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A Molecular Phylogeny of the Genus Bonamia Based on Sequence Data of the Ribosomal RNA (rRNA) Gene Complex

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A Molecular Phylogeny of the Genus *Bonamia* Based on Sequence Data of the

Ribosomal RNA (rRNA) Gene Complex

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A Thesis

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Science

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By

Delonna M. White

2008

APPROVAL SHEET
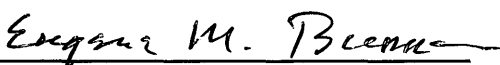
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ABSTRACT

Oyster parasites of the genus *Bonamia* have been described from Europe (*Bonamia ostreae*), Australia (*B. roughleyi*), New Zealand (*B. exitiosa*), and North America (*B. perspora*). In addition, various parasites have been observed in the following oysters on five continents: *Ostrea angasi*—NSW, Australia; *O. puelchana*—San Antonio Bay, Argentina; *O. chilensis*—Chiloe Island, Chile; and *Crassostrea ariakensis*—North Carolina, USA. *Bonamia* parasites are members of the phylum Haplosporidia and are generally characterized as intracellular microcells between 2-3 μm in diameter containing haplosporosomes and mitochondria. Previous studies have used the small subunit ribosomal RNA (SSU rRNA) gene to resolve the phylogenetic relationships among the characterized *Bonamia* spp. This study used the nucleotide sequences of the SSU rRNA gene, the internal transcribed spacer (ITS) regions, and the 5.8S rRNA gene to examine the taxonomic relationships among previously characterized and recently discovered *Bonamia* parasites. A total of 1060 oysters were sampled from North Carolina, USA, Maine, USA, Argentina, Chile, New Zealand, and Australia. Of this total, parasite sequences from 22 sampled oysters were used for the phylogenetic study. Collectively, the described *Bonamia* parasites and the unnamed *Bonamia* parasites were sequenced using *Bonamia*-specific SSU primers, haplosporidian-specific ITS primes, and *Bonamia*-specific ITS primers. Sequences were aligned using ClustalW in MacVector and phylogenetically analyzed in PAUP*4.0b10. Parsimony bootstrap and parsimony jackknife analyses of the SSU gene, the ITS region, and the combined SSU/ITS dataset suggest that a single *Bonamia* species may occur in New Zealand, Australia, Argentina, and North Carolina, USA. The *Bonamia* sp. infecting oysters from Argentina, Australia, and North Carolina, USA are phylogenetically indistinguishable from the New Zealand *O. chilensis Bonamia* sp. for the ITS and combined ITS/SSU data. There were some differences observed between the group of *Bonamia* spp. found in New Zealand, Australia, Argentina, and North Carolina, USA, and the *B. exitiosa* and *B. roughleyi* sequences for the SSU data. Results also suggest that a separate species of *Bonamia* might exist in Chile, although further study is necessary. Additionally, this study did not observe a correlation between winter mortality in *Saccostrea glomerata* in Australia and the presence of *B. roughleyi*, casting some doubt on *B. roughleyi* as the cause of winter mortality.

A Molecular Phylogeny of the Genus *Bonamia* Based on Sequence Data of the

Ribosomal RNA (rRNA) Gene Complex

INTRODUCTION

Farming and transporting oysters to new locations has been widely practiced across the globe in response to overharvesting and disease of local oyster stocks. The transport of *Crassostrea gigas* in 1970 into Europe in response to disease and mass mortality of Portuguese oysters, *C. angulata,* by an iridovirus is a more recent example (Comps et al. 1976 in Bougrier et al. 1986). In some instances, the introduction of non-native oysters has led to transmission of parasites destructive to native stocks (Burreson et al. 2000). In the 1970s, European countries such as France, England, Ireland, the Netherlands, and Spain sought to increase their oyster stocks by importing *Ostrea edulis* (Comps et al. 1980, Balouet et al. 1983; Van Banning 1987; McArdle et al. 1991; Beare et al. 1998). Shortly thereafter, these countries experienced mortalities in their *O. edulis* stocks. By 1979, it was known that a *Bonamia* parasite had infected and caused mortality in *O. edulis* stocks from France. Originally, *O. edulis* seed oysters had been transported from California to France (Comps et al. 1980; Elston et al. 1986), and it is believed that these transported oysters were infected with *Bonamia ostreae*. Similarly, it was considered possible that the *Bonamia* parasite reported in *O. edulis* oysters grown in Maine in the early 1990s might have resulted from the transport of infected *O. edulis* oysters from California (Friedman and Perkins 1994). Currently the true source of *B. ostreae* introduction into Maine is not known since *O. edulis* has been introduced many times and from locations such as the Netherlands, France, and California (Heinig and Tarbox 1985; Friedman and Perkins 1994).

Over the past two decades, molecular analyses have been increasingly applied to characterize and classify closely related organisms. Cochennec et al. (2000) write that phylogenetic relationships that are constructed solely using morphological and ultrastructural data can sometimes result in the taxonomic misplacement of a species, or the application of broad, generalized definitions of a group of organisms. In addition, there are very few homologous morphological characters that can be compared among a vast number of living organisms and used to determine phylogenetic relationships (Hillis and Dixon 1991). By contrast, there are a number of genes essential to all life forms that are present in all species, and can be analyzed to clarify phylogenetic relationships among closely related and morphologically similar organisms (Hillis and Dixon 1991). The ribosomal RNA (rRNA) gene complex has been used previously to address recent evolutionary events and formulate hypotheses about the origins of life (Mindell and Honeycutt 1990). Collectively, the rRNA genes and their spacer regions comprise the rRNA gene complex. One reason why the rRNA gene complex region is so useful in clarifying phylogenetic relationships is due to the conserved, semi-conserved, and non-conserved domains within the complex (Hillis and Dixon 1991). The functional rRNA encoding regions, the small-subunit (SSU), large-subunit (LSU), and 5.8S rRNA genes, are comparatively well conserved, while the transcribed and non-transcribed spacers are quite variable. In addition, the rRNA gene complex is a multi-copy region, further increasing the sensitivity of detection methods that target this region of the genome (Sogin and Silberman 1998). The SSU rRNA gene has been commonly used as a target for polymerase chain reaction (PCR) primers and in situ hybridization probes (McCarroll et al. 1983; Siddal et al. 1995; Flores et al. 1996; Carnegie et al. 2000b; Cavalier-Smith

and Chao 2003; Cochennec-Laureau et al. 2003; Reece and Stokes 2003; Burreson et al. 2004; Reece et al. 2004; Carnegie et al. 2006).

Molecular approaches have proven to be useful for reconstructing phylogeny (Hillis 1987). McCarroll et al. (1983) suggested that rRNA gene sequences appear to be better suited than cytochrome b sequences for revealing phylogenetic relationships because they are universally distributed across all living systems, and are functionally equivalent in each cell. In addition, pairwise comparisons of the SSU rRNA gene result in more reliable phylogenies because of the gene's large size (1500-2000 bp), and because it contains numerous conserved functional domains (McCarrol et al. 1983). The SSU rRNA gene has been used numerous times before to assess phylogenetic relationships within and among protistan taxa, including molluscan parasites (McCarroll et al. 1983; Siddal et al. 1995; Flores et al. 1996; Carnegie et al. 2000a,b; Cochennec et al. 2000; Cavalier-Smith and Chao 2003; Reece and Stokes 2003; Cochennec-Laureau et al. 2003; Burreson et al. 2004; Reece et al. 2004; Carnegie et al. 2006; Corbeil et al. 2006a,b).

In *Bonamia* species, the SSU rRNA gene consists of a few domains that are highly conserved amongst *Bonamia* species, but are distinct from the other haplosporidians. These highly conserved, yet distinctively unique, regions within the genus *Bonamia* have been used for design of genus-specific PCR primers as diagnostic tools for detection of *Bonamia* parasites in oyster tissue (Carnegie et al. 2000a; Cochennec et al. 2000). In the past decade, PCR amplification of the SSU rRNA gene became a commonly used method for rapid detection of *Bonamia* sp. DNA in oyster tissue, and SSU rDNA sequences were used to establish phylogenetic relationships

between the genus *Bonamia* and other closely related haplosporidians (Carnegie et al.

2000; Cochennec-Laureau et al. 2003). The SSU rRNA gene does have limitations,

however. Because of its highly conserved nature, the SSU rRNA gene is less useful at

evaluating phylogenetic relationships of taxa that diverged more recently. Pelandakis et

al. (2000) determined that analysis of the SSU rRNA gene fragment could not

differentiate two species of aquatic parasites in the genus *Naegleria*, indicating that

variability in this gene region was insufficient to resolve relationships among these

parasites. In contrast, the spacer regions, (internal transcribed spacer (ITS), non-

transcribed spacer (NTS), and external transcribed spacer (ETS)), have often been used to

infer phylogeny among more closely related taxa due to the relatively rapidly evolving

nature of these regions of the complex (Hillis and Davis 1986; Mindell and Honeycutt

1990; Hillis and Dixon 1991). These non-coding regions are not under the same

evolutionary pressures as the genes that encode functional RNAs, and therefore are less

conserved than the rRNA coding genes and thus are more suitable for phylogentic

characterization of closely related organisms than the conserved regions of the rRNA

gene complex (Hillis and Dixon 1991; De Jonckheere 1998; Pelandakis et al. 2000;

Lange and Medlin 2002). Goggin (1994) used sequence data from the more variable ITS

region of the rRNA gene complex, which includes ITS-1, ITS-2 and the 5.8S rRNA gene,

to identify molluscan parasites in the genus *Perkinsus* originating from a variety of hosts

and from various geographical locations. The non-coding ITS-1 and ITS-2 regions flank

the relatively small 5.8S rRNA gene, which in *Bonamia* spp is roughly 150bp in length.

Objectives

The objective of this research was to construct a molecular phylogeny of the genus *Bonamia* using the ITS-1/5.8S/ITS-2 region of the rRNA gene complex as well as the SSU rRNA gene. This project tests the following hypotheses: 1) the *Bonamia* sp. discovered in *Crassostrea ariakensis* oysters deployed for research studies in North Carolina is closely related to other *Bonamia* species found in the northern hemisphere 2) the Chilean and Argentinean *Bonamia* spp. are conspecific, and 3) the species of *Bonamia* infecting *O. angasi* oysters in Australia is a novel *Bonamia* sp.

LITERATURE REVIEW

Parasitic Diseases of oysters

Oyster farming has stimulated increased research on development of methods to rapidly identify and classify the types of parasites that infect oysters. Management of these parasites has important practical implications including management of mortality from parasitic diseases, and increasing the quality and appearance of a half-shell oyster (Elston 1997). Most serious oyster parasites are protists, which are generally unicellular, motile organisms measuring 1.0 μm to 5 μm in size, although some protistan taxa are known to exhibit complex multicellular forms (Sogin and Silberman 1998). Protists reproduce sexually or asexually, inhabit a large variety of environments, and are either autotrophs or heterotrophs (Sogin and Silberman 1998). The degree of variation among the protists is greater than that in any other eukaryotic kingdom. As such, no single trait is both universal and exclusive to all protists. Classifying these organisms solely on morphological characteristics is problematic (Sogin and Silberman 1998). The following genera contain protists that are molluscan parasites: *Haplosporidium, Urosporidium, Minchinia,* and *Bonamia* of the phylum Haplosporidia; *Mikrocytos, Perkinsus, Marteilia, Marteilioides, Nematopsis, Sphenophrya, and Stegotricha.* The phylum Haplosporidia will be the focus of this next section as it contains the genus *Bonamia*.

Phylum Haplosporidia

The phylum Haplosporidia represents a group of endoparasites that predominantly infect marine invertebrates (Perkins 2000). An exception is *Haplosporidium pickfordi,* a freshwater species that inhabits North American lakes and parasitizes snails (Burreson

2001). Both freshwater and marine species of haplosporidians are found all across the globe in areas including Portugal, France, Australia, New Zealand, Japan, and the east and west coasts of the United States (Burreson and Ford 2004). The phylum Haplosporidia contains four genera: *Urosporidium*, *Minchinia*, *Haplosporidium*, and *Bonamia* (Burreson and Ford 2004). The phylum includes 36 recognized species, although many of these have not been identified or reported since their first description and some may not be valid species (Burreson and Ford 2004). Additionally, there are probably numerous undescribed species (Burreson and Ford 2004). Currently, haplosporidians are classified as a phylum, although the relationship of this group to other protists is not well understood (Reece et al. 2004).

Morphologically, members of the phylum Haplosporidia display mitochondria and haplosporosomes in cell forms that include multinucleate plasmodia, various sporogonic stages, and an ovoid spore. The spore stage has a thick wall and an anterior orifice protected by a hinged lid or a flap of cell wall material, but lacks polar filaments and capsules (Flores et al. 1996; Perkins 2000; Burreson and Ford 2004). It is generally accepted that spore ornamentation, tubular and filamentous projections on the external spore surface, is the best morphological characteristic for distinguishing species within the phylum (Burreson and Ford 2004). Perkins (1987) considered *Bonamia* to be a haplosporidian because it contains haplosporosomes, even though a spore stage had not been observed at the time. Recently, molecular data supported placement of the genus *Bonamia* in the phylum Haplosporidia (Carnegie et al. 2000b; Reece and Stokes 2003; Reece et al. 2004), and discovery of a spore stage with an orifice and lid in *B. perspora* (Carnegie et al. 2006) further supported this placement.

Background on *Bonamia* spp.

Members of the genus *Bonamia* are protistan parasites that infect a wide range of oysters including *O. edulis* (Pichot et al. 1980; Friedman et al. 1989; McArdle et al. 1991), *O. chilensis* (=*Tiostrea lutaria;* O' Foighil et al. 1999) (Dinamani et al. 1987; Hine et al. 2001), *O. angasi* (Bougrier et al. 1986) *C. ariakensis* (= *C. rivularis*) (Cochennec et al. 1998; Burreson et al. 2004), *Saccostrea glomerata* (= *S. commercialis*) (Farley et al. 1988; Cochennec-Laureau et al. 2003), *O. puelchana* (Kroeck and Montes 2005), and *Ostreola equestris* (Carnegie et al. 2006). *Bonamia* species are generally characterized as uninucleate, intrahemocytic "microcells" (Katkansky et al. 1969) that are less than 5µm in diameter. Ultrastructurally unlike the superficially similar oyster parasite *Mikrocytos mackini* (Hine et al. 2001) *Bonamia* spp. posses both haplosporosomes and mitochondria (Pichot et al. 1980; Perkins 1987). Besides uninucleate forms, a binucleate stage, a multinucleate plasmodial stage, and in the case of *B. perspora* (Carnegie et al. 2006), a spore stage may also occur at a frequency depending on the *Bonamia* species (Dinamani et al. 1987; Bucke 1988; Hine 1991b, Hine et al. 2001; Carnegie and Cochennec-Laureau 2004; Carnegie et al. 2006). An intermediate host is likely necessary for those haplosporidian species, including *B. perspora*, that produce spores during their life cycle (Haskin and Andrews 1988; Powell et al. 1999). Most *Bonamia* species, however, are thought to be transmitted directly from one host to the next (Carnegie and Cochennec-Laureau 2004). *Bonamia perspora* is also unique among *Bonamia* spp. in infecting the connective tissues of its host *O. equestris* (Carnegie et al. 2006). *Bonamia perspora* can also be found parasitizing gut epithelial cells and hemolymph sinuses (Carnegie et al. 2006). Montes et al. (1994) reported a single record

in which *B. ostreae* was found in the epithelial cells of gill tissue in an unspecified number of oysters with heavy infections. Although the significance of this finding is uncertain, the gill epithelium may serve as a portal of entry and exit into and out of the host (Carnegie and Cochennec-Laureau 2004).

Bonamia species are simple morphologically, and thus are extremely difficult to distinguish taxonomically using light microscopy (Carnegie and Cochennec-Laureau 2004). In addition, their small size makes them difficult to detect using standard histopathology and histocytology techniques (Carnegie and Cochennec-Laureau 2004). Previously, diagnosis of a *Bonamia* infection in a host consisted of preparing routine histological sections or staining smears of gill and heart tissue (Boulo et al. 1989). These methods, however, are not very reliable in detecting light infections (Culloty et al. 2003). Molecular diagnostic techniques such as PCR, real-time PCR, restriction fragment length polymorphism (RFLP) analysis, and *in situ* hybridization (ISH), have been used to detect *Bonamia* spp. nucleic acids in oyster tissues (Adlard and Lester 1995; Carnegie et al. 1999; Carnegie et al. 2000a,b; Carnegie et al. 2001; Hine et al. 2001; Carnegie et al. 2003; Diggles 2003; Carnegie et al. 2006; Corbeil et al. 2006a,b).

The infection intensity, prevalence and seasonality of each *Bonamia* species vary, and are dependent on certain environmental and host conditions. *Bonamia ostreae* has its highest intensity and prevalence during the warmer months, but is infective year-round (Balouet et al. 1983; Carnegie and Cochennec-Laureau 2004). *Bonamia roughleyi* is present only for a short

period of time during the late austral winter from August to September (Adlard and Lester 1995). *Bonamia exitiosa* has its highest prevalence and intensity in April (Hine

1991a). Within a population, the prevalence of each of the *Bonamia* parasites, however, is usually high and can range anywhere between 40% and 100% (Burreson et al. 2004; Cranfield et al. 2005). An exception is *B. perspora*, which has a prevalence of 1-2% in *O. equestris* (Carnegie et al. 2006).

Sequence data currently available for *Bonamia* species include complete and partial sequences for the SSU rRNA gene for *B. roughleyi*, *B. perspora*, *B. ostreae*, and *B. exitiosa*. Partial ITS data for *B. ostreae*, the *O. chilensis Bonamia* sp. in Chile, and the *O. angasi Bonamia* sp. in Australia are also available in GenBank.

Bonamia ostreae: History, Host Affiliation, and Geographic Distribution

Bonamia ostreae was the first identified and described species of *Bonamia* and is known to infect populations of the flat oyster, *O. edulis* found in California (Friedman et al. 1989), Washington (Elston 1986), Maine (Friedman and Perkins 1994; Zabaleta and Barber 1996), British Columbia (Marty et al. 2006), and Europe including Ireland, Spain, France, and the Netherlands (Comps et al. 1980; Pichot et al. 1980; Van Banning 1987; Bucke 1988; McArdle et al. 1991). *Bonamia ostreae* is believed to infect the following hosts experimentally: *O. chilensis* (Bucke and Hepper 1987; Grizel et al. 1983), *O. denselamellosa* (Le Borgne and Le Pennec 1983), *O. angasi* (Bougrier et al. 1986), *O. puelchana* (Pascual et al. 1991), and *C. ariakensis* (= *C. rivularis*) (Cochennec et al. 1998).

In the late 1960s, a parasite described as a "microcell" was observed infecting stocks of *O. edulis* transported from Milford, Connecticut to Drakes Estero, Morro Bay, and Elkhorn Slough in California, USA (Katkansky et al. 1969). Seed stocks of *O. edulis*

grown in California were transported to France. Almost a decade later, mortality of European flat oyster, *O. edulis*, led researchers in Brittany, France to conclude that the parasite responsible for the disease outbreak was a microcell similar to the one described by Katkansky et al. (1969), and was given the name *B. ostreae* (Pichot et al. in 1980). It is currently accepted that the microcell found in *O. edulis* stocks transported from California was *B. ostreae,* and the transport of *O. edulis* stocks from California to France led to the introduction of this parasite into France (Elston et al. 1986). Limited documentation of *O. edulis* transportations in North America reveal that stocks of *O. edulis* have been transported between the following states and countries: from California to Washington, from France to Maine, from Maine to California, from California to Maine, and from the Netherlands to Maine by way of Milford, Connecticut and from Washington to British Columbia, Canada (Heinig and Tarbox 1985; Elston et al. 1986; Friedman and Perkins 1994; Marty et al. 2006). Incomplete data as it relates to infection of *O. edulis* by *B. ostreae* before each importation has made it difficult to determine the true origin of *B. ostreae.*

Bonamia ostreae became established in the Netherlands in 1980 when it was introduced to Dutch farms growing *O. edulis* in the Yerseke Bank (Van Banning 1987). Shortly after the introduction of the parasite, *O. edulis* stocks plummeted in that area. *B. ostreae* spread quickly to other oysters and persisted throughout the year and among low densities of oysters (Van Banning 1987). In Ireland, *B. ostreae* was present among *O. edulis* oyster stocks as early as 1986, when oysters from Cork Harbour were found to be infected (McArdle et al. 1991). The parasite quickly spread to other growing areas and

was observed in Clew Bay oyster stocks in 1988 and Galway Bay oyster stocks the following year (McArdle et al. 1991).

Bonamia exitiosa: History, Host Affiliation, and Geographic Distribution

Between 1985 and 1993, massive mortality events occurred among the dredge oyster, *O. chilensis,* in Foveaux Strait, New Zealand (Hine et al. 2001). During the New Zealand autumn of 1986, *Bonamia* sp. infection was discovered in *O. chilensis* after oystermen reported dead, gaping, and moribund oysters, and mortality of 63% (Dinamani et al. 1987). In 1990, populations of the dredge oyster were reduced by 67%, and by 1992, stocks had decreased to 91% of the oysters present in 1975 (Doonan et al. 1994). In 1993, the fishery closed for a period of time, which had a tremendous impact on the economy of the local communities (Doonan et al. 1994). *Bonamia exitiosa* is now known to be the cause of the mortality events. This parasite has been present in Foveaux Strait since 1964 (Hine 1991a,b).

Bonamia roughleyi: History, Host Affiliation, and Geographic Distribution

Bonamia roughleyi infects the Sydney rock oyster, *Saccostrea glomerata,* and has been observed in the Georges River and other areas in New South Wales, Australia (Adlard and Lester 1995). Previously placed in the genus *Mikrocytos* (Farley et al. 1988), molecular data provided support for placement of this parasite in the genus *Bonamia* (Cochennec-Laureau et al. 2003). *Bonamia roughleyi* is thought to be associated with a disease syndrome in its host known as "winter mortality" (Farley et al. 1988; Cochennec-Laureau et al. 2003; Diggles 2005). Since the 1920s, mortality of the

three-year age class in *S. glomerata* oysters has been observed during the austral winter months (Roughley 1926). It was not until the discovery of *B. roughleyi* in the late 1980s that an agent of "winter mortality" was presumed to have been found (Farley et al. 1988; Adlard and Lester 1995). Farley et al. (1988) noted that the parasite thrives in salinities ranging from 30 to 35 ppt and is unknown in salinities below this range. High intensities of the parasite occur over narrow seasonal time intervals with an incubation period of about 2 months (Adlard and Lester 1995). Evidence also suggests that oyster mortality is strongly connected to environmental factors such as an increase in salinity and a decrease in temperature (Adlard and Lester 1995). Mortality has not been observed in animals less than 3 years of age (Farley et al. 1988). *Bonamia roughleyi* and its disease signs (focal lesions) are most often observed between August and September (Roughley 1926; Adlard and Lester 1995). It is not clear whether the parasite has a cryptic stage in oyster tissue during the summer months, or if there are other hosts serving as reservoirs for this parasite (Adlard and Lester 1995).

Bonamia perspora: History, Host Affiliation, and Geographic Distribution

Bonamia perspora is a recently described spore-forming species, and was first discovered in the crested oyster *O. equestris* in Morehead City and Wilmington, North Carolina USA (Carnegie et al. 2006). *Ostreola equestris* are found in euryhaline to polyhaline waters from North Carolina to Argentina, but are small and commercially unimportant (Harry 1985).

Bonamia sp. in *Crassostrea ariakensis* in USA.

In 2003, juvenile *C. ariakensis* were transplanted from Gloucester Point, Virginia to Bogue Sound, North Carolina (Burreson et al. 2004). Oyster mortality was observed one month later (Burreson et al. 2004). Histological analysis of these samples indicated the presence of a large number of cells resembling *Bonamia* parasites (Burreson et al. 2004). DNA sequencing of part of the SSU rDNA gene from this parasite revealed an affinity not to *B. ostreae* but to two other *Bonamia* parasites already characterized—*B. roughleyi* and *B. exitiosa,* two species of *Bonamia* that have been described from the southern hemisphere. The question of whether the *Bonamia* sp. infecting *C. ariakensis* was one of the species described from the southern hemisphere or a third undescribed species was not resolved from this study.

Bonamia sp. in *Ostrea chilensis* in Chile

The farming of *O. chilensis* in Chile has developed considerably over the past several decades (Campalans et al. 2000). Nonetheless, very little attention has been paid to diseases affecting these molluscs, and even less information exists on the diseases that affect Chilean bivalve molluscs reared in aquaculture environments (Campalans et al. 2000). Between late fall of 1996 and early summer of 1997, Campalans et al. (2000) conducted a study on Chiloe Island in Chile. A total of 167 oysters were collected from five aquaculture facilities, and tissue samples from the mantle, digestive gland, and gills were reserved for histological analysis (Campalans et al. 2000). Two of the oysters showed significant hemocytosis, with a number of parasites, 2-3μm in size, observed in the cytoplasm of these hemocytes (Campalans et al. 2000). It was not possible to ascribe

this organism to a particular genus although the authors stated that the organism

resembled a *Bonamia* sp.

Bonamia sp. in *Ostrea puelchana* in Argentina

The practice of farming *O. puelchana* is fairly new in Argentina and began in the

1980s. This oyster is found geographically from southern Brazil to northern Patagonia,

Argentina. The commercial cultivation of *O. puelchana* oysters began in Banco Garzas

of San Antonio Bay, Argentina, a protected site within the bay, between March 1995 and

December 1997 (Kroeck and Montes 2005). The goal was to export these oysters to

Europe. In 1997, a 33% mortality of *O. puelchana* was reported (Kroeck and Montes

2005). About 34 months from the initial time of culture, 95% of *O. puelchana* oysters

had died. Histologic evaluation of moribund oysters indicated the presence of a

Bonamia-like parasite associated with hemocytosis of the mantle, gill, and digestive

gland although oysters generally showed no clinical signs of infection. Hemocytes

contained between 1 and 8 parasites measuring 2-3 µm in diameter (Kroeck and Montes

2005). The characteristics of this parasite were similar to those of the parasite infecting

O. chilensis in Chile and New Zealand, and *O. angasi* of Australia. Kroeck and Montes

(2005) suggested that this parasite is a *Bonamia* sp., and should be treated as being

different from *B. ostreae*, *B. exitiosa*, and the Australian *Bonamia* sp. until more studies

can verify its taxonomic placement (Kroeck and Montes 2005).

Bonamia sp. in *Ostrea angasi* in Australia

Organisms morphologically resembling *Bonamia* species have been associated with mortalities of *O. angasi* oysters from Port Philip Bay, Victoria (1991), Georges Bay, Tasmania (1992), and Albany, Western Australia (1993) (Hine and Jones 1994). Sequencing of the SSU rRNA gene from the *Bonamia*-like organism from Victoria and *B. exitiosa* from New Zealand suggest that the parasites from these two locations are identical (Hine and Jones 1994). Corbeil et al. (2006a) conducted a study using infected *O. angasi* collected from New South Wales (NSW), Australia to identify and characterize this Australian *Bonamia* isolate using molecular techniques that would presumably establish the relationship of this isolate to other described *Bonamia* species. The SSU rRNA gene and ITS-1 region of the rRNA gene complex were used in the analysis (Corbeil et al. 2006a). Comparison of three NSW *Bonamia*-like parasites with *B. exitiosa* and *B. ostreae* revealed a greater degree of nucleotide similarity between the NSW *Bonamia*-like parasites and *B. exitiosa* than was observed between the NSW *Bonamia*-like parasites and *B. ostreae* (Corbeil et al. 2006a).

It is possible that this parasite originated in New Zealand, but spread to Australia during the transport of commercial New Zealand dredge oysters to Victoria and Tasmania, Australia during attempts to replenish *O. angasi* stocks that had suffered from high mortality during the late 1800s (Diggles 2005: Corbeil et al. 2006a). There are no known movements of New Zealand oysters to Western Australia, however, which is another location wherein a *Bonamia* sp. has been described in *O. angasi* (Diggles 2005). This information may indicate that the *Bonamia*-like parasite infecting *O. angasi* is

endemic to Australia, and might also explain the mortality of *O. angasi* in the late 1800s

before the introduction of New Zealand oysters into Australian waters (Diggles 2005).

MATERIALS AND METHODS

Sample Collection

The following oyster species collected from the indicated locations (Figure 1), were used in this study: *C. ariakensis* (deployed to and collected from upweller systems located on Bogue Sound and a field site in Wilmington, North Carolina, USA), *O. puelchana* (San Antonio Bay, Argentina), *O. chilensis* (Chiloe Island, Chile and Foveaux Strait, New Zealand), *O. edulis* (Maine, USA), *O. equestris* (Bogue Sound, North Carolina, USA) *O. angasi* (Pambula River, NSW, Australia), and *S. glomerata* (Georges River and Port Stephens, NSW, Australia). All of the samples, except *O. angasi* and *S. glomerata*, were collected prior to 2006. Oyster sample sizes are listed in Table 1. In November 2006, 176 wild and hatchery-reared *S. glomerata,* 2-3 years of age, were collected from the Pambula River, NSW, Australia. Also collected at the time were 42 *O. angasi*. The *S. glomerata* collected from Pambula River, NSW, Australia displayed some gross lesions, characteristic of "winter mortality", although mortality was not observed during the time these oysters were sampled. A total of 218 *S. glomerata* and *O. angasi* were processed for DNA extraction. In August 2007, 200 *S. glomerata* were collected from Georges River, NSW, Australia, and 232 *S. glomerata* were collected from Port Stephens, NSW, Australia. These areas are "winter mortality"-enzootic, and sampling was conducted at the time of year when disease would typically peak. A total of 280 oysters—200 from Georges River and 80 from Port Stephens—were processed for DNA extraction.

Oysters were shucked, and roughly 25 mg of gill tissue was collected from each oyster and placed in a 1.5 mL microcentrifuge tube containing 1000 µl of 95% ethanol.

The remaining tissue was fixed in Davidson's (samples from 2006 and earlier) or

Carson's fixative (2007 samples) for histology. Scalpels and forceps were sterilized with

95% ethanol and flamed in between each sample to minimize the potential for cross

contamination of DNA.

Isolation of DNA and Estimation of Concentration

To lyse the tissue, ethanol was pipetted from each tube and tissues were allowed

to dry in their respective tubes with lids open for two hours. For tissue roughly 25mg in

size, about 180 µl of lysis buffer and 20 µl of proteinase K was added to each sample.

For larger tissue, an additional 10 or 20 µl of proteinase K was added after tissue had

been allowed to lyse for several hours. All samples were incubated overnight in a 56°C

water bath. Total genomic DNA was extracted the following day using the QIAamp

DNA Mini Kit tissue protocol (QIAGEN, Valencia, CA). DNA was re-dissolved in 100-

150 µl of elution buffer and held at either 4°C for short-term storage, or -20°C for long-

term storage. AGeneQuant *pro* spectrophotometer (Amersham Biosciences, Piscataway,

NJ) was used to determine the concentration of DNA in each sample.

Generic PCR Assay for Bonamia sp.

To verify the presence of *Bonamia* spp., two PCR protocols that target different

portions of the SSU rRNA gene were used. The first protocol from Carnegie et al. (2000)

and modified as described below results in a product size of 760 bp, and was used on the

following oysters: *C. ariakensis, O. puelchana, O. chilensis, O. equestris,* and *O. edulis.*

The 25-µl reaction contained 200-250 ng template DNA, 10X PCR buffer, dH_2O, 10

mg/ml BSA, 200 mM dNTPs, 100 pm/µl of primers C_F and C_R (Table 2, Figure 2), and 5

U/µl of Ampli*Taq* polymerase (Applied Biosystems, Foster City, CA). PCR reaction

conditions consisted of an initial denaturation at 94°C for 4 minutes followed by 35

cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 1 minute, and

extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes.

The second protocol (VIMS unpublished) also targets the DNA of *Bonamia* spp.,

but yields a 206 bp product and is more sensitive than the Carnegie et al. (2000) protocol

(unpublished data). This PCR was performed on *O. angasi* and *S. glomerata* samples.

The 25-µl reaction volume consisted of 10X PCR buffer, dH$_2$0, 10 mg/ml BSA, 200 mM

dNTPs, 100 pm/µl of primers BON319F+ BON524R (Table 2, Figure 2), 5 U/µl of

Ampli*Taq* polymerase (Applied Biosystems), and 200-250 ng template DNA. PCR

reaction conditions consisted of an initial denaturation at 94°C for 4 minutes followed by

35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and

extension at 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes.

To visualize the PCR products, samples were loaded and electrophoresed on a 2%

agarose gel at 100V for roughly 30 minutes, stained using ethidium bromide, and viewed

under UV light exposure.

Bonamia SSU rDNA PCR

Amplifying the complete SSU rRNA gene required that the 5' end of the

sequence be amplified separately from the 3' end of the sequence, and resulted in an

overlap of about one hundred base pairs. The following primers were used to amplify the

5' end of the SSU rRNA gene and the 3' end of the SSU rRNA gene for all of the

Bonamia parasites except for the *O. angasi Bonamia* and the *S. glomerata Bonamia*: 16S-A forward primer and BON-1110r reverse primer and BON-925f forward primer and 16S-B reverse primer respectively (Table 2, Figure 2). The product of the first primer set yields an1110bp amplicon, whereas the product of the second primer set yields an 850bp amplicon. Initially primers 16S-A, BON-1110r, BON-925f, and 16S-B (Table 2, Figure 2) were used to attempt to amplify the complete SSU rRNA gene of the *Bonamia* sp. infecting *O. angasi*; however, only primers BON-925f and 16S-B resulted in amplification of the correct gene product. A number of other primer pairs were attempted before primers BON-319f and BON-990r were used to amplify the 5' end of the *O. angasi Bonamia* SSU rRNA gene (Table 2, Figure 2). The use of primer BON-319f resulted in a loss of the first 319bp of the SSU rRNA gene sequence for the *O. angasi Bonamia* sp.

The 25-μl reaction volumes for each PCR mixture consisted of 10X PCR buffer, dH$_2$0, 10 mg/ml BSA, 200 mM dNTPs, 100 pmol/μl of each primer, 5 U/μl of AmpliTaq polymerase, and 200 ng of template DNA. PCR reaction conditions were the same for each assay and consisted of the following: an initial denaturation at 94°C for 4 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 1.5 minutes, and a final extension at 72°C for 5 minutes. PCR reaction conditions differed only for the *O. angasi Bonamia* sp. such that an annealing temperature gradient of 53-59°C was used to amplify the 5' end of the SSU rRNA gene. PCR products were visualized as previously described.

Bonamia ITS region *PCR*

To amplify the ITS-1 and ITS-2 regions and the 5.8S rRNA gene for the majority

of the *Bonamia* parasites, the forward primer haploITSf (Table 2, Figure 2), which targets

the 3' end of the haplosporidian SSU rRNA gene was paired with a reverse primer

BonITS-R3 (Table 2, Figure 2) that is specific to *Bonamia* species. The product of these

primers is roughly 760 bp. As before, the 25-µl assay consisted of 10X PCR buffer,

dH_2O, 10 mg/ml BSA, 25 mM $MgCl_2$, 200 mM dNTPs, 100 pmol/µl of primers

(haploITSf+BonITS-R3), 5 U/µl of Ampli*Taq* polymerase (Applied Biosystems), and

200-250 ng of template DNA. PCR reaction conditions were as follows: initial

denaturation at 95°C for 7 minutes followed by 35 cycles of denaturation at 95°C for 1

minute, annealing at 51°C for 1 minute, extension at 72°C for 1.5 minutes succeeded by a

final extension at 72°C for 7 minutes.

The *O. angasi Bonamia* sp. was the only *Bonamia* sp. for which two separate

primer pairs were used to amplify the ITS region of the parasite's genome. As with the

SSU gene, difficulties arose when amplification of the ITS regions of the *Bonamia* sp.

infecting *O. angasi* was attempted. After experimenting with a number of different

primer pair arrangements and PCR conditions, it was discovered that by targeting the ITS

regions 1 and 2 of the *O. angasi Bonamia* sp. separately, it was possible to amplify the

parasite DNA. To amplify the ITS-1 region, the forward primer RA2 (Adlard and Lester

1995) (Table 2, Figure 2) and the reverse primer 5.8SREV (this study) (Table 2, Figure 2)

were used, while to amplify the ITS-2 region, the forward primer 5.8SFOR (Table 2,

Figure 2) and the reverse primer ITS2.2 (Adlard and Lester 1995) (Table 2, Figure 2)

were used. Both primer sets were used in the following PCR mixture: 1.5 mM of 10X

PE Buffer, dH$_2$0, 10 mg/ml BSA, 200 mM dNTPs, 100 pmol/µl of each primer, 5 U/µl of

Ampli*Taq* polymerase (Applied Biosystems), and 200-250 ng of template DNA. PCR

reaction conditions consisted of an initial denaturation at 95°C for 7 minutes followed by

35 cycles of denaturation at 95 °C for 1 minute, annealing at 59°C for 1 minute, and

extension at 72°C for 1.5 minutes followed by a final extension at 72°C for 7 minutes.

PCR products were visualized as previously described. All PCR experiments were

performed using the MJ Research PTC-200, DNA Engine/Peltier thermal cycler

(Cambridge, MA). Unfortunately, both the reverse (5.8SREV) and forward (5.8SFOR)

primers (Table 2, Figure 2) used to amplify the ITS regions anneal to the same region in

the parasite's DNA. Since primers were excised from all sequences prior to aligning

them in MacVector, all clones contributing to the ITS sequence of the *Bonamia* sp.

infecting *O. angasi* are missing 23 nucleotide bases representative of the 5' end of the

5.8S gene. Nonetheless, it was still possible to align the *O. angasi Bonamia* sp. ITS

sequences with the other *Bonamia* spp. sequences despite the missing data.

Sequencing of SSU rDNA & the ITS-1/5.8S/ITS-2 region

　　　　Triplicate PCR amplicons of each infected sample were pooled and purified using

the QIAquick PCR purification kit protocol (QIAGEN, Valencia, CA). Pooled and

purified PCR products were ligated into the pCR4-TOPO plasmid vector using the TOPO

TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). One Shot® TOP10

competent *E. coli* cells were chemically transformed to take up the plasmid vector

(Invitrogen, Carlsbad, CA).

Transformed cell mixtures were plated onto Luria Bertani (LB) agar plates containing ampicillin for selection of colonies that had successfully taken up recombinant plasmids, and were grown overnight in an incubator at 37°C. Roughly 15-20 colonies from each *Bonamia*-infected sample were additionally screened for plasmids that contained the correct-sized insert. Screening of the plasmids was accomplished by resuspending plasmid cells in 40 µl of TE solution, adding 40 µl of phenol chlorophorm isoamyl alcohol, vortexing for 30 seconds, and then centrifuging at high speed (13,000 rpm) for 5 minutes. The centrifugation step helps to separate the bacterial and recombinant plasmid DNA from lipids, proteins, and other cell debris. Subsequently, the extracted DNA was electrophoresed on a 1.5% agarose gel to determine which of the selected clones contained the correct-sized insert. Clones containing the insert of interest were grown overnight at 37°C in 4 mL of LB liquid broth media containing ampicillin. Recombinant plasmids were later isolated from the *E. coli* DNA and purified using the QIAprep Spin Miniprep Kit Protocol following the manufacturer's instruction (QIAGEN, Valencia, CA). The concentration of plasmid DNA was determined using the DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, CA).

Recombinant plasmids were sequenced using the LI-COR or Applied Biosystems (ABI) automated sequencers. For the LI-COR protocol, plasmid preps underwent simultaneous bi-directional sequencing (SBS) using the Thermo Sequenase™ Primer Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ). A master mix containing 1.5 µl each of the infrared (IR) labeled M13 forward and M13 reverse primers, plasmid DNA, and distilled water for a total reaction volume of 15 µl was made for each plasmid clone. A volume of 2 µl of the corresponding nucleotide base

(A,T,G,C) was added individually to a strip tube containing four tubes. To each tube in a strip, 3.5 µl of the designated master mix was added. Sequencing reactions were accomplished using a thermal cycler and required the following PCR conditions: initial denaturation at 92°C for 2 minutes followed by 30 cycles of denaturation at 92°C for 30 seconds, annealing at 54°C for 30 seconds, and an extension at 70°C for 30 seconds. DNA fragments were separated via electrophoresis on a 4% acrylamide gel using the LI-COR Model 4200L automated sequencer.

Using the ABI protocol, recombinant plasmids were sequenced separately in both the forward and reverse directions using the following master mix: 0.25 µl of Big Dye® Terminator (Applied Biosystems), 0.875 µl of 5X Buffer, 0.16 µl M13 forward or M13 reverse primer, and 2.715 µl of distilled water (dH$_2$0). A volume containing 1 µl of plasmid DNA was added to 4 µl of the master mix for a total reaction volume of 5 µl. Sequencing reaction conditions were as follows: initial denaturation at 96°C for 1 minute followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and an extension at 60°C for 4 minutes. Each product from the sequencing reaction was precipitated using the following reagents and amounts: 0.75 µl of 3M sodium acetate (pH 5.2), 15.63 µl of nondenatured 95% ethanol, and 3.63 µl of sterile PCR water for a total volume per reaction of 20 µl. Following a series of centrifugation spins and the addition of 37.5 µl of 70% ethanol to each of the tubes, samples were resuspended in 20 µl Hi-Di formamide (Applied Biosystems). A volume of 10 µl of each resuspended sample was added to a 96-well plate and reactions were denatured for 2 minutes. After denaturation, the 96-well plate was placed into the ABI PRISM® 3130 Genetic Analyzer and sequenced by capillaries.

Sequence Alignment/Editing

Initial PCR primers sequences (Table 2) were removed from both forward and

reverse raw sequences using the MacVector® 8.02 software package (Oxford Molecular).

Forward and reverse sequences were aligned using the AssemblyLIGN™ 1.0.9c software

package, however, final consensus sequences were generated in MacVector® 8.02.

Consensus sequences were uploaded into BLAST, which is a basic local alignment

search tool, to verify that the sequenced DNA was that of a *Bonamia* parasite. Multiple

alignments were generated for the SSU rRNA gene sequences and the ITS region

sequences using the ClustalW algorithm (European Bioinformatics Institute) in

MacVector® 8.02. The following default settings were used: pairwise alignment—open

gap penalty (10.0), extended gap penalty (5.0)—multiple alignment—open gap penalty

(10.0), extended gap penalty (5.0), delay divergent (40%), with transitions weighted.

Clones that were sequenced from the same oyster host and contained the exact nucleotide

sequence were represented as one sequence. Clones that had one or more nucleotide

differences or polymorphisms from the rest of the clones in that group were considered to

be single, distinct sequences. Each sequence generated in this study was also deposited

into GenBank and assigned an accession number (EU709021-EU709133, EU723225-

EU723231, EU780686-EU780692). The following GenBank sequences were also used

in this study: AF337563 (accession number for *B. exitiosa* type sequence), AF508801

(accession number for *B. roughleyi* type sequence), AF262995 (accession number for *B.*

ostreae type sequence), DQ356000 (accession number for *B. perspora* type sequence),

and AY542903 (accession number for *C. ariakensis Bonamia*). Pairwise distance

measurements were constructed for both the SSU rRNA gene and the ITS-1/5.8S/ITS-2 region.

Partition homogeneity test

A partitioned homogeneity test was used to test for compatibility between the ITS region and SSU rRNA gene data (Farris et al. 1995). Incongruence length differences (ILDs) were examined using a heuristic search of 1000 replicates and 100 random sequence additions to determine if there would be complications with combining the two datasets.

Phylogenetic Analysis

Phylogenetic relationships of the *Bonamia* parasites were hypothesized using independent maximum parsimony analyses of the SSU rRNA gene and the ITS-1/5.8S/ITS-2 region, and a combined analysis of the SSU rRNA gene sequence and the ITS-1/5.8S/ITS-2 region. All maximum parsimony analyses were conducted in PAUP*4.0b10 (Swofford 2002). A heuristic search algorithm was conducted, and the tree-bisection reconnection (TBR) branch swapping algorithm was used to construct topologies. Two consensus trees, one generated using the bootstrap method and the other generated using the jackknife method, were constructed for each data set. All nucleotide positions were considered unordered, transitions and transversions were equally weighted, and all characters were given the same weights. Gaps in the data set were classified as missing data. For each bootstrap analysis 1,000 replicates with 100 random step-wise additions was conducted to produce a 50% majority rule, consensus tree. For

each jackknife analysis, 10,000 replicates with 1000 random step-wise additions were completed. The percentage of characters deleted in each jackknife replicate equaled 25 percent. Two *Minchinia* sequences (Accession numbers AY449711 and U20320) were chosen as outgroups for construction of the SSU tree only. *Bonamia ostreae* sequences were chosen as the outgroup for construction of the ITS region and the combined SSU/ITS region trees since there are currently no ITS region sequences available for *Minchinia* species; although, either *B. ostreae* or *B. perspora* sequences could have been used as the outgroup. Uncorrected ("p") distances (Tables 3 and 4) were also calculated in PAUP*4.0b10 for both the SSU rRNA gene and ITS region sequences using the pairwise distance option under the data analyses tab.

Table 1. Summary of collection dates, sampling locations, sampling sizes and prevalence of *Bonamia* parasites based on PCR for all samples used for this study.

SSU Data

Species Name	Location	Sampling Date	Sample Size (n)	*Bonamia* prevalence (PCR)
Ostrea puelchana	San Antonio Bay	March 22, 2005 and April 14, 2005	57,59	(6) 10.5% and (1) 1.7% respectively
Ostrea chilensis	Foveaux Strait, New Zealand	NA	1	(1) 100%
Ostrea edulis	Maine, USA	July 2003	1	(1) 100%
Ostreola equestris	Bogue Sound, North Carolina, USA	Summer 2004	96	(1) 1.0%
Crassostrea ariakensis	Bogue Sound (CCC upwellers), North Carolina, USA	November 25, 2003	30	(18) 60%
Ostrea chilensis	Chiloe Island, Chile	October 2003	32	(6) 18.8%
Ostrea angasi	NSW, Australia	November 2006	42	(1) 2.4%
Saccostrea glomerata	Pambula River, NSW, Australia	November 2006	176	*Bonamia roughleyi* not detected (0%)
Saccostrea glomerata	Port Stephens, NSW, Australia	August 2007	232	*Bonamia roughleyi* not detected (0%)
Saccostrea glomerata	Georges River, NSW, Australia	August 2007	200	*Bonamia roughleyi* not detected (0%)

ITS Data

Species Name	Location	Sampling Date	Sample Size (n)	*Bonamia* prevalence (PCR)
Ostrea puelchana	San Antonio Bay	March 22, 2005 and April 14, 2005	57,59	(6) 10.5% and (1) 1.7% respectively
Ostrea chilensis	Foveaux Strait, New Zealand	NA	1	(1) 100%
Ostrea edulis	Maine, USA	July 2003	1	(1) 100%
Ostreola equestris	Bogue Sound, North Carolina, USA	Summer 2004	96	(1) 1.0%
Crassostrea ariakensis	Bogue Sound, North Carolina, USA	July 12, 2005, Sept. 20, 2005, Nov. 29, 2005	25,16,25	(14) 56%, (10) 62.5%, and (8) 32% respectively
Crassostrea ariakensis	Wilmington, North Carolina, USA	November 1, 9, and 22, 2005	23,25,20	(19) 82.5%, (8) 32%, and (10) 50% respectively
Ostrea chilensis	Chiloe Island, Chile	October 2003	32	(6) 18.8%
Ostrea angasi	NSW, Australia	November 2006	42	(1) 2.4%
Saccostrea glomerata	Pambula River, NSW, Australia	November 2006	176	*Bonamia roughleyi* not detected (0%)
Saccostrea glomerata	Port Stephens, NSW, Australia	August 2007	232	*Bonamia roughleyi* not detected (0%)
Saccostrea glomerata	Georges River, NSW, Australia	August 2007	200	*Bonamia roughleyi* not detected (0%)

CCC = Carteret Community College; NA = indicates the sampling date is unknown

Table 2. List of primer sequences used for identifying presence of *Bonamia* DNA (C$_F$, C$_R$, BON 319, BON 524), and those used to amplify *Bonamia* ITS-1,2 regions and the 5.8S gene (haploITS-f, Bon-ITS-R3, Bon-ITS-F2, RA2-B, 5.8SREV, 5.8SFOR, and ITS2.2), along with the SSU rRNA gene (16S-A, BON-1110r, BON-319F, BON-925f, BON-990R and 16S-B).

Primer Name	Sequence 5'-3'	Assay	Reference
C$_F$	CGGGGGCATAATTCAGGAAC	*Bonamia*-generic PCR	Carnegie et al. (2000)
C$_R$	CCATCTGCTGGAGACACAG	*Bonamia*-generic PCR	Carnegie et al. (2000)
BON 319F	TTTGACGGGTAACGGGGAATGCG	*Bonamia*-generic PCR	VIMS unpublished
BON 524R	CTTGCCCTCCGCTGGAATTC	*Bonamia*-generic PCR	VIMS unpublished
haplo-ITSf	GGGATAGATGATTGCAATTRTTC	Haplosporidian-generic PCR	VIMS unpublished
Bon-ITS-F2	TTGAATAATGAGGTGAATTAGG	*Bonamia*-generic PCR	VIMS unpublished
Bon-ITS-R3	CTTAAATTCAGCGGGTCGC	*Bonamia*-generic PCR	VIMS unpublished
RA2-B	GTCCCTGCCCTTTGTACACA	*Bonamia*-generic PCR	Adlard and Lester (1995)
5.8SREV	GAGCCTAGTCATCCATTGCAAAG	*Bonamia*-generic PCR	Corbeil et al. (2006)
16S-A	AACCTGGTTGATCCTGCCAGT	Universal SSU PCR primer	Medlin et. al. 1988
BON-1110r	CCTTTAAGTTTCACTCTTGCGAG	*Bonamia*-specific PCR	VIMS unpublished
BON-925f	ATTCCGGTGAGACTAACTTAT G	*Bonamia*-specific PCR	VIMS unpublished
BON-990R	CTTAGTCGACATCGTTTATGGTT GGG	*Bonamia*-specific PCR	VIMS unpublished
16S-B	GATCCTTCCGCAGGTTCACCTAC	Generic PCR primer	Medlin et al. 1988
5.8SFOR	CTTTGCAATGGATGACTAGGCTC	*Bonamia*-specific PCR	This Study
ITS2.2	CCTGGTTAGTTTCTTTTCCTCCGC	*Bonamia*-generic PCR	Adlard and Lester (1995)

RESULTS

Parasite prevalence

A total of 1060 oysters were used in this analysis with prevalence of parasite based on PCR amplification indicated in parentheses: 164 total *C. ariakensis* (prevalence between 32.0% and 82.0%), 116 *O. puelchana* (two samples with 1.7% and 10.5%), 32 *O. chilensis* from Chile (18.2%), 1 *O. chilensis* from New Zealand (100%), 96 *O. equestris* (1.0%), 1 *O. edulis* (100%), 42 *O. angasi* (2.0%), and 608 *S. glomerata* (0.0%) (Table 1). *Bonamia* DNA was detected in some but not all animals in each sample using either one or both of the *Bonamia* genus PCR protocols. *Bonamia* sp. parasite sequences from 22 of the sampled oysters—7 *C. ariakensis*, 3 *O. puelchana*, 7 *O. chilensis*, 3 *O. equestris*, 1 *O. edulis*, and 1 *O. angasi*—were used in the phylogenetic study.

B. roughleyi PCR and Sequencing Results

Despite the appearance of gross physical signs such as green pustules on the mantle, erosion of the gills, abductor muscle lesions, and tissue emaciation among several *S. glomerata* collected in 2006, PCR assays were unable to identify the presence of *B. roughleyi* DNA. Likewise in 2007, PCR amplification of oysters collected during peak winter mortality season did not reveal the presence of *B. roughleyi* DNA.

Remaining Bonamia sp. PCR and Sequencing Results

Complete SSU rRNA gene sequences were generated prior to this study for the parasites infecting *O. edulis*, *O. equestris*, *O. chilensis*, *O. puelchana*, and *C. ariakensis*. PCR products and sequencing results of the above mentioned parasites indicated the

amplified DNA closely resembled DNA from *Bonamia* parasites. Additionally, SSU rDNA PCR and sequencing results for the *Bonamia* sp. infecting *O. angasi* indicated that its DNA closely resembled DNA from a *Bonamia* parasite. Three of the parasites, *B. ostreae* in *O. edulis* from ME, USA, *B. perspora* in *O. equestris* from NC, USA, and the *Bonamia* sp. in *C. ariakensis* from NC, USA had their sequences submitted to GenBank by individuals associated with this project prior to this study, and are represented by the following accession numbers: AF262995, DQ356000, and AY542903 respectively. Type sequences for *B. roughleyi* and *B. exitiosa* (accession numbers AF508801 and AF337563 respectively) were generated previously by individuals not associated with Virginia Institute of Marine Science and were submitted previously to GenBank. The remaining parasites had their SSU rDNA sequences submitted to GenBank during the time of this study and contain the following accession numbers: EU709021-EU709023, and EU709133).

Strong PCR products were obtained from all of the *Bonamia*-infected oyster samples using the ITS assays described above in the methods section. Most of the sequences that were produced from the PCR clones showed little to no ambiguity in their nucleotide bases for both the forward and reverse nucleotide strands. All of the samples that were used in this study contained 7-10 clones that could be used for further analyses; between 9 and 10 clones were originally sequenced for each sample. Internal transcribed spacer region sequences submitted to GenBank contain the following accession numbers: EU709024-EU709132, EU723225-EU723231, EU780686-EU780692).

SSU rDNA Sequence Alignment

In aligning the SSU rDNA sequences, two *Minchinia* sequences (accession numbers: AY449711 and U20320), a *B. roughleyi* sequence of 1000 bp (accession number: AF508801), and a *B. exitiosa* sequence of 1600 bp (accession number AF337563) were included. Additionally the *O. angasi Bonamia* from Australia (EU709133), the *O. chilensis Bonamia* from Chile (EU709021), the *O. chilensis Bonamia* from New Zealand (EU709023), the *O. puelchana Bonamia* from Argentina (EU709022), the *C. ariakensis Bonamia* from NC, USA (AY542903), *B. ostreae* (AF262995), and *B. perspora* (DQ356000) were also included in the SSU multiple alignment. Few adjustments were made when the SSU rRNA gene sequences were aligned using the ClustalW algorithm in MacVector ® 8.02. The *Bonamia* sp. detected in *O. angasi* did not have the first 300 bp of the SSU rRNA gene sequenced. Therefore, alignment adjustments were made such that the *O. angasi Bonamia* sp. sequence began 300 bp from the start of the alignment. Overall there were few differences between the *Bonamia* ssp. SSU rRNA gene sequences. A total of nine *Bonamia* consensus sequences were used in the SSU phylogenetic analysis in addition to the two outgroup sequences. Of the 1769 characters, 1383 characters (78%) were constant, 196 characters (11%) were parsimony-uninformative, and 190 characters (11%) were parsimony-informative.

ITS Sequence Alignment

The ITS sequence alignment did, however, require some adjusting by eye. When the ITS sequences were first aligned using the default parameters in MacVector® 8.02, there was little consistency in the 5' end of the sequence. The sequences were adjusted

so that homologous bases would align at the 5' end of the sequence. A distinct sequence of base pairs, which represent the beginning of *Bonamia* ITS-1 (GATCATTA), were identified and used as an anchor to align the sequences in this region. Since two of the ITS primers used for this study annealed to the 5' end of the 5.8S gene, this was used as an anchor point to determine the starting sequence for the 5.8S gene. Finally, the alignment was adjusted such that all of the sequences came into register near the LSU rRNA gene or the 3'end of the alignment. Some adjustments were made to the ITS-1 and ITS-2 to include gap insertions and/or a gap extensions. This was done so that the sequences would align at the SSU, 5.8S, and LSU rRNA gene locations. The Chilean *Bonamia* sp. sequences varied distinctly from the *C. ariakensis Bonamia* sp., the *O. puelchana Bonamia* sp., the *O. angasi Bonamia* sp., and the *O. chilensis Bonamia* sequence from New Zealand. In addition, the Chilean *Bonamia* sp. sequences did not match the *B. perspora* or the *B. ostreae* sequences. The *C. ariakensis Bonamia* sp., the *O. puelchana Bonamia* sp., the *O. angasi Bonamia* sp., and the *O. chilensis Bonamia* sp. from New Zealand, contained sequences that were highly similar to one another. A total of 116 distinct nucleotide sequences representing 565 characters of the ITS-1/5.8S/ITS-2 region were used for the PAUP analysis. Of the 565 characters, 248 characters (44%) were constant, 32 variable characters (6%) were parsimony-uninformative, and 285 characters (50%) were parsimony-informative.

Combined SSU and ITS Sequence Alignment

The combined ITS region and SSU rRNA gene data sets resulted in an alignment with 2337 positions (characters). A total of 1941 characters (83%) were constant, 30

variable characters (1%) were parsimony-uninformative, and 366 characters (16%) were parsimony-informative.

SSU rDNA Phylogenetic and Distance Analyses

Phylogenetic analyses of the SSU rRNA gene, with *Minchinia* sequences indicated as outgroups, produced two main ingroup clades (Figure 3). The first clade connsisted of *B. perspora* and *B. ostreae* sequences. This clade had a relatively low bootstrap support of 71% and moderate jackknife support of 86%. The second clade consisted of sequences from the *O. chilensis Bonamia* sp. from Chile, the *C. ariakensis Bonamia* sp., the *O. angasi Bonamia* sp., the *O. puelchana Bonamia* sp., the *O. chilensis Bonamia* sp. from New Zealand, *B. exitiosa* type sequence, and *B. roughleyi* type sequence with bootstrap and jackknife support of 100%. Embedded within this larger clade was a subclade consisting of the *B. roughleyi* and the *B. exitiosa* type sequences with a low bootstrap and jackknife support values of 60% and 71% respectively. Support for the entire *Bonamia* clade was 100% for both bootstrap and jackknife analyses (Figure 3).

Uncorrected ("p") distances of 670 characters for the SSU *Bonamia* spp. sequences showed no divergence (p = 0.000) among the *B. exitiosa*-like group, which consisted of the Argentinean *Bonamia* sp., the *C. ariakensis Bonamia* sp., the *O. angasi Bonamia* sp., and the *O. chilensis Bonamia* sp. from New Zealand (Table 3). Between the Chilean *Bonamia* sp. sequence and the *B. exitiosa*-like group sequences, a p-value of 0.00156 was measured (Table 3). Between the Chilean *Bonamia* sp. sequence and the *B. exitiosa* type sequence, the p-value was 0.00465. The distance between *B. perspora* and

B. ostreae was 0.0261 (Table 3). The distance between *B. roughleyi* and the *B. exitiosa-*like group was 0.00772 (Table 3). Greater distances were observed, however between *B. roughleyi* and *B. perspora* (0.0437) and between *B. roughleyi* and *B. ostreae* (0.0422) (Table 3). The genetic distance between *B. roughleyi* and the Chilean *Bonamia* sp. was 0.00931 (Table 3). Divergence between *B. ostreae* and the Chilean *Bonamia* sp. was 0.0423 whereas divergence between the Chilean *Bonamia* sp. and *B. perspora* equaled 0.0377 (Table 3). Distance values between *B. perspora* and the *B. exitiosa-like* group was 0.0359 and between *B. ostreae* and the *B. exitiosa-*like group a distance of 0.0406 was recorded (Table 3). Distance values between the *B. exitiosa* type sequence and the *B. exitiosa-*like group was 0.00305. Difference between the *B. exitiosa* type sequence and *B. roughleyi* type sequence was 0.00460. Divergence between *B. ostreae* and the *B. exitiosa* type sequence equaled 0.0433. Likewise the *B. perspora* sequence and the *B. exitiosa* type sequence differ by 0.0447.

ITS Phylogenetic and Distance Analyses

Phylogenetic analyses of the ITS region data resolved the *Bonamia* sequences into four main clades with a few sub clades in each group (Figure 4). *Bonamia ostreae* sequences were selected to serve as the outgroup in this analysis, and they formed a clade with support of 100 for both the bootstrap and jackknife analyses. The *Bonamia perspora* sequences formed a clade of their own with bootstrap and jackknife support of 100 for the main clade. Falling sister to the *B. perspora* clade were the ITS sequences derived from the *O. chilensis Bonamia* sp. from Chile, and the *B. exitiosa-*like sequences which consisted of the *C. ariakensis Bonamia* sp., the *O. puelchana Bonamia* sp., the *O.*

angasi Bonamia sp., and the *O. chilensis Bonamia* sp. from New Zeland (Figure 4).

Within this larger clade, the Chilean *Bonamia* sp. sequences comprised their own clade,

which fell sister to the clade containing the *B. exitiosa*-like sequences listed above

(Figure 4). Support for the Chilean *Bonamia* sp. as well as the *B. exitiosa*-like sp. clades

were both 100 for the bootstrap and jackknife analyses (Figure 4).

Uncorrected ("p") distances (Table 4) were calculated in PAUP using 12

sequences chosen to represent the 116 distinct ITS sequences that were used in the

analysis, and ranged between 0.002 and 0.39 (Table 4). The greatest distance was

observed between the *B. exitiosa*-like sequences and *B. perspora*, between the *B.*

exitiosa-like sequences and *B. ostreae*, between the Chilean *Bonamia* sp. and *B. ostreae*,

and between the Chilean *Bonamia* sp. and *B. perspora* with distances ranging between

0.34 and 0.39 (Table 4). Additionally, distances between *B. perspora* and *B. ostreae*

ranged from 0.35 and 0.37 (Table 4). Distances amongst the *B. exitiosa*-like sequences,

the *C. ariakensis Bonamia* sp., the *O. angasi Bonamia* sp., the *O. puelchana Bonamia* sp.

and *O. chilensis Bonamia* sp. from New Zealand, ranged between 0.002 and 0.006 (Table

4). Distances between the Chilean *Bonamia* sp. and the *B. exitiosa*-like group equaled

0.16 (Table 4). Distances among the Chilean *Bonamia* sp. sequences ranged between

0.007 and 0.011.

Incongruence Test

The incongruence heterogeneity test yielded a p-value of 0.530000, which

confirmed that the two datasets, both the SSU and the ITS datasets, shared enough

congruency in their characters such that the phylogenetic information produced from both

data sets would converge toward the same phylogenetic tree.

Combined SSU and ITS Phylogenetic Analyses

Phylogenetic trees that were produced from the combined SSU and ITS dataset

(Figure 5) did not differ much from the trees that were produced from the ITS analysis

(Figure 4). As in the ITS tree topology, support for the four main clades equaled 100 for

both the bootstrap and the jackknife analysis. Support for the sub clades, however,

ranged between 52% and 90% for the jackknife analysis and between 57% and 78% for

the bootstrap analysis. The support values for the bootstrap and the jackknife analyses

with the SSU/ITS combined dataset did not differ substantially from those obtained in the

analyses based on the ITS dataset.

Table 3. Uncorrected ("p") distance matrix of SSU sequence data using 668 continuous characters.
This distance matrix contains only the region of the SSU gene that is present for all *Bonamia* parasites.

	1	2	3	4	5	6	7
1) *B. perspora*	--						
2) *B. ostreae*	0.0261	--					
3) Chilean *Bon.* sp.	0.0377	0.0423	--				
4) Argentinean *Bon.* sp.	0.0359	0.0406	0.00156	--			
5) *C. ariakensis Bon.* sp.	0.0359	0.0406	0.00156	0.00000	--		
6) New Zealand *Bon. sp*	0.0359	0.0406	0.00156	0.00000	0.00000	--	
7) *O. angasi Bon.* sp.	0.0336	0.0406	0.00156	0.00000	0.00000	0.00000	--
8) *B. roughleyi*	0.0437	0.0422	0.00931	0.00772	0.00772	0.00772	0.00772
9) *B. exitiosa*	0.0447	0.0433	0.00465	0.00305	0.00305	0.00305	0.00305
10) *M. teredinis*	0.148	0.158	0.153	0.156	0.156	0.156	0.156
11) *M chitonis*	0.171	0.177	0.175	0.177	0.177	0.177	0.177

Table p: Uncorrected ("p") distance matrix of SSU sequence data continued

	8	9	10	11
8) *B. roughleyi*	--			
9) *B. exitiosa*	0.0046	--		
10) *M. teredinis*	0.163	0.165	--	
11) *M chitonis*	0.179	0.18432	0.0985	--

Table 4: Uncorrected ("p") distance matrix of ITS region sequence data using 567 continuous characters

	1	2	3	4	5	6	7	8
1) *O. angasi Bon.* sp.	--							
2) *C. ariakensis Bon.* sp.	0.00203	--						
3) *Argentinean Bon.* sp.	0.00408	0.00195	--					
4) *New Zealand Bon.* sp	0.00614	0.00395	0.00591	--				
5) *Chilean Bon.* sp.1	0.162	0.153	0.155	0.154	--			
6) *Chilean Bon.* sp. 2	0.164	0.155	0.157	0.156	0.0110			
7) *Chilean Bon.* sp. 3	0.162	0.153	0.155	0.154	0.00730	0.00734	--	
8) *B. ostreae 1*	0.383	0.363	0.361	0.366	0.354	0.358	0.356	--
9) *B. ostreae 2*	0.370	0.351	0.349	0.354	0.346	0.349	0.347	0.0142
10) *B. perspora 1*	0.383	0.363	0.361	0.357	0.389	0.383	0.386	0.355
11) *B. perspora 2*	0.380	0.360	0.358	0.354	0.388	0.382	0.385	0.361
12) *B. perspora 3*	0.385	0.365	0.363	0.358	0.390	0.384	0.387	0.355

Table p: Uncorrected ("p") distance matrix of ITS region sequence data continued

	9	10	11	12
9) *B. ostreae 2*	--			
10) *B. perspora 1*	0.367	--		
11) *B. perspora 2*	0.370	0.00812	--	
12) *B. perspora 3*	0.364	0.0102	0.00607	--

Figure 1. World map of the sampling locations for the oysters collected for this study. The abbreviations stand for the following sites: NC (Bogue Sound and Wilmington, NC, USA), ME (Maine), CH (Chile), AR (Argentina), NZ (Foveaux Strait, New Zealand), and NSW/PS (New South Wales/ Port Stephens, Australia). Also represented are areas where *Bonamia* spp. have been identified, but were not included in this sampling experiment. What follows is a list of abbreviations: CA (California, USA), WA (Washington, USA), BC (British Columbia), SP (Spain), IR (Ireland), NE (Newtherlands), TA (Tasmania), and VC (Victoria).
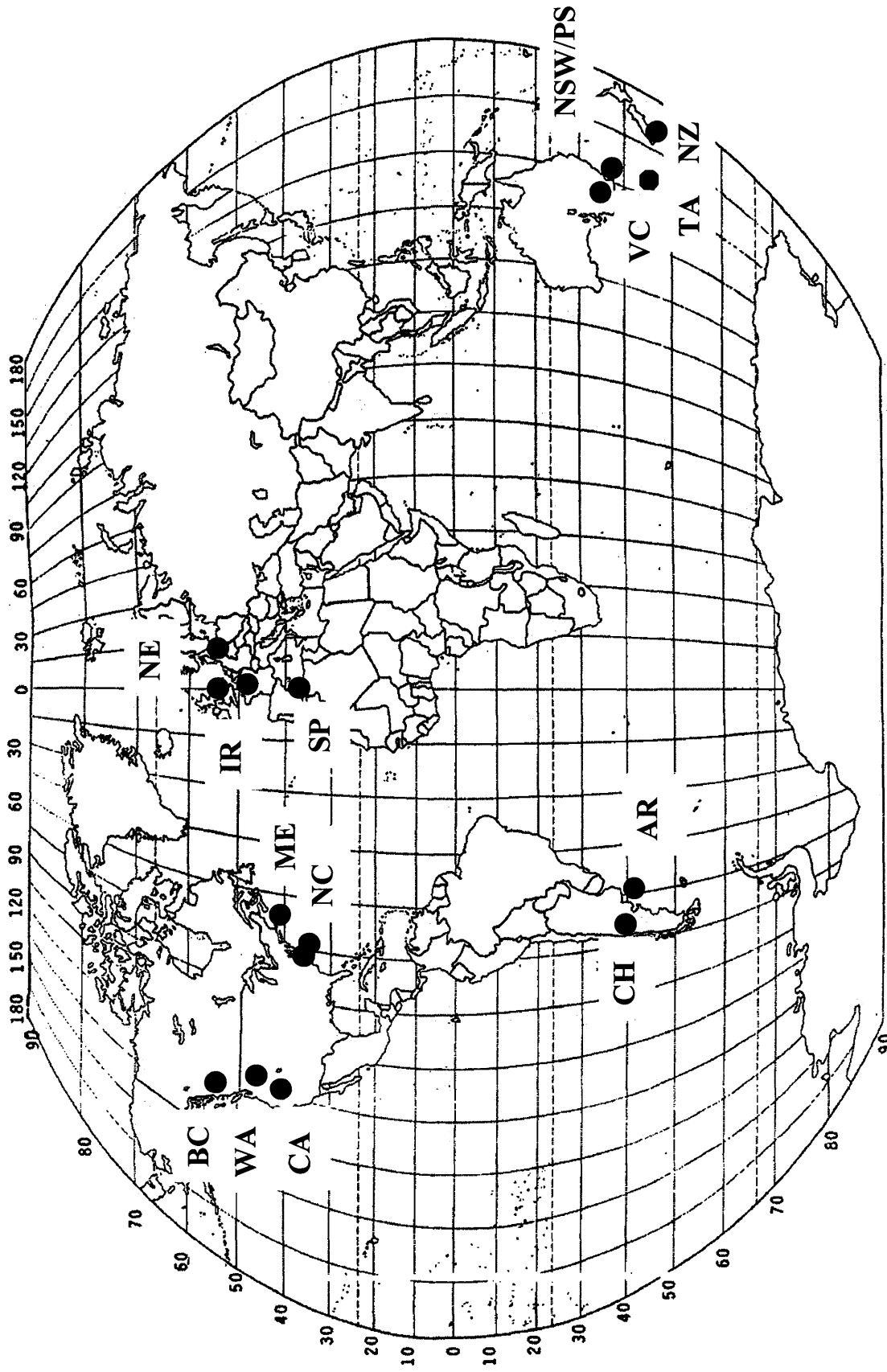
Figure 2: Diagram of the multi-copy ribosomal RNA gene complex indicating the location of the external transcribed spacer (ETS) region, the small subunit (SSU) ribosomal RNA (rRNA) gene, the internal transcribed spacer (ITS) regions 1 and 2, the 5.8S rRNA gene, the large subunit (LSU) rRNA gene, the non-transcribed spacer (NTS) regions 1 and 2, and the 5S rRNA gene. PCR primer binding sites for those used in this study are also indicated. (Dimensions are not drawn to scale).
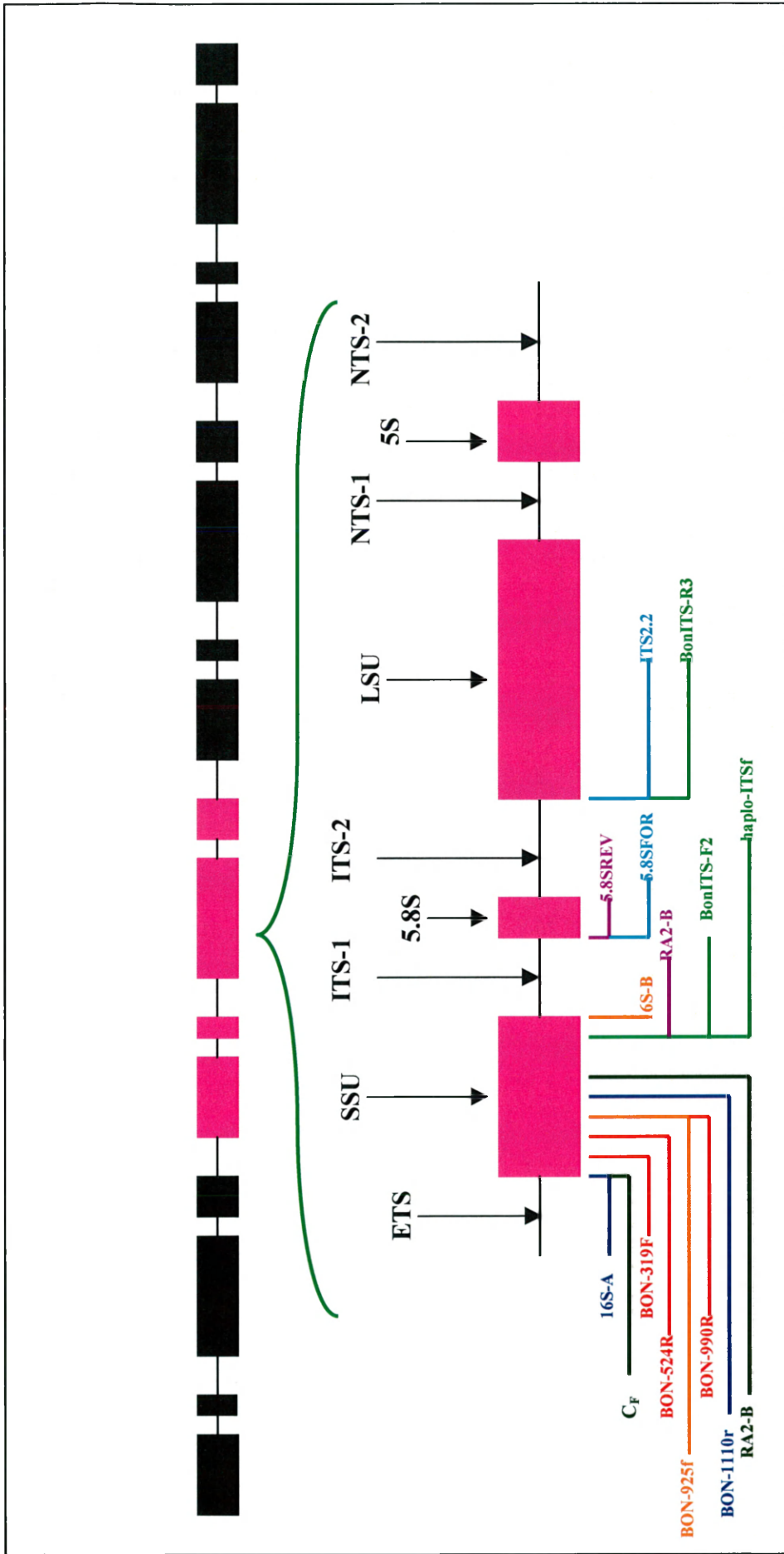
Figure 3: Maximum parsimony consensus tree resulting from bootstrap and jackknife analyses of small subunit ribosomal (SSU) RNA (rRNA) gene sequences from *Bonamia* spp. Each sequence is a consensus of *Bonamia* sp. sequences from a particular oyster host. 1,000 bootstrap pseudoreplicates with 100 random sequence additions were conducted as well as 1,000 jackknife pseudoreplicates with 100 random sequence additions, with 25% deletion of characters. Jackknife values are depicted above the bipartition and bootstrap values are depicted below the bipartition. *Minchinia chitonis* (AY449711) and *M. teredinis* (U20320) were used as outgroups for the analyses.

O. chilensis Bonamia, Chile

C. ariakensis Bonamia, NC, USA

O. puelchana Bonamia, Argentina

O. angasi Bonamia, Australia

O. chilensis Bonamia, New Zealand

B. exitiosa type seq., New Zealand

B. roughleyi type seq., Australia

B. perspora, NC, USA

B. ostreae, ME, USA

M. teredinis

M. chitonis

100
100

100
100

71
60

86
71

100
100
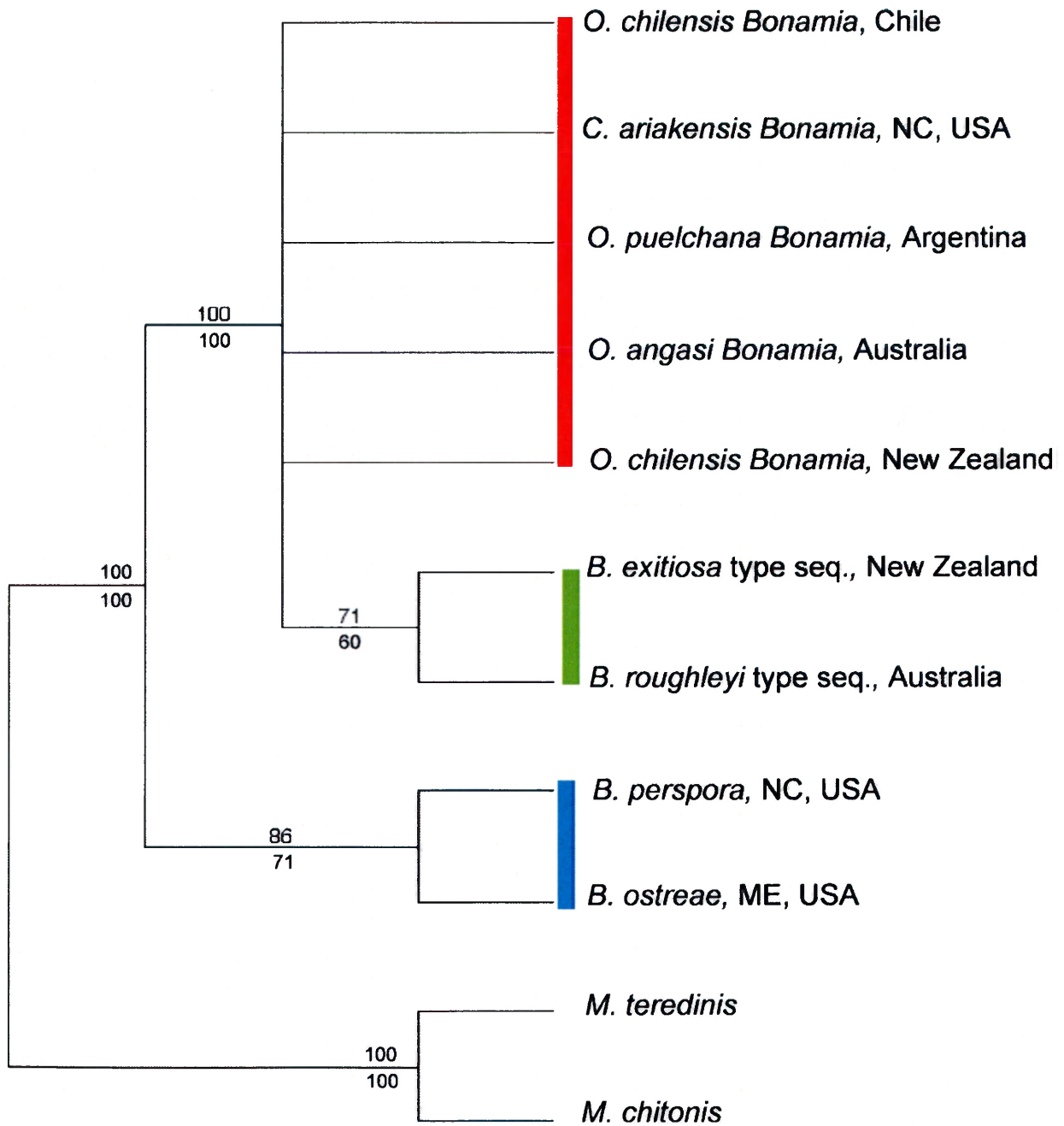
Figure 4: Maximum parsimony consensus tree resulting from bootstrap and jackknife analyses of internal transcribed spacer (ITS) region sequences of *Bonamia* spp. Tree branch support was assessed using 1,000 bootstrap replicates and 100 random additions, and 10,000 jackknife replicates and 1,000 random additions with 25% deletion of characters in each replicate. Jackknife support values are shown above the bipartition whereas bootstrap support values are shown below the bipartition.

C. ariakensis Bonamia, NC, USA
O. angasi Bonamia Australia
C. ariakensis Bonamia, NC, USA
O. puelchana Bonamia, Argentina
O. angasi Bonamia Australia
C. ariakensis Bonamia, NC, USA
O. puelchana Bonamia, Argentina
O. angasi Bonamia Australia
O. chilensis Bonamia, New Zealand
O. chilensis Bonamia, Chile
B. perspora, NC, USA
B. ostreae, ME, USA
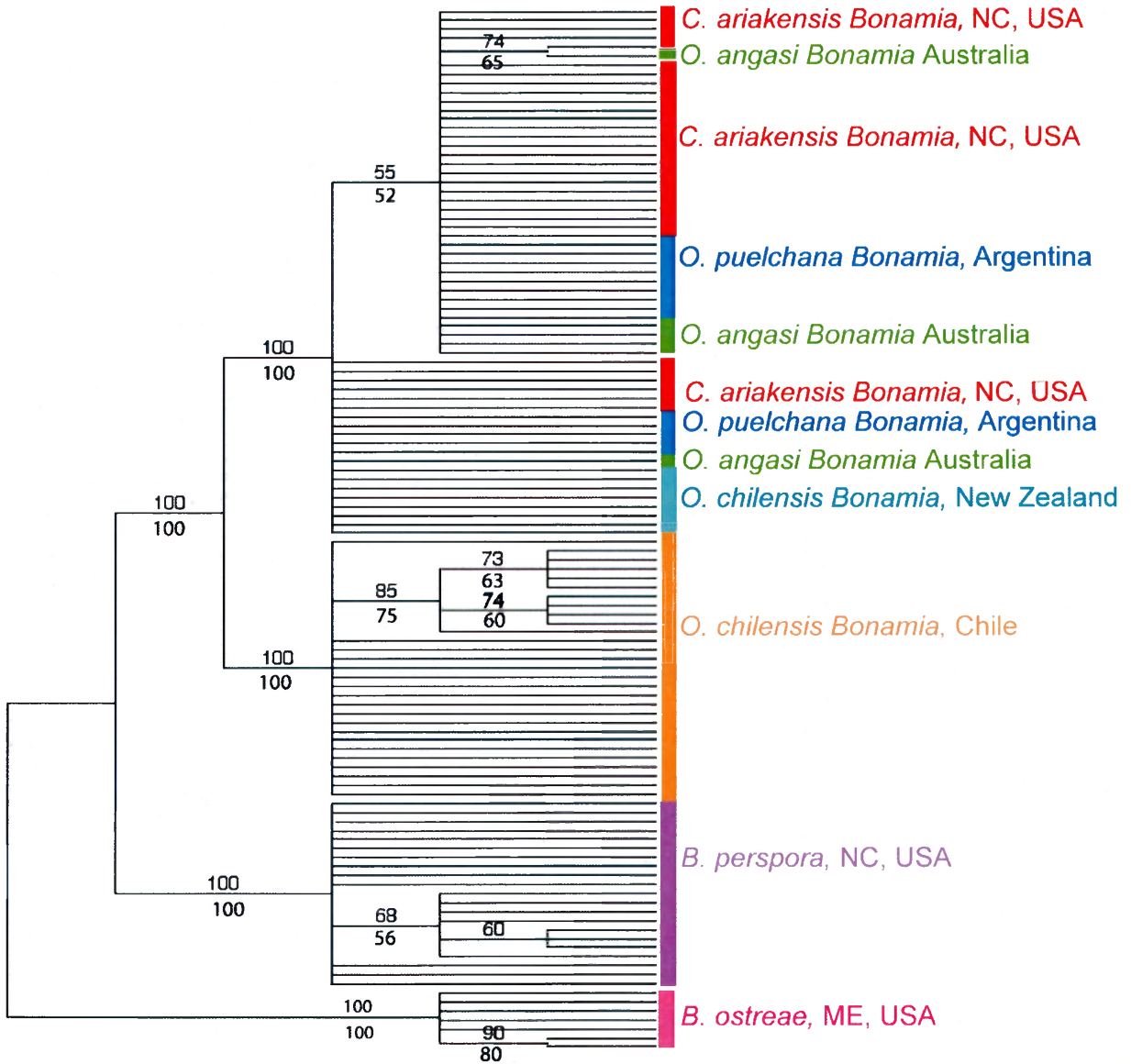
Figure 5: Maximum parsimony consensus tree resulting from bootstrap and jackknife analyses of concatenated internal transcribed spacer (ITS) region and small subunit (SSU) ribosomal RNA (rRNA) gene sequences of *Bonamia* spp. Tree branch support was assessed using 1,000 bootstrap replicates and 100 random additions and 10,000 jackknife replicates with 1,000 random additions with 25% of characters deleted in each replicate. Jackknife support values are shown above the bipartition, and bootstrap support values are shown below the bipartition.

C. ariakensis Bonamia, NC, USA
O. angasi Bonamia Australia
C. ariakensis Bonamia, NC, USA
O. puelchana Bonamia, Argentina
O. angasi Bonamia Australia
C. ariakensis Bonamia, NC, USA
O. puelchana Bonamia, Argentina
O. angasi Bonamia Australia
O. chilensis Bonamia, New Zealand
O. chilensis Bonamia, Chile
B. perspora, NC, USA
B. ostreae, ME, USA
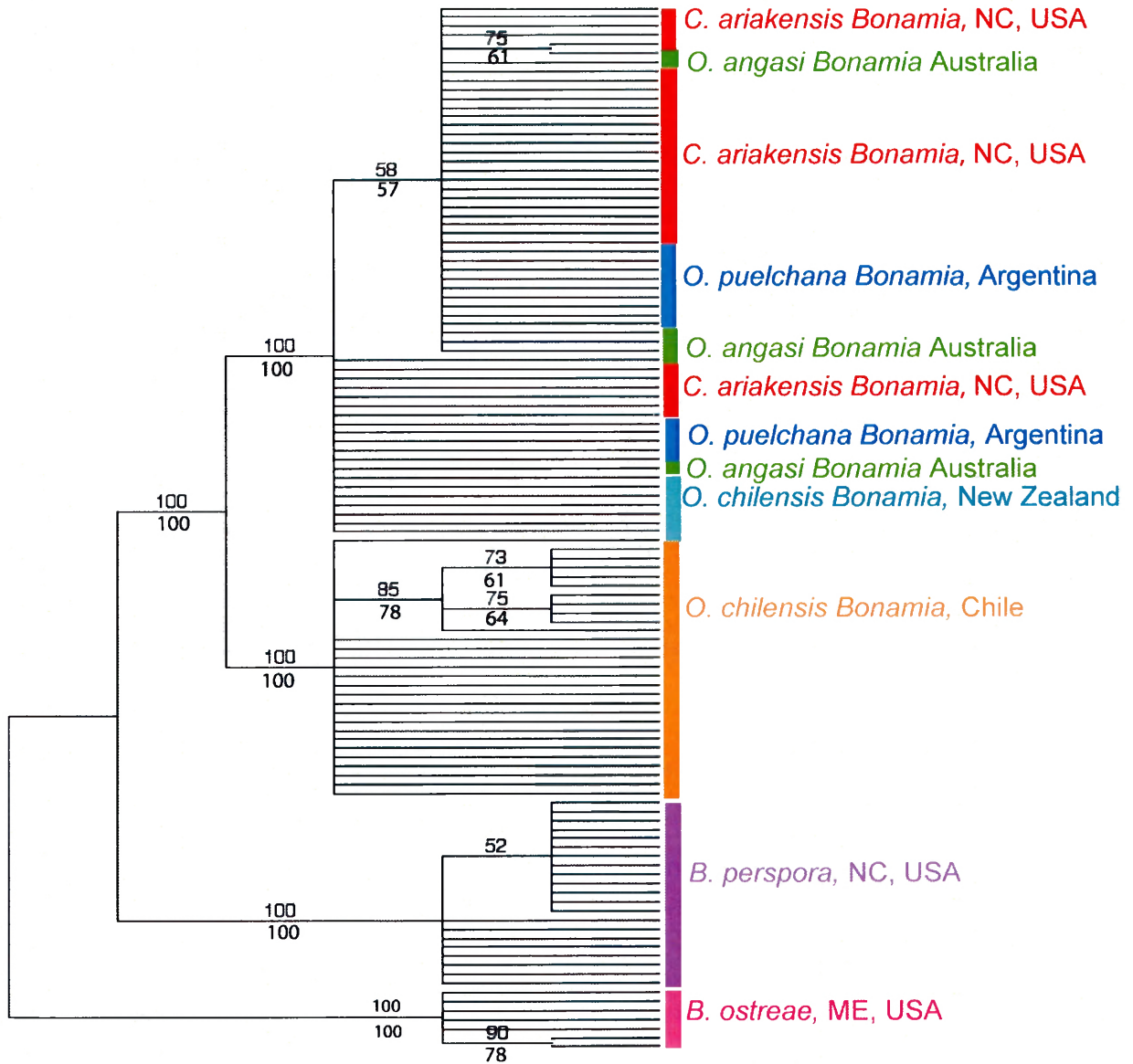
DISCUSSION

A phylogenetic analysis of the genus *Bonamia* was carried out in this study using information from the SSU gene and the ITS region of the ribosomal RNA (rRNA) gene complex. Phylogenetic relationships of the *Bonamia* parasites were inferred by examining the phylogenetic trees of each region of the rRNA gene complex separately, and also by assessing the relationship using the combined data. Both the ITS region analysis and the combined ITS/SSU analysis resulted in trees with similar topologies hypothesizing the same general relationships among the taxa. The SSU analyses gave strong support (100%) for the overall grouping of the *B. exitiosa*-like sequences, the Chilean *Bonamia* sequence, and the *B. roughleyi* and *B. exitiosa* type sequences. The SSU tree topologies gave low bootstrap support for the grouping of *B. ostreae* with *B. perspora* (71%), but yielded moderate support (86%) in the jackknife analysis. Also, by adding a *Minchinia* outgroup, the SSU tree topologies were able to support the placement of all of the experimental sequences within the genus *Bonamia*.

Small Subunit Ribosomal RNA gene

The multiple alignment of the *Bonamia* spp. SSU rRNA gene sequences depicted a few polymorphisms among the sequences; however, overall the sequences were highly conserved. The greatest differences among the SSU sequences occurred between the *B. perspora* sequence and the *B. exitiosa*-like group, the *B. ostreae* sequence and the *B. exitiosa*-like group, between the *B. perspora* sequence and the Chilean *Bonamia* sp. sequences, and between the *B. ostreae* and the Chilean *Bonamia* sp. sequences. There were also differences observed between the *B. perspora* and *B. ostreae* sequences. Slight

differences were observed between the *B. exitiosa* type sequence and the *B. exitiosa*-like

sequences, between the *B. exitiosa* type sequence and the *B. roughleyi* type sequence,

between the *B. roughleyi* type sequence and *the B. exitiosa*-like sequences, between the

Chilean *Bonamia* sp. sequence and the *B. exitiosa*-like sequences, between the Chilean

Bonamia sp. sequence and the *B. roughleyi* type sequence, and between the Chilean

Bonamia sp. sequence and the *B. exitiosa* type sequence.

There were, however, limitations to using the SSU rRNA gene, as was discovered

by the lack of resolution between the Chilean *Bonamia* sequences and the *B. exitiosa*-like

group (Figure 3). Apparent in both the jackknife and bootstrap analyses was a sub-clade

consisting of *B. roughleyi* and *B. exitiosa*-type sequences; though there was extremely

low support for this relationship (bootstrap support-60%, jackknife support-71%).

Although the *B. exitiosa* type sequence did not group amongst the *B. exitiosa*-like

sequences in the main clade, the pairwise distance analysis suggests the *B. exitiosa* type

sequence is more similar to the *B. exitiosa*-like sequences (difference of 0.31%) than the

B. exitiosa type sequence is to the *B. roughleyi* type sequence (0.46%). It should be

noted that the pairwise distance analysis is based on 668 characters, whereas the

phylogenetic analyses are based on 1769 characters, which is likely the cause for the

discrepancy. Nonetheless, both the bootstrap and jackknife analyses of the SSU rRNA

gene produced similar tree topologies and differed only by their jackknife and bootstrap

values (Figure 3). Hillis and Dixon (1991) suggested that SSU rDNA sequences are

better at depicting divergences that took place nearly 500 million years ago during the

Precambrian period or for more distantly diverged taxa. In addition, they suggested that

regions most suitable for phylogenetic analyses are greater than 70 percent, but less than

100 percent similar. Table 3 depicts uncorrected ("p") distances for nine SSU *Bonamia*

sequences. None of the *Bonamia* sequences differ from one another by more than 4.5%.

Similarity among the *B. exitiosa*-like sequences is 100%. In addition, the Chilean

Bonamia sp. sequence and the *B. roughleyi* sequence differ from the *B. exitiosa*-like

group by only 0.16% and 0.77% respectively. Comparing *B. perspora* and *B. ostreae* to

the *B. exitiosa*-like *Bonamia* spp. sequence led to differences of 3.6% and 4.1%,

respectively. Comparing *B. perspora* and *B. ostreae* to the Chilean *Bonamia* sp. sequence

resulted in differences of 3.8% and 4.2% respectively. The greatest pairwise distance

values occurred between the *B. exitiosa* type sequence and *B. perspora* (4.5%) and

between the *B. exitiosa* type sequence and *B. ostreae* (4.3%). When *B. perspora* and *B.*

ostreae were compared to the *B. roughleyi* type sequence differences of 4.4% and 4.2%

were observed. Comparison between *B. ostreae* and *B. perspora* resulted in a difference

of 2.6%. With such a high degree of similarity between the *B. exitiosa*-like sequences

and the Chilean *Bonamia* sequence, it is difficult to separate these sequences beyond the

genus level. Nonetheless, the results from the SSU rRNA analyses and the pairwise

distance values were sufficient to reject the hypothesis that the *Bonamia* sp. found in

North Carolina is closely related to *B. perspora* and/or *B. ostreae*. The results of the SSU

rRNA gene sequences were used in this study, however, to confirm, that the *Bonamia*-

like parasites that were sequenced from varying locations of the world do share a

common ancestor with the other identified *Bonamia* species and should be placed within

the genus *Bonamia*.

ITS regions 1 and 2 and the 5.8S gene

This study is the first time a broad-scale phylogenetic study has been conducted on the ITS region of *Bonamia* spp. infecting oysters. Only one previous study constructed a molecular phylogeny of the genus *Bonamia* using ITS-1 data (Corbiel et al. 2006a). Corbiel et al (2006) identified the parasite infecting *O. angasi* oysters in Australia by sequencing the SSU rRNA gene and the ITS-1 region. In the present study, analyses of the ITS-1/5.8S/ITS-2 region for both characterized and uncharacterized *Bonamia* parasites indicate the *Bonamia* from North Carolina, Argentina, NSW, Australia, and New Zealand are closely related to one another. As indicated by the results of the bootstrap and jackknife trees, the *C. ariakensis*, *O. puelchana*, *O. chilensis* from New Zealand, and *O. angasi Bonamia* spp. form a monophyletic clade. Support values for this grouping were 100 when both the jackknife and bootstrap trees were analyzed. Likewise, the ITS analyses indicate the Chilean *Bonamia* sp. is not as closely related to the *O. chilensis Bonamia* sp. from New Zealand as are the *C. ariakensis Bonamia* sp., the *O. puelchana Bonamia* sp., and the *O. angasi Bonamia* sp. The Chilean *Bonamia* sp. is also neither closely related to *B. perspora* or *B. ostreae*. What follows is a separate discussion of the ITS data of each of the *Bonamia*-like parasites that were analyzed in this study.

Bonamia sp. in Crassostrea ariakensis in NC, USA

This study indicates that the *C. ariakensis Bonamia* sp. ITS sequences are closely related to the *O. chilensis B. exitiosa*-like sequence from New Zealand and to the other *B. exitiosa*-like ITS sequences. Bootstrap and jackknife branch support for the main *B.*

exitiosa-like clade comprised of sequences from the *Bonamia* sp. infecting *C. ariakensis*,

O. puelchana, *O. angasi*, and *O. chilensis* from New Zealand were 100%. Also, the *B.*

exitiosa-like clade was distinct from the Chilean *Bonamia* sp., the *B. perspora*, and the *B.*

ostreae clades, which further supports the close relationship between the *C. ariakensis*

Bonamia sp. and the *O. chilensis B. exitiosa*-like sp. from New Zealand and not to either

B. perspora or *to B. ostreae*, two *Bonamia* spp. that have been described from the

northern hemisphere. The results of the phylogenetic trees, however, do support the

observation that the *C. ariakensis Bonamia* sp. is closely related to a species of *Bonamia*

that is found in the southern hemisphere.

Also, pairwise distances, of the ITS region sequences, between the *C. ariakensis*

Bonamia sp. and the *O. chilensis B. exitiosa*-like sp. from New Zealand equaled 0.4%.

Comparing this value with the values given by the pairwise distances between the *C.*

ariakensis Bonamia sp. and *B. ostreae* (35.1%-36.3%) and between the *C. ariakensis*

Bonamia sp. and *B. perspora* (36.0-36.5%%), it is apparent that the *C. ariakensis*

Bonamia sp. is relatively closely related to the *O. chilensis B. exitiosa*-like sequence from

New Zealand. Additionally, since the ITS region is a highly variable region in the rRNA

gene complex, highly divergent sequences would be expected to have a distance value

greater than 0.4% if in fact the sequences belonged to two different species (Hillis and

Dixon, 1991; Pélandakis et al. 2000).

Bonamia sp. in *Ostrea angasi* in Australia

This study indicates that the *O. angasi Bonamia* sp. ITS sequences are more

closely related to the New Zealand *O. chilensis B. exitiosa*-like sequence and to the other

B. exitiosa-like ITS sequences than to any of the remaining *Bonamia* species ITS

sequences. Based on the ITS tree topologies, the *O. angasi Bonamia* sp. groups with the

B. exitiosa-like sequences, with bootstrap and jackknife support of 100%. There are also

three locations within the *B. exitiosa*-like clade wherein *O. angasi Bonamia* sp. sequences

can be found. Two of the *O. angasi Bonamia* ITS sequences fall out amongst the main

clade, which contains the *O. chilensis B. exitiosa*-like sp. from New Zealand ITS

sequences; however, 4 of the *O. angasi Bonamia* sp. ITS sequences fall within a sub

clade that includes the *O. puelchana Bonamia* sp., the *O. angasi Bonamia* sp., and the *C.*

ariakensis Bonamia sp. sequences and are supported by the relatively low bootstrap and

jackknife values of 55% and 52%, respectively. Embedded in this clade is another clade

that is comprised only of two *O. angasi Bonamia* sp. sequences and is supported by a

bootstrap value of 65%, and a jackknife value of 74%. These relationships indicate the

close resemblance of the *O. angasi Bonamia* sp. ITS sequences to the *O. chilensis B.*

exitiosa-like sp. and other *B. exitiosa*-like ITS sequences. Thus, the results of the ITS

phylogenetic analyses fail to support the third hypothesis in this study, which states that

the *Bonamia* sp. infecting *O. angasi* oysters from Australia is a novel *Bonamia* sp.

Instead, the results of this study suggest that the *O. angasi Bonamia* sp. is closely related

to the other *B. exitiosa*-like sequences used in this study, although further data from

additional loci is needed to confidently make this assertion.

Pairwise distances (Table 4), also suggest that the *O. angasi Bonamia* sp. is

closely related to the *C. ariakensis Bonamia* sp., the *O. puelchana Bonamia* sp., as well

as to the New Zeland *O. chilensis B. exitiosa*-like sp. It has not yet been determined

whether or not *B. exitiosa* was present in Australia prior to its initial observation in the

1990s (Diggles 2005). Studies claim the parasite might have existed as far back as the late 1800s; however, no conclusive evidence exists to support this hypothesis (Diggles 2005).

Bonamia sp. in *Ostrea puelchana* in Argentina

Kroeck and Montes (2005) described a *Bonamia* sp. infecting *O. puelchana* stocks in San Antonio Bay, Argentina. They described physical signs in oysters including hemocytic infiltration of connective tissue of the mantle, gills, and digestive gland and concluded that bonamiasis was the cause of mortality; however, they were unable to specifically identify the parasite. The molecular analyses of the SSU and ITS regions presented in this study, suggest that the Argentinean *Bonamia* parasite is more closely related to the *B. exitiosa*-like sequences than to any other formerly characterized *Bonamia* sp.

The clade that was comprised of the *O. puelchana Bonamia* sp., the New Zealand *O. chilensis B. exitiosa*-like sp., the *C. ariakensis Bonamia* sp., and the *O. angasi Bonamia* sp. ITS sequences was supported by a value of 100% for both bootstrap and jackknife analyses. *Ostrea puelchana Bonamia* sp. ITS sequences were only observed among the *B. exitiosa*-like clade, which is contrary to the hypothesis proposed in this thesis that the *O. puelchana Bonamia* sp. is closely related to the Chilean *Bonamia* sp.

Pairwise distance analysis of the ITS region also suggest the *O. puelchana Bonamia* sp. is more closely related to the *O. chilensis B. exitiosa*-like sp. with a distance of 0.6% than to the Chilean *Bonamia* sp, which has a distances of 15.5% and15.7%. Pairwise distances between the *O. puelchana Bonamia* sp. and the *O. angasi Bonamia*

sp., as well as between the *O. puelchana Bonamia* sp. and the *C. ariakensis Bonamia* sp., equaled 0.2% for each comparison. These results suggest that the *O. puelchana Bonamia* sp. ITS sequences are more similar to the *C. ariakensis Bonamia* sp. and to the *O. angasi Bonamia* sp. ITS sequences than to the *O. chilensis B. exitiosa*-like sp. ITS sequences although more sampling would need to be done to confirm this observation.

In summary, the *Bonamia*-like sequences from *C. ariakensis*, *O. puelchana*, and *O. angasi*, are closely related to, or may even be the same species as the New Zealand *O. chilensis B. exitiosa*-like sp. To determine if the *O. chilensis B. exitiosa*-like sp.sequenced for this project is *B. exitiosa*, more molecular data is needed for the *B. exitiosa* type sequence. In addition, the recent discovery of a *B. exitiosa*-like parasite in *O. edulis* from Spain may further extend the range and host specificity of *B. exitiosa*, if these are all indeed the same species (Abollo et al. 2008).

Bonamia sp. in *Ostrea chilensis* in Chile

Previous analyses of complete ITS data have not been published for the Chilean *Bonamia* parasite, although partial sequence data for this species have been deposited in GenBank (accession number AY539840). Corbeil et al. (2006), suggested ITS-1 sequence information for the Chilean *Bonamia* sp. was more similar to *B. exitiosa* and the *O. angasi Bonamia* sp. sequences than to a *B. ostreae* sequence.

Range extension of *O. chilensis* by rafting was hypothesized by O' Foighil et al. (1999) for *O. chilensis* oysters. Several scenarios were proposed to explain how *O. chilensis* oysters arrived in Chile. Given that New Zealand *O. chilensis* populations are ancestral to those in Chile, that *O. chilensis* oysters lack an extended pelagic larval phase,

and that Chile and New Zealand are separated by 7000km of open-ocean, it was hypothesized that oysters rafted on pumice from New Zealand to Chile (O' Foighil et al. 1999). This was thought to support claims that the *Bonamia* sp. in Chile is closely related to *B. exitiosa* found in New Zealand.

Based on these previous studies, I hypothesized that the Chilean *Bonamia* sp. would group with the other *Bonamia* spp. parasites that have been described from the southern hemisphere. Bootstrap and jackknife analyses, however, indicated that the Chilean *Bonamia* sp. forms a sister clade to the *B. exitiosa*-like clade. Support for the Chilean *Bonamia* sp. clade was 100% for both the bootstrap and jackknife analyses. Additionally, the Chilean *Bonamia* sp. sequences did not fall among the *B. perspora* clade.

Pairwise distances between the Chilean *Bonamia* sp. ITS sequences and the *B. exitiosa*-like ITS sequences are between 15.3% and 16.4%. However, if pairwise differences between the *O. chilensis Bonamia* sp. from Chile and *B. ostreae* and between the *O. chilensis Bonamia* sp. from Chile and *B. perspora* are compared, the differences are between 34.6% and 35.8% and between 38.2% and 39.0% respectively. This suggests that the *Bonamia* sp. infecting Chilean *O. chilensis* oysters is not closely related to *B. perspora* or *B. ostreae*. The results from this study also support rejection of the second hypothesis in which it was proposed the Chilean *Bonamia* sp. and the *O. puelchana Bonamia* sp. are closely related. The results do, however, support placement of the Chilean *Bonamia* sp. as a sister clade to the *B. exitiosa*-like clade.

Taking a further look at the hypothesis of range extension by rafting, O' Foighil et al. (1999) hypothesized that post-metamorphic New Zealand *O. chilensis* oysters were

rafted on pumice, which is thought to be a suitable substrate for long-distance transport of

hard-bottom, suspension-feeding, epibenthic organisms. Radiocarbon dating of *O.*

chilensis oysters from an oyster midden site in southern Chile estimates *O. chilensis*

oysters were present 2998-3383 years ago, which predates human existence in the area

and nullifies anthropogenic influences on the transport of *O. chilensis* oysters to Chile (Ó

Foighil et al. 1999). If *B. exitiosa* has been present in New Zealand for thousands of

years, it is possible that when *O. chilensis* oysters were rafted from New Zealand to

Chile, they carried the parasite with them. Although this prediction is not entirely

impossible, the ITS data from this study suggests that 3000 yrs is not nearly enough time

for two populations of *Bonamia* to accumulate differences of 15.3% and 16.4% in the

nucleotide sequences of their ITS regions (Kimura 1968). This evidence thus leads to

rejection of the hypothesis of rafting of *B. exitiosa* from New Zealand to Chile by way of

infected *O. chilensis* oysters.

The addition of *B. roughleyi* and *B. exitiosa* type ITS sequence information to this

data set would help support any claims that the Chilean *Bonamia* sp. might in fact be a

new species of *Bonamia*. Although there are clear differences in the SSU sequences of

all three parasites, we cannot say that the Chilean *Bonamia* parasite represents a distinct

species until more molecular information becomes available for both the *B. roughleyi* and

B. exitiosa type species.

Bonamia roughleyi in *Saccostrea glomerata* in Australia

Bonamia roughleyi DNA was not detected in any of the *S. glomerata* oysters

sampled in this study, and it is worth determining if *B. roughleyi* is the actual cause for

winter mortality of *S. glomerata*. Neither of two attempts to obtain *B. roughleyi* sequence information was successful. Even when oysters were sampled in August, when infections are expected to be most prevalent, no evidence of *B. roughleyi* was found. In addition, Adlard and Lester (1995) conceded that diagnosis of *B. roughleyi* is problematic because intensity of infection is low in natural infections. Our inabilities to detect *B. roughleyi* infection in oysters support the view that if the parasite is present in *S. glomerata*, it most likely occurs at very low intensity and prevalence. It is also possible that *B. roughleyi* is not the causative agent of winter mortality in *S. glomerata*.

Various other factors, not addressed in this study, might also be the source for winter mortality. Elevation in temperature has been documented to cause summer seed mortality of *Crassostrea gigas* grown in Tomales Bay, California (Burge et al. 2007). Additionally, mortality of marine organisms in Offatts Bayou in Galveston has been linked to dredging in the inner Bayou in conjunction with the incorporation of a chemical change, such as hydrogen sulfide, into the environment (Gunter, 1942). Samain et al. (2007) identified environmental stressors, to include hydrogen sulfide (H2S), ammonium (NH$_4^+$), and ammonia (NH$_3$), which are present in the sediment, as potential sources of summer mortality of *C. gigas* in California. Just as in summer mortality, various environmental factors and stressors might also serve as potential sources for winter mortality in *S. glomerata* oysters in NSW, Australia; although additional research is needed to determine the exact source(s) of winter mortality.

SUMMARY

This molecular study combined sequence information from the ITS region and the SSU rRNA gene to develop a phylogeny of haplosporidian oyster parasites belonging to the genus *Bonamia*. Previous molecular studies of this genus analyzed the complete SSU rDNA of the four recognized *Bonamia* species and various congeneric species. This is the first study that produced a phylogeny of the genus *Bonamia* using complete ITS region sequence data, including the 5.8S gene, combined with SSU data. The phylogentic trees that were generated indicated the ITS data were more useful than the SSU data at resolving the relationships among the *Bonamia* species. Future phylogenetic studies of this group might focus on developing sequences of the actin genes, the cytochrome oxidase 1 (COI) gene, and/or the non-transcribed spacer (NTS) region. Both the actin and the COI genes have been used successfully in the past to study relationships among closely related organisms (Reece et al. 1997; Otranto et al. 2003; Reece et al. 2004; Ames et al. 2006). The non-transcribed spacer (NTS) region has a much higher rate of evolutionary change than the ITS region, and might help resolve small genetic differences among members of the *B. exitiosa*-like clade (Hillis and Dixon 1991; Robledo et al. 1999). In addition, including more gene regions into a phylogenetic analysis helps to increase the support of certain relationships, especially if the information provided by different genes results in a consistent evolutionary signal (Mattern 2004). More importantly, future analyses of the genus *Bonamia* should integrate morphological and life cycle information with molecular data to provide a better understanding of the evolutionary relationships among *Bonamia* species.

Molecular tools can provide a rapid and specific method for parasite detection. *Bonamia perspora* has a low prevalence in nature (between 1.0-2.0%). Current populations of *O. edulis* in Europe and in the United States have exhibited a lower prevalence of *B. ostreae* (26.0%) compared to historic populations (60.0%) (Elston et al. 1987; Friedman et al. 1989). It is thought that a decline in the prevalence of *B. ostreae* might be due to advanced oyster culturing methods, or the development of resistance to the parasite (Friedman et al. 1989). It is also possible that *S. glomerata* has developed resistance to *B. roughleyi*, and that *B. roughleyi* is present in this oyster, but at very low prevalence levels. This statement might explain why no signal of *B. roughleyi* was found during the two times oysters from NSW, Australia were sampled and tested for *B. roughleyi* infections. In contrast, the *B. exitiosa*-like species that were found in *C. ariakensis* and *O. puelchana* oysters have a high level of intensity and prevalence of infection (Burreson et al. 2004; Kroeck and Montes 2005). An explanation for this observation may be that *C. ariakensis* and *O. puelchana* are recent hosts for *Bonamia* parasites, and so have not had much time to adapt to the *Bonamia* spp. parasites.

To revisit the Chilean *Bonamia* sp. sequences, this sequence showed differences in its nucleotide sequence relative to the other *Bonamia* sp. sequences. However, to provide an answer to the question of whether or not the Chilean *Bonamia* sp. is a novel species, one would have to look at multiple datasets to arrive at a conclusion with adequate data to support or refute such a statement.

To explain how several *B. exitiosa*-like parasites have been found in multiple species of oysters from different locations, it is possible that commercial transport, cultivation and husbandry techniques, or even recreational activities might be

contributing causes. There are many examples in the literature of unintentional introduction of a species (Raloff 1999; Mack et al. 2000; Elston 1986). As stated earlier, *B. ostreae* was introduced into Europe after infected *O. edulis* oysters were imported to help stabilize the dwindling native oyster populations (Elston 1986). In some instances, nonindigenous species are introduced via ballast water of commercial ships or through biofouling (Chesapeake Bay Commission, 1995). In order to determine if such a mode of dispersal is possible for *Bonamia* species, a thorough examination of the life cycle, traffic records of commercial vessels, and information concerning human importations of aquatic species is necessary.

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Delonna Michelle White was born in Cheyenne, Wyoming on July 20, 1982 to parents Michael Eugene and Donna Gail White. Delonna graduated in the top ten percent of her graduating class of 200 from North Pole High School, North Pole, AK in May of 2000. Delonna earned her B.S. degree in Biology Education from Hampton University, Hampton, VA in May of 2004, graduating as the valedictorian with a 4.06 GPA. During the spring of 2000, Delonna was awarded the Hall-Bonner doctorial fellowship award. Delonna began her Masters of Science program at the College of William and Mary, School of Marine Science in the spring of 2005. In the summer of 2005, Delonna participated in the Multicultural Students at Sea Together (MAST) program, where she learned about sailing, conducted scientific experiments, and was exposed to a variety of cultural experiences. Delonna has been a member of the Minorities Striving and Pursuing Higher Degrees of Success (MSPHDS) in earth system sciences program since the fall of 2006.