve bivalves. During the second inoculation experiment, standard *Perkinsus* spp. culture media was supplemented with *C. ariakensis* homogenate as a protein source in an attempt to increase the natural virulence that may have been lost by the *P. olseni* cells when cultured in artificial media. An additional bath challenge experiment was performed in which naïve bivalves were exposed to *P. olseni* derived from *Ruditapes decussatus* clams procured from a *P. olseni*-endemic region of Spain. During the first inoculation experiment, the triploid *C. ariakensis* obtained for use in the disease challenge were briefly exposed to York River water where they were

unintentionally exposed to *P. marinus,* a *Perkinsus* sp. endemic to that area. As the experiment progressed, the acquisition of moderate and heavy infection intensities of *P. marinus* in *C. ariakensis* was observed in oysters being maintained in the laboratory. Results suggest that there may be some risk of mortality from *P. marinus* if *C. ariakensis* is held under stressful conditions in a hatchery or laboratory setting. PCR and RFTM assay results compiled from the inoculation experiments and bath challenge experiments sugest that *M. mercenaria* and *C. virginica* may be susceptible to the exotic pathogen, *P. olseni,* therefore highlighting a serious disease risk associated with introduction of *C. ariakensis* to Chesapeake Bay.

INTRODUCTION

The eastern oyster, *Crassostrea virginica,* an integral part of the economy and ecology of the Chesapeake Bay, has been in a severe state of decline in recent decades. Two pathogens, *Haplosporidium nelsoni* and *Perkinsus marinus*, the parasites responsible for the diseases known as MSX and dermo, respectively, have contributed significantly to the decimation of the oyster populations in Chesapeake Bay since the 1950s (Sindermann 1990).

The decline in oyster production in Virginia led to the formation of a number of panels in the early 1990s to make recommendations on restoring oyster populations. One option being seriously considered currently is the introduction and use of a non-native oyster in Chesapeake Bay. A 1998 field-based study on the Asian oyster, *Crassostrea ariakensis*, documented rapid growth and survival in that species, as compared to *C. virginica*, even when endemic diseases were prevalent (Calvo et al. 2001).

Based on significant impacts that non-native introductions of various aquatic species have had around the world, the International Council for the Exploration of the Seas (ICES) developed a Code of Practice on Introductions and Transfers of Marine Organisms (ICES 2005). Recommendations put forth by ICES and other organizations, were the impetus behind a survey conducted on the parasites of *Crassostrea ariakensis* and other sympatric oysters in the *C. ariakensis* native range of China, Japan and Korea.

A *Perkinsus* spp. genus-specific PCR assay targeting the internal transcribed spacer region (ITS) of the ribosomal RNA complex was used to test Asian oyster samples for the presence of parasite DNA. Sequencing of positive amplification products from the genus-specific assay revealed that *P. olseni* was present in *C. ariakensis* in Japan and in *C. ariakensis* and *C. honkongensis* in northern China (Chapter 1). This result raised concern that there was potential for an accidental introduction of the parasite to the Chesapeake Bay region either through infected oysters or from ballast water entering Bay waters through Asian shipping traffic. Therefore, an investigation of the potential pathogenicity of *P. olseni* to local Chesapeake Bay bivalve species, *C. virginica* and *Mercenaria mercenaria,* as well as to *C. ariakensis,* was initiated.

The first experiment consisted of a direct inoculation of a standard dose of *P. olseni* cultured cells into the pallial cavity of the above listed bivalves. Due to an unfortunate exposure of *C. ariakensis* to the Chesapeake Bay native parasite, *Perkinsus marinus,* results of the first inoculation experiment were confounded. Although, in the first experiment, *C. ariakensis* acquired moderate and heavy infection intensities of *P. marinus*, there was little evidence of disease in the bivalves challenged with the cultured *P. olseni* cells. There was evidence from several previous experiments conducted by other researchers (Ford et al. 2002) that virulence is compromised in cultured *Perkinsus*

spp. cells. Therefore, a second challenge experiment was undertaken to analyze if supplementing standard culture media with oyster homogenate resulted in differential transmission or pathogenicity when compared to a direct inoculation of cells cultured in the absence of oyster homogenate. Previous studies conducted with *P. marinus* have demonstrated that virulence of cultured cells may be increased by supplementing the culture media in this manner (MacIntyre et al. 2003, Earnhart et al. 2004). The third experiment in the *P. olseni* challenge series was a bath experiment in which the three bivalve species were exposed to *P. olseni* cells purified directly from naturally infected Spanish *Ruditapes decussatus* clams.

Experiment 1. First challenge experiment

MATERIALS AND METHODS

Quarantine procedures. A quarantine facility in Byrd Hall at the Virginia Institute of Marine Science (on the upper campus, physically separated from the Aquaculture Facility/shellfish hatchery on the lower campus) has been established for the experimental culture of shellfish infected with non-endemic pathogens. This is a restricted-access facility, and great care is taken to prevent the escape of potential pathogens from this facility to the environment. Those working in this lab are required to log in all activities, wash hands and arms thoroughly and spray with ethanol before leaving, and use a sanitizing footbath upon exiting. All experiments involving exotic Asian *Perkinsus* spp. were conducted using strict quarantine protocols. As is customary in the quarantine facility, two thirds of the water from tanks used in the *Perkinsus* sp. experiments was renewed three times a week. Bleach was added to the waste tank at a final concentration of greater than 300 ppm Cl_2. According to Bushek et al. (1997), this

concentration of bleach kills all parasite cells within 30 minutes. After 24 h, chlorine was neutralized with sodium thiosulfate. The water was pumped into the drain, allowing this water to be additionally treated by Hampton Roads Sanitation District facilities. Any whole animals, portions of animals, or shell disposed of during the duration of these experiments were immersed in 70% ethanol for 24 h, or autoclaved, before being discarded into the garbage in sealed plastic bags.

Experimental design. On January 24, 2005, 120 triploid *C. ariakensis* (74.4 ± 8.9 mm shell length) were received from the Aquaculture Genetics and Breeding Technology Center hatchery at VIMS. On February 15, 2005, 120 hard clams, *Mercenaria mercenaria* (36.1 ± 2.7 mm shell length) were obtained from Mobjack Bay Seafood Company, Ware Neck, Virginia. On February 4, 2005, 120 eastern oysters, *Crassostrea virginica* (83.7 ± 8.6 mm), were obtained from Hog Island Oyster Company, Tomales Bay, California. The *C. ariakensis* were held for 4 d inside a plastic mesh bag in a holding tank prior to bringing them into the laboratory aquaria. The holding tank was not covered and there was flow-through of non-filtered York River water of approximately 9 °C and 16 ppt salinity.

When the *M. mercenaria* and *C. virginica* were received, the *C. ariakensis* were removed from the holding tank and 20 individuals of each species were sacrificed. Whole wet body weights were obtained and gill and mantle was excised aseptically from each animal for DNA extraction. Genomic DNA of each oyster was used in a PCR-based molecular diagnostic assay (Casas et al. 2002) to examine the animals for the presence of DNA from *Perkinsus* spp. parasites.

The animals were subsequently held for 59 days in 10 gallon glass aquaria that were maintained at 20 °C and 25 ppt salinity at a density of approximately 25 individuals

per tank and were fed 0.1 g per oyster algal food daily (Reed Mariculture, San Jose, CA). After 50 days, on March 24, 2005, five individuals of each species were sacrificed and gill and mantle were excised aseptically from each animal to be used in *Perkinsus* spp. screening by PCR as described below. This was to insure that all hosts remained *Perkinsus* spp.-free prior to the start of the experiment.

On March 28, 2005, the remaining *C. virginica, M. mercenaria* and *C. ariakensis* were used in an experiment designed to evaluate the pathogenicity of *P. olseni* to these bivalve species. Twenty *C. ariakensis*, 37 *M. mercenaria* and 25 *C. virginica* received single pallial cavity injections of 100 µl of 25 ppt sterile artificial seawater (SASW) through a notch in the shell. Those *C. ariakensis* inoculated with 25 ppt SASW were maintained in one 10 gallon aquarium, the *M. mercenaria* were split into two aquaria, with 18 and 19 clams in each, respectively, and the *C. virginica* were also split into two aquaria, with 12 and 13 oysters in each, respectively. Thirty-nine *C. ariakensis*, 36 *C. virginica* and 57 *M. mercenaria* were inoculated with 10^5 log phase cultured *P. olseni* cells, suspended in 100 µl of 25 ppt SASW, per gram body weight. The average body weight of each host (wet, excised from shell) was 12.2 g for *C. ariakensis*, 7.3 g for *C. virginica* and 4.3 g for *M. mercenaria*, translating to inoculations of approximately 12.2 x 10^6, 7.3 x 10^6, and 4.3 x 10^6 cells per host, respectively, in a 100 µl volume. For all *Perkinsus* spp. inoculations, cell counts and viability were recorded using neutral red and an Improved Neubauer, 1/400 Square mm counting chamber (Hausser Scientific, Horsham, PA). After injection, animals were wrapped with a rubber band and held at 4 °C for 5--6 h. The clonal *P. olseni* culture used for this experiment was isolated from a Japanese *Venerupis* (=*Tapes*) *philippinarum* clam that was obtained from Mr. Chris Dungan of the Maryland Cooperative Oxford Laboratory, Oxford Maryland, (ATCC

PRA-181). The *C. ariakensis* were divided into three aquaria with 20, 20 and 19 oysters in each. The *C. virginica* were divided into three aquaria with 12 oysters in each. The *M. mercenaria* were divided into three aquaria with 18 clams in each. As a positive control, 17 *C. virginica* were inoculated with 10^5 log phase cultured *Perkinsus marinus* cells per gram body weight in a 100 µl volume. The clonal *P. marinus* culture (ATCC 50439) was also obtained courtesy of Mr. Chris Dungan. These *C. virginica* were divided evenly between two aquaria, 8 and 9 animals each. Thirty additional untreated, control *C. ariakensis* were neither notched nor inoculated, but held in separate aquaria and otherwise maintained and fed in the same manner as the experimental animals for the duration of the experiment. All aquaria were identical and each tank was covered with a plexiglass lid.

All aquaria environments were maintained at 20--22 °C and contained 25 ppt aerated, 1-µm filtered York River water. When the experimental hosts were first introduced to the aquaria, the aquaria were only half full of water. After 1 h, 1 ml of algae feed (Reed Mariculture) was added to each aquarium. These steps were taken to instigate feeding in order to promote uptake of any purged cells that may have been released. Eighteen hours later, the aquaria were filled entirely with 25 ppt filtered York River water. Starting four days post inoculation, water changes took place 2--3 times weekly, during which two thirds of the water was removed each time and replaced with an equal volume of clean, 25 ppt, 1-µm filtered York River water. Animals were fed daily a single dose of 0.1 g algal feed per animal.

Aquaria were checked daily for mortality and moribund animals were removed. When moribund or dead animals were discovered, and if sufficient undegraded tissue remained it was processed for analysis. Gill and mantle tissues were removed with a

portion preserved in 95% ethanol for DNA extraction and molecular diagnostics, and gill, mantle and rectal tissues were processed for Ray's fluid thioglycollate medium (RFTM) assay (Ray 1952). Following 27 °C incubation for 5--6 d in RFTM, tissues were removed from the culture tubes, macerated on microscope slides and stained with Lugol's iodine. Stained, cover-slipped tissue preparations were examined under a light microscope and *Perkinsus* sp. tissue burdens were enumerated on a scale from rare (R) to very heavy (VH) based on the categories of Ray (1952, 1954). Visceral mass tissue sections were preserved in Davidson's solution for histological analysis (Shaw & Battle 1957).

Experimental sampling. Because of space constraints in the laboratory, the untreated *C. ariakensis* that were remaining after 37 d were sacrificed and tissues were taken and preserved for DNA, RFTM, and histological analyses of disease status as described above. For those tanks in which hosts were inoculated with either 25 ppt SASW or with *P. olseni,* two randomly chosen individuals from each tank were removed and sacrificed for disease diagnosis on days 21, 44 and 59 post-inoculation. The challenge experiment was terminated on day 72 and all remaining animals were sacrificed and tissues preserved for DNA, RFTM and histological analysis.

Nucleic acid extraction. Genomic DNA was extracted from the excised mantle and gill snips, using a DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA) as in Chapter 1.

SSU gene and genus-specific *Perkinsus* sp. PCR assays. In order to assure that PCR amplifiable DNA was present in all extracted samples, genomic DNAs were tested using a universal small subunit (SSU) ribosomal RNA gene assay and screening for *Perkinsus* sp. DNA was performed using a *Perkinsus* genus-specific PCR assay, both described in Chapter 1.

***Perkinsus* species-specific assays.** Identification of the *Perkinsus* species that was infecting animals shown to have positive amplification products with the *Perkinsus* genus-specific assay was accomplished through the use of *P. marinus* and *P. olseni* species-specific PCR assays. *Perkinsus marinus*-specific primers PmarITS-70F (5′ CCTTTGYTWGAGWGTTGCCAGATG 3′) and PmarITS-600R (5′ CGAGTTTGCGAGTACCTCKAGAG 3′) (Audemard et al. 2004) and *P. olseni*-specific primers designed for this study Pols-140F (5′ GACCGCCTTAACGGGCCGTGTT 3′) and PolsITS-600R (5′ GGRCTTGCGAGCATCCAAAG 3′) were used in separate 25 µl reactions. PCR reactions for the *P. marinus* ITS region contained the following: 20 mM Tris-HCl (pH8.4), 50 mM KCl, $MgCl_2$ 1.5 mM, 0.2 mM each dNTP, each primer at 0.1 µM, 0.025 U μl^{-1} *Taq* polymerase, 0.05 mg per ml BSA and 0.5 µl genomic DNA (~10--50 ng). Amplifications were performed with an initial denaturation of 95 °C for 4 min followed by 40 cycles of 94 °C for 1 min, 57 °C for 1 min, 65 °C for 3 min, with a final elongation of 65 °C for 10 min. PCR reactions for the *P. olseni* ITS region contained the following: 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.0 mM $MgCl_2$, 0.2 mM each dNTP, each primer at 0.1 µM, 0.025 U μl^{-1} *Taq* polymerase, 0.05 mg per ml BSA and 0.5 µl genomic DNA (~10--50 ng). Thermocycling parameters were as follows: an initial denaturation of 95 °C for 4 min followed by 40 cycles of: 94 °C for 1 min, 64 °C for 1 min, 68 °C for 3 min, all followed by a final elongation step of 68 °C for 10 min. Following amplification, for each species-specific reaction, 4 µl of PCR product were analyzed as described above.

Specificity of *P. olseni* primers was tested against *P. marinus*, *P. chesapeaki, P. mediterraneus,* and *P. honshuensis* DNAs. In addition, selected amplification products from positive *P. olseni*-specific reactions were sequenced. PCR products were cloned

into the plasmid pCR®4-TOPO® and transformed into *E. coli* using a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. Cloned fragments were sequenced according to methods described previously (Chapter 1).

Histological analysis. Tissues preserved for histological analysis in Davidson's solution (Shaw & Battle 1957) were dehydrated in a series of ethanol baths, infiltrated with paraffin and embedded in paraffin blocks prior to sectioning. Sections of 5 μm thickness were stained with Harris-hematoxylin and eosin. Histological sections of infected oysters were examined using light microscopy to visualize *Perkinsus* sp. parasite cells in situ.

In situ hybridization. Representative tissue sections from three *C. ariakensis* oysters determined to have *P. marinus* or mixed infections of both *P. marinus* and *P. olseni* by species-specific PCR (see Results) were evaluated by ISH. In addition, those *C. virginica* and *M. mercenaria* determined to contain *P. olseni* DNA by PCR assay were also evaluated. A genus-specific 5′ end digoxigenin-labeled *Perkinsus* probe (Elston et al. 2004) was used to specifically target SSU rRNA sequences. *Perkinsus* species-specific probes, PmarLSU-181DIG (5′ GACAAACGGCGAACGACTC 3′), specific to *P. marinus,* and PolsLSU-464DIG (5′ CTCACAAGTGCCAAACAACTG 3′), specific to *P. olseni*, were designed by locating unique regions in aligned available *Perkinsus* species LSU rRNA gene sequences. Digoxigenin-labeled oligonucleotides were obtained from Operon Biotechnologies, Inc. (Huntsville AL). The protocol followed for ISH was that previously described (Stokes and Burreson 1995) with the modifications published by Elston et al. (2004). Pronase at a final concentration of 125 μg mL^{-1} was used for permeabilization during a 30 min incubation, and a probe concentration of 7 ng μl^{-1} was used for hybridization. The putative species-specific probes were tested for specificity

with numerous *Perkinsus* sp.-infected reference tissues, including *P. marinus* in *C. virginica*, *P. chesapeaki* in *Mya arenaria, P. olseni* in *Haliotis laevigata* and *P. mediterraneus* in *Chamelea gallina.* Controls for each *Perkinsus* species-specific probe were tested identically except that they received hybridization buffer lacking probe during the hybridization step.

RESULTS – Experiment 1

Genus-specific PCR and RFTM assay results. All tissue samples used for DNA extraction yielded high quality genomic DNA as indicated by strong 1800 bp amplification products that were clearly visible by UV illumination of agarose gels following PCR assays with the universal SSU rRNA gene primers. *Perkinsus* genus-specific PCR-based diagnostic screening of an initial baseline subset of 20 individuals of each host type showed that all animals were free of *Perkinsus* sp. DNA. After being held in the laboratory aquaria for 59 days, prior to inoculation, the five *C. ariakensis* sacrificed for genus-specific PCR screening, however, indicated 100% prevalence of *Perkinsus* sp. DNA, while no *Perkinsus* sp. DNA was detected in either *M. mercenaria* or *C. virginica.*

***Crassostrea ariakensis* results.** RFTM and *Perkinsus* genus-specific PCR screening results for the *C. ariakensis* that were inoculated with SASW or *P. olseni* cells are shown in Table 1. RFTM data were not collected during the first day of sampling, day 21, however, the PCR screening indicated the presence of *Perkinsus* sp. DNA in both individuals sampled from those inoculated with SASW and in three of the four oysters sampled from the *P. olseni* injected group. There were no *Perkinsus* cells observed by RFTM assays on the day 44 in the SASW oysters, although *Perkinsus* DNA was found by the PCR assay in one of the two oysters sampled. Very light to light RFTM rankings were observed for two of the four *P. olseni*-inoculated oysters sampled on day 44 and

three of the four were positive in the *Perkinsus* genus-specific PCR assay. On day 59 both of the SASW-inoculated oysters sampled had moderate-moderate/heavy *Perkinsus* sp. tissue burdens and *Perkinsus* DNA was detected in both animals by the PCR assay. Three of the four *P. olseni*-inoculated oysters that were sampled on day 59 had light-moderate infections as indicated by the RFTM assay and *Perkinsus* sp. DNA was detected in all four of the sampled oysters. On day 72, when the experiment was terminated, six SASW- and 15 *P. olseni*-treated oysters remained and were sacrificed. *Perkinsus* sp. DNA was found by the PCR assay in all of the oysters remaining from the two treatment groups (Table 1). *Perkinsus* cells were not detected, however, by the RFTM assay in three of the SASW-inoculated or in one of the *P. olseni*-inoculated oysters. The other three remaining SASW-inoculated oysters had rare RFTM rankings. Rare-light/moderate tissue burdens were found in nine *P. olseni*-inoculated oysters at the end of the experiment and four *P. olseni*-inoculated oysters had heavy-very heavy infections as indicated by the RFTM assay.

RFTM and *P. marinus* PCR assay results for the 29 untreated *C. ariakensis* that were still alive and were sacrificed on day 37 of the challenge experiment are shown in Table 2. Eighteen of these oysters were ranked as having rare to light *Perkinsus* sp. tissue burdens, two were ranked as light/moderate and two as moderate-moderate/heavy tissue burdens. Seven had no observable *Perkinsus* sp. cells in the RFTM assay, however five of these were positive in the PCR assay. *Perkinsus marinus* DNA, as indicated by the PCR assay, was found in 23 of these 29 untreated oysters (Table 2), with no *P. marinus* DNA detected in three of the oysters with a rare RFTM ranking and in one with a light infection as indicated by the RFTM assay.

***Crassostrea virginica* results.** RFTM and *Perkinsus* genus-specific PCR screening results for the *C. virginica* that were inoculated with *P. marinus* or *P. olseni* cells are shown in Table 3.

Positive RFTM or PCR results were not observed during the course of the experiment for any of the SASW treated *C. virginica.*

RFTM data were not collected during the first day of sampling, day 21, for any of the *Perkinsus marinus*-inoculated *C. virginica.* On day 21, PCR screening indicated the presence of *Perkinsus* sp. DNA in three of the four oysters sampled from those inoculated with *P. marinus.* On Day 44, none of the *C. virginica* in the *P. marinus* treatment were PCR positive for *Perkinsus* sp., though *Perkinsus* sp. cells were observed in one individual (light to moderate RFTM ranking). On Day 59, two *C. virginica* were PCR positive for *Perkinsus* sp., however no cells were observed in RFTM assays of these individuals. On the last sampling, day 72, two of the four *C. virginica* sampled were PCR positive for *Perkinsus* sp. and no *Perkinsus* sp. cells were observed in these individuals by RFTM assay.

RFTM data were not collected on day 21 for any of the *Perkinsus olseni*-inoculated *C. virginica.* For the *Perkinsus olseni* inoculated *C. virginica* treatment, *Perkinsus* sp. DNA was detected in four of the six oysters sampled on day 21. On Day 44, *Perkinsus* sp. cells were observed in one individual from the *P. olseni* treatment by RFTM (rare ranking), however it was not positive for *Perkinsus* sp. DNA by PCR. On day 59, *Perkinsus* sp. cells were observed in one *C. virginica,* however this sample was not *Perkinsus* sp. positive by PCR. On the last sampling, day 72, *Perkinsus* sp. cells were observed in one *C. virginica* oyster from the *P. olseni* treatment (light RFTM ranking), however it was not PCR positive for *P. olseni* DNA.

Table 1. RFTM ranking and PCR-based *Perkinsus* genus-specific assay screening results of the first challenge study *C. ariakensis* oysters that were notched and inoculated with either SASW or cultured *P. olseni* cells and sampled from the experimental aquaria on days 44, 59 and 72.

RFTM Ranking	Day 44 (n=2) SASW		Day 44 (n=4) *P. olseni*		Day 59 (n=2) SASW		Day 59 (n=4) *P. olseni*		Day 72 (n=6) SASW		Day 72 (n=15) *P. olseni*	
	# RFTM pos	# PCR pos	# RFTM pos	# PCR pos	# RFTM pos	# PCR pos	# RFTM pos	# PCR pos	# RFTM pos	# PCR pos	# RFTM pos	# PCR pos
None (N)	2	1	2	1			1	1	3	3	1	1
Rare (R)									3	3	1	1
Very Light (VL)			1	1							2*	2
Light (L)			1	1			2	2			6	6
Light/Moderate (LM)											1	1
Moderate (M)					1	1	1	1				
Moderate/Heavy (MH)					1	1						
Heavy (H)											1	1
Very Heavy (VH)											3	3

* Species-specific PCR assays indicated that one of these individuals, as well as one *P. olseni*-inoculated individual collected on day 21 (data not shown), contained DNA from both *P. marinus* and *P. olseni*. Only *P. marinus* DNA was detected in all other individuals.

Table 2. RFTM ranking and PCR-based *P. marinus* assay screening results of control untreated *C. ariakensis* (unnotched and uninoculated) on day 37 of the first challenge study. (Note: *P. olseni* DNA was not detected in any of these oysters.)

	Day 37 (n=29)	
RFTM Ranking	**# individuals**	**# *P. marinus* PCR positive**
None (N)	7	5
Rare (R)	7	4
Very Light (VL)	3	3
Light (L)	8	7
Light/Moderate (LM)	2	2
Moderate (M)	1	1
Moderate/Heavy (MH)	1	1
Heavy (H)		
Very Heavy (VH)		

Table 3. RFTM ranking and PCR-based *Perkinsus* genus-specific assay screening results of the challenge study *C. virginica* oysters that were notched and inoculated with cultured *P. marinus* or *P. olseni* cells and sampled from the experimental aquaria on days 44, 59 and 72.

RFTM Ranking	**Day 44 (n=4)** ***P. marinus***		**Day 44 (n=6)** ***P. olseni***		**Day 59 (n=4)** ***P. marinus***		**Day 59 (n=6)** ***P. olseni***		**Day 72 (n=4)** ***P. marinus***		**Day 72 (n=17)** ***P. olseni***	
	# RFTM	# PCR pos	# RFTM	# PCR pos	# RFTM	# PCR pos	# RFTM	# PCR pos	# RFTM	# PCR pos	# RFTM	# PCR pos
None (N)	3		5		4	2	5		4	2	16	1
Rare (R)			1	0			1	0				
Very Light (VL)												
Light (L)											1	0
Light/Moderate (LM)	1	0										
Moderate (M)												
Moderate/Heavy (MH)												
Heavy (H)												
Very Heavy (VH)												

***Mercenaria mercenaria* results.** Positive RFTM or PCR results were not observed during the course of the experiment for any of the SASW treated *M. mercenaria.* RFTM and *Perkinsus* sp. PCR screening results for the *M. mercenaria* that were inoculated with *P. olseni* cells are shown in Table 4. RFTM data was not collected during the first sampling day 21, however two of the four sampled clams were positive for *P. olseni* DNA on that day. On day 44, one clam was observed with a rare RFTM ranking, however *Perkinsus* sp. DNA was not detected. On day 56, all clams were negative by RFTM and PCR. On day 72, one clam had a rare ranking by RFTM and another a very light ranking by RFTM, although only the individual with the very light RFTM ranking was found to be positive for *Perkinsus* sp. DNA.

Mortality data. Mortality was observed in untreated *C. ariakensis* oysters and in those injected with either SASW or *P. olseni* (Table 5). For the untreated *C. ariakensis,* two died during the 37-day period that they were held in the aquaria. For the SASW treatment and the *P. olseni* treatment, cumulative mortality for the *C. ariakensis* after 72 days was 40.0% and 46.2%, respectively. Of the 27 dead *C. ariakensis* removed from the untreated tank and the experimental aquaria during the course of the experiment, it was possible to conduct RFTM analysis on only 11 oysters and PCR analysis on 22 because tissues rapidly degraded in the small oysters. All tissues taken from dead *C. ariakensis* were PCR positive for *P. marinus* DNA. Of these 11 oysters examined by RFTM, two had none, or rarely observable *Perkinsus* sp. cells, two had very light or light tissue burdens, one had a light to moderate tissue burden and six had moderate to heavy tissue burdens of *Perkinsus* sp. cells.

The observed mortality was minimal for the *C. virginica* and *M. mercenaria.* Two *C. virginica* from the SASW treatment and one from the *P. olseni* treatment died

Table 4. RFTM ranking and PCR-based *Perkinsus* genus-specific assay screening results of the first challenge study *M. mercenaria* clams that were notched and inoculated with cultured *P. olseni* cells and sampled from the experimental aquaria on days 44, 59 and 72.

RFTM Ranking	**Day 44 (n=4)** ***P. olseni***		**Day 59 (n=4)** ***P. olseni***		**Day 72 (n=35)** ***P. olseni***	
	# RFTM	# PCR pos	# RFTM	# PCR pos	# RFTM	# PCR pos
None (N)	3		4		33	4
Rare (R)	1	0			1	0
Very Light (VL)					1	1
Light (L)						
Light/Moderate (LM)						
Moderate (M)						
Moderate/Heavy (MH)						
Heavy (H)						
Very Heavy (VH)						

Table 5. Daily observed mortalities during the course of the first challenge experiment.

Column headings indicate the sample treatments.

	Crassostrea ariakensis			*Crassostrea virginica*			*Mercenaria mercenaria*	
Day	**Untreated**	**25 ppt SASW**	***Perkinsus olseni***	**25ppt SASW**	***Perkinsus olseni***	***Perkinsus marinus***	**25 ppt SASW**	***Perkinsus olseni***
2	1							
6					1			
11	1							
37		1		1				
42				1				
43	na*		1					
45	na							1
49	na	4	1					
53	na		1					
55	na		4					
58	na	1	1					
59	na	1						
64	na	1	2					
66	na		1					
72	na		6					

*na=not applicable as the *C. ariakensis* from the untreated tank were sacrificed on day 37.

during the course of the study. There was no tissue remaining to process for the SASW treatment oyster that died on day 42, however for the other SASW or *P. olseni* treatment oysters, neither was either RFTM or PCR positive for *Perkinsus* sp. Only one clam died during the course of this experiment. It was an animal from the *P. olseni* treatment and there was no tissue left to process and analyze for the presence of *Perkinsus* sp. DNA.

Species-specific PCR screening for *Perkinsus marinus* and *Perkinsus olseni.* All bivalve species shown to be PCR positive with the *Perkinsus* genus-specific assay were analyzed using both *P. marinus*-specific and *P. olseni*-specific primers. As the *P. olseni* primers were new for this study, specificity was tested against *P. marinus* and *P. chesapeaki* DNAs. The *P. olseni* primers did not amplify DNA from these other *Perkinsus* species. Sequencing of amplification products from the control DNA samples further confirmed the specificity as sequences of amplification products from all positive *P. olseni*-specific reactions matched those of GenBank deposited *P. olseni* sequences. The five *C. ariakensis* taken as a baseline sample immediately prior to the start of the study, and all untreated oysters had only *P. marinus*, not *P. olseni,* DNA. In addition, all but two of the *C. ariakensis* that were SASW- or *P. olseni*-inoculated and that were *Perkinsus* sp. positive with the genus-specific primers, were positive for only *P. marinus* DNA. Two *C. ariakensis* that were inoculated with *P. olseni* at the start of the challenge and were sampled on days 21 and 72 were positive for both *P. marinus* and for *P. olseni* DNA. Tissue from all dead oysters collected from either the experimental or untreated aquaria were found to be PCR-positive for *P. marinus* only.The six *C. virginica* from the *P. olseni*-inoculated treatment that were *Perkinsus* sp. positive with the genus-specific primers, were positive only for *P. olseni* DNA. The seven PCR positive oysters from the *P. marinus*-inoculated treatment were found to be positive only for *P. marinus* DNA.

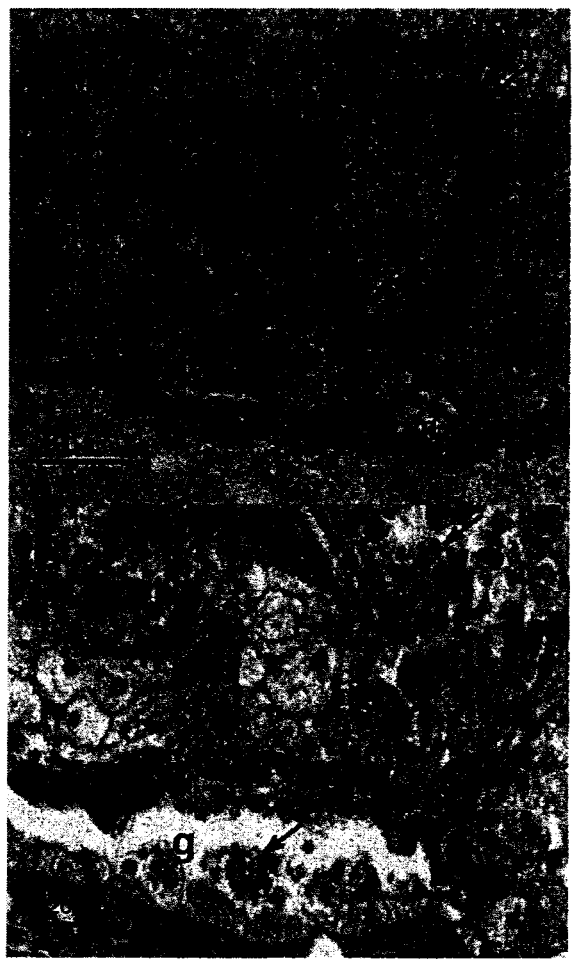
In total, seven *M mercenaria* were *Perkinsus* sp.-positive from the *P. olseni* treatment and all of these clams were found to be positive only for *P. olseni* DNA.

RFLP results. The *P. olseni* and *P. marinus* positive control DNA isolated from cultured cells, and a plasmid-containing *Perkinsus chesapeaki* ITS region DNA, were amplified in the *Perkinsus* genus-specific assay. ITS region amplification products were digested with *Mbo* I restriction endonuclease. Results confirmed that *P. marinus* ITS region DNA has a unique digestion profile when digested with *Mbo* I and the observed fragment sizes of approximately 17 bp, 226 bp and 264 bp were consistent with the fragment sizes expected for *P. marinus*. With this enzyme, ITS region amplification products of *P. chesapeaki* would remain uncut while *P. olseni* ITS region amplification products would be cut, resulting in a 64 bp and a 262 bp fragment.

Histology. Ten *C. ariakensis* shown by PCR to have *Perkinsus* sp. DNA, and whose tissues showed light to very heavy RFTM rankings were chosen for further histological examination. A tissue section from one animal with a very heavy RFTM ranking that had died during the experiment showed gross *Perkinsus* sp. lesions (Fig. 1A) and observable parasite cells when stained with Harris-hemotoxylin and eosin (Fig. 1B). Parasite cells were numerous and observed systemically throughout the visceral mass. Obvious lesions due to *Perkinsus* sp. infection were not observed by histology in tissue sections taken from animals with light or moderate *Perkinsus* sp. tissue burdens as determined by the RFTM assays.

Figure 1. H&E-stained histological section of *Perkinsus marinus* lesions in the gonad of *Crassostrea ariakensis*. (A) Low power photomicrograph showing the extent of the lesions (arrows) in the vicinity of the gonoduct (g). Square represents the area shown in B. (B) Higher magnification in the vicinity of a gonoduct (g) showing *P. marinus* cells (arrows) in the lesions.

Figure 1.



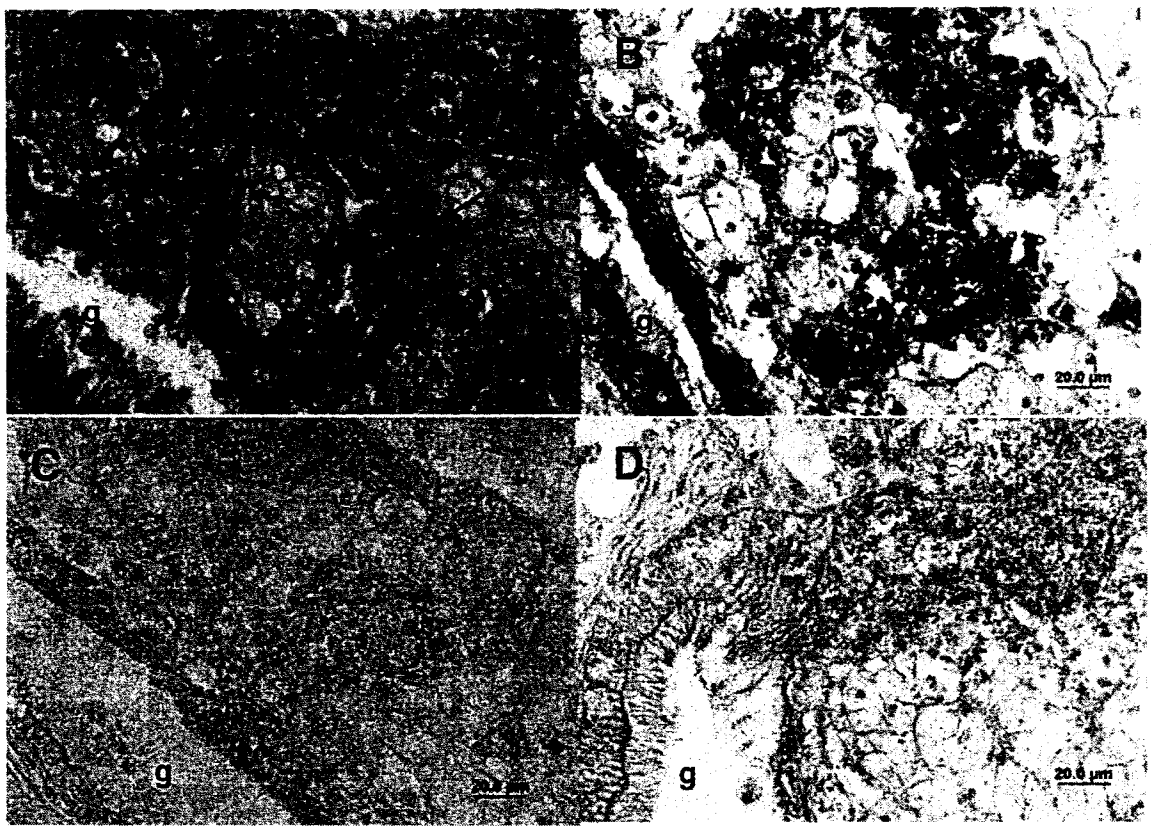
All *C. virginica* and *M. mercenaria* observed to be *P. olseni* positive or RFTM positive (within the *P. olseni* treatment only) were examined histologically. *Perkinsus* sp. cells were not observed in any tissue sections examined.

In situ hybridization results. In situ hybridization probes designed to target the LSU rRNA gene of *P. marinus* or *P. olseni* exhibited specificity for the targeted *Perkinsus* species. In the specificity tests, the *P. marinus* probe hybridized only to the *Perkinsus* cells in the sample of *P. marinus* in *C. virginica* and the *P. olseni* probe hybridized to *Perkinsus* sp. cells in the control sample of *P. olseni* in *Haliotis laevigata.* The probes did not cross-react with non-targeted *Perkinsus* species or host tissues in other control samples.

Figure 2A-D shows four consecutive sections from the heavily infected *Crassostrea ariakensis* oyster shown in Fig. 1. Figure 2A is an H&E-stained section. Fig. 2B shows positive in situ hybridization with the PmarDIGLSU-181 probe to *Perkinsus* sp. cells indicating that the lesions were caused by *P. marinus*. The *P. marinus*-specific probe bound to cells throughout the digestive epithelium, gonads and gonoducts. In situ hybridization reactions conducted without probe (Fig. 2C), or with the *P. olseni*-specific probe, PolsDIGLSU-464, (Fig. 2D), produced no signal in host tissues of this oyster. The *P. olseni*-specific probe was also tested against two *C. ariakensis* that were inoculated with *P. olseni* and found to harbor both *P. marinus* and *P. olseni* DNA by the PCR assays. The *P. marinus* probe hybridized to *Perkinsus* sp. cells in the dually infected oysters, however, no binding of the *P. olseni* probe was observed, suggesting that infections by *P. olseni* were extremely light, or that only *P. olseni* DNA was present and not viable cells.

Figure 2. *Perkinsus marinus* in *Crassostrea ariakensis*. (A) H&E-stained histological section in the vicinity of a gonoduct (g) showing *Perkinsus marinus* lesions (arrows). (B) In situ hybridization with the *P. marinus*-specific probe, Pmar181LSUDIG, illustrating strong binding to the *Perkinsus* sp. cells (arrows). (C) No probe negative control showing no binding to the *C. ariakensis* tissue. (D) In situ hybridization with the *P. olseni*-specific probe, Pols464LSUDIG, illustrating no binding to the *Perkinsus* sp. cells.

Figure 2.



The tissue sections of the six *C. virginica* and seven *M. mercenaria* that were PCR positive for *P. olseni* DNA produced no hybridization of the *P. olseni* probe, suggesting that there were not enough *P. olseni* cells in those individuals to be detected using this technique.

EXPERIMENT 2. Supplementation of *Perkinsus olseni* culture media

MATERIALS AND METHODS

Experimental hosts. Two hundred *Crassostrea virginica* were obtained from Hog Island Oyster Company, Tomales Bay, California, 200 *Mercenaria mercenaria* were obtained from Mobjack Bay Seafood Company, Ware Neck, Virginia, and 200 triploid *Crassostrea ariakensis* were obtained from the hatchery at the Virginia Institute of Marine Science (VIMS), Gloucester Point, Virginia. Prior to the start of the experiment, 20 individuals of each species were sacrificed in order to confirm that they were *Perkinsus* sp.-free. Tissue from the subsampled hosts was processed for DNA, RFTM and histological analysis as above.

Remaining *C. ariakensis*, *C. virginica* and *M. mercenaria* were acclimated for two weeks in separate, 50 gallon glass aquaria to 25 ppt, 1-μm filtered York River water. Aquaria water temperature was maintained at 20--20 °C and animals were fed 0.2 g per oyster algal food daily. Two thirds of the aquaria water was removed three times each week and replaced with an equal volume of clean, 25 ppt, 1-μm filtered York River water.

***Perkinsus olseni* culture.** A clonal, cryopreserved *Perkinsus olseni* culture, (ATCC PRA-181) isolated from a Japanese *Venerupis philippinarum* clam was thawed, propagated, and continually expanded over two weeks at the Maryland Cooperative

Oxford Laboratory, Oxford Maryland by Chris Dungan and Rosalee Hamilton. *Perkinsus* sp. medium consisted of DME: Ham's F12 media, 2mM L-glutamine, 25 mM HEPES, 7 mM $NaHCO_3$, carbohydrates (glucose, glactose, trehalose) and 3% (v/v) fetal bovine serum (FBS). The final osmolality was 850 mOsm kg^{-1} (29 ppt) and hereafter will be indicated as DME/F12-3 (Burreson, Reece and Dungan 2005). Medium was additionally supplemented with penicillin (100 ug/ml), streptomycin (100 ug/ml) and gentamicin (100 ug/ml). Absence of microbial contamination was microscopically confirmed in all flasks and cell viability was determined using the neutral red assay. Two weeks later, cells were harvested by pipetting 180 ml each into four 250 ml conical centrifuge bottles. Cells were centrifuged for 15 min at 20 °C, 300 x g, to recover four, ~3 ml volume cell pellets. Cell pellets were resuspended to wash in 50 ml of 30 ppt sterile artifical seawater (SASW), and were repelleted by centrifugation for 5 min at 20°C, 300 x g. Supernatant was removed and each cell pellet was then resuspended in 132 ml of serum free DME/F12-3 medium and resulting suspensions were distributed 22 ml each to 24, 125 cm^2 culture flasks. Flasks were tightly sealed and sent to VIMS. Upon arrival, 2 ml of fresh media was added to each flask and they were incubated at 20 °C.

Homogenate preparation. Approximately 250 small (25.9 ± 4.08 mm average shell height) triploid *C. ariakensis* were aseptically shucked and homogenized in 25 ppt SASW in batches using sterile blenders. *C. ariakensis* homogenate was pooled and divided into 12 Sorval ultracentrifuge tubes held on ice. Ultracentrifuge tubes containing homogenate were balanced using 25 ppt SASW and were initially centrifuged for 1 h at 4 °C, 16,000 x g. The supernatant was removed using sterile Pasteur pipettes and pooled into sterile 50 ml polystyrene falcon tubes on ice. The supernatant was then aliquoted

into six new ultracentrifuge tubes and centrifuged for an additional 45 min at 4 °C, 100,095 x g. Resulting supernatant was removed using sterile Pasteur pipettes and pooled into 15 ml polystyrene falcon tubes on ice. Pooled supernatant was sterile filtered three times using sterile 3 ml syringes fitted with 0.22 μm filters (Costar, Whatman, Clifton, N.J.) and placed at 4 °C overnight. An aliquot of sterile *C. ariakensis* homogenate was run in triplicate in a bicinchoninic acid assay (BCA) against BSA standards to determine the protein concentration of the homogenate.

***P. olseni* culture supplementation.** Propagating *P. olseni* cells were observed microscopically for absence of microbial contamination and pooled into six sterile 250 ml conical centrifuge bottles. One milliliter aliquots of cells were removed from each bottle for cell counts and determination of viability. The pooled cells were then centrifuged for 15 min at 20 °C, 300 x g. The supernatant culture media was removed and cells resuspended in a volume of fresh, serum-free DME/F12 media as necessary to yield a final concentration of 10^8 cells/ml. Six 225 cm^2 polystyrene culture flasks were designated for fetal bovine serum (FBS) culture treatment and were inoculated with 237 ml of fresh DME/F12 media, 2.4 ml of *P. olseni* cells at 10^8 cells/ml, and 0.7 ml of FBS (35 mg/ml protein) to yield cell cultures supplemented at 0.1 mg/ml with FBS at 10^6 cells/ml seeding density. Six additional 225 cm^2 polystyrene culture flasks were designated for the *C. ariakensis* homogenate supplemented treatment and were inoculated with 236 ml of fresh DME/F12 media, 2.4 ml of *P. olseni* cells and 1.6 ml of *C. ariakensis* homogenate (14.8 mg/ml protein), to yield cell cultures supplemented at 0.1 mg/ml with *C. ariakensis* homogenate at 10^6 cells/ml seeding density.

All flasks of cells were then incubated at 22 °C, for 96 h. Every 24 h, all flasks were observed microscopically to confirm the absence of microbial contaminants and at

the same time, 2 ml of cells were removed from each flask. These subsamples of cells were centrifuged for 10 min at 20°C, 300 x g. Supernatant culture media was removed from the cell pellet using sterile 3 ml syringes (needle removed) and passed through 0.22 um filters (Costar, Whatman, Clifton, N.J.) into sterile 1.5 ml centrifuge tubes. Sterile cell-free media taken from each flask was frozen at -80°C for later analysis of protease activity.

After the 96 hour incubation period, four flasks of cells for each treatment were then pooled separately into sterile 250 ml conical centrifuge bottles and centrifuged for 15 min at 20°C 300 x g. The remaining two flasks for each treatment were combined into a new flask and held for an additional 25 d. Cell free media was taken as described above from each of these flasks at 5, 13, 20 and 27 d. The supernatant media from the pooled, centrifuged *P. olseni* cells was removed and 50 ml of 25 ppt SASW were added to each centrifuge bottle. Cells were washed and repelleted for 10 min at 20 °C, 500 x g. The supernatant was removed and cells were resuspended in 50 ml of 25 ppt SASW. Washed cells were centrifuged for an additional 10 min at 20 °C, 500 x g. Supernatant was removed and cells were resuspended in 25 ml of fresh 25 ppt SASW. Cell counts and viability were performed on aliquots of cells for each supplementation treatment as described above.

After the cell counts were performed, *P. olseni* cells for each supplementation treatment were then separately diluted in 25 ppt SASW in sterile 25 cm^2 culture flasks as needed for each culture treatment. Inoculum concentrations were manipulated so that a 100 µl volume of cells at 10^6 cells per gram body weight of host could be administered to each host. Average body weights of *C. ariakensis, C. virginica* and *M. mercenaria* were

0.56 ± 0.24, 5.91 ± 1.69 grams and 7.71 ± 1.80 grams, respectively. Inocula in 25 ppt SASW were held at 20°C overnight prior to injection.

On the same day that the *P. olseni* cells were washed and resuspended in 25 ppt SASW (96 h post supplementation), all experimental hosts were removed from the acclimation aquaria. Using a Dremel tool fitted with a stone cutting disk, the *C. virginica* were notched at the posterior valve margin. *Mercenaria mercenaria* were notched along the valve margin in the vicinity of the mouth. Due to the small size and fragile nature of the *C. ariakensis* shells, a sterile razor blade was used to notch individuals at the posterior valve margin. All animals were returned to the acclimation aquaria where they remained overnight.

The following day all animals were removed from the acclimation aquaria. Fourty-five individuals of each host species were given a single, 100 µl volume inoculation of 10^6 log phase *P. olseni* cells per gram body weight, cultured previously for 96 hours in 0.1 mg/ml FBS supplemented DME/F12 culture medium. An additional 45 individuals of each host type received an identical inoculation of *P. olseni* cells cultured previously with DME/F12 media supplemented with 0.1 mg/ml *C. ariakensis* homogenate. As negative controls, thirty individuals of each host received a single 100 µl inoculation of 25 ppt SASW only. Inoculated animals were placed on plastic trays, notch side up, and held for 5 h in a humidified room at 4°C. For each group of hosts inoculated with the designated *P. olseni* culture inocula, the 45 individuals were then divided into three aquaria, 15 individuals per aquaria. For each sham-inoculated group of hosts, the 30 individuals were then divided into two aquaria, 15 individuals per aquaria.

When the experimental hosts were first introduced to the aquaria, the aquaria were only half full of water. After one hour, 1ml of algae feed was added to each

aquaria. These steps were taken in order to promote uptake, by feeding, of any purged cells that may have been released. Eighteen hours later the aquaria were filled entirely. All animals were held in 10 gallon glass aquaria maintained at 20—22 °C and 25 ppt aerated, 1-μm filtered York River water, and were fed 0.2 g algal food/oyster twice daily. The first water change occurred 5 days post injection so that any purged *P. olseni* cells were not immediately removed from the aquaria. From this point forward, two thirds of the aquaria water was removed three times each week and replaced with an equal volume of clean, 25 ppt, 1-μm filtered York River water.

Aquaria were checked daily for mortality and moribund animals were removed. When moribund or dead animals were discovered, and if sufficient undegraded tissue remained it was processed for analysis. Gill and mantle tissues were removed, preserved and processed as described above for RFTM, PCR and histological analyses.

Analysis of *P. olseni* proteolytic enzyme expression. Supernatants from the two *P. olseni* culture treatments and media controls were analyzed for the presence of proteolytic activity. Ten microliters of culture supernatant or control media was mixed with 5 μl of Laemmli non reducing sample buffer (Biorad, Hercules, CA) and electrophoresed under non-reducing conditions on a 10% sodium dodecyl sulfate polyacrylamide (SDS) resolving gel containing 0.1% porcine gelatin (G-8150; Sigma-Aldrich, Inc.) with a 4% stacking gel. One lane per gel contained 10 μl of a broad range, unstained protein standard (Biorad, Hercules, CA). Electrophoresis was performed on a Bio-Rad mini-Protean apparatus using a Tris-glycine buffer system at 120 volts and 4°C for approximately 1.5 h. Following electrophoresis, gels were vigorously washed three times for 10 min at 4 °C in 250 ml of 4°C 2.5% Triton X-100 in order to renature the proteins and then washed once in 250 ml of room temperature 100mM Tris HCl (pH 8.0).

Gels were incubated overnight at 37 °C in 250 ml fresh 100mM Tris HCl (pH 8.0). Use of activating buffer at pH 8.0 was chosen as it had been previously determined to be optimal for detection of protease expression in *P. marinus* (La Peyre et al. 1995). Following incubation, gels were stained for at least 4 h in Coomassie brilliant blue G-250 in 40% methanol-10% acetic acid. Gels were destained in 40% methanol, 10% acetic acid and analyzed for the presence of clear bands corresponding to the location of protease activity. Gel images were recorded on an Alpha Innotech FlourChem® (SanLeandro, CA) imaging system.

Experimental sampling – animals. Three animals were removed from each aquarium on days 14, 28, 42, 56 and on day 70, the final day of the experiment, as long as enough live animals remained. Sampled animals were aseptically sacrificed and tissues were subdivided as described above.

Nucleic acid extraction, test for genomic DNA quality and *P. olseni* – specific PCR. Genomic DNA was extracted and analyzed as described above to determine that PCR amplifiable DNA was present in all extracted samples.

DNA from all animals as well as from all water samples (see below) was analyzed using the PCR-based molecular diagnostic assay specific for *P. olseni* using primers Patl140F and Patl600R, described above, that targets the ITS region of the ribosomal RNA gene complex. Following amplification, 5 µl of PCR product was analyzed by agarose gel electrophoresis (2%), stained with ethidium bromide and visualized as above.

Enumeration and DNA extraction of cultured *P. olseni* cells. Enumeration and DNA extraction of cultured *P. olseni* cells was performed according to methods in Audemard et al. (2004) with slight modification. Approximately 10 ml of *Perkinsus olseni* (ATCC PRA-181) cells in DME F/12 culture media were centrifuged for 10

minutes at 20 °C, 200 x g, to pellet the cells. The supernatant was removed and cells were resuspended in 10 ml of 25 ppt SASW in a 15 ml polystyrene falcon tube. An aliquot of cells was removed and eight cell counts were performed as described above on a subsample of cells stained with neutral red. Based on the mean cell concentration from eight cell counts, six replicates of 25 ppt SASW were spiked with cells to obtain a final concentration of 1,000 cells/ml and a final volume of 100 ml (100,000 cells total per 100 ml sample). Each *P. olseni* spiked replicate was filtered under vacuum filtration onto a sterile, 47 mm diameter, 3 μm pore size Nucleopore filter (Costar, Whatman, Clifton, N.J.) with a disposable apparatus (Nalgene Nunc International, Rochester, N.Y.). Membranes were handled with sterile disposable forceps and placed into 180 μl of QIAmp DNA Stool Mini Kit lysis buffer with 20 μl (100 mg/ml) of proteinase K (Qiagen, Valencia California) and incubated overnight at 55 °C to lyse the cells prior to DNA extraction.

DNA was extracted from the spiked water samples using a QIAmp DNA Stool Mini Kit (Qiagen, Valencia, California) according to the manufacturer's protocol with the modifications published by Audemard et al. (2004). Modifications included the overnight lysis step, decreasing by half the volume of ASL buffer and using half of an InhibitEX tablet. DNA was eluted by three 100 μl loadings of elution buffer onto the column with 5 min of incubation before centrifugation (eluates combined).

Real-time PCR conditions. Quantitative real-time PCR was performed using the Light Cycler from Roche Diagnostics (Mannheim, Germany). Amplified PCR product was quantified on a cycle-by-cycle basis through the acquisition of a fluorescent signal generated by the binding of the molecule, Sybr Green I (Roche Diagnostics) to double stranded DNA. DNA from the six replicates of *P. olseni*-spiked water (1,000 cells/ml),

was diluted serially by six orders of magnitude using elution buffer. This corresponded to a dilution series ranging from 333 x 10^0 to 3.3 x 10^{-4} cells/μl. From the dilution series, a standard curve was generated in which the threshold cycle was plotted versus the logarithm of the starting concentration of DNA (corresponding to a known number of cells). This standard curve, generated from known concentrations of cells, allowed quantification of the number of *P. olseni* cells in the water collected from experimental aquaria when sample cycle threshold values were plotted against the standard curve.

Real-time PCR reactions were performed using a LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche Diagnostics) in 10 μl volumes and *P. olseni*-specific PCR primers Pols140F and Pols600R. Each PCR reaction contained 1 U Fast Start DNA Master^{PLUS} polymerase mixture (contains $MgCl_2$), 0.5 μm of high performance liquid chromatography purified primers, Pols140F and Pols600R (Invitrogen), 0.4 mg/l bovine serum albumin, and 1 ul of DNA. The amplification program was as follows: the reaction was heated at 95 °C for 10 min to activate the DNA polymerase, followed by 50 cycles of increasing the temperature 20 °C/s to 95 °C, holding the temperature at 95 °C for 10 s, decreasing the temperature 20 °C/s to 67 °C, holding this temperature for 10 s, increasing the temperature 20 °C/sec to 72 °C, and holding this temperature for 18 s. Flourescence acquisition was acquired at 72 °C at the end of each cycle. A melting curve was acquired by heating the PCR products at 20 °C/s to 95 °C, cooling it at 20 °C/s to 60 °C, and slowly heating it at 0.1 °C/s to 95 °C, with fluorescence values collected at 0.1 °C intervals. The melting temperature of *P. olseni* ITS PCR products was determined to be 83 °C. The serial dilutions used to create a standard curve and all experimental samples were run in triplicate. The reproducible

limit of sensitivity for the *P. olseni* real-time PCR reaction was 3.3 x 10^{-2} cells in a 10 µl reaction volume or ~1000 cells/liter.

Experimental sampling – water. On days 5, 14, 28, 42, 56 and on day 70, the final day of the experiment, duplicate 100 ml samples of water were taken from each aquarium using a sterile 50 ml serological pipette and were dispensed into 100 ml sterile coliform bottles. Water samples were filtered and DNA was extracted following the protocol described above for enumeration and DNA extraction of *P. olseni* spiked water samples used to create the standard curve.

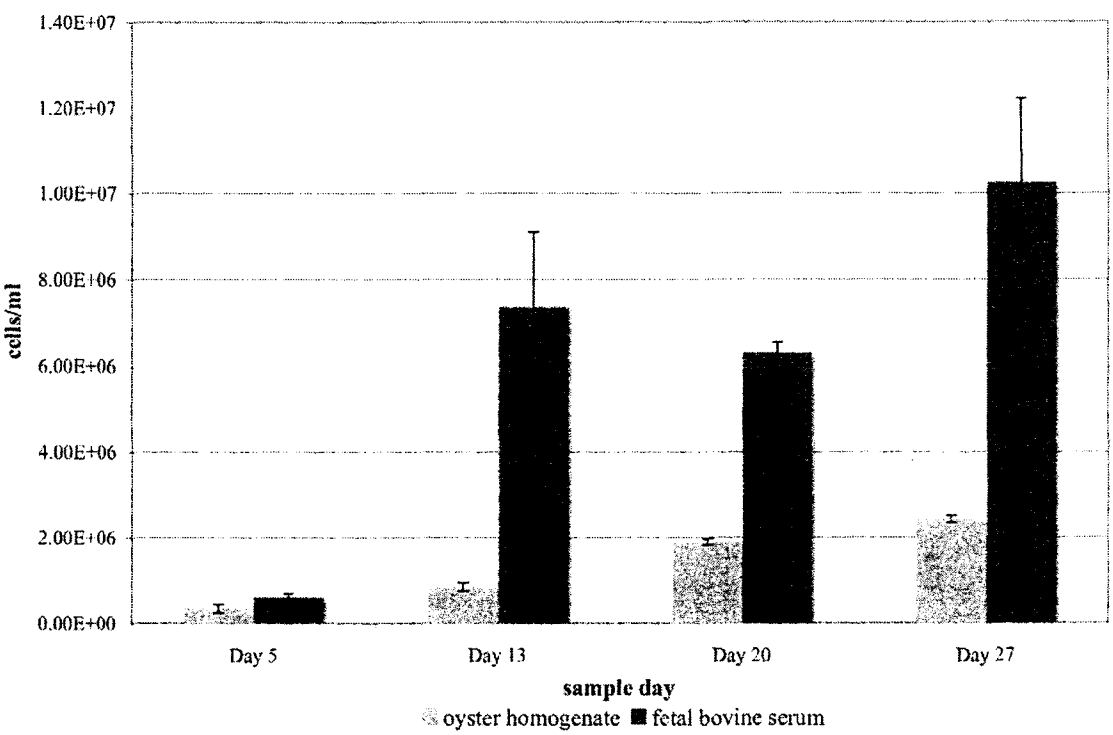
Data analysis. Acquisition of *P. olseni,* as determined by PCR results, was analyzed for all hosts and treatments in Systat (Systat 11, San Jose California) using logistic regression. In particular, the questions of interest were 1) is there a difference in number of PCR positive samples seen in experimental hosts depending on the culture method used and 2) is there a difference in the number of *P. olseni* PCR positive samples depending on host species inoculated. In addition, *P. olseni* cell counts taken on day 5, day 13, day 20 and day 27 for each treatment were analyzed using a two way analysis of variance in MiniTab version 14 (State College, Pennsylvania) in order determine if culture supplementation had an effect on *P. olseni* cell proliferation. In all analyses, differences were considered significant at $\propto < 0.05$.

RESULTS – Experiment 2

***P. olseni* culture cell population & analysis protease activity.** *Perkinsus olseni* cell counts taken on days when cell free supernatant was acquired revealed a marked difference among days (Figure 3) and subsequently the frequency of certain cell types between the media treatments. On day 5 (days following supplementation), there were

Figure 3. *Perkinsus olseni* cell counts taken over a 27-day incubation in media supplemented with either 0.1 mg/ml FBS or 0.1 mg/ml *Crassostrea ariakensis* homogenate.

Figure 3.



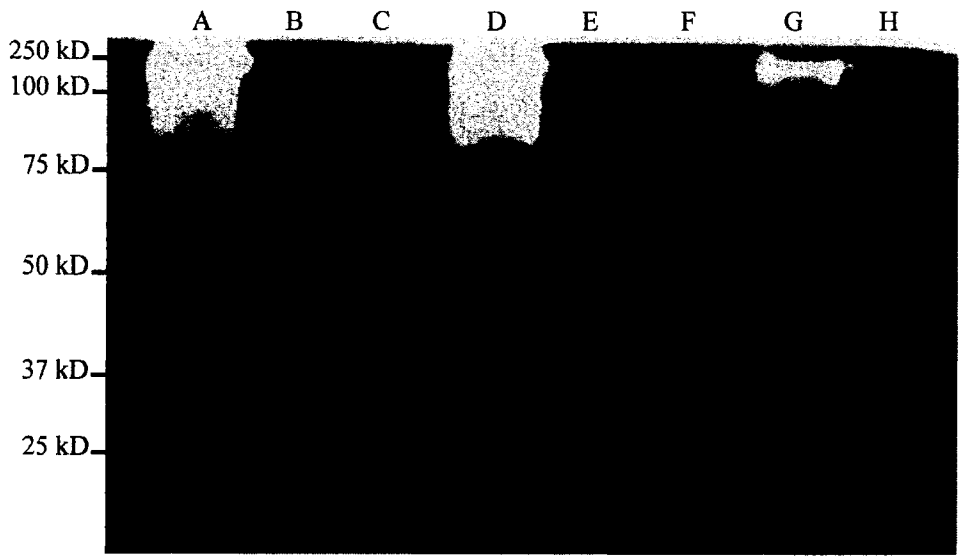
approximately 1.8 times the number of cells in the FBS treatment compared to the oyster homogenate treatment. By day 13, that difference reached a maximum, with the *P. olseni* cells in FBS treatment being 8.7 times more dense than the cells in the oyster homogenate treatment. On days 20 and 27, the difference in cell density between the FBS and oyster homogenate was 3.3 and 4.2 times, respectively. Though individual counts of the various life stages were not taken, throughout the 27-day incubation, flasks containing cells in the oyster homogenate treatment had visibly more zoosporangia than the FBS treatment flasks. Zoosporulation of *P. olseni* in culture is often inversely proportional to the density of the culture, therefore proliferating stages in the oyster homogenate flasks were dominated by zoosporangium whereas proliferating stages in the FBS flasks were dominated by schizonts.

A number of the *P. olseni* supernatant samples taken during the first 96 hours of incubation were unfortunately lost after being stored at -80°C. Samples analyzed included those taken after 96 hours and on day 8 and day 20. High molecular weight protease activity was observed for the oyster homogenate supplemented control media sample, but not for the FBS supplemented control media sample. High molecular weight protease activity was observed for samples taken from the oyster homogenate supplemented flasks 96 hours post-supplementation, as well as on days 8 and 20, with an observed increase in strength of proteolytic activity over time. No protease activity was seen for FBS-supplemented culture samples (Fig 4).

Experimental sampling – animals. All animals sampled from the negative control treatments were negative for *P. olseni* by PCR and RFTM assays. Individuals from all three host species inoculated with *P. olseni* cells from either the FBS supplemented or oyster homogenate supplemented media, except the clams from the

Figure 4. Effect of 0.1 mg/ml *C. ariakensis* homogenate versus 0.1 mg/ml FBS on protease expression by *P. olseni*. SDS-PAGE gelatin gel showing clearing in the presence of proteolytic enzymes. A) Day 8 oyster homogenate culture supernatant B) Day 8 FBS culture supernatant C) empty lane D) Day 20 oyster homogenate culture supernatant E) Day 20 FBS culture supernatant F) empty lane G) oyster homogenate media control H) FBS media control.

Figure 4.



oyster homogenate treatment, showed the presence of *P. olseni* DNA by PCR (Table 6) at the first sampling point, week 2. Many *C. virginica* died within the first two weeks of the experiment, therefore, the PCR and RFTM data from these mortalities was combined with the week 2 sampling data. At week 4, individuals of all host types in both treatments were PCR positive for *P. olseni*, however, no cells were observed by the RFTM assay. At week 6, some *C. virginica* and *M. mercenaria* individuals were PCR positive for *P. olseni* in both treatments, however, only one *C. ariakensis* from the oyster homogenate treatment was found to contain *P. olseni* DNA. In addition, only one *C. virginica* in each of the treatments was positive by RFTM assay, while no *Perkinsus* sp. cells were observed in any of the sampled *M. mercenaria* or *C. ariakensis*. At week 8, *C. virginica* and *M. mercenaria* were PCR positive for *P. olseni* in both treatments, however, only one *C. ariakensis* was positive for *P. olseni* DNA from the oyster homogenate treatment. Due to previous mortality, there were very few *C. virginica* remaining by week 10. At the week 10 sampling, although some *M. mercenaria* were PCR positive in each treatment, no *Perkinsus* sp. cells were observed in any of the clams by the RFTM assay. *Crassostrea virginica* and *C. ariakensis* were found to be PCR positive for *P. olseni* only in the oyster homogenate treatment and *Perkinsus* sp. cells were observed by RFTM in only one sampled *C. virginica* from this treatment.

When examining cumulative or percent PCR positive and RFTM positive for each host species and treatment, higher values for both assays were observed for *C. virginica* and *M. mercenaria* from the FBS treatment compared to the oyster homogenate treatment (Table 6), however the differences observed were not statistically different ($p = 0.67$). *Perkinsus olseni* DNA was detected most frequently in those *C. virginica* inoculated with

Table 6. PCR and RFTM data for the three bivalve species studied during the course of the second *P. olseni* challenge experiment in which culture media was supplemented with *C. ariakensis* homogenate or fetal bovine serum. Data is divided into the treatments indicative of how the *P. olseni* culture media was supplemented prior to inoculation. Fractions indicate the number of animals either PCR or RFTM positive over the total number sampled at that time point.

	Fetal Bovine Serum Supplemented Treatment			**Oyster Homogenate Supplemented Treatment**		
Week 2	**PCR positive**	**RFTM positive**	**RFTM ranking**	**PCR positive**	**RFTM positive**	**RFTM ranking**
Crassostrea virginica	12/14	11/14	N-L	12/13	8/13	R-L
Mercenaria mercenaria	8/9	3/9	N-VL	5/9	0/9	N
Crassostrea ariakensis	4/9	1/9	N-VL	8/9	1/9	N-VL
Week 4	**PCR positive**	**RFTM positive**	**RFTM ranking**	**PCR positive**	**RFTM positive**	**RFTM ranking**
Crassostrea virginica	7/10	0/10	N	5/9	0/9	N
Mercenaria mercenaria	5/9	0/9	N	7/9	0/9	N
Crassostrea ariakensis	4/9	0/9	N	2/9	0/9	N
Week 6	**PCR positive**	**RFTM positive**	**RFTM ranking**	**PCR positive**	**RFTM positive**	**RFTM ranking**
Crassostrea virginica	8/10	1/10	R	3/9	1/9	R
Mercenaria mercenaria	7/9	0/9	N	3/9	0/9	N

Crassostrea ariakensis	0/9	0/9	N	1/9	0/9	N
Week 8	**PCR positive**	**RFTM positive**	**RFTM ranking**	**PCR positive**	**RFTM positive**	**RFTM ranking**
Crassostrea virginica	6/9	1/9	R	3/9	1/9	R
Mercenaria mercenaria	2/9	0/9	N	4/9	1/9	R
Crassostrea ariakensis	0/9	0/9	N	1/9	1/9	R
Week 10	**PCR positive**	**RFTM positive**	**RFTM ranking**	**PCR positive**	**RFTM positive**	**RFTM ranking**
Crassostrea virginica	0/2	0	N	2/4	1/4	R
Mercenaria mercenaria	1/9	0	N	2/9	0/9	N
Crassostrea ariakensis	0/7	0	N	1/9	1/9	N
totals (%)	**PCR positive**	**RFTM positive**		**PCR positive**	**RFTM positive**	
Crassostrea virginica	**33/45 (73%)**	**12/45 (27%)**		**25/45 (56%)**	**12/45 (27%)**	
Mercenaria mercenaria	**23/45 (51%)**	**3/45 (7%)**		**21/45 (47%)**	**1/45 (2%)**	
Crassostrea ariakensis	**8/45 (18%)**	**1/45 (2%)**		**13/45 (29%)**	**3/45 (7%)**	

cells cultured in FBS (73%) of which 27% were RFTM positive. In comparison, 56% of the *C. virginica* in the oyster homogenate treatment were PCR positive for *P. olseni,* (27% RFTM positive). *Mercenaria mercenaria* had the second highest values for cumulative PCR and RFTM positive samples. Fifty one percent of the *M. mercenaria* in the FBS treatment were positive for *P. olseni* DNA (7% RFTM positive) and in the oyster homogenate treatment, a similar percentage of clams (47%) were PCR positive (2% RFTM positive). *Crassostrea ariakensis* sampled from either treatment were the least likely to have the presence of *P. olseni* by DNA analysis or the presence of *Perkinsus* sp. cells by RFTM. Eighteen percent of the *C. ariakensis* were PCR positive (2% RFTM positive) in the FBS treatment, and 29% were PCR positive (7% RFTM positive) in the oyster homogenate treatment. Though the observed difference between treatments for *C. ariakensis* was not statistically significant with a p-value of 0.093, an odds ratio of 2.953 --10.451 suggests that a trend may exist in that *C. ariakensis* are more likely to be PCR positive when inoculated with *P. olseni* cells cultured in media supplemented with oyster homogenate. Within each host species, there was not a significant relationship between being PCR positive for *P. olseni* versus host size; however, there was a significant negative relationship observed between time following inoculation and being PCR positive for *P. olseni* in that the longer it had been since inoculation, the less likely the individuals of any host species were to be PCR positive.

Mortality data. Mortality was observed beginning three days post inoculation in the *C. virginica* oysters and was observed until day 35. Mortality occurred in all treatments and 6, 7 and 9 oysters died in the control, oyster homogenate supplemented and FBS supplemented treatments, respectively. None of the control oysters was positive

for *P. olseni,* however many of those oysters from both inoculation treatments were PCR, as well as RFTM, positive for *Perkinsus* sp. (Table 7).

No mortality occurred in any of the *M. mercenaria* treatments and only two *C. ariakensis* were found dead upon sampling at week 10, both from one of the FBS supplemented treatment tanks. No tissue remained in these two oysters for DNA or RFTM analysis.

In situ hybridization analyses. All RFTM positive animals from all host types were analyzed by in situ hybridization. Hybridization to *P. olseni* cells was observed in only one sample, a *M. mercenaria* clam sampled from the FBS treatment at week 8, with a very light ranking by RFTM (Figure 5). *P. olseni* cells were observed in small clusters in a section of gill only and were not detected in any other organs. *Perkinsus* sp. cells could not be found in an H&E stained tissue section from the same animal.

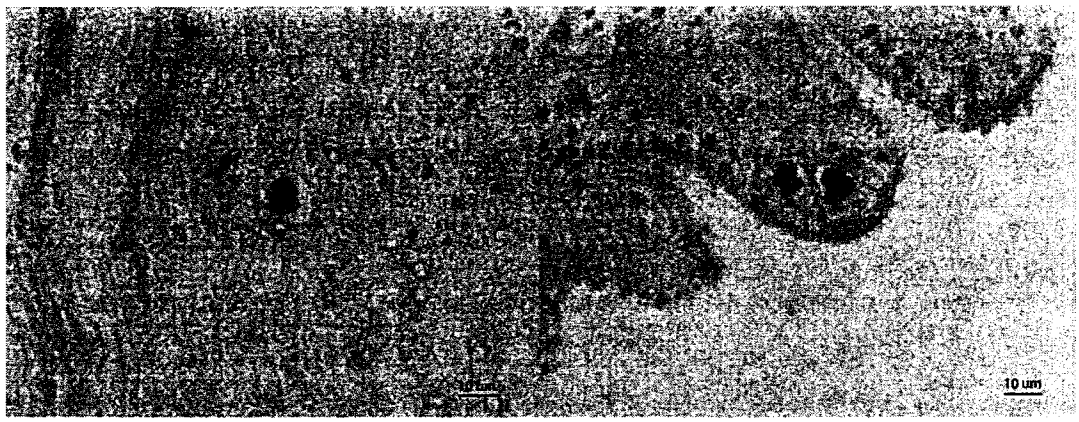
Experimental sampling – water. Standard PCR analysis of DNA extracted from water samples taken 5 d post –inoculation (before the first water change) detected *P. olseni* DNA in one *C. virginica* tank in the oyster homogenate treatment and in one *C. virginica* and two *M mercenaria* tanks in the FBS treatment. Two weeks post-inoculation, *P. olseni* DNA was amplified from water taken from one *C. ariakensis* tank and one *C. virginica* tank from the oyster homogenate treatment and from one *M. mercenaria* tank from the FBS treatment. Water from one *C. ariakensis* tank from the oyster homogenate treatment and water from one *C. virginica* tank from the FBS treatment were PCR positive for *P. olseni* DNA on week 4. On week 6, only water from one *M. mercenaria* tank was positive for *P. olseni* DNA. No water samples tested positive for *P. olseni* DNA on weeks 8 and 10.

Table 7. PCR and RFTM data for *Crassostrea virginica* mortality occurring during the second *P. olseni* challenge experiment.

Date	Days Post Inoculation	Control Treatment			FBS Treatment				Oyster Homogenate Treatment			
		# dead	# PCR pos	# RFTM	# dead	# PCR pos	# RFTM	RFTM ranking	# dead	# PCR pos	# RFTM	RFTM ranking
3/25/06	3	1	0	0								
3/27/06	5	1	0	0	3	3	3	VL, L, L	1	1	1	L
3/28/06	6	1	0	0								
3/29/06	7				1	1	1	VL	1	1	1	R
3/31/06	9	1	0	0	1	1			1	1		
4/2/06	11								1	1	1	L
4/3/06	12								1	1		
4/4/06	13								2	1	1	R
4/5/06	14				1	1	0					
4/6/06	15				1	0	0					
4/16/06	25	1	0									
4/19/06	28	1	0		1	1	0					
4/26/06	35				1	1	1	R				
	Totals	6	0	0	9	8	5		7	6	4	

Figure 5. In situ hybridization of the *P. olseni*-specific probe, Pols689DIG, illustrating binding to *Perkinsus* sp. cells in the gill of a *Mercenaria mercenaria* clam collected at week 8 from the FBS treatment.

Figure 5.



Real time PCR analysis using *P. olseni* specific primers was performed on all water samples that were positive using standard PCR primers, however no samples had reliable replication of quantification that was above the previously determined threshold limit of 3.0×10^{-2} cells per 10 µl reaction.

Experiment 3. Bath challenge with *Perkinsus olseni*

MATERIALS AND METHODS

Experimental hosts. On September 28, 2006, 200 *Mercenaria mercenaria* (36.6 ± 2.5 mm) were received at VIMS from Mobjack Bay Seafood Company, Ware Neck, Virginia. On November 1, 2006, 200 *Crassostrea virginica* (81.8 ± 8.9 mm) were received from Hog Island Oyster Company, Marshall, California. On November 15, 2006, 200 triploid *Crassostrea ariakensis* (37.5 ± 6.0 mm) were received from the hatchery at the University of Maryland's Center for Environmental Science. Upon arrival, 20 individuals of each host type were sacrificed, and gill and mantle was excised aseptically from each animal for DNA extraction. Additional gill, mantle and rectal tissues were excised for RFTM assay. Genomic DNA of each oyster was used in the PCR-based molecular diagnostic assay to examine the animals for the presence of DNA from *Perkinsus* spp. parasites. All remaining animals were put in separate 10 gallon glass (20--30 animals individuals each) aquaria at equivalent salinity to which they came from and were acclimated over a period of weeks to 1-µm filtered, 25 ppt York River water. Animals were fed 0.2 g oyster^{-1}algal food daily and 2/3 of the water was refreshed three times per week with clean 1-µm filtered, 25 ppt York River water.

Ruditapes decussatus. On November 26, 2006, 100 *Ruditapes decussatus* collected from Ría de Pontevedra, Galicia, Spain arrived at VIMS. They were delayed by 15 d during refrigerated shipment and most were moribund or dead upon arrival. The clams had been collected by commercial harvesters in Spain per request by Dr. Antonio Villalba of the Centro de Investigaciones Marinas, Galacia, Spain. A sample of clams was previously collected and analyzed using RFTM assays by Dr. Villalba. Results from RFTM assay of gill tissues indicated the prevalence of *Perkinsus olseni* was 80% and the clams had a *Perkinsus* sp. weighted prevalence of 2.17. In previous attempts to collect *R. decussatus* or *R. philippinarum* for this experiment, most clams had either been dead on arrival or died within a few days after being placed in acclimation aquaria. It was determined to keep the November 26th shipment in a humidified, refrigerated holding room and use the moribund or dead animals to harvest live *P. olseni* cells for a bath challenge experiment.

Purification of *Perkinsus olseni*. On November 29th, December 3rd, December 8th and December 11th, *R. decussatus* clams were used to create a partially purified homogenate of *Perkinsus olseni* cells using a method modified from La Peyre and Chu (1994). Fifteen clams were aseptically shucked onto sterile plastic weigh boats. Foot and siphon tissue was removed using a sterile razor blade. All remaining tissue was macerated with a sterile razor blade and then homogenized in a sterile blender with approximately 200 ml of 4 °C, 25 ppt SASW. Homogenized tissue was passed through a 1 mm mesh sieve and subsequently through a 90 μm and 73 μm sieve. The filtered homogenate was dispensed to 50 ml polystyrene falcon tubes and centrifuged at 300 rpm for 15 min at 15 °C. The supernatant was removed and an equal volume of fresh 25 ppt SASW was added to each cell pellet. The cell pellet was gently disturbed and washed

followed by centrifugation and washing twice more. After the final centrifugation, the supernatant was removed and the cell pellet was resuspended in 15 ml of fresh 25 ppt SASW. *Perkinsus* sp. cell counts were performed and viability determinations were performed as described above.

Experimental design. For each host species, 120 individuals were divided among 6 tanks, 20 individuals per tank. Three tanks for each host received doses of live *P. olseni* and the other three received an equal volume of 25 ppt SASW only. All aquaria environments were maintained at 20--22 °C and contained 25 ppt aerated, 1-μm filtered York River water.

When the experimental hosts were first introduced to the aquaria, the aquaria were entirely full of water. Prior to being dosed with the partially purified *P. olseni,* half of the water was removed from the aquaria and 1ml of algae feed was added to each aquarium. These steps were taken in order to promote feeding by the host bivalves and uptake of *P. olseni* cells added to the water. Eighteen hours later, the aquaria were filled entirely. Starting four days after each dose of cells, water changes took place 2--3 times weekly, during which two thirds of the water was removed each time and replaced with an equal volume of clean, 25 ppt, 1-μm filtered York River water. Animals were fed twice daily a single dose of 0.2 g per oyster algal feed.

Aquaria were checked daily for mortality and moribund animals were removed. When moribund or dead animals were discovered, and if sufficient undegraded tissue remained, it was processed for analysis. Gill and mantle tissues were removed and processed for DNA and RFTM analysis and visceral mass tissue sections were preserved in Davidson's solution for histological analysis as described above.

Challenge with *Perkinsus olseni*. On November 29th, 4 ml of partially purified *P. olseni* were added to each of the treatment aquaria and 4 ml of 25 ppt SASW were added to the control aquaria. Cell counts indicated a *P. olseni* density of 2.3×10^5 cells per ml. This was equivalent to 9.2×10^5 cells per tank or 4.6×10^4 cells per animal. On December 1st, 8 ml newly purified *P. olseni* were added to each aquarium and 8 ml of 25 ppt SASW were added to the control aquaria. Cell counts on December 1st indicated a *P. olseni* density of 1.1×10^5 cells per ml. This was equivalent to 8.9×10^5 cells per tank or 4.5×10^4 cells per animal. Cell counts taken after sacrificing more clams and homogenate preparation on December 8th and December 11th indicated that few viable *Perkinsus* sp. cells remained in the necrotic clam tissue, therefore aquaria were not dosed with these homogenate preparations.

Experimental sampling. On 30 and 60 d post inoculation (PI), 5 animals from each aquarium were sacrificed. Tissues were aseptically subdivided from each animal for DNA, histology and RFTM analysis as above. The experiment terminated after 90 d and all remaining animals were sacrificed and tissues preserved as above for DNA, RFTM and histological analysis.

RESULTS - Experiment 3

Experimental sampling. PCR and RFTM results for the 20 animals of each host species sacrificed prior to the start of the experiment were negative in all cases.

On day 30 PI, six *M. mercenaria* sampled from the three *P. olseni* dosed aquaria were PCR positive for *P. olseni*. Of these, one individual had visible *Perkinsus* sp. cells in the RFTM assay (light ranking), while no other animals had visible *Perkinsus* sp. cells. On day 60 PI, no clams were PCR positive for *P. olseni*, however two clams had visible

Perkinsus sp. cells in the RFTM assay. One had a rare ranking and the other a very light ranking. No clams were PCR positive on day 90 PI. (Table 8). After 90 d PI, no *C. ariakensis* or *C. virginica* oysters were PCR positive for *P. olseni.* Tissue from one *C. ariakensis* sacrificed on day 30 had visible *Perkinsus* sp. cells, however, this animal was found to be positive for *P. marinus* and not *P. olseni.* This suggests that the animal had acquired *P. marinus* cells prior to being used in the experiment, most likely during their holding at the University of Maryland, in waters where *P. marinus* is endemic.

Mortality. Seven *C. ariakensis* died during the experiment, two from the *P. olseni* dosed treatment and five from control tanks. None of these oysters was positive for *Perkinsus* sp. by RFTM or PCR. One *C. virginica* from a control aquarium died and it was also negative for *Perkinsus* sp. by RFTM and PCR.

Histological analysis. All *M. mercenaria* that were either PCR or RFTM positive for *Perkinsus* sp. were analyzed by ISH utilizing the *P. olseni*-specific probe as described above. No tissue sections showed binding of the *P. olseni* probe to *Perkinsus* sp. cells.

Table 8. PCR and RFTM data for *Mercenaria mercenaria* sampled during the *Perkinsus olseni* bath challenge experiment.

	Day 30 (n=15)	**Day 60 (n=15)**	**Day 90 (n=30)**
RFTM ranking	**# RFTM**	**# RFTM**	**# RFTM**
None (N)	14	13	30
Rare (R)	0	1	0
Very Light (VL)	0	1	0
Light (L)	1	0	0
Light/Moderate(LM)	0	0	0
Moderate (M)	0	0	0
Moderate/Heavy(MH)	0	0	0
Heavy (H)	0	0	0
Very Heavy (VH)	0	0	0
# *Perkinsus olseni* PCR positive	6	0	0

DISCUSSION

Perkinsus olseni, though widespread geographically and found on at least four continents, Asia, Europe, Australia and South America, is not currently found on the east coast of the USA and has not been reported from either the eastern oyster, *Crassostrea virginica* or the hard clam, *Mercenaria mercenaria*. This parasite has been found previously in South Korea in the Venus clam *Protothaca jedoensis* (Park et al. 2006) and in *Ruditapes philippinarum* (Choi and Park 1997), in *R. philippinarum* in Japan (Hamaguchi et al. 1998) and northern China (Liang et al. 2001), and in *Crassostrea ariakensis* and *Crassostrea hongkongensis* in northern China and Japan (Moss et al. 2007, Chapter 1). The research leading to the discovery of *P. olseni* in Asian oysters occurred during a recent survey of *C. ariakensis* populations in Asia (Moss et al. 2007, Chapter 1), motivated by a proposed introduction of *C. ariakensis* to Chesapeake Bay in order to restore an ecologically functional and commercially viable oyster population to the Bay. The detection of *P. olseni* in a natural population of *C. ariakensis* from potential Asian broodstock sites prompted initiation of challenge studies to examine the pathogenicity of *P. olseni* to *C. ariakensis, C. virginica* and *M. mercenaria* for evaluation of potential impacts in case of accidental introduction of the parasite with Asian oysters. *Perkinsus olseni* has been implicated in mortality of shellfish populations (Villalba et al. 2005, Park et al. 2006), however the host and environmental dynamics that lead to pathogenicity of *P. olseni* have not been extensively studied (Miossec et al. 2006). The fact that researchers in northern China and Japan have not reported disease signs or mortality in *C. ariakensis* populations associated with a *Perkinsus* sp. infection, may suggest that *P. olseni* is a rare or benign parasite in these oysters.

At the termination of the first challenge experiment conducted here, PCR based diagnostics suggested that a relatively low number of the *P. olseni*-inoculated *C. ariakensis* (n=2) harbored both *P. marinus* and *P. olseni* DNA. Many *C. ariakensis* were PCR positive for *P. marinus* only and *P. marinus*-specific ISH assays confirmed the presence of *P. marinus* in each of the three oysters analyzed. RFTM and PCR assay results for the *C. virginica* and *M. mercenaria* inoculated with *P. olseni* also did not suggest that transmission of *P. olseni* readily occurred. Although on day 21 there were five *C. virginica* oysters that were PCR positive for *P. olseni*, no viable *Perkinsus* sp. cells, either by H&E or ISH, were seen in those oysters. During the rest of the 72 -day experiment, *Perkinsus* sp. cells were observed in only three *C. virginica*, however negative PCR and histological analysis of these individuals makes the suggestion of transmission inconclusive. On Day 21 there were two clams that were PCR positive for *P. olseni*, yet no viable cells were seen histologically or by RFTM. One clam was RFTM positive on day 44 and two on day 72, however only one clam, on day 72 was both RFTM and PCR positive for *P. olseni.* Histological analysis of all RFTM or *P. olseni* PCR-positive oysters and clams was negative, therefore I could not conclusively demonstrate during this first experiment that transmission of this parasite occurs to these hosts.

Data concerning the effect of *P. marinus* on *C. ariakensis* collected during the first challenge was unexpected and enlightening given that one of the reasons for the proposed introduction of this oyster species is its reported resistance to *P. marinus* (Calvo et al. 2001). Initial screening using a PCR-based diagnostic method on a sample of the *C. ariakensis* oysters that were obtained for the first challenge study suggested that they did not harbor *P. marinus.* A small sample (n = 5) of these oysters screened 59 days later,

however, had 100% *P. marinus* DNA prevalence as indicated by the *P. marinus*-specific PCR assay. I suggest that the *C. ariakensis* oysters either acquired a small number of *P. marinus* cells that were not numerous enough to be detected by the initial PCR assay of gill/mantle tissue while in the VIMS hatchery before collection for this experiment, or while being held in unfiltered York River water for 4 days prior to being brought into the aquaria where they were subsequently held only in filtered York River water.

Warm water temperatures, 20--25 °C, and high salinity >15 ppt, have been shown to correlate with times of maximal proliferation of parasite cells, as well as highest incidences of infection in oysters (Andrews and Hewatt 1957, Chu et al. 1994). Due to the effects of salinity and temperature on the parasite, the infections caused by *P. marinus* are seasonal in the Chesapeake Bay, with maximum parasite prevalence recorded in late summer and minimum prevalence observed during the winter months (Burreson and Ragone Calvo 1996). York River water conditions during the short time that the oysters were in the holding tank were approximately 9 °C and 16 ppt salinity. Environmental conditions of the York River were not likely favorable for *P. marinus* proliferation at the start of the first experiment; however, it has been shown that parasite cells can remain viable in over wintering conditions of temperatures as low as 4 °C and a salinity of 4 ppt (Chu & Greene 1989, Ragone Calvo & Burreson 1994, Chu 1996). Therefore, I cannot discount the possible presence of parasite cells in the York River water during the holding period, when the oysters were in unfiltered water.

The experimental conditions under which the *C. ariakenis* were held, 20 °C and 25 ppt salinity, would have favored the proliferation of *P. marinus* cells present in the oysters, accounting for the increase in infection prevalence observed after the baseline sampling. The *P. marinus* proliferated to PCR detectable levels after 59 days in aquaria

with disease developing and mortality subsequently occurring in not only those oyster groups that had been subjected to notching and inoculation with either SASW or *P. olseni* for the challenge study, but also in the control group of unnotched and untreated oysters that were simply held for an additional 37 days during the challenge experiment. RFTM and PCR assay results for the *C. ariakensis* initially inoculated with either 25 ppt SASW or cultured *P. olseni* cells for the challenge study revealed a progression of *P. marinus* infection in the oyster tissues. Sampling and subsequent PCR-based screening of both the 25 ppt SASW and *P. olseni*-inoculated oysters on days 21 and 44 indicated that *P. marinus* DNA was found in 83.3% and 66.7% of the oysters, respectively. I believe that the observed decrease in *P. marinus* prevalence on day 44 was unlikely an actual decrease in the prevalence among the oysters, but rather was a function of the small sample sizes (n = 6) assayed to determine PCR-based *Perkinsus* sp. prevalence at each time point. Although *P. olseni* DNA was found in two of the *P. olseni*-inoculated oysters (one each on days 21 and 72), there was an increase in the observed PCR prevalence of *P. marinus* DNA to 100% in samples of all treated oysters collected on days 59 and 72. Likewise, as determined by RFTM assays, the tissue burdens for the two positive individuals were light in the *P. olseni*-treated oysters collected on day 44 of the experiment. However, among samples of these same oyster groups that were collected on days 59 and 72, not only light infections, but also moderate to very heavy tissue burdens were observed. Interestingly, disease progression in those *C. ariakensis* not notched or inoculated, mimicked that seen in the manipulated experimental oysters based on the RFTM-based tissue burdens. By day 37 when the control oysters were sacrificed, the observed *Perkinsus* sp. tissue burdens ranged from light to moderate/heavy.

During the course of the first challenge experiment, there were many instances where dead *C. ariakensis* were discovered in the various treatment groups and there was little or no tissue available for either PCR or RFTM analysis. It is important to note, however, that of the 11 dead oysters with available tissue, more than half (55%) had moderate to heavy *Perkinsus* sp. tissue burdens.

In the first experiment, cumulative mortalities for *C. ariakensis* were highest in those treatments subjected to the extra stress of notching and injection, and the highest *Perkinsus* sp. tissue burdens were seen in dead *C. ariakensis.* Minimal mortality occurred with the other oyster and clam species and could not be attributed to one treatment. Higher cumulative mortalities may have been observed in the untreated control oysters, as well, had they been held for a longer time period. In *C. virginica*, digestive gland epithelia and the stomach are often heavily colonized with *P. marinus* and damaged. As parasite proliferation increases to lethal levels, massive tissue sloughing occurs, which eventually contributes to the death of the animals (Mackin, 1951). In the first experiment, tissue sections from a moribund *C. ariakensis* with a very heavy RFTM rating showed dense, systemic *P. marinus* infection and the *in situ* hybridization analyses confirmed that all of the observable *Perkinsus* sp. cells were *P. marinus.* Collectively, the results presented here strongly suggest that *P. marinus* was an important contributing factor to the death of these oysters.

The first experiment provided valuable information regarding the potential for advanced *P. marinus* infections to occur in *C. ariakensis*. Prior field studies conducted in Chesapeake Bay have indicated that although *C. ariakensis* is capable of acquiring *P. marinus* infections (Calvo et al. 2001), there was no evidence that *C. ariakensis* was susceptible to the advanced parasite infections known to occur in *C. virginica*. The

experimental environmental conditions, under which the oysters were held for a total of five months, combined with the stress of the unnatural aquaria environment and experimental manipulation, may have promoted the development of the intense *P. marinus* infections in *C. ariakensis* that were observed here. The first experiment provides valuable information on potentially problematic disease issues including parasite proliferation that can arise if *P. marinus*-infected *C. ariakensis* encounter stress challenges in the wild or aquaculture environment, or if they are held in hatcheries or laboratories under stressful conditions.

In the first experiment, *C. virginica* that were inoculated with *P. marinus* served as an indicator that the notching and inoculation of *Perkinsus* sp. was performed correctly. By the termination of the first experiment, 44% of those animals initially inoculated were either RFTM or PCR positive for *P. marinus,* a pathogen which we know naturally infects the eastern oyster. In that experiment, an order of magnitude fewer *P. marinus* cells were inoculated into naïve *C. virginica* than in the second study. After 72 days, 55% of the *C. virginica* acquired *P. marinus* infections (PCR and RFTM data combined). It is widely known that *P. marinus* is a lethal pathogen to *C. virginica*, and the first experiment suggested that even when cultured under artificial conditions, it was still capable of causing infection in its native host.

The results of the first experiment suggested that the three bivalve species tested might not be readily susceptible to *P. olseni*, or that virulence attenuation of the parasite may have occurred during the culturing period prior to the use of the parasite as inoculum. The goal of the second experiment was to see if *P. olseni* would become more infective to the three bivalve species if cultured under altered media conditions. In addition, I hypothesized that protease activity would be higher in cultures where *P. olseni*

cells were supplemented with oyster homogenate compared to cells cultured with fetal bovine serum (FBS). *Perkinsus marinus* virulence factors include proteases that are believed to play a role in parasite invasion and spreading in host tissues (Anderson et al. 1996), evasion of host defenses (Garreis et al.1995, Tall et al. 1999) and have been shown to increase infectivity of *P. marinus in vivo (*La Peyre et al. 1996). Virulence attenuation with *in vitro* cultured *P. marinus* cells has been well documented (Ford et al. 2002). Recent studies, however, have demonstrated that *P. marinus* virulence, correlated with protease activity, is enhanced by supplementing *Perkinsus* sp. culture media with oyster homogenate (MacIntyre et al. 2003, Earnhart et al. 2004) and therefore, a second experiment was undertaken in which standard *P. olseni* culture media was supplemented with either *C. ariakensis* oyster homogenate or FBS as a protein source.

The results of the second experiment suggest that there is no significant difference in virulence of *P. olseni* cells cultured in media containing *C. ariakensis* homogenate as compared to those cultured with FBS, at least for the supplementation concentration tested, the bivalve species used, and under the environmental conditions at which the experiments were performed. The cumulative percentage of *P. olseni* PCR positive individuals in the oyster homogenate treatment for *C. virginica, M. mercenaria* and *C. ariakensis* was 56%, 47% and 29%, respectively. For the FBS treatment, the cumulative percentage of *P. olseni* PCR positive individuals for *C. virginica, M. mercenaria* and *C. ariakensis* was 73%, 51% and 18%, respectively. There was a weak trend for *C. ariakensis* to be more prone to be *P. olseni* PCR positive when inoculated with cells cultured in oyster homogenate supplemented media. The opposite was true, though not statistically significant, for *C. virginica* and *M. mercenaria,* both of which had more *P. olseni* PCR positive individuals when inoculated with cells cultured in FBS supplemented

media. All three hosts inoculated with live cells appeared to harbor cells, or at least *P. olseni* DNA, for approximately 6 weeks post inoculation, after which the number of PCR positive individuals found at each sampling generally decreased. The RFTM assay detected live cells in many instances at week 2 and rarely after that time point. This trend may indicate that initial infections may have been resolved in the animals as the experiment progressed.

Many *C. virginica* died during the early weeks of the experiment, both in the control and experimental treatments, suggesting that of the three species, the *C. virginica* oysters may be more susceptible than the *C. ariakensis* or *M. mercenaria* to stress induced by the notching and inoculation process. *Perkinsus olseni* was not detected in any of the dead *C. virginica* using molecular or histological techniques.

In situ hybridization analysis of the animals sampled during the second experiment indicated the presence of *P. olseni* cells in only one animal, a *M. mercenaria* clam, found also to contain *P. olseni* DNA by PCR. In this clam, the *P. olseni* –specific probe hybridized to a few clusters of cells in the gill. The ISH result confirms that *P. olseni* was transmitted to this clam. The presence of *P. olseni* DNA only must not be used as a definitive diagnostic assay for infection unless microscopic examination of preserved tissue confirms the existence of parasite cells. Lacking histological data to confirm the positive *P. olseni*-specific PCR results for the other samples/hosts reported here, it is hard to comment on the pathogenicity of *P. olseni* to these host species. It is possible that cells are infective, but if so, they are not abundant enough to be detected in representative 5 μm histological sections. Likewise, it has been reported previously that RFTM assays using only a small piece of gill, mantle and rectal tissues, as was performed here, will only detect *Perkinsus* sp. cells when the total body burden exceeds 10^3 cells per

gram wet weight of oyster tissue (Choi et al. 1989, Bushek et al. 1994). It is equally plausible, therefore, that the PCR results reflect the persistence of cells within the host tissues that are below the detection limit for RFTM and histological analyses.

Combined analysis of data collected in the two inoculation experiments suggests that *P. olseni* cultured in the laboratory and artificially inoculated into naïve hosts does not promote the development of advanced *P. olseni* infections in *C. virginica, M. mercenaria* or *C. ariakensis*. This may indicate that either *P. olseni* will not readily infect these hosts in nature, or, as is known with *P. marinus*, *P. olseni* cultured in artificial media may lose its virulence attributes and will not readily cause infection even when re-introduced to its natural host. In this study, 0.1 mg/ml oyster homogenate or 0.1 mg/ml FBS (final concentration) was chosen as a protein supplement to standard DME:Ham's F12 *Perkinsus* spp. media in an attempt to promote increased virulence of *P. olseni.* The concentration of oyster homogenate used to supplement the cultures was chosen for two reasons. The first is that previous studies (Earnhart et al. 2004) found that higher concentrations of *C. virginica* and *C. gigas* homogenate, 1.0 mg/ml, for example, decreased the *P. marinus* cell numbers below the seeding density and *C. ariakensis* homogenate at that concentration yielded the *P. marinus* cells non viable. Lower homogenate concentrations tested (down to 0.004 mg/ml) were not inhibitory to cell growth yet still elicited an induction of low molecular weight proteases in the homogenate supplemented cell cultures. Secondly, a collaborator, Chris Dungan at the Cooperative Oxford Laboratory, Maryland, performed a preliminary experiments supplementing *P. olseni* growing in DME:Ham's F12 media with 0.1 mg/ml *C. ariakensis* homogenate and found that it was not significantly inhibitory to cell proliferation. Due to the large number of cells needed for this experiment, it was

important to use a concentration of oyster homogenate that had the potential to stimulate protease activity but that would not be prohibitive to achieving the necessary cell densities.

I believed that ninety-six hours was an adequate amount of time for *P. olseni* to grow in the various media formulations and to react to the protein supplement prior to inoculation. In fresh DME:Ham's media with 5% FBS, growth rates of *P. olseni* are high and in log phase within the first week, after which growth slows and cell cultures fall into a stationary state with cells not actively replicating (Ordas and Figueras, 1998). Protease activity of 1-day old *P. marinus* cultured in a standard media formulation has been shown to equal that of *P. olseni* held in phosphate buffered saline without nutritional supplement for 3 days (Ordas et al. 1999). If *Perkinsus* sp. cells are replicating at a logarithmic rate and are capable of expressing protease activity within 24 hours (see also La Peyre et al. 1995), it was reasoned that within 96 hours, *P. olseni* cells should be able to react and respond to stimuli present in the media.

In examining the ECP enzyme activities of *P. olseni,* Casas et al. (2002b) detected numerous enzymes including, among others, esterase, esterase lipase, acid phosphatase, β-glucosidase, lipase, β-galactosidease and α-glucosidase. They did not find activity of the serine proteases, trypsin and α-chymotrypsin, detected in ECP of cultures of *P. marinus* (La Peyre et al. 1995, Brown and Reece 2003) and suggested that the lack of (or undetected) serine protease production by *P. olseni* could explain a relatively lower virulence of *P. olseni* to *Ruditapes decussates* than is exhibited by *P. marinus* in *C. virginica.* In the second experiment, *P. olseni* media was supplemented with a low concentration of *C. ariakensis* homogenate. Supernatant analyzed from *P. olseni* cultures 20 days old indicated high molecular weight proteolytic activity in the presence of oyster

homogenate, however protease bands of low molecular weight (LMP) were not detected. This observation was in contrast to that observed for cultures of *P. marinus* supplemented with *C. virginica* oyster plasma (Earnhart et al. 2004) where both high and low molecular weight protease forms were recorded. Low molecular weight protease activity of *P. marinus* was also recorded previously by La Peyre et al. (1995) when cultured in JL-ODRP-1 media without bovine serum albumin. The LMPs were identified as serine proteases. MacIntyre et al. (2005), however, reported that LMPs were not produced when *P. marinus* was cultured in media supplemented with *C. gigas* or *C. ariakensis* homogenate, two hosts which are relatively more resistant to *P. marinus* than *C. virginica.*

Other than molecular detection of *P. olseni* in *C. ariakensis* in northern China and Japan (Chapter 1), there has been no report of this *Perkinsus* sp. as a problematic disease agent in these bivalves. It could be argued that the protease profile described here, suggesting absence of LMP and serine protease activity, in concert with lack of concrete evidence of *P. olseni* transmission to *C. ariakensis,* as further indication that *P. olseni* may not be a severe pathogen of *C. ariakensis* in nature. On the other hand, the minimum infective dose for *P. olseni* to infect *C. virginica, M. mercenaria* or *C. ariakensis* may not have been achieved in the inoculations or bath challenge conducted for this study (see below). In previous studies on *P. marinus* disease processes, Chu and Volety (1996) suggested that the dosage required for parasite transmission was between 10 and 100 cells via oyster cavity injection and Mackin (1962) similarly found that injection of 10 to 500 *P. marinus* cells was enough to cause disease. In the environment oysters likely come into contact with *Perkinsus* sp. cells through filtration, however after ingestion, cells may also be released in the feces and pseudofeces (Bushek et al. 1994). If

this scenario holds true for *P. olseni,* then it is possible that even though $>10^6$ cells were injected into each animal, many may have been purged or eliminated. These cells would have been removed during the process of water changes, thereby diluting the number of potentially infective cells in the aquaria.

Using molecular techniques, *P. olseni* cells were sporadically detected in the aquaria of the supplemented *P. olseni* media challenge experiment until week eight, suggesting that cells were continually being shed into the water. It was necessary to exchange 2/3 of the aquaria water three times a week during the experiment to maintain water quality, so it is likely that shed parasite cells were removed from the aquaria as initial infections were resolved or as digested cells were shed in feces. Standard PCR detected *P. olseni* DNA in the water, however the amount of *P. olseni* cells present was unable to be quantified using the real time PCR assay developed here, because the quantity of cells was at or below the detection limit for this assay.

Crassostrea virginica, M. mercenaria and *C. ariakensis* may not be readily susceptible to *P. olseni* when exposed by a single concentrated dose of cultured *P. olseni* cells. The fact that potentially infective cells are removed from the experimental aquaria with water changes suggests the injection experiments may not adequately reproduce the prolonged exposure that might take place in nature. Repeated exposure to potentially infective cells would likely more accurately reflect a natural scenario, therefore, a bath challenge experiment was undertaken in which *P. olseni* cells harvested directly from naturally infected clams were added to aquaria with *P. olseni*-free hosts.

Initially a cohabitation experiment using naturally infected *Ruditapes philippinarum* or *R. decussatus* from Spain as a source of *P. olseni* was intended. It was reasoned that by using naturally infected animals, exposure to the parasite would more

closely mimic exposure that occurs with naïve animals in nature. If the Spanish clams died, *P. olseni* cells would be released into the aquaria and could be transmitted by normal feeding activity to the experimental animals. Three shipments of Spanish clams were received, however it was not possible to use any of these clams in cohabitation experiments. They either died upon arrival or shortly thereafter, most likely because of the combined stress of having a high body burden of parasite to start with, along with being shipped overseas. It was preferred to use naïve *R. philippinarum* obtained from the west coast, USA as control animals for the bath challenge experiment. On two occasions clams from Hog Island Oyster Company, California, were received, and clams from both shipments died within days of being put into acclimation aquaria. The cause for the mortality was never investigated, although it was later learned that the hatchery from which they came had reported unexplained mortality events earlier that year. Healthy *R. philippinarum* from Taylor Shellfish, Washington, were eventually obtained; however, by the time they arrived and had acclimated for a week, there were no more live *P. olseni* cells to harvest from my last shipment of clams, therefore it was impossible to expose these animals to *P. olseni* in the same manner as the *C. virginica, M. mercenaria* and *C. ariakensis.* The unfortunate series of events admittedly compromised the integrity of the third exposure experiment.

Results of the bath challenge experiment suggested that transmission of *P. olseni* occurred to naïve *M. mercenaria*, although this could not be proven histologically. *Perkinsus olseni*-positive PCR results were obtained for a few clams and live *Perkinsus* sp. cells were additionally observed in a few instances. Unlike the first two experiments, no suggestion of transmission was observed for *C. ariakensis* or *C. virginica* during the bath exposure. The dosage of cells used in the bath challenge experiment was much less

than what was administered to each host in the previous two experiments. The fact that PCR and RFTM assays were positive for *M. mercenaria* only may indicate that the hard clam could be more susceptible to *P. olseni.* As described above, an increase in the parasite dose, perhaps under different environmental conditions or along with physiological stress of the hosts, may have led to a different outcome. This experiment, however, is valuable in revealing the relative susceptibility of these hosts when *P. olseni* is administered by a more natural means.

The data presented here, suggest that *P. olseni* is not a problematic pathogen in *C. ariakensis* in Asia. Combined results of the three experiments, however, suggests that *C. virginica* and *M. mercenaria* may be more susceptible to *P. olseni* than *C. ariakensis.* Consistently there was more *P. olseni*-specific PCR and RFTM data suggesting transmission of *P. olseni to C. virginica* and *M. mercenaria,* and additionally, the only histological confirmation of *P. olseni* acquisition was seen in a *M. mercenaria* clam. One must caution that laboratory manipulation of *Perkinsus* sp. cells and laboratory holding of animals does not represent outcomes that are possible in nature. Ford et al. (2006) found that different isolates of *P. marinus* may have different temperature and salinity optima, therefore it is possible that aquaria conditions chosen for these experiments were not optimal for *P. olseni* isolates that were used. Infection dynamics must also consider the status and immune response capability of the host at different environmental conditions and the interaction between *P. olseni* and these three hosts at a range of environmental parameters was not conducted in this study.

Hayward and Lester (2004) reported that *P. olseni* did not initially appear to be a virulent pathogen to blacklip abalone, *Haliotis rubra*; however, six weeks after a temperature stress, *P. olseni* prevalence as determined by RFTM assay, increased from

0% to 82%. They suggested that *Perkinsus*-associated disease did not develop in abalone until after a significant disturbance. They also suggested that pollution from sewage outfalls, resulting in elevated levels of nitrogen and phosphorus, may have been linked to hotspots of disease. These findings of Haywood and Lester (2004) are relevant to the above described studies, as they suggest that *P. olseni* can appear to be benign in some hosts but can become virulent when hosts are physiologically stressed. In addition it was clear in the first experiment that *C. ariakensis* may acquire advanced and likely lethal infections of *P. marinus* under stress, something not observed in previous field trials (Calvo et al. 2001). The three experiments conducted here indicate that *P. olseni* may be transmitted to *C. virginica* and *M. mercenaria* and the evidence that physiological stress may increase the pathogenicity of *Perkinsus* sp. to infected hosts validates further that there is a recognizable risk associated with exposure of *P. olseni* to native Chesapeake Bay bivalves.

Chapter 4. *Perkinsus beihaiensis* n. sp. cohabitation studies

ABSTRACT

A novel *Perkinsus* sp. parasite, *Perkinsus beihaiensis,* has been discovered in *Crassostrea ariakensis, C. hongkongensis* and other bivalve mollusc species in southern China (Moss et al. 2007, Chapters 1 and 2). Molecular analysis of multiple DNA loci including the internal transcribed spacer (ITS) region and the large subunit (LSU) ribosomal RNA (rRNA) gene of the ribosomal RNA gene complex and type 1 actin gene(s), confirm that it is a member of the genus *Perkinsus,* however it is distinct from other known *Perkinsus* spp. *Crassostrea ariakensis* is currently under consideration for introduction to Chesapeake Bay, USA, in order to restore a commercially viable and sustainable oyster population to that region. Research is ongoing to examine, among other things, the potential disease impacts that this non-native oyster could have on Chesapeake Bay shellfish populations. In order to assess the potential for transmission of *P. beihaiensis* to Chesapeake Bay oysters and clams, experiments were performed in which Asian oysters, specifically the species *C. ariakensis* and *C. hongkongensis*, which were naturally exposed to *P. beihaiensis* in native Asian waters, were cohabitated with naïve *C. ariakensis* and *C. virginica* oysters and *Mercenaria mercenaria* clams during two separate experiments lasting three and six months. At pre-determined intervals, a subset of the oysters and clams were removed from the cohabitation aquaria and sacrificed. In order to establish the pathogenicity of *P. beihaiensis* to these host species,

tissues from the necropsied bivalves were subdivided for PCR-based molecular diagnostics, for cell culture-based Ray's fluid thioglycollate media (RFTM) assays (Ray 1952) and for histological analyses. PCR and RFTM assay evidence highlighted that *P. behaiensis* may be transmitted to *C. virginica* and *M. mercenaria.* Additionally, many of the Chinese oysters used in these experiments developed severe *P. beihaiensis* infections suggesting that this parasite may be a problematic pathogen for *C. ariakensis* and *C. hongkongensis.*

INTRODUCTION

A novel *Perkinsus* sp. parasite, *Perkinsus beihaiensis,* has been discovered in *Crassostrea ariakensis, C. hongkongensis* and other bivalve mollusc species in southern China (Moss et al. 2007, Chapters 1 and 2). Molecular analysis of the internal transcribed spacer (ITS) region and the large subunit (LSU) ribosomal RNA gene of the ribosomal RNA gene complex, and type 1 actin gene(s) of this parasite confirm that it is a member of the genus *Perkinsus,* however it is distinct from other known *Perkinsus* spp. Using a *P. beihaiensis*-specific PCR assay (Chapter 2), in southern China the distribution of *P. beihaiensis* extends at least from Tong'an, Fujian Province, to waters surrounding Beihai, Guangxi Province, on China's southern border with Vietnam. PCR results from a 2003 oyster sample from Zhanjiang, Guangdong suggest that the prevalence in *Crassostrea* spp. oysters of infections by *P. beihaiensis* n. sp. can be as high as 46.5%. *Perkinsus beihaiensis* cell morphology and pathology in infected oysters is consistent with what has been described for known *Perkinsus* spp., however nothing is known about its natural pathogenicity within Asian bivalve mollusc populations.

The proposed introduction of *C. ariakensis* to Chesapeake Bay ignited research into the potential disease impacts that this oyster could have on Bay bivalve molluscs. The current International Council for Exploration of the Seas (ICES) protocols (ICES, 2005) require that adult oysters from Asia be imported into designated quarantine facilities prior to conditioning and spawning. Once adult oysters are spawned, they are required to be destroyed and only F1 or later generations of oysters originating from the Chinese broodstock can be deployed. Strictly executed quarantine and breeding procedures would not effectively limit vertically transmitted pathogens, however. Rogue introductions of native Asian oysters or accidental failure of quarantine facilities could also expose Chesapeake Bay waters to other types of exotic pathogens. Furthermore, a concern is that introduced *C. ariakensis* could serve as vectors for exotic pathogens introduced through ballast water. The discovery of a new *Perkinsus* sp. in oysters from potential broodstock sites in Asia therefore necessitates further research, both into its effects on its native hosts in Asia and its potential impact on those mollusc species native to Chesapeake Bay.

In order to assess the potential for transmission of *P. beihaiensis* to Chesapeake Bay oysters and clams, experiments were performed in which Asian oysters naturally exposed to *P. beihaiensis* in native Asian waters were cohabitated with naïve *C. ariakensis* and *C. virginica* oysters and *Mercenaria mercenaria* clams during two separate experiments lasting three and six months. At pre-determined intervals, a subset of the oysters and clams were removed from the cohabitation aquaria and sacrificed. In order to establish the pathogenicity of *P. beihaiensis* to these host species, tissues from the necropsied bivalves were subdivided for PCR-based molecular diagnostics, for cell culture-based Ray's fluid thioglycollate media (RFTM) assay (Ray 1952) and for

histological analysis. During these experiments, PCR analysis detected *P. beihaiensis* DNA in all host species (*C. virginica, C. ariakensis* and *M. mercenaria*) and RFTM assays detected *Perkinsus* spp. cells in a number of samples. Confirmatory histological evidence would have provided additional support that transmission of *P. beihaiensis* occurred; however, *P. beihaiensis* cells could not be detected using *P. beihaiensis*–specific DNA probes when in situ hybridization assays were performed on tissue sections from *P. beihaiensis*-specific PCR or RFTM positive samples. Although the evidence of transmission of *P. beihaiensis* to the naïve bivalves used in the two experiments was somewhat limited, this does not rule out the possibility that Chesapeake Bay oysters and clams could be more readily infected by *P. beihaiensis* when exposed at higher doses, or for longer time periods. At the termination of the cohabitation experiments, many of the Chinese oysters had histological evidence of *P. beihaiensis* infection and examination of these Chinese *C. ariakensis* and *C. hongkongensis* suggested that *P. beihaiensis* can establish systemic and likely lethal infections in these bivalve hosts (see also Chapter 2).

Experiment 1

MATERIALS AND METHODS

Quarantine procedures. Quarantine procedures were conducted as previously described in Chapter 3.

Chinese oysters. In April 2006, oysters were collected from the Dafen River, Beihai, and Qinzhou, Guangxi Zhuang, People's Republic of China. Oysters were shipped in refrigerated containers to the Virginia Institute of Marine Science on April 19, 2006, and were transported directly to the quarantine facility where they were allowed to warm to room temperature (~20 °C). The oysters were divided among four 50-gallon

glass aquaria, two per sample location, containing aerated 1-μm filtered York River water at 21 ppt and 20 °C. Oysters were fed twice daily 0.1 grams^{-1} oyster algae feed (Reed Mariculture, San Jose, CA) and two-thirds of the water was refreshed three times weekly. Many oysters were dead on arrival or died within a few days of arrival and for all such individuals, tissues were aseptically excised and preserved for DNA extraction, Ray's fluid thioglycollate media assay (RFTM) or histological analysis (see below), as sufficient undegraded tissue allowed.

Experimental hosts. Specific pathogen free (SPF) *C. virginica* (19.6 ± 2.7 mm shell height) and triploid *C ariakensis* (36.8 ± 7.7 mm shell height) were received from Haskin Shellfish Research Lab, Rutgers University, Port Norris, New Jersey. Upon arrival, eight *C. virginica* and ten *C. ariakensis* were sacrificed, and gill and mantle was excised aseptically for DNA extraction. Genomic DNA of each oyster was used in the *Perkinsus* genus-specific PCR assay in order to detect the presence of *Perkinsus* spp. parasites (see below).

Experimental design. On April 21, 2006, the *C. ariakensis* and *C. virginica* were divided among three 10-gallon aquaria, 23--27 individuals per tank, with 4--6 Chinese oysters from Dafen River, Beihai, also added to each tank. As controls, 48 naïve *C. ariakensis* and *C. virginica* were each divided into four separate aquaria, 24 of each host type per tank, and no Chinese oysters were added. All aquaria were maintained in aerated 1-μm filtered York River water at 21 ppt and 18--21 °C and oysters were fed 0.1 grams^{-1} oyster algae feed twice daily. Two-thirds of the water was exchanged three times per week with fresh, 1-μm filtered York River water at 21 ppt and 18--21 °C.

Perkinsus spp.–naïve *C. ariakensis* and *C. virginica* were cohabitated with Chinese oysters for a total of 95 d. On day 47, 5--6 more Chinese oysters, this time from

both the Dafen River site and from Qinzhou, were added to each cohabitation aquaria. For the duration of the experiment, aquaria were checked daily for mortality and moribund animals were removed. When moribund or dead animals were discovered, and if sufficient tissue remained, it was processed for *P. beihaiensis* analysis. Gill and mantle tissues were removed with a portion preserved in 95% ethanol for DNA extraction and molecular diagnostics and gill and mantle tissues were processed for the RFTM assay (Ray 1952) (see below.) Visceral mass tissue sections were preserved in Davidson's solution for histological analysis (Shaw & Battle 1957).

Five *C. ariakensis* or *C. virginica* were sampled from all aquaria on days 20 and 69, and all remaining oysters were sampled on day 95 when the experiment was terminated. All remaining Chinese oysters from the cohabitation aquaria were removed and sacrificed five days later.

RFTM assay. Gill, mantle and rectal tissues of dead, moribund or sacrificed oysters were taken for RFTM analysis as previously described (Chapter 3). Stained, cover-slipped tissue preparations were examined under a light microscope and *P. beihaiensis* tissue burdens were enumerated on a scale from rare (R) to very heavy (VH) based on the categories of Ray (1952, 1954). To calculate weighted prevalence (WP) of *P. beihaiensis* in the Chinese oysters, categorical data was converted to numerical data and averaged for hosts within each aquarium and then collectively for each host. Conversion of categorical data to numerical data was as follows: none (N) = 0, rare (R) to light (L) = 1, light to moderate (LM) = 2, moderate (M) = 3, moderate to heavy (MH) = 4, heavy to very heavy (H) = 5.

Nucleic acid extraction. Genomic DNA was extracted from the excised mantle and gill snips, using a DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA) as described in Chapter 1.

SSU genes. In order to assure that PCR amplifiable DNA was present in all extracted samples, genomic DNAs were tested using universal small subunit (SSU) ribosomal RNA gene primers (Chapter 1). Following amplification, 3 µl of PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized under UV light. Images were recorded with an Alpha Innotech FluorChem® (San Leandro, CA) imaging system.

***Perkinsus* genus-specific PCR assay.** Screening for *Perkinsus* sp. DNA was performed using *Perkinsus* genus-specific primers that were slightly modified from those of Casas et al. (2002) (Chapter 1). Following amplification, 4 µl of PCR product was electrophoresed on a 2% agarose gel, and visualized as described above.

***Perkinsus beihaiensis* -specific PCR.** A previously developed (Moss et al. 2007, Chapter 2) *Perkinsus beihaiensis*- specific PCR assay was used to screen sampled oyster tissues for the presence of *P. beihaiensis* DNA. PCR reaction conditions using the modified previously published PerkITS-85 primer (above and Chapter 1) with a unique reverse primer, PerkITS-430R (5' TCTGAGGGGCTACAATCAT 3'). The targeted fragment was approximately 460 bp in length. Each PCR reaction contained the following: 20 mM Tris-HCl (pH8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, each primer at 0.1 µM, 0.0125 U μl^{-1} *Taq* polymerase, 0.2 mg ml^{-1} BSA and 0.5 µl genomic DNA (~10--50 ng total). Amplifications were performed with an initial denaturation of 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 57 °C for 45 sec, 68 °C for 90 s, with a final elongation of 68 °C for 5 min. Following amplification, 6

μl of PCR product was electrophoresed on a 2% gel and visualized under UV light. Gel images were recorded as above.

Histological analysis. Tissues preserved for histological analysis in Davidson's solution (Shaw and Battle 1957) were dehydrated in a series of ethanol baths, infiltrated with paraffin and embedded in paraffin blocks prior to sectioning. Sections of 5 μm thickness were stained with Harris-hematoxylin and eosin. Histological sections of oysters were examined using light microscopy to visualize *Perkinsus* sp. parasite cells in situ.

In situ hybridization. Representative tissue sections of those oysters and clams positive for *P. beihaiensis* DNA by species-specific PCR or RFTM analysis, were evaluated by in situ hybridization (ISH) using separately a *Perkinsus* genus-specific probe, Perksp700DIG (5' CGCACAGTTAAGTRCGTGRGCACG 3') (Elston et al. 2004) and a *P. beihaiensis*-specific DNA probe (Chapter 2), PerkBehLSUDIG (5' GTGAGTAGGCAGCAGAAGTC 3'). The digoxigenin-labeled 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 published by Elston et al. (2004). Pronase at a final concentration of 125 $\mu g\ mL^{-1}$ was used for permeabilization during a 30-min incubation, and a probe concentration of 7 ng μl^{-1} was used for hybridization. Negative control tissue sections were tested identically except that they received hybridization buffer lacking probe during the hybridization step.

PCR-RFLP identification of oyster host species. Species identification of Chinese oysters was carried out using a molecular diagnostic key based on the PCR

amplification and restriction enzyme digestion of the first internal transcribed spacer gene region (ITS-1) (Cordes and Reece 2005, Chapter 2).

RESULTS – Experiment 1

Experimental sampling. Five oysters were sampled from each of the aquaria on days 20 and 69 and all remaining oysters were sacrificed on day 95. All PCR and RFTM data collected for sampled and dead or moribund oysters found during the experiment is listed in Table 1. In addition, the *P. beihaiensis*-specific PCR screening and weighted prevalence data for the Chinese oysters used in the cohabitation aquaria are also listed in Table 1. No negative control oysters were PCR or RFTM positive for *P. beihaiensis*. On Day 20, no initially *P. beihaiensis*-naïve *C. ariakensis* or *C. virginica* were PCR or RFTM positive for *P. beihaiensis*. On Day 69, all five *C. virginica* sampled from cohabitation tank 1 were PCR positive (5/15); however, none was RFTM positive for *P. beihaiensis*. On day 95, one *C. ariakensis* in each of the three cohabitation tanks was RFTM positive (3/15); however, only those in tank two and tank three (2/15) were also PCR positive for *P. beihaiensis*. One *C. virginica* in cohabitation tank one (1/15) was PCR positive, although it was not RFTM positive.

After 95 days, the final *P. beihaiensis* prevalence, as determined by positive PCR results in the initially naïve hosts were as follows: for *C. ariakensis,* prevalence was 0.0% (0/16), 4.8% (1/21) and 4.5% (1/22) for cohabitation tanks one, two and three, respectively; for *C. virginica*, prevalence was 26.1% (6/23), 27.2% (6/22) and 0.0% (0/18) respectively, for tanks one, two and three.

Chinese oysters. The final number of Chinese oysters cohabitated with naïve hosts varied between aquaria. For *C. ariakensis* cohabitation tanks one, two and three,

Table 1. PCR and RFTM data for oysters sampled during the first cohabitation experiment. Final *Perkinsus* sp. prevalence values are calculated based on the number of PCR positive samples out of the total number that had tissue available for PCR. Weighted prevalence (WP) for Chinese oyster *Perkinsus* sp. RFTM ranking includes data collected on dead oysters.

	Day 20		Day 69		Day 95		Total mortality			Final host *Perkinsus* sp. prevalence	Final Chinese oyster *Perkinsus* sp. prevalence		
Cohabitation Tank	**# PCR pos**	**# RFTM**	**# PCR pos**	**# RFTM**	**# PCR pos**	**# RFTM**	**# dead**	**# PCR pos**	**# RFTM**	**# PCR pos**	**Total #**	**# PCR pos**	**WP**
C. ariakensis 1	0/5	0/5	0/5	0/5	0/13	1/13 (R)	6	0/3	0/3	0/16 (0.0%)	13	3/10 (30%)	0
C. ariakensis 2	0/5	0/5	0/5	0/5	1/7	1/7 (L)	5	0/4	0/4	1/21 (4.8%)	8	6/6 (100%)	0.75
C. ariakensis 3	0/5	0/5	0/5	0/5	1/11	1/11 (R)	3	0/1	0/0	1/22 (4.5%)	10	8/8 (100%)	0
C. virginica 1	0/5	0/5	5/5	0/5	1/12	0/12	1	0/1	0/1	6/23 (26.1%)	10	9/9 (100%)	2.0
C. virginica 2	0/5	0/5	0/5	0/5	0/3	0/3	15	6/9	0/7	6/22 (27.2%)	15	8/11 (72.7%)	0.18
C. virginica 3	0/5	0/5	0/5	0/5	0/7	0/7	7	0/1	0/0	0/18 (0.0%)	13	4/11 (36.4%)	0.86

13, 8 and 10 Chinese oysters, respectively, were included. For *C. virginica* cohabitation tanks one, two and three, 10, 15 and 13 Chinese oysters, respectively, were included with naïve oysters. Five Chinese oysters died during the experiment, and a few small 'spat' oysters attached to the shells of mature oyster were found dead when the experiment was terminated. Using only those Chinese oyster samples where enough tissue remained for PCR and RFTM analysis, the *P. beihaiensis* prevalence based on the *P. beihaiensis*-specific PCR assay, as well as weighted prevalence based on the RFTM assay, is listed in Table 1. *Perkinsus beihaiensis* PCR-based prevalence was 30% (3/10), 100% (6/6) and 100% (8/8) in the Chinese oyster/naïve *C. ariakensis* cohabitation tanks one, two and three, respectively and RFTM-based weighted prevalence was 0.0, 0.75 and 0.0. *Perkinsus beihaiensis* PCR-based prevalence in the Chinese oyster/naïve *C. virginica* tanks one, two and three was 100% (9/9), 72.7% (8/11) and 36.4% (4/11), and weighted prevalence values were 2.0, 0.18 and 0.86, respectively. Oyster species identification revealed that the majority of the oysters used in the experiment were *C. hongkongensis,* and 4/68 individuals (5.8%) were *C. ariakensis.* There were four oysters that could not be identified using the PCR and RFLP key described above because of either lack of PCR amplification or because the RFLP pattern was unrecognizable. In all cases, the unidentifiable oysters were small 'spat' oysters, possibly *Saccostrea* sp., as suggested by small denticles observe on the edge of the shell.

Mortality data. Significant mortality was observed in both of the initially naïve oyster species and sporadically in the Chinese oysters serving as the potential parasite source in the cohabitation tanks. Many of the small spat Chinese oysters that were sampled at the end of the experiment contained no tissue; however, the death of these animals either was not noticed during the experiment or those shells were empty prior to

the start of the experiment, but not discovered at the time. For this reason, final PCR and RFTM data for the Chinese oysters is based on the number of individuals that had tissue available for processing. For the Chinese oysters, two deaths occurred in the *C. ariakensis* cohabitation tank one, of which none was PCR positive for *P. beihaiensis.* Two Chinese oysters died in *C. ariakensis* cohabitation tank three, of which one was PCR positive for *P. beihaiensis.* One Chinese oyster died in *C. virginica* cohabitation tank 1, and it was both PCR and RFTM positive for *P. beihaiensis* with an RFTM ranking of moderate.

Cumulative mortality of the initially naïve *C. ariakensis* was 18.7% (14/75) and for *C. virginica* was 30.7% (23/75). Of those oysters that died, many did not have tissue remaining for PCR and/or RFTM analysis. Six of nine (66.7%) dead *C. virginica* from cohabitation tank two were PCR positive for *P. beihaiensis,* however, none was RFTM positive. None of the *C. ariakensis* that had suffered mortality tested positive for *P. beihaiensis* using either RFTM or PCR assays.

Histological analysis. In situ hybridization analysis was performed on all those initially naïve *C. virginica* and *C. ariakensis* oysters sampled during the experiment that were either PCR or RFTM positive for *P. beihaiensis* (n = 15). Hybridization of the *P. beihaiensis* species-specific probe to *P. beihaiensis* cells was not observed in any of the tissue sections analyzed.

Histopathological analyses of hematoxylin-eosin stained tissue sections were performed on 16 of the Chinese oysters used in the experiment that were species-specific PCR assay -positive for *P. beihaiensis.* In addition, RFTM data were collected for these as well as all other Chinese oysters sacrificed at the termination of the experiment. Twelve of 16 (75%) Chinese oysters were RFTM positive, having infection intensities

ranging from 1-5 (Table 2). *Perkinsus* sp. cells were observed commonly in the epithelia of the stomach and intestine, as well as in the connective tissue of the visceral mass. On rare occasion, cells were observed in the gill and mantle and epithelia of digestive tubules. In one instance, a few cells were observed in gonadal tissue. Eight of the sixteen Chinese oysters used as a parasite source were analyzed using the *P. beihaiensis*-specific ISH probe. Hybridization of the *P. beihaiensis*–specific probe to *P. beihaiensis* sp. cells was observed in all Chinese oyster tissue sections analyzed (Table 2). (See also Chapter 2).

Table 2. Histological data for a subset of Chinese oysters used in the first cohabitation experiment. The tank that the Chinese oyster was cohabitated in is indicated in the first column. When in situ hybridization analysis was not performed on a sample, it is indicated as "n/d."

Cohabitation Tank	RFTM 0-5	PCR Assay	Histopathology Analysis +/ -	Histopathology Analysis 0-5	Oyster Species ID	ISH Analysis
C. ariakensis 1	0	+	+	0	*C. hongkongensis*	n/d
C. ariakensis 1	0	+	+	1	*C. hongkongensis*	n/d
C. ariakensis 2	4	+	+	2	*C. hongkongensis*	+
C. virginica 1	1	+	+	0	*C. hongkongensis*	n/d
C. virginica 1	2	+	+	0	unknown sp.	n/d
C. virginica 1	5	+	+	5	*C. hongkongensis*	+
C. virginica 1	4	+	+	4	*C. ariakensis*	+
C. virginica 1	3	+	+	3	*C. hongkongensis*	+
C. virginica 1	1	+	+	1	*C. hongkongensis*	+
C. virginica 2	1	+	+	2	*C. hongkongensis*	n/d
C. virginica 2	1	+	+	3	*C. hongkongensis*	+
C. virginica 2	0	+	+	0	*C. hongkongensis*	n/d
C. virginica 2	0	+	+	1	*C. hongkongensis*	n/d
C. virginica 3	3	+	+	3	*C. hongkongensis*	+
C. virginica 3	2	+	+	0	*C. hongkongensis*	n/d
C. virginica 3	4	+	+	2	*C. hongkongensis*	+

Experiment 2

MATERIALS AND METHODS

Chinese oysters. In November 2006, oysters were collected from Beihai, Guangxi Zhuang, People's Republic of China. Oysters were shipped in refrigerated containers to the Virginia Institute of Marine Science, arriving on November 13, 2006 and were transported directly to the quarantine facility where they were allowed to warm to room temperature (~20 °C). The oysters were divided among 10-gallon glass aquaria, containing aerated 1-μm filtered York River water at 23--24 ppt at 20 °C. Oysters were fed twice daily 0.1 grams^{-1} oyster algae feed (Reed Mariculture, San Jose, CA) and two thirds of the water was refreshed three times weekly with 1-μm filtered York River water at 23--24 ppt and 20 °C.

Experimental hosts. On December 2, 2006, *Crassostrea ariakensis* (37.54 ± 6.03 mm shell height) were received from the University of Maryland's Center for Environmental Science (UMCES). *Crassostrea virginica* (72.3 ± 6.5 mm shell height) were obtained from Taylor Shellfish, Shelton, Washington, on December 5, 2006 and *Mercenaria mercenaria* clams (35.5 ± 3.0 mm shell height) were obtained from Mobjack Bay Seafood Company, Ware Neck, VA, on December 7, 2006. Upon arrival, twenty individuals of each bivalve species were sacrificed, and gill and mantle was excised aseptically for DNA extraction. Genomic DNA of each oyster or clam was used in the *Perkinsus* genus-specific PCR assay to determine the presence of *Perkinsus* spp. parasites (see below). Remaining oysters and clams were divided among 10-gallon glass aquaria, containing aerated 1-μm filtered York River water at 23--24 ppt and 20 °C. Oysters were fed twice daily 0.1 grams^{-1} oyster algae feed (Reed Mariculture, San Jose, CA) and 2/3 of

the water was refreshed three times weekly with 1-μm filtered York River water at 23--24 ppt and 20 °C.

Experimental design. On December 14, 2006, 60 naïve *C. virginica, C. ariakensis* and *M. mercenaria* were divided evenly among three 10-gallon aquaria per host species, 20 individuals per tank, with seven oysters from Beihai, China (9 cohabitation tanks total). Sixty *C. virginica, C. ariakensis* and *M. mercenaria* were divided evenly among three 10-gallon aquaria per host species, 20 individuals per tank without Chinese oysters (9 negative control tanks total). All aquaria were maintained in aerated 1-μm filtered York River water at 23--24 ppt and 21--24°C and were fed 0.1 grams^{-1} oyster algae feed twice daily. Two-thirds of the water was exchanged three times per week with 1-μm filtered York River water at 23--24 ppt and 21--24°C.

Perkinsus beihaiensis–naïve *C. virginica, C. ariakensis* and *M. mercenaria* were cohabitated with Chinese oysters for a total of six months. For the duration of the experiment, aquaria were checked daily for mortality and moribund animals were removed. When moribund or dead animals were discovered, and if sufficient tissue remained, it was processed for analysis. Gill and mantle tissues were removed with a portion preserved in 95% ethanol for DNA extraction and later *Perkinsus* spp. molecular diagnostics and gill and mantle tissues were processed for RFTM assays (Ray 1952). Visceral mass tissue sections of each sample were preserved in Davidson's solution for histological analysis (Shaw & Battle 1957).

Five *C. virginica, C. ariakensis* and *M. mercenaria* were sampled from all aquaria after one, two, and three months, and all remaining oysters and clams were sampled at the end of six months. All remaining Chinese oysters from the cohabitation aquaria were removed and sacrificed when the experiment was terminated.

Sample processing. Nucleic acid extraction, amplification of SSU genes, *Perkinsus* genus-specific PCR assays, *Perkinsus beihaiensis*–specific PCR assays, in situ hybridization analysis using *P. beihaiensis*–specific oligonucleotide probes, and PCR RFLP identification of Chinese oyster hosts were performed exactly as described in the first experiment (see above and Chapter 1, 2 and 3). RFTM assays using gill, mantle and rectal tissues as described above were performed on all clams and oysters sampled during the first three months and on all dead or moribund animals that were removed from the aquaria during the course of the experiment. A change to previously mentioned protocols included the RFTM analysis performed when the experiment was terminated (see below).

Because occasionally *Perkinsus* sp. cells were observed by RFTM in control animals, or in cohabitated initially naïve animals in the absence of *P. beihaiensis*–specific PCR results, all DNA samples were screened using the *Perkinsus* sp. genus-specific PCR assay and then positive samples were tested using *P. beihaiensis, P. chesapeaki, P. marinus* and *P. olseni*–specific PCR assays. *Perkinsus chesapeaki* and *P. marinus* are endemic to Chesapeake Bay and therefore could be a likely possible contaminant of the oysters and clams obtained from Maryland or Virginia waters. Additionally, because *P. olseni* has been found in northern China and Japan, it was necessary to test for the presence of *P. olseni* in both the Chinese oysters and subsequently in naïve oysters and clams sampled from cohabitation aquaria.

Modified whole body burden RFTM assay. When the experiment was terminated, remaining clams and oysters, including all remaining Chinese oysters, were processed for the presence of *Perkinsus* spp. using a modification of a previously described whole body burden RFTM method (Bushek et al. 1994, Choi et al. 1989). Oysters and clams were aseptically shucked and replicate gill and mantle samples were

taken for DNA analysis and for preservation in 95% ethanol. A transverse visceral section was removed from each animal and preserved for histological analysis. All remaining tissues for each individual were then macerated on a sterile plastic weigh boat using a sterile scalpel. Macerated *C. virginica* and *M. mercenaria* tissues were separately incubated in 25 ml of RFTM media in sterile polystyrene falcon tubes while the initially naïve *C. ariakensis* tissues were incubated in 10 ml of RFTM media in sterile polystyrene falcon tubes. All samples were incubated for 5--7 d at 27 °C. After incubation, samples were centrifuged at 1,600 x g for 20 min and RFTM media was removed. Remaining tissue including trace remaining RFTM media was weighed and approximately 10 ml of 2M NaOH was added per gram wet tissue weight. Samples were vortexed and incubated at 50 °C for 1 h or until tissue was completely digested. Samples were centrifuged at 1,600 x g for 10 min and the 2M NaOH was removed. An equal volume to that of the NaOH of 1X phosphate buffered saline (PBS) was added to each sample followed by vortexing. Each sample was then centrifuged at 1,600 x g for 10 min, supernatant removed and an equal volume of 1X PBS was added. Vortexing, centrifugation and removal of supernatant PBS was repeated. After this final washing step, 2 ml of 10% Lugol's iodine solution was added and the sample was vortexed. Stained cell preparations were vacuum filtered onto a 47 mm, 0.45 μm filter and the *Perkinsus* sp. cells were counted. If previous PCR analysis of a sample indicated it was *P. beihaiensis* –positive, three 100 μl aliquots of the sample were first counted. Triplicate aliquots were counted or diluted as necessary and an average of the counts was multiplied according to the final volume of the stained sample. Data were recorded as total number of cells per sample. For the Chinese oysters only, wet cell pellet weight was recorded after initial removal of RFTM media was reduced by 10% (to account for the approximate weight of

the residual RFTM media) and cell counts were also converted to Mackin's scale values based on Choi et al. (1989).

***Perkinsus marinus* and *P. olseni* PCR assays.** PCR primers PmarITS-70F and PmarITS-600R (Audemard et al. 2004) were used in a *P. marinus*-specific PCR reaction as described previously (Moss et al. 2006, Chapter 3). Following amplification, 4 µl of PCR product was analyzed as described above.

PCR primers Pols-140F and PolsITS-600R were used in a *P. olseni*-specific PCR reaction as described previously (Moss et al. 2006, Chapter 3). Following amplification, 4 µl of PCR product was analyzed as described above.

***Perkinsus chesapeaki* PCR assay.** A previously published PCR assay (Burreson et al. 2005) targeting the conserved areas of the *P. chesapeaki* ITS region was used to screen oyster and clam genomic DNA for the presence of *P. chesapeaki.* The forward primer sequence was (5' AAACCAGCGGTCTCTTCTTCGG 3') and the reverse primer sequence was (5' CGGAATCAACCACAACACAGTCG 3'). PCR reagents for the 25 µl reaction *P. chesapeaki*–specific PCR reaction contained 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 mM each primer, 0.625 U per µl *Taq* polymerase, 0.2 mg per ml BSA and 0.5 µl genomic DNA (~10--50 ng total). Amplifications were performed with an initial denaturation of 94 °C for 4 min, followed by 10 cycles at 94 °C for 30 s, 64 °C for 30 s (with a decrease of 1 °C per cycle), and 72 °C for 90 s. After ten cycles, amplification included 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 90 s with a final elongation of 72 °C for 5 min. Following amplification, 5 µl of PCR product was analyzed by agarose gel electrophoresis (2%), stained with ethidium bromide and visualized under UV light. Gel images were recorded as above.

RESULTS – Experiment 2

Experimental sampling. During the six-month experiment, no negative control animals tested positive for *Perkinsus beihaiensis* DNA using the *P. beihaiensis*-specific PCR assay, however four *Mercenaria mercenaria* tested positive for *P. chesapeaki.* No negative control or cohabitated clams or oysters, or any Chinese oyster ever tested positive for *P. marinus* or *P. olseni* DNA.

PCR and RFTM data for initially naïve oysters and clams, in addition to the Chinese oysters used during the second experiment as parasite vectors, are shown in Table 3. After one month in cohabitation with *P. beihaiensis*–infected Chinese oysters, no *Crassostrea virginica, Crassostrea ariakensis* or *M. mercenaria* were PCR positive when analyzed using the *P. beihaiensis*-specific PCR assay, nor did any samples exhibit *Perkinsus* sp. cells by RFTM analysis. After two months, no samples tested positive for *P. beihaiensis* DNA, however three *M. mercenaria* had visible *Perkinsus* sp. cells by RFTM. These three clams were sampled from cohabitation tanks two and three.

Table 3. PCR and RFTM data for oysters and clams sampled during the second cohabitation experiment. The symbol "*" next to RFTM values is a reminder that *Perkinsus chesapeaki* was detected using PCR in some *Mercenaria mercenaria* clams, but *Perkinsus beihaiensis* was never detected by PCR or in situ hybridization, therefore the presence of visible *Perkinsus* sp. cells as seen by RFTM could be either *P. chesapeaki* or *P. beihaiensis.* For the Chinese oyster data, *P. beihaiensis* PCR positive and RFTM positive sample data is given as the number of positive samples over the number of samples where tissue was available for processing. "WP" refers to *P. beihaiensis* weighted prevalence for Chinese oysters within the specified aquaria.

	Month 1		**Month 2**		**Month 3**		**Month 6**		**Mortality**			**Chinese oysters**		
Cohabitation Tank	# PCR pos	# RFTM	# PCR pos	# RFTM	# PCR pos	# RFTM	# PCR pos	# RFTM	# PCR pos	# RFTM	Total # dead	# PCR pos	# RFTM	WP
C. ariakensis 1	0/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	1/1	0/1	1	1/7 (14.3%)	5/7 (71.4%)	0.43
C. ariakensis 2	0/5	0/5	0/5	0/5	0/5	0/5	0/4	1/4	0/3	0/3	2	1/7 (14.3%)	5/7 (71.4%)	0.71
C. ariakensis 3	0/5	0/5	0/5	0/5	0/5	0/5	0/3	1/3	0/1	0/1	3	4/6 (66.7%)	3/6 (50.0%)	0.88
C. virginica 1	0/5	0/5	5/5	0/5	0/5	0/5	1/5	4/5	0/0	0/0	1	6/7 (85.7%)	5/5 (100%)	0
C. virginica 2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/0	0/0	0	1/7 (14.3%)	4/7 (57.1%)	0
C. virginica 3	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/0	0/0	0	3/7 (42.9%)	4/5 (80.0%)	0.21
M. mercenaria 1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	4*/5	0/0	0/0	0	5/7 (71.4%)	7/7 (100%)	0.94
M. mercenaria 2	0/5	0/5	0/5	1*/5	0/5	0/5	0/5	3*/5	0/0	0/0	4	1/7 (14.3%)	2/7 (28.6%)	0.04
M. mercenaria 3	0/5	0/5	0/5	2*/5	0/5	0/5	0/5	2*/5	0/0	0/0	1	3/6 (50.0%)	4/6 (66.7%)	0.42

One clam from tank one tested positive for *P. chesapeaki* DNA during the sampling at two months, however *Perkinsus* sp. cells were not observed in that clam by RFTM. When sampling was performed after three months, no previously naïve oysters or clams tested positive for *P. beihaiensis* DNA, however one *C. ariakensis* from cohabitation tank one was found to contain one *Perkinsus* sp. cell by RFTM assay.

The final sampling of the experiment occurred after six months exposure to *P. beihaiensis*-infected Chinese oysters. At that time, one previously naive *C. ariakensis* (cohabitation tank two) and one *C. virginica* (cohabitation tank one) were PCR positive according to the *P. beihaiensis*-specific PCR assay. The *C. ariakensis* was dead and necrotic when sampled and had no tissue left to analyze histologically, though three *Perkinsus* sp. cells were observed in tissue of this sample when analyzed by RFTM. The tissue processed for RFTM analysis from the *P. beihaiensis*-PCR positive *C. virginica* was found to contain 1,300 *P. beihaiensis* cells. An additional two *C. ariakensis,* five *C. virginica* and nine *M. mercenaria* were *Perkinsus* sp.-positive using the RFTM assay. Two of these clams, however, were positive for *P. chesapeaki* DNA.

Mortality data. There was little mortality in the *P. beihaiensis* –naïve *C. virginica, C. ariakensis* and *M. mercenaria* during the six-month experiment. Two *C. ariakensis* in the control treatment and four *C. ariakensis* from the cohabitation treatment died. Only one of these oysters (described above) tested positive for *Perkinsus* sp. DNA and it was determined to be *P. beihaiensis* DNA by the *P. beihaiensis*-specific PCR assay.

Chinese oysters. All of the Chinese oysters imported to use as the parasite source that had tissue and DNA available to analyze were determined to be *Crassostrea hongkongensis.* In total, 63 oysters were used in the experiment and over six months, 12

oysters died. Of these, seven were RFTM and/or PCR positive for *P. beihaiensis.* In total, 41.0% (25/61) were positive for *P. beihaiensis* DNA using PCR and 68.4% (39/57) were positive by RFTM. The difference between the total number of animals processed using either PCR or RFTM reflects that in some instances tissue was not available to be processed for one or both of the assays because the oyster had died and remaining tissue was severely degraded or absent.

All of the Chinese oysters sacrificed at the end of the experiment were analyzed using the modified whole body burden RFTM assay and many contained thousands of *P. beihaiensis* cells. For example, for those *C. hongkongensis* used in *C. ariakensis* cohabitation tank one, there were on average, 71,886 ± 190,191 *P. beihaiensis* cells counted per oyster based on the amount of tissue examined when they were finally sacrificed. Similarly, for *M. mercenaria* cohabitation tank one, there were on average 225,106 ± 576,162 *P. beihaiensis* cells counted per oyster based on the modified whole body burden RFTM assay.

Combined PCR and RFTM analysis of the Chinese oysters indicated that after six months, 61.9% (13/21) of the oysters cohabitated with *M. mercenaria,* and 66.7% (14/21) of those oysters cohabitated with *C. virginica* or *C. ariakensis* were infected with *P. beihaiensis.* The *P. beihaiensis* weighted prevalence (WP) of the Chinese oysters cohabitated with *C. ariakensis* was 0.52, for the Chinese oysters cohabitated with *M. mercenaria* it was 0.47 and for those oysters cohabitated with *C. virginica* it was 0.07.

Histological analysis. All of those initially naïve oysters or clams found to contain *P. beihaiensis* DNA or where *Perkinsus* sp. cells were observed by RFTM during the course of, or at the end of the experiment, were analyzed by ISH using both *Perkinsus* genus-specific and *P. beihaiensis*–specific oligonucleotide probes. No binding of either

the genus or species probe was found in any of the tissue sections from analyzed. Both probes bound to *P. beihaiensis* cells in the tissue sections of three *C. hongkongensis* from the experiment used as positive controls for the ISH assay.

DISCUSSION

The discovery of the new *Perkinsus* species, *Perkinsus beihaiensis,* in southern China during a disease survey of bivalve mollucs from China, Japan and Korea (Chapter 1 and 2), in light of the proposed introduction of *Crassostrea ariakensis* to the Chesapeake Bay, prompted the investigation into the potential for transmission of this exotic pathogen to native Chesapeake Bay bivalve species. Transmission studies of *P. beihaiensis* to *C. virginica, Mercenaria mercenaria,* as well as to naïve triploid *C. ariakensis* were undertaken in order to assess this risk.

These experiments were undertaken using naturally-infected Chinese oysters collected from Dafen River, Qinzhou, and Beihai, China. Significant mortality had occurred during transport of the first set of oysters from China to Virginia in the spring of 2006, therefore the likelihood that they would survive re-acclimation to aquaria in the quarantine facility was tenuous. The fragile state of the oysters discouraged non-lethal subsampling of gill tissue or hemolymph in order to assess *P. beihaiensis* presence in each oyster prior to the start of the cohabitation. Additionally, only with histological analysis conducted later (Chapter 2) was information gained about the tissue tropisms of this *Perkinsus* sp., therefore it would have been possible for sampling of selected tissues to not have been truly diagnostic of the body burden of parasite for an oyster at the start of the cohabitation. For both of the experiments, it would have been ideal to cohabitate an equal number of infected Chinese oysters in each of the experimental aquaria at the

beginning. Especially during the first experiment, due to the high early mortality in the imported oysters, it was necessary to start the cohabitation with naïve hosts quickly, rather than try to screen the oysters for *P. beihaiensis* individually, risking that they may die during the stress of experimental manipulation. Lacking a non-lethal and proven diagnostic method, prevalence of *P. beihaiensis* in the Chinese oysters could be obtained only at the end of the experiments.

In the first experiment averaging *Perkinsus* sp. weighted prevalence (WP) among the Chinese oysters for each host treatment, *C. ariakensis* and *C. virginica*, the WP of the oysters cohabitated with the *C. ariakensis* was 0.25, whereas for those cohabitated with the *C. virginica*, WP was 1.01. Weighted prevalence data were recorded for the Chinese oysters at the termination of the experiment, therefore one might argue that these data does not reflect the *P. beihaiensis* levels at the beginning of the experiment. All aquaria were maintained under similar conditions, and there is no reason to hypothesize that cells of this *Perkinsus* species reproduce at variable rates. For this reason, the starting body burdens of parasite should be proportionately less at the beginning of the cohabitation experiment than after 95 days. With this consideration, overall, the *C. virginica* were likely exposed to a greater number of *P. beihaiensis* cells than were the *C. ariakensis.*

The fact that on average, *C. ariakensis* were exposed to a fewer number of cells than *C. virginica* may explain why final average *P. beihaiensis* PCR prevalence for *C. virginica* was 17.8%, whereas average prevalence for *C. ariakensis* was 6%. Weighted prevalence data of the Chinese oysters and putative transmission to the naive hosts, based only on positive *P. beihaiensis* PCR results, were not correlated. For example, within the *C. ariakensis* treatment, no parasite cells were recorded in Chinese oysters that had been in tanks one and three, however *P. beihaiensis* PCR prevalence by the end of the

experiment in the initially naive *C. ariakensis* was 0.0% in tank one, and 4.5% in tank three. In addition, a WP of 0.18 was recorded for Chinese oysters from *C. virginica* tank two while PCR prevalence data in initially naïve *C. virginica* became 27.2%. A WP of 0.86 in the Chinese oysters was recorded in *C. virginica* tank 3 and those *C. virginica* had a *P. beihaiensis* PCR prevalence of 36.4%.

Proliferation of *P. beihaiensis* within the Chinese oysters or horizontal transmission of the parasite during the experimental challenge is suggested because baseline *Perkinsus* sp. prevalence data taken for Dafen River oysters was approximately 33% and 10% for Qinzhou oysters, whereas a mixture of oysters from both locations sampled at the end of 95 days had a *P. beihaiensis* PCR prevalence of 73%. The animals used in the experiment were randomly sampled from those collected in Asia, therefore the starting *P. beihaiensis* prevalence in those oysters should have been similar to that recorded in the initial sample.

The oysters received from Beihai, China in November 2006 stabilized once put into aquaria at VIMS and only occasional mortality was observed. The original *P. beihaiensis* prevalence in those oysters that were dead or moribund on arrival indicated that approximately 10% of the oysters were infected with the parasite. Initially, the second cohabitation experiment was scheduled to last for only four months; however, when there was little evidence of *P. beihaiensis* transmission in the first three months, perhaps because of the low initial prevalence of *P. beihaiensis* in the Chinese oysters, the experiment was extended for an additional two months. Based on histological analysis of Chinese oysters used in the first experiment, while the second experiment was already in progress, it became apparent that gill and mantle tissues sampled for DNA and RFTM analysis were likely not representative of the body burden of the oysters. Many Chinese

oysters were found to contain *P. beihaiensis* cells in the visceral mass connective tissues, and cells were observed in gill or mantle connective tissues in the histological sections (Chapter 2). In order to increase the potential for *P. beihaiensis* detection, a modified whole body burden RFTM assay was used on the final six-month samples in order to screen any remaining tissues that were not used in DNA analysis or for histological preservation. This modification may have detected *P. beihaiensis* cells that would have been missed employing the previously used RFTM method or by performing PCR-based molecular diagnostics on DNA extracted from the gill and mantle tissues alone.

In the second cohabitation experiment, the PCR assay amplified *P. beihaiensis* DNA from only one of the initially naive *C. virginica* and one of the *C. ariakensis,* both collected when the experiment was terminated. When using the body burden RFTM assay, *Perkinsus* sp. cells were observed in nine *M. mercenaria,* six *C. virginica* and three *C. ariakensis* sampled from cohabitation aquaria at the six-month sampling whereas *Perkinsus* sp. cells had been observed only in three *M. mercenaria* and in no *C. ariakensis* or *C. virginica* in prior samplings. The increase in the observed number of *Perkinsus* sp. cells may indicate that the modified RFTM assay was in fact more sensitive than the previously used method, and/or that it took at least six months for transmission of *P. beihaiensis* to occur. *Perkinsus chesapeaki* DNA was detected in five *M. mercenaria* clams, therefore in the absence of confirmatory *P. beihaiensis*-specific PCR or in situ hybridization results, the *Perkinsus* sp. cells found in clams could be either *P. chesapeaki* or *P. beihaiensis* or represent a mixed infection of both *Perkinsus* species. *Perkinsus* sp. DNA, other than *P. beihaiensis* DNA, was never detected in *C. ariakensis* or *C. virginica.* The *C. virginica* came from Washington, USA, where *Perkinsus* sp. has never been reported, therefore it is plausible that the *Perkinsus* sp. cells observed in *C.*

virginica could be *P. beihaiensis* cells and the PCR assay, targeting genomic DNA isolated only from gill and mantle tissue, was not sensitive enough to detect the *P. beihaiensis* present. The *C. ariakensis* used in the second experiment originated from a hatchery in Maryland, from waters where *P. marinus* has been previously detected (data not shown); however, *P. marinus* never was detected in this batch of oysters. There does exists a slight chance, due to the origin of the oysters, that some *P. marinus* cells could be harbored by the *C. ariakensis* and only after 6 months in an aquaria environment where *P. marinus* has been shown to proliferate (Chapter 3), did the cells reach a detectable level, especially using the more sensitive RFTM assay. *Perkinsus marinus* has been shown to actively replicate in *C. ariakensis* and can reach lethal levels within two months (Chapter 2), so if the oysters were infected with *P. marinus* at the start of the experiment, it is very unlikely that the presence of *P. marinus* would have gone undetected for six months. This suggests that the *Perkinsus* sp. cells observed in the *C. ariakensis* at the end of the experiment are likely *P. beihaiensis.* If this is true, then during the six-month experiment 13.3% (6/45) of the *C. virginica*, and 6.7% (3/45) of the *C. ariakensis* may have become infected with *P. beihaiensis.*

In hindsight, an aliquot of the pellet left from the whole body burden RFTM technique should have been used for DNA extraction and PCR analysis, and the remainder of the pellet then stained and examined for the presence of *P. beihaiensis* cells. This may have increased the chances of detecting *P. beihaiensis* DNA, although it is unclear how residual NaOH or organic compounds associated with the visceral mass (common inhibitors of PCR) would have effected DNA extraction and subsequent PCR amplification of *P. beihaiensis* DNA if present.

Only gill, mantle and rectal tissues were analyzed for those Chinese oysters that died during the experiment, whereas the more comprehensive RFTM technique that surveys a larger portion of the total tissue of the oyster was used for those oysters that survived until the end of the experiment. The relationship between Mackin's scale ranking (based on a small amount of tissue such as that used here) and the number of *P. marinus* hypnospores per gram wet tissue weight has been characterized (Choi et al. 1989). Cell counts taken for Chinese oysters were converted to Mackin's scale values in order to indicate the weighted prevalence for those vector oysters in each of the cohabitation aquaria (Table 3) and able to compare the relative *P. beihaiensis* exposure of host bivalves in this experiment to those used in the first experiment. According to Choi et al. (1989), *Perkinsus* sp. cells would go undetected until infection intensity reached at least 1,000 cells per gram wet tissue weight (Mackin rank of 0). In this study, smaller increments (between 0 and 1) were used here to more accurately portray cell counts between 0 and 1,000 that were observed in the Chinese oysters. The PCR data suggest that overall, the initially naïve clams and oysters may have been equally exposed to *P. beihaiensis* cells because combined PCR and RFTM analysis (positive or negative for cells) of the Chinese oysters used in the second cohabitation experiment indicated that after 6 months, 61.9% (13/21) of the oysters cohabitated with *M. mercenaria,* and 66.7% (14/21) of those oysters cohabitated with *C. virginica* or *C. ariakensis* had *P. beihaiensis* DNA. The weighted prevalence data for the second cohabitation experiment, however, suggests that the *C. ariakensis* were exposed to the greatest number of cells (Chinese oyster WP of 0.52), followed by *M. mercenaria* (Chinese oyster WP of 0.47) and *C. virginica* (Chinese oyster WP of 0.07).

With exposure being variable for each host species in these experiments, a drawback of using Chinese oysters naturally infected with variable levels of *P. beihaiensis*, it is not possible to comment on the relative apparent susceptibility of *C. virginica, C. ariakensis* or *M. mercenaria*. Further experiments using equal exposure rates and confirmatory histological evidence of transmission would be required; however, lacking pure cultures of *P. beihaiensis* handicaps efforts to conduct direct inoculation studies or to standardize dosages of cells to *P. beihaiensis*-free bivalve molluscs in bath challenge experiments.

Use of oysters naturally infected with *P. beihaiensis* to serve as vectors of the parasite in cohabitation could be ideal, however, as previously mentioned, the sensitivity of non-lethal versus more invasive methods to detect *P. beihaiensis* cells have not been studied. Histological evidence collected thus far on naturally infected animals and those used in these cohabitation experiments suggests that lesions most commonly occur in the epithelia of the stomach and intestine as well as in the connective tissues of the visceral mass. Only rarely were cells detected in the gill and mantle. The presence of *P. beihaiensis* cells circulating in the hemolymph was not examined. Being that parasite cells are only rarely observed in the gill and mantle with this *Perkinsus* sp., and it is only possible to take these tissues non-lethally from anesthetized animals, it makes non-lethal pre-screening of potentially infected hosts virtually impossible until another means of diagnostics is developed.

As was discussed in Chapter 3, it was not possible to test a wide range of salinity and temperature regimes during these cohabitation experiments. *Perkinsus beihaiensis* has been found in oysters taken from waters of 21--23 ppt therefore these conditions were used in the cohabitation experiments. Without proliferating pure cultures of this

Perkinsus sp. it is difficult to determine the optimal conditions under which the parasite proliferates, or the host is susceptible, and therefore likely is most infective to potential hosts.

The results of these cohabitation experiments suggest that *P. beihaiensis* may be transmitted to *C. virginica, C. ariakensis* and *M. mercenaria.* PCR and RFTM analysis detected *Perkinsus* sp. cells in oysters and clams that were exposed to *P. beihaiensis,* however histological evidence of parasite acquisition is lacking. PCR detection of *P. beihaiensis* DNA cannot solely be used for evidence of transmission because DNA-based analysis can detect cells that are not viable or may only be superficial. For example, parasite cells on the gills or passing through the gut do not necessarily represent infections by a parasite. To verify infections, therefore, PCR data must always be confirmed by histological evidence of parasite cells within an organism.

Additionally, although the bivalve mollusc species tested here do not appear to be highly susceptible to this parasite, especially when considering that *P. beihaiensis* appeared to readily proliferate within Chinese oyster hosts, the data reported here should be interpreted that *P. beihaiensis* does represent a risk to Chesapeake Bay bivalves and steps must be taken to prevent introduction of this non-native parasite to the Bay.

CONCLUSION

The proposed introduction of *Crassostrea ariakensis* to Chesapeake Bay has incited intense debate regarding the economic viability and risks associated with introducing a non-native species to the mid-Atlantic, USA. Funded research priorities have been identified and incorporated guidance from the International Council for Exploration of the Seas (ICES) Code of Practice on the Introductions and Transfers of Marine Organisms. ICES recommends, among other things, that the "ecological, genetic and disease relationships of the species in its natural range and environment" be examined prior to introduction of a non-native organism.

The basis for the research presented here began with a pathogen survey of *C. ariakensis* throughout its natural range in Korea, Japan and China (Chapter 1). In addition to *C. ariakensis,* a sympatric *Crassostrea* species, *C. hongkongensis,* distinguishable from *C. ariakensis* only by genetic techniques (Cordes and Reece, 2005), as well as other *Crassostrea* spp., and pearl oysters collected in the same area from 1999--2006 were screened for molluscan herpes virus and for *Perkinsus* spp. parasites using PCR-based molecular diagnostics. Histological analysis was performed by the VIMS shellfish and pathology lab, in conjunction with molecular screening performed personally, on oysters collected in 1999 from eight locations in China. As a result of that parasite survey, multiple genetic strains of molluscan herpes virus and two *Perkinsus* spp., *P. olseni* and a novel *Perkinsus* sp., *Perkinsus beihaiensis,* were reported. *Steinhausia*-like microsporidians, viral gametocytic hypertrophy, *Clamydia*-like

organisms, *Nematopsis* sp., cestodes, and ciliates were observed in histological analyses performed on the 1999 samples.

The report of a new *Perkinsus* sp. in southern China was initially based solely on phylogenetic analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA gene complex. In order to confirm the uniqueness of this parasite, additional DNA loci were targeted for examination, and histological analysis of *Perkinsus beihaiensis* infected oysters was also performed (Chapter 2). Large subunit rRNA gene and actin gene DNA fragments were amplified from genomic DNA of infected oysters using newly developed PCR assays, and additional ITS region sequences were also obtained. Combined phylogenetic analysis of ITS region, LSU rRNA gene and type 1 actin gene nucleotide sequences yielded trees with similar topologies. In all analyses, sequences of this new parasite showed that it was clearly a member of the genus *Perkinsus;* however, it was unique from all currently accepted *Perkinsus* species. Histological analysis performed on *Perkinsus beihaiensis* PCR positive oysters revealed cells with typical *Perkinsus* morphology and recognizable *Perkinsus* sp. life stages in tissue sections examined. In some oysters, systemic *Perkinsus beihaiensis* infections were observed suggesting that this species may reach lethal intensities under certain conditions. A unique PCR assay, as well as a *Perkinsus beihaiensis* -specific oligonucleotide probe used for in situ hybridization, was developed for this new *Perkinsus* species. Using these genetic tools, the range of this new *Perkinsus* sp. has been determined to be at least from Fujian Province to Guangxi Province in southern China, and it is associated with *C. ariakensis* and *C. hongkongensis* (PCR and histological confirmation) as well as pearl oysters, *Pinctada martensii* and *P. margaritifera* (based on PCR results only).

Those organisms discovered by histological analysis during the parasite survey were observed infrequently and likely are not significant pathogens of those *Crassostrea* spp. examined. Molluscan herpesvirus can be a serious problem leading to severe economic losses, particularly in hatcheries where it can cause massive mortalities in larvae and spat (Hine 1992, Le Deuff et al.1994, Arzul et al. 2001b); furthermore, it is implicated as a possible cause of summer mortality in *C. gigas* in California (Friedman et al. 2005). Previous research suggests that molluscan herpesvirus can be both horizontally (Arzul et al. 2001b and 2001c) and vertically transmitted (Renault et al. 1994, Arzul et al. 2002). Vertical transmission of *Perkinsus* spp. protozoans has not been documented, though vertical transmission of microsporidians has been demonstrated previously in invertebrates such as amphipod crustaceans and *Daphnia* (Kelly et al. 2003, Galbreath et al. 2004, Haine et al. 2004, Vizoso and Ebert 2004). Current quarantine methods and import procedures as outlined by ICES (ICES, 2005) should minimize or eliminate the risk of exposure of native Asian broodstock oysters, or associated pathogens, to the waters and shellfish of Chesapeake Bay. Current protocols, however, will not be effective against vertically transmitted pathogens. In order to understand the potential disease impacts associated with an introduction of *C. ariakensis* to Chesapeake Bay, we must attempt to predict the outcome of exposure of Asian pathogens to naïve local bivalve species.

Perkinsus olseni is geographically widespread and is known to be especially problematic to the carpet shell clam, *Ruditapes decussatus,* in Spain (Casas et al. 2002, Villalba et al. 2005). The discovery of *P. olseni* in *C. ariakensis* and *C. hongkongensis* in northern China (Chapter 1) is not surprising given that previous reports of *P. olseni* have documented its occurrence in bivalve shellfish from northern China (Liang et al. 2001),

Japan (Hamaguchi et al. 1998) and Korea (Choi and Park 1997, Park et al. 2006). The pathogenicity of *P. olseni* to *C. ariakensis* is unknown, therefore this question and the potential for transmission to native Bay bivalve shellfish, the hard clam, *M. mercenaria,* and the eastern oyster, *C. virginica*, were the motivation behind conducting two direct inoculation experiments, using cultured *P. olseni* cells, as well as a bath challenge experiment, using *P. olseni* cells harvested directly from naturally-infected Spanish *R. decussatus* (Chapter 3).

During the first inoculation experiment, there was little evidence of *P. olseni* transmission to naïve hosts; however, *C. ariakensis* briefly exposed to *P. marinus* developed heavy and lethal infections. This provided valuable information on potentially problematic disease issues including parasite proliferation that could arise if *P. marinus*-infected *C. ariakensis* encountered stress challenges in the wild or aquaculture environments, or if they are held in hatcheries or laboratories under stressful conditions.

It is known that *Perkinsus* sp. may lose virulence in culture (Ford et al. 2002a). Therefore, in the second experiment, *P. olseni* culture media was supplemented with oyster homogenate. Oyster homogenate has been shown to increase protease activity of *P. marinus* in culture (Earnhart et al. 2004, MacIntyre et al. 2003). Proteolytic enzyme analysis of *P. olseni* cell free supernatant showed high molecular weight protease activity in the presence of oyster homogenate, but none in the presence of fetal bovine serum. The lack of low molecular weight proteases suggested that *P. olseni* may not secrete serine proteases, as does *P. marinus*, or that they were not detected. *Crassostrea virginica* followed by *M. mercenaria* and *C. ariakensis* had the highest number of PCR positive and RFTM positive samples following the challenge, though the presence of *P. olseni* cells was only detected in one clam when analyzed using in situ hybridization.

A *P. olseni* bath challenge experiment was undertaken due to the inability to acquire live, *P. olseni*-infected oysters or clams for cohabitation experiments. Crudely purified *P. olseni* cells obtained from *R. decussatus* from Spain were added to aquaria containing the above listed host species on two occasions. After three months, 6/60 (10%) of the *M. mercenaria* were PCR positive for *P. olseni* and RFTM analysis detected *Perkinsus* sp. cells in 3/60 (5%) clams, although none of these three clams was of the six that were PCR positive for *P. olseni*.

Combined results of the three *P. olseni* experiments provided genetic evidence, as seen by positive *P. olseni*-specific PCR assays, as well as RFTM evidence of *P. olseni* transmission to naïve hosts of all three species. Histological confirmation of infection was limited, however, and only in situ hybridization performed on one clam ever showed hybridization to *P. olseni* cells in situ. Histological examination of one or a few 5 μm tissue sections may not be sensitive enough to detect rare or focal *Perkinsus* spp. infections. It is also imperative to understand that for *P. olseni,* neither the minimum infective dose of cells, nor the optimal environmental conditions for transmission and disease development are known (Miossec et al. 2006). The experiments were conducted over a very small temperature and salinity range and replacing water each week in the experimental aquaria also removed potentially infective cells from the system. In addition to obtaining evidence that *C. virginica* and *M. mercenaria* may be susceptible to *P. olseni,* important diagnostic methods were developed for these experiments that can be applied to future research. A *P. olseni*-specific PCR assay was optimized, a quantitative real time PCR assay was developed and *P. marinus* and *P. olseni*-specific in situ hybridization probes were developed.

Along with describing the new *Perkinsus* sp., cohabitation experiments were performed in order to assess the pathogenicity and likelihood of transmission of this exotic *Perkinsus* sp. to the hard clam and the eastern oyster. Live oysters were imported from the Beihai, China region on two occasions and placed in aquaria with naïve *C. ariakensis, M. mercenaria* or *C. virginica.* The experiments lasted four and six months. Modified RFTM assays were performed during the second cohabitation experiment and appeared to be more sensitive than previous RFTM assays that used only gill, mantle or rectal tissues for detection of *Perkinsus* spp. Combined PCR and RFTM analysis suggested that *Perkinsus* n. sp. may be transmitted to both the eastern oyster and the hard clam, but this cannot be confirmed because *Perkinsus* n. sp. cells could not be found in tissue sections examined histologically. The Chinese oysters used in these experiments appeared to be highly susceptible to *Perkinsus* n. sp. with infections becoming more prevalent and intense during the course of each experiment.

This research suggests that transmission of exotic *Perkinsus* spp. to *M. mercenaria* and *C. virginica* may be possible and results reported herein should be interpreted with the understanding that it is impossible to emulate natural conditions in the laboratory, nor is it possible to mimic each environmental scenario possible along the expansive range of conditions under which these parasites may survive, proliferate, or become pathogenic. As was demonstrated in the first *P. olseni* inoculation experiment, *C. ariakensis*, considered to be relatively resistant to *P. marinus* as compared to *C. virginica,* can develop lethal *P. marinus* infections. This finding is especially important because it is contrary to what was found in previous field trials undertaken to understand how well *C. ariakensis* might perform under the disease pressure naturally present in the Bay (Calvo et al. 2001). *Crassostrea hongkongensis* and *C. ariakensis* can also develop

systemic infections of *Perkinsus* n. sp. The apparent *Perkinsus* spp. susceptibility also indicates that these oysters could act as reservoirs for non-native *Perkinsus* spp., should they become introduced through discharge of Asian ballast water into Chesapeake Bay or by failure of quarantine protocols or facilities holding Asian brood stock oysters. There clearly are dangerous viral and protozoan pathogens associated with *C. ariakensis* that may be transmitted to naïve populations of Bay oysters and clams.

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