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Understanding the Origins, Dispersal, and Evolution of Bonamia Species (Phylum Haplosporidia) Based on Genetic Analyses of Ribosomal RNA Gene Regions

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Understanding the Origins, Dispersal, and Evolution of *Bonamia* Species (Phylum Haplosporidia) Based on Genetic Analyses of Ribosomal RNA Gene Regions

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ABSTRACT

Bonamia species (Haplosporidia), protistan parasites of oysters, are notorious for causing devastating mortality in commercially important oyster species such as *Ostrea edulis* and *Ostrea chilensis*. Described species were originally thought to have fairly circumscribed host and geographic ranges: *Bonamia exitiosa* infecting *O. chilensis* in New Zealand, *Bonamia roughleyi* infecting *Saccostrea glomerata* in Australia, and *Bonamia ostreae* infecting *O. edulis* in Europe and North America. The discovery of a *B. exitiosa*-like parasite in experimental *Crassostrea ariakensis* in North Carolina, and the observation of this parasite and a novel species, *Bonamia perspora,* in non-commercial *Ostrea equestris* there, altered this perception and prompted a wider evaluation of the global diversity of *Bonamia* parasites in heretofore uncharacterized oyster hosts. Samples of 14 oyster species from 21 locations were screened for *Bonamia* spp. by polymerase chain reaction (PCR), and small subunit (SSU) and internal transcribed spacer (ITS) regions of *Bonamia* sp. ribosomal DNA were sequenced from PCR-positive individuals. Infections were confirmed histologically. Phylogenetic analyses using parsimony and Bayesian methods revealed one species, *B. exitiosa,* to be a host generalist with a cosmopolitan distribution: it was found in nine oyster species from Australia, New Zealand, Argentina, the southeastern and western coasts of the USA, and Tunisia. The more limited host and geographic distributions of *B. ostreae* and *B. perspora* were confirmed, but nothing genetically identifiable as *B. roughleyi* was found in Australia or elsewhere. Newly discovered diversity included a *Bonamia* sp. in *Ostrea sandvicensis* from Hawaii that is basal to the other *Bonamia* species, but which has not been observed to display spores despite its basal position; and a *Bonamia* sp. in *O. edulis* from Tomales Bay, California, that is closely related to *B. exitiosa* and the previously observed *Bonamia* sp. from *O. chilensis* in Chile.

In order to better understand how *B. exitiosa* reached its current distribution, TCS gene genealogies were constructed using ITS region rDNA sequencing data. The analyses revealed population structure in the form of four well-defined clusters of sequences, three corresponding to geographic regions (temperate Atlantic and Pacific waters of the Southern Hemisphere, California, and the western Atlantic along the coast of the Americas), and the fourth cosmopolitan in distribution. Dispersal of the southern hemispheric cluster among New Zealand, Australia, and South America *B. exitiosa* sequences may plausibly reflect natural dispersal via rafting with oyster hosts. The California cluster, conversely, may reflect a limited anthropogenic introduction. Wide distribution of *B. exitiosa* parasites in the cosmopolitan and Atlantic coast groups may relate partly to both natural and anthropogenic dispersal with one host, *O. stentina*, which is conspecific with *O. auporia* and *O. equestris* and is distributed from the eastern Americas to the Mediterranean and African coast to New Zealand—that is, in most regions where *B. exitiosa* has been found to occur. These analyses highlight the utility and importance of ITS region rDNA sequencing for studies of *Bonamia* parasites, though development of additional loci for the study of *Bonamia* phylogenetics and phylogeography remains essential.

Understanding the Origins, Dispersal, and Evolution of *Bonamia* Species (Phylum Haplosporidia) Based on Genetic Analyses of Ribosomal RNA Gene Regions

INTRODUCTION

Taxonomy and Life History

Bonamia species (phylum Haplosporidia) are protozoan parasites of oysters whose characteristic cell form is a uninucleate, 2- to 3-μm "microcell" (Pichot et al. 1980) associated with the disease bonamiasis. There are four described species, which were described from four different oyster hosts: *Bonamia ostreae* Pichot et al., 1980 in *Ostrea edulis* L., *Bonamia roughleyi* (Farley et al. 1988) in *Saccostrea glomerata* (Gould, 1850), *Bonamia exitiosa* Hine et al., 2001 in *Ostrea chilensis* Philippi, 1845, and *Bonamia perspora* Carnegie et al., 2006 in *Ostrea equestris* Say, 1834.

Taxonomically, *Bonamia* species are within the phylum Haplosporidia, which is a small group of endoparasitic protists of mainly marine invertebrates. There are about 38 recognized species in the phylum, which contains three other genera, *Urosporidium*, *Haplosporidium*, and *Minchinia* (Sprague 1979). Additional species have been reported in a number of invertebrate hosts, though they have not yet been identified specifically (Burreson and Ford 2004). Morphologically, haplosporidians parasitizing molluscs are characterized by having 1) multinucleate plasmodia and 2) ovoid, walled spores lacking polar filaments or polar tubes with an orifice at one pole, which is covered either by a hinged operculum externally or an internal flap made of wall material (Burreson and Ford 2004). *Bonamia* species were suspected by Perkins (1987) to be haplosporidians based ultrastructurally on the presence of haplosporosomes (membrane-bound organelles with an unknown function), but at that time spores had yet to be observed in any described *Bonamia* species (Perkins 2000). The placement of the genus *Bonamia* in the Haplosporidia was later confirmed molecularly using SSU rDNA (Carnegie et al. 2000;

Cochennec-Laureau et al. 2003; Reece et al. 2004) and actin gene (López-Flores et al. 2007) sequences in phylogenetic analyses. Then, in 2006, further morphological support for the placement of *Bonamia* in the phylum Haplosporidia came with the description of *B. perspora*, which exhibits sporogony (Carnegie et al. 2006). Carnegie et al. (2006) suggested that other *Bonamia* species might also be able to produce spores, but perhaps only under certain conditions or only within their primary hosts.

Still, *Bonamia* parasites are atypical haplosporidians, as they tend to display only small (< 5 μm), uninucleate cells as opposed to multinucleate plasmodia, and unlike typical haplosporidians, which are usually extracellular in their hosts, parasitize host hemocytes. Additionally, typical haplosporidians are presumed to have indirect life cycles with intermediate hosts, while both *B. exitiosa* and *B. ostreae* are presumed to be directly transmitted among oyster hosts (Hine et al. 1996, Elston et al. 1987); though it is not certain that direct transmission fully characterizes their life histories.

The type species of the genus is *B. ostreae*. The first presumed observation of this parasite was in 1969 by Katkansky et al. with their report of a "microcell" disease in *O. edulis* in California, USA (Elston et al. 1986). It was not until 1979, when the parasite caused massive mortalities in native French *O. edulis*, that the parasite was named (Pichot et al. 1980). *Bonamia ostreae* has since been attributed to catastrophic mortality in other European *O. edulis* populations from the British Isles to Spain (Van Banning 1982, Bucke et al. 1984, Polanco et al. 1984, Rogan et al. 1991, Grizel et al. 1988) and has also been detected in Italy (Narcisi et al. 2010). In North America, in addition to California, *B. ostreae* has been reported in *O. edulis* from Washington State, British Columbia, and Maine (Elston et al. 1986, Marty et al. 2006, Friedman and Perkins 1994).

Bonamia ostreae has also been detected in other oyster species including *Ostrea puelchana* (Kroeck and Montes 2005) and *O. chilensis* (Bucke and Hepper 1987) in France, *Ostrea lutaria* (= *O. chilensis*) in the United Kingdom (Bucke and Hepper 1987), and *Crassostrea gigas* in Ireland and Spain (Lynch et al. 2010), indicative of a potentially wide host range.

Bonamia perspora is the most recently described *Bonamia* species. It was discovered in Bogue Sound, North Carolina, USA in *O. equestris*—a little-studied, non-commercial oyster species that inhabits euhaline to polyhaline waters from North Carolina south to Argentina (Carnegie et al. 2006). Burreson et al. (2004) were examining a bonamiasis epizootic in *Crassostrea ariakensis* in Bogue Sound, North Carolina, and while doing so, evaluated *O. equestris* as a possible source and/or reservoir for the *Bonamia* sp. observed in *C. ariakensis* (Carnegie et al. 2006). They found *O. equestris* to be co-infected by the *C. ariakensis*-pathogenic *Bonamia* sp. and a second, novel *Bonamia* species. This novel species, named *B. perspora,* displays more typical haplosporidian characters than that of the other *Bonamia* species, including the presence of an ornamental spore with an orifice covered by a hinged operculum as mentioned above (Carnegie et al. 2006). *Bonamia perspora* is rarely observed within hemocytes and instead seems to reside extracellularly in connective tissues. It also exhibits a wide diversity of cell forms that are not seen in other *Bonamia* species including sporonts, sporocysts, and spores. Carnegie et al. (2006) also postulated that after systemically invading the connective tissues as uninucleate or small plasmodial forms, *B. perspora* appears to sporulate synchronously like another oyster parasite, *Haplosporidium costale.* Despite these more typical haplosporidian traits, phylogenetic parsimony analysis of SSU

rDNA data supported the monophyly of the *Bonamia* genus with 100% jackknife support for the inclusion of *B. perspora* in the clade. Results also suggested that *B. perspora* may be sister to the type species of the genus, *B. ostreae* (Carnegie et al. 2006).

Bonamia roughleyi (Farley et al. 1988) is the presumed causative agent of winter mortality of Sydney rock oysters, *Saccostrea glomerata*, in southeastern Australia, which has occurred since at least 1924 (Roughley 1926). Winter mortality is greatest in higher salinity waters (30-35 ppt) with mortality occurring primarily in oysters greater than three years old (Farley et al. 1988). *Bonamia roughleyi* was initially placed in the genus *Mikrocytos*, an unrelated genus of microcell parasites, based on gross pathology and host specificity (Farley et al. 1988). *Mikrocytos* parasites were said to be associated with focal lesions in the gill, connective, and gonadal tissues and to occur in crassostreid oysters, as opposed to *Bonamia* species, which were thought to be associated with "systemic, non-abscess type disease manifestations" in ostreid oyster hosts (Farley et al. 1988). These characteristics, however, were insufficient to distinguish the two genera. With the advent of molecular techniques, Cochennec-Laureau et al. (2003) set out to clarify the taxonomic relationship between *M. roughleyi* and other microcell parasites. They sequenced part of the SSU rRNA gene of *M. roughleyi* and found 95.2% sequence similarity to *B. ostreae* and 98.4% similarity to *B. exitiosa* (Cochennec-Laureau et al. 2003). Furthermore, parsimony analysis showed 100% bootstrap support for the inclusion of *M. roughleyi* in the *Bonamia* clade (Cochennec-Laureau et al. 2003). Based on this molecular evidence, Cochennec-Laureau et al. (2003) suggested the species be reclassified as a *Bonamia* species. Recently, Hill et al. (2010) argued that there was no molecular, morphological,

or ultrastructural basis for delineating species boundaries between *B. roughleyi* and *B. exitiosa*.

Bonamia exitiosa was described by Hine et al. in 2001 and has caused large-scale mortalities in *O. chilensis* occurring in Foveaux Strait, New Zealand since 1964 (Hine et al. 2001). Again, ultrastructurally and morphologically, *B. exitiosa* is indistinguishable from other described species, but molecular analysis of SSU rDNA suggests that *B. exitiosa* is distinct (Hine et al. 2001).

Few studies have examined the transmissibility of *Bonamia* parasites, but it appears that they pass from an infected oyster into the water column, and then nearby oysters ingest the parasite while feeding (Elston et al. 1986; Lynch et al. 2006). Both *B. ostreae* and *B. exitiosa* can be detected throughout the year, but prevalence and intensity of infection peak post-spawn in September/October for *B. ostreae* (Lynch et al. 2006). Studies done in the late 1990s by Culloty and colleagues demonstrated direct transmission in a cohabitation study in which naïve oysters became infected upon exposure to *B. ostreae*-infected oysters (Lynch et al. 2006). By 4-6 weeks post-exposure, infections could be observed (Lynch et al. 2006). For *B. exitiosa*, parasites are presumed to be released from dead and dying oysters' tissues (gonad, kidney, gills, gut) (Hine 1991a, 1991b). Half of these particles are thought to survive for about 48 hours in 18°C seawater and during this time are ingested by nearby oysters (Diggles and Hine 2002). Once the parasite enters an oyster host, it is phagocytosed by hemocytes. Here, the parasite avoids destruction via an unknown mechanism and proliferates via cell division. The hemocytes eventually lyse releasing the microcells into the cytoplasm where the microcells are then phagocytosed by new hemocytes and the cycle begins again. As

infection intensities increase, host cell membranes degrade. The host can no longer remain turgid or extend its gills for feeding and dies (Hine 1996).

Direct transmissibility could facilitate and expedite the spread of these parasites to other ecosystems. Parasites with assumed indirect life cycles, like typical haplosporidians, would require the presence of a series of hosts at each location in order to establish successful populations of the parasite. Therefore, these parasites with indirect life cycles might not be as easily distributed as those with direct life cycles, especially if the directly transmissible parasites are host generalists. Species like *B. ostreae* and *B. exitiosa*, then, may be a cause for greater concern in this respect given their presumed direct transmission and history of devastating oyster populations. Incorporate anthropogenic and other natural processes that aid in the movement of oyster hosts, and the probability of parasite dispersal likely increases.

Bonamia exitiosa and *B. exitiosa*-like parasites, in particular, have been observed in a wide range of hosts and locations. They have been detected in *Ostrea angasi* from New South Wales, Australia (Corbeil et al. 2006); *O. puelchana* from Argentina (Kroeck and Montes 2005; Kroeck et al. 2008); *O. chilensis* from Chile (White 2008); *O. edulis* from Spain (Abollo et al. 2008), Italy (Narcisi et al. 2010), and the Mediterranean coast of France (Arzul et al. 2010); *O. equestris* from North Carolina (Burreson et al. 2004); and *Ostrea stentina* from Tunisia (Hill et al. 2010). Additionally, *B. exitiosa* and *B. exitiosa*-like parasites have been detected in experimental *C. gigas* in Spain (Lynch et al. 2010) and experimental *C. ariakensis* (=*Crassostrea rivularis*) from North Carolina (Burreson et al. 2004) and France (Cochennec et al. 1998). Many of these oyster species,

with the exception of *O. stentina* and *O. equestris,* are important for fisheries, aquaculture, and/or restoration activities.

Emerging Perspectives: Bonamia Species Distribution and Diversity

Crassostrea ariakensis was recently being considered for use in restoration and aquaculture in the mid-Atlantic region of the United States and in particular the Chesapeake Bay (STAC 2004). Because of the apparent susceptibility of *C. ariakensis* to bonamiasis (Cochennec et al. 1998), the introduction prompted concern, especially since *B. ostreae* is already present in Maine in *O. edulis* populations (Burreson et al. 2004). In 2003, small (<25 mm), pathogen-free, triploid *C. ariakensis* deployed to upweller systems in Bogue Sound, North Carolina displayed high mortality after only a month (Burreson et al. 2004). Histopathological analysis revealed the presence of 2-3 μm intrahemocytic microcells resembling those of *Bonamia* species, and both partial sequence of SSU rDNA and *in situ* hybridization (ISH) analysis confirmed this identification (Burreson et al. 2004). Phylogenetic analysis of partial SSU rDNA revealed that this *Bonamia* sp. was less closely related to *B. ostreae*, however, and more closely related to *B. exitiosa* (Burreson et al. 2004).

Until this observation, it was thought that the three described *Bonamia* species were more or less confined to their respective hosts and geographic locations—*B. ostreae* in *O. edulis* in the temperate Northern Hemisphere, and closely related *B. exitiosa* and *B. roughleyi* in the temperate Southern Hemisphere. Therefore, finding a *B. exitiosa*-like parasite in the Northern Hemisphere challenged earlier perceptions of the parasites' limited geographical ranges. At the same time, our perception of *Bonamia* diversity changed with the description of *B. perspora*, a more typical haplosporidian. As

mentioned above, bonamiasis has been implicated in epizootics in commercially important oyster species such as *O. edulis*, *S. glomerata*, and *O. chilensis*, and in each case, the disease has caused both ecological and economic harm. Epizootics such as these also restrict aquaculture production, which in turn negatively affects the economy of affected countries (Berthe et al. 1999); therefore it is imperative to better understand the dispersal and distribution of these parasites.

Historically, oyster species have been intentionally introduced to novel locations worldwide, usually in an effort to restore a fishery diminished by overfishing and/or disease (for summary see Ruesink et al. 2005). One of the ecological implications of these translocations has been the introduction of pathogens, such as *B. ostreae* and the notorious eastern oyster (*Crassostrea virginica*) parasite, *Haplosporidium nelsoni*. Elston et al. (1986) proposed that *B. ostreae* was introduced to Europe via infected *O. edulis* seed from the West Coast of the United States in the late 1970s. Seed *O. edulis* from supposed *B. ostreae*-enzootic areas was imported into both France and Spain, where the parasite spread and caused catastrophic mortality from the British Isles to Spain (Elston et al. 1986). It is thought that *H. nelsoni*, a parasite native to Asia that has devastated native *C. virginica* populations along the Mid-Atlantic coast of the USA, was introduced via the experimental introduction of *C. gigas* sometime prior to 1957 (Burreson et al. 2000).

Similar theories with respect to the movement of oyster hosts exist regarding the introduction of *B. exitiosa* to supposed non-enzootic areas. Abollo et al. (2008) detected a *B. exitiosa*-like parasite in *O. edulis* in Galicia, Spain and hypothesized that the parasite could have been inadvertently introduced through the legal or illegal importation of

oysters from *B. exitiosa*-endemic areas. The authors also suggested the possibility of a recent introduction via the ballast water and outer hulls of ships, which was a hypothesis suggested by Bishop et al. (2006) regarding the possible introduction of the *B. exitiosa*-like species found in North Carolina. However, with increasing observations of *Bonamia* species around the world, the origin of the parasite is becoming less clear—challenging some of these widely-accepted dispersal hypotheses.

Morphological examination, while being extremely useful, has its limitations. Morphological phylogenetic analyses for haplosporidians, for example, have been unsuccessful in determining placement of these taxa among other protists (Flores et al. 1996), and the above findings emphasize and expose the limitations of histopathological methods for discriminating species of microcell parasites, like *Bonamia* species. Morphology also is limited in its ability to distinguish differing cell forms of the same species. Plasticity of cell forms occurs in space and time: differing among hosts (primary vs. alternate) or during times of stress or poor environmental conditions, e.g. spores. Presence or absence of spores as a character, therefore, might not be best for classification. Ultrastructural methods also have their limits, because some important ultrastructural characters used to classify protists (e.g., haplosporosomes) appear to have arisen several times during evolution (Cochennec-Laureau et al. 2003), which could confound morphological phylogenies.

Using Molecular Tools to Discover Bonamia Species Distribution, Diversity, and Evolution

Molecular analyses have been increasingly used to characterize and classify closely related taxa. DNA analyses have limitations as well, but when taxa are indistinguishable morphologically such as *Bonamia* species, great strides can be made in the clarification of taxonomy and phylogenetics using these techniques. Regarding *B. roughleyi* in particular, ultrastructural analysis placed this species within *Mikrocytos,* but analysis of SSU rDNA sequence revealed this species' evident placement within the genus *Bonamia* (Cochennec-Laureau et al. 2003). Genomic DNA sequences also do not vary with life stage or developmental phase or with varying hosts and/or tissue locations (Berthe et al. 1999). These analyses are less subjective compared to morphological and ultrastructural methods and would therefore decrease the chances of misidentification (as shown above with the application of ISH).

The rRNA gene complex has been used most commonly in examining the relationships of members within the phylum Haplosporidia (Flores et al. 1996; Carnegie et al. 2000; Reece and Stokes 2003; Cochennec-Laureau et al. 2003; Reece et al. 2004; Burreson and Reece 2006; Carnegie et al. 2006). This gene complex comprises both conserved and variable regions, reflecting variation in rates of molecular evolution (Hillis and Dixon 1991). The small-subunit (SSU), large-subunit (LSU), and 5.8S ribosomal RNA (rRNA) genes are relatively well conserved as they encode functional RNAs, while the internal (ITS-1, ITS-2) and external (ETS) transcribed spacer regions and non-transcribed or intergenic spacers (NTS or IGS) are more variable (Hillis and Dixon 1991) (Figure 1).

Also, rRNA genes are multi-copy genes, often making them the targets of diagnostics because the many copies will help ensure sensitivity (Berthe et al. 1999). For *Bonamia* parasites, the SSU rRNA gene has been the target of polymerase chain reaction (PCR) diagnostics (Carnegie et al. 2000) and *in situ* hybridization probes (Cochennec et al. 2000), and further used to determine relationships between *Bonamia* species and other haplosporidians (Carnegie et al. 2000; Cochennec-Laureau et al. 2003; Reece et al. 2004; Abollo et al. 2008). However, because the SSU rRNA gene is relatively well-conserved analyses based on this region may not resolve relationships at the species level.

Increasingly, spacer regions of the rRNA gene complex are used to develop phylogenies of closely related taxa (e.g., Litaker et al. 2007). These regions are more variable due to significant divergence during speciation (Hillis and Dixon 1991; Litaker et al. 2007), and because of this variability, the relationships of more closely related taxa can be examined using the ITS, ETS, and NTS regions (Hillis and Dixon 1991). In White's Masters thesis (2008), the ITS-1, 5.8S rRNA gene, and ITS-2 (collectively the ITS rDNA region) was used to construct a molecular phylogeny of *Bonamia* in order to determine the relationships between the *Bonamia* species observed in *C. ariakensis* in North Carolina, *O. chilensis* in Chile, *O. puelchana* in Argentina, and *O. angasi* in Australia. Based on SSU rDNA parsimony analysis, she found that all of these species fell within the same clade as *B. exitiosa* (White 2008). However, ITS rDNA analysis revealed that while most were still within the *B. exitiosa* clade, the *Bonamia* sp. found in *O. chilensis* from Chile appeared to be distinct (White 2008; Hill et al. 2010), demonstrating the usefulness of this region for phylogenetic analysis. This question remains: are all of the *Bonamia* species found within variable hosts and locations and

within the *B. exitiosa* clade conspecific? If so, how did the parasite achieve its current distribution?

Prompted by emerging insights regarding the distribution and diversity of all *Bonamia* species, the objective of Chapter One of my thesis project was to assess the global distribution of *Bonamia* parasites and determine interspecific evolutionary relationships to better understand their origins and evolution using molecular markers. Chapter Two presents a description of a novel species discovered during this global survey. Chapter Three presents results of analyses on the intraspecific genetic variation and the geographic distribution of *B. exitiosa* genotypes. *Bonamia exitiosa* ITS region rDNA sequences from multiple hosts and locations around the world were examined using statistical parsimony/networking models to 1) determine if phylogeographic population structure exists, and if so 2) to develop dispersal hypotheses regarding how *B. exitiosa* came to achieve its wide distribution. By analyzing population structure, phylogeographic hypotheses can be developed, enabling us to better understand the current distribution of ecologically, and sometimes economically, important parasites.

In summary, the overall goal of my thesis was to develop a better understanding of the origins, dispersal, and evolution of the haplosporidian parasites of oysters in the genus *Bonamia.* By assessing and understanding the relatedness and dispersal of *Bonamia* species, we can better understand the origins of the parasites, which could ultimately help in management of bonamiasis. With new *Bonamia* parasites being observed outside of their type localities, this study should continue to alter the current view of *Bonamia* parasites' geographic and host ranges and will lead to additional, and perhaps alternative, hypotheses on the dispersal of *Bonamia* species.

CHAPTER ONE: Phylogenetics of *Bonamia* Species Based on Small Subunit Ribosomal DNA (SSU rDNA) and Internal Transcribed Spacer (ITS) Region rDNA Sequence Data

OBJECTIVES

Objective 1: Assess the global distribution and diversity of *Bonamia* parasites in hosts and locations worldwide.

Objective 2: Develop a better understanding of the origins and evolution of the genus *Bonamia* through phylogenetic analyses of small subunit (SSU) ribosomal DNA (rDNA) and internal transcribed spacer (ITS) region rDNA sequences.

INTRODUCTION

Bonamia species are protozoan parasites of oysters within the phylum Haplosporidia whose characteristic cell form is a 2-3 μm "microcell" (Pichot et al. 1980). There are four described species, which were described from four different oyster hosts: *Bonamia ostreae* Pichot et al., 1980 in *Ostrea edulis* L., *Bonamia roughleyi* (Farley et al. 1988) in *Saccostrea glomerata* (Gould, 1850), *Bonamia exitiosa* Hine et al., 2001 in *Ostrea chilensis* Philippi, 1845, and *Bonamia perspora* Carnegie et al., 2006 in *Ostrea equestris* Say, 1834. Species boundaries between *B. roughleyi* and *B. exitiosa*, however, were questioned in Hill et al. 2010, who concluded that there was no morphological, ultrastructural, or molecular basis for the distinction of these two species.

Other questions regarding the diversity of *Bonamia* species were also raised in conjunction with new discoveries of the geographic and host ranges of these parasites. Prior to 2004, it was thought that *Bonamia* species were more or less confined to their respective hosts and geographic locations—*B. ostreae* in *O. edulis* in the temperate Northern Hemisphere, and closely related *B. exitiosa* and *B. roughleyi* in the temperate Southern Hemisphere. However, this perspective was found to be suspect when Burreson et al. (2004) found a *B. exitiosa*-like species infecting experimental *Crassostrea ariakensis* (Fujita, 1913) in Bogue Sound, North Carolina. This parasite was subsequently observed infecting native oyster *O. equestris* in North Carolina, and this oyster was additionally found to be infected by a second, novel species that was described as *B. perspora* (Carnegie et al. 2006, Hill et al. 2010). Earlier perceptions of the host range, geographic distribution, and diversity of *Bonamia* species were, therefore, considerably altered.

A phylogeographic study of *Bonamia* parasites is important given the ecological and economic destruction these parasites can cause in oyster hosts. *Bonamia ostreae,* for example, contributed greatly to the collapse of *O. edulis* populations and fisheries in Europe (Grizel et al. 1988), and *B. exitiosa* has devastated *O. chilensis* populations in New Zealand (Hine et al. 2001). The objective of the research presented in this chapter was to assess the global distribution of *Bonamia* parasites by collecting potential oyster hosts from around the world to better understand the geographic and host ranges and the genetic diversity of these parasites. Separate phylogenies were constructed based on the small subunit ribosomal RNA gene (SSU rDNA) and internal transcribed spacer (ITS) region rDNA (defined as ITS-1 rDNA, the 5.8S rRNA gene, and ITS-2 rDNA) to better understand the evolutionary relationships of *Bonamia* species that were found in nearly every oyster host and every location examined.

MATERIALS AND METHODS

Sample Collection

Fourteen oyster species were collected from twenty-one locations around the world (Table 1.1). Most oysters were collected from natural habitats, however samples of *C. ariakensis* from North Carolina and Florida, USA were cultured and experimentally deployed to these locations; *Ostrea denselamellosa* from the Okayama Prefecture, Japan were cultured as part of a restoration program; and *O. angasi* from Australia, *S. glomerata* from Australia, and one sample of *O. chilensis* from Chile were obtained from commercial culture. Oysters were shucked, and small pieces of gill and mantle tissue (~3-5 mm^3) were either preserved individually in 95% ethanol or placed directly in lysis

solution (QIAamp DNA Kit; QIAGEN, Valencia, CA) for molecular analyses. The only exceptions were *O. edulis* and 2004 *Ostrea conchaphila* tissue samples collected from California. Tissues from *O. conchaphila* individuals were stored at -80°C in pools of three or four oysters/tube, then preserved in 100% ethanol for shipping. The California *O. edulis* samples were preserved in 95% ethanol, but again pooled: fourteen pools of four individuals, and one pool of two individuals in one sample. All instruments used for dissection were sterilized with 95% ethanol and flamed between each sample.

Gill, mantle, and visceral mass tissues were fixed in Davidson's fixative (Shaw and Battle 1957) for standard histopathology in most cases. Exceptions were the *Isognomon* sp. from Florida, *O. edulis* from the Netherlands, *O. chilensis* from Chile, and the 2006 *Ostrea sandvicensis* samples, in which tissues were collected for molecular analyses only.

DNA Extraction

Genomic DNA from each oyster sample was extracted using a QIAamp DNA Kit (QIAGEN). DNA was eluted from the QIAGEN column in 100–225 µl of elution buffer and stored at 4°C. For the pooled *O. edulis* samples from California, each pool was divided into 2 individuals per extraction (except one pool that had larger pieces of tissue allowing for division of the 4 individuals into 4 individual extractions). This was done in order to obtain a better estimate of prevalence. After each extraction, DNA was quantified using a GeneQuant *pro* spectrophotometer (Amersham Biosciences, Piscataway, NJ).

Bonamia-generic PCR

Oysters were screened for *Bonamia* spp. DNA using either generic PCR primers BON-319F and BON-524R (Hill et al. 2010), which target a 206-bp portion of *Bonamia* spp. SSU rDNA, or generic primers C_F and C_R (Carnegie et al. 2000), which amplify a 760-bp portion of *Bonamia* spp. SSU rDNA. For the BON-319F + BON-524R PCR, a 25-µl total volume reaction contained 1x PCR buffer (Applied Biosystems, Carlsbad, CA), 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.4 µg/µl bovine serum albumin (BSA), 0.25µM primer mix, 0.024 units/µl Ampli*Taq* DNA polymerase (Applied Biosystems) and 200-250 ng (=0.5-1.6 µl) template DNA. A 4-min initial denaturation at 94° C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, and then by a final extension at 72°C for 7 min. Products were electrophoresed on 2.5% agarose gels (100 V, 30 min), subsequently stained with ethidium bromide, and visualized under a UV light.

The 25-µl C_F + C_R reaction contained the same reagents and concentrations, but the thermal cycling program differed slightly: a 4-min initial denaturation at 94°C was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min, and then by a final extension at 72°C for 10 min. These products were electrophoresed on 2% agarose gels (100 volts, 30 minutes), stained with ethidium bromide, and visualized under UV light.

Bonamia spp. SSU rRNA Gene Sequencing

PCR products from infected oysters were used to generate *Bonamia* spp. SSU rDNA sequence. In order to generate sequence for the entire SSU rDNA region, which is

approximately 1750 bp in length, multiple PCR amplifications had to be performed. Several primer pairs were tested, and the pair that yielded a single amplicon of appropriate size was used for subsequent cloning reactions. In order to amplify the 5' end of *Bonamia* spp. SSU rDNA region, a reverse *Bonamia*-generic primer (either Bon-745R, Bon-927R, Bon-990R, Bon-1110R, or Bon-1050R) was paired with primer 16S-A (Medlin et al. 1988), a universal SSU rDNA-specific primer that amplifies eukaryotic rDNA (Table 1.2, Figure 1.1). The 3' end of *Bonamia* spp. SSU was generated using a forward *Bonamia*-generic primer (Bon-925F, or Bon-1310F) paired with primer 16S-B (Medlin et al. 1988) (Table 1.2). In most cases, a third PCR had to be done in order to complete the SSU rDNA region. Either $C_F + C_R$ or Bon-319F + Bon-990R primer pairs were used to generate sequencing data for the gap (Table 1.2). For the *Bonamia* sp. found in *O. sandvicensis* in Hawaii, however, a new primer, HIBon-620F, had to be designed in order to obtain the middle portion of the SSU rDNA sequence (Table 1.2). HIBon-620F was designed using MacVector 8.0 (Oxford Molecular Ltd., Oxford, UK) and was paired with Bon-1110R (White 2008). For each PCR, a 25-µl total reaction volume contained the same reagents at the same concentrations as the $C_F + C_R$ PCR described above. For these PCRs (except the $C_F + C_R$ reactions, which were done as described above), a 4-minute initial denaturation was followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 54-58°C for 45 s, extension at 72°C for 1 min (for products < ~ 800 bp) or 1.5 min (for > ~ 800-bp products), and then by a final extension at 72°C for 6 min. The selected amplification products from triplicate PCR reactions were pooled and purified using a QIAquick PCR purification kit (QIAGEN).

The only exceptions to the above were the reactions to obtain the *Bonamia* sp. SSU rDNA found in *O. edulis* from California. A 25-µl total reaction volume contained 1x PCR buffer (Invitrogen Corporation, Carlsbad, CA), 2mM $MgCl_2$, 0.2mM dNTPs, 0.25µM forward primer, 0.25µM reverse primer, and 0.05 units/µl Platinum *Taq* DNA polymerase (Invitrogen), and 200-250 ng template DNA. The thermal cycling program was as stated above. Duplicate reactions were individually purified; amplification products were not pooled as for other samples.

Purified PCR products were cloned into the plasmid vector pCR4-TOPO using the TOPO TA Cloning kit (Invitrogen), and then transformed into One Shot TOP10 competent *E. coli* cells (Invitrogen). The transformed cells were plated onto Luria Bertani (LB) agar plates containing 50 µg/ml ampicillin for selection of successful recombinant cells and incubated overnight at 37°C.

Colonies were screened using either phenol/chloroform/isoamyl-alcohol (PCI) extractions or PCR analyses. For screening by PCI extraction, a toothpick scraping from a single colony re-streak was placed in a microcentrifuge tube containing 40 µl 1x STE (100mM NaCl, 20mM Tris (pH =7.5), 10mM EDTA) using a sterile toothpick. Forty microliters of PCI (50% phenol/48% chloroform/2% isoamyl-alcohol) was added, and samples were vortexed briefly and then centrifuged at 13,000 rpm (5 min). The aqueous (top) layer was then removed to a clean tube. One microliter of RNase A (1 mg/ml) was added to 15 µl of lysate, which was then incubated for ~ 2 min at room temperature. Two microliters of gel loading dye was added to each sample, and the samples were loaded onto a 1.5% agarose gel. The products were electrophoresed (100 V, 60-75 min), stained

in ethidium bromide, and visualized under UV light to determine which selected clones contained inserts of appropriate size.

For direct screening using PCR, M13 Forward (5'-GTAAAACGACGGCCAG-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3') primers (Invitrogen), which flank the vector cloning site, were used. A toothpick scraping of a single colony re-streak was placed into 50 µl PCR dH_2O and boiled at 100°C (10 min). One microliter of this "boiled prep" was used in a 25-µl reaction containing 1x PCR buffer (Applied Biosystems), 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.4 µg/ml BSA, 0.2µM M13 Forward, 0.2µM M13 Reverse, and 0.024 units/µl Ampli*Taq* DNA polymerase (Applied Biosystems). A 2-min initial denaturation at 94°C was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min, and then by a final extension at 72°C for 5 min. PCR products were loaded onto 1.5% or 2% agarose gels, and subsequently electrophoresed (100 V, 30 min), stained with ethidium bromide, and visualized using UV light.

Clones with inserts of desired size were cultured in 4 ml 2x YT media plus ampicillin (0.05 mg/ml) overnight in a 37°C shaking water bath. The plasmids were extracted using the QIAprep Spin Miniprep Kit protocol (QIAGEN), and sequenced on either a LI-COR 4200L (LICOR, Lincoln, NE) or a 16-capillary Applied Biosystems 3130*xl* Genetic Analyzer.

For sequencing on the LI-COR 4200L, the concentration of each plasmid was determined using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, CA). Simultaneous bidirectional cycle-sequencing reactions were performed using a Thermo Sequenase Sequencing Kit (Amersham Biosciences) and M13 forward

and reverse infrared-labeled primers (LI-COR) following the manufacturer's protocol. Products of the sequencing reactions were electrophoresed and detected on the LI-COR sequencer.

For sequencing on the Applied Biosystems 3130*xl* Genetic Analyzer, plasmids underwent bidirectional sequencing reactions using a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) using 1/8 of the reaction size recommended by the manufacturer's instructions with unlabelled M13/pUC forward or reverse sequencing primers (New England Biolabs, Ipswich, MA). The products of the sequencing reactions were cleaned using an ethanol/sodium acetate protocol (ABI User Bulletin, April 11, 2002). The precipitated sequences were re-suspended in 20 μL of Hi-Di formamide (Applied Biosystems). Ten microliters of resuspended DNA was added to a 96-well plate and electrophoresed on the sequencer. Sequencing Analysis 5.2 software (Applied Biosystems) was used for base calling.

Bonamia spp. Internal Transcribed Spacer (ITS) Region rDNA Sequencing

PCR products from oysters that tested positive for *Bonamia* spp. were used to determine the sequence of *Bonamia* sp. ITS region rDNA from each host species using primers HaploITSf (Hill et al. 2010) and ITS-B (= reverse primer D, Goggin 1994) (Table 1.2). This primer pair amplifies partial SSU rDNA (~220 bps of the 3' end of the SSU rRNA gene), complete ITS-1, 5.8S, and ITS-2 region rDNA, and partial large subunit (LSU) rDNA of most haplosporidians. Prior to June 2008, cloning reactions were done in triplicate and pooled as described above for SSU rDNA sequencing. These reactions were done using Applied Biosystems reagents at the same concentrations as

described above for the $C_F + C_R$ PCR. After June 2008, duplicate reactions were performed on each individual or pool of individuals, so that each reaction could be individually sequenced and compared. A 25-µl total reaction contained 1x PCR Buffer (Invitrogen), 2-2.5mM $MgCl_2$, 0.2mM dNTPs, each primer at 0.25µM, 0.05 U/µl Platinum *Taq* DNA polymerase (Invitrogen), and 200-250 ng (=0.5-1.6 µl) template DNA. Regardless of reagents used, a 7-min initial denaturation was followed by 35 cycles of denaturation at 95°C for 1 min, annealing between 55-61°C for 1 min, and extension at 72°C for 1.5 min, and then by a final extension at 72°C for 7 min. These products were purified, cloned, transformed, screened, and sequenced as in the above description of SSU rDNA sequencing.

Oyster Mitochondrial 16S Ribosomal RNA Gene Sequencing

All oysters were presumptively identified based on morphological characteristics and sample locations. In some cases, universal primers 16Sar and 16Sbr (Kessing et al. 1989; Table 1.2) were used to amplify a portion of the mitochondrial 16S (mt16S) rRNA gene of *Bonamia*-positive host species to confirm oyster identity: *Ostrea auporia* from New Zealand, *O. conchaphila* and *O. edulis* from California, *O. equestris* from Argentina, *O. stentina* from Tunisia, and *O. sandvicensis* from Hawaii. A 25-µl total reaction contained 1x PCR Buffer (Applied Biosystems), 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.4 µg/µl BSA, 0.20µM primer mix, 0.024 units/µl Ampli*Taq* DNA polymerase (Applied Biosystems), and 200-250 ng (=0.5–2.5 µl) template DNA. Initial denaturation at 95°C for 4 min was followed by 38 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min, and by a final extension at 72°C for 7 min.

Products were electrophoresed and visualized as above. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and quantified using either a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc.) or a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) as per manufacturers' instructions. Three to ten nanograms of the purified PCR product was then added to the reagents from a BigDye® Terminator v3.1 cycle sequencing kit (using 1/8 of the reaction size recommended by the manufacturer's instructions) and the 16Sar and 16Sbr primers (Kessing et al. 1989). Reactions were then cleaned, precipitated, and sequenced as above. Primers were cropped from the resulting sequences, and complementary sequences were compared to one another and to their chromatograms using MacVector 8.0 (Oxford Molecular Ltd., Oxford, UK) or CodonCode Aligner (CodonCode Corp., Dedham, MA). These were then compared, using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997), to the National Center for Biotechnology Information (NCBI) GenBank database. *Ostrea equestris* samples from Argentina were sequenced by Dr. Ami Wilbur at the University of North Carolina Wilmington using the same primer set.

Bonamia spp. Sequence Alignments and Molecular Phylogenetics

Primers were removed from the resulting sequences, and complementary sequences were compared to one another and to their chromatograms using MacVector 8.0 or CodonCode Aligner. For SSU rDNA sequences, a consensus sequence was generated for each *Bonamia* sp. found in a host species. Because the SSU rDNA had to be sequenced in two or three fragments, the consensus sequence for each segment was pieced together by aligning the sequences in MacVector 8.0 to generate a single SSU

rDNA sequence for each presumptive *Bonamia* sp. found in an oyster host. These were then compared to the NCBI GenBank database using BLAST. The newly generated SSU rDNA *Bonamia* spp. sequences from *O. edulis* and *O. conchaphila* from California, *O. equestris* from Argentina, *S. glomerata* from Australia, *O. auporia* and *O. chilensis* from New Zealand, *C. ariakensis* from Florida, and *O. sandvicensis* from Hawaii were then aligned with published SSU rDNA sequences. These included *B. ostreae* (GenBank accession numbers AF262995 and AF192759), *B. exitiosa* (AF337563), *B. roughleyi* (AF508801), and *B. perspora* (DQ356000); the *Bonamia* spp. from *O. stentina* from Tunisia (GQ385242), *C. ariakensis* from North Carolina (AY542903), *O. edulis* from Spain (EU016528) and Italy (EU598800 and EU598801), *O. angasi* from Australia (DQ312295 and JF495408), *O. chilensis* from Chile (AY860060 and GQ366703), and *O. puelchana* from Argentina (JF495409); and outgroup species *Minchinia tapetis* (AY449710), *Minchinia teredinis* (U20319), *Minchinia chitonis* (AY449711), and *Minchinia mercenariae* (FJ518816). *Minchinia* spp. were chosen for the outgroup because *Minchinia* is sister to *Bonamia* in the haplosporidian phylogeny (Reece et al. 2004). Alignments were generated using MAFFT v. 6 (Katoh and Toh 2008) using the automatic setting (which determines the best algorithm given the dataset).

Bonamia spp. ITS region rDNA sequences amplified from *C. ariakensis* from Florida, *O. angasi* and *S. glomerata* from Australia, *O. auporia* and *O. chilensis* from New Zealand, *O. chilensis* from Chile, *O. conchaphila* and *O. edulis* from California, *O. edulis* from the Netherlands, *O. equestris* and *O. puelchana* from Argentina, *O. equestris* from North and South Carolina, *O. stentina* from Tunisia, and *O. sandvicensis* from Hawaii were aligned with VIMS Shellfish Pathology Laboratory archival *Bonamia* spp.

ITS region rDNA sequences from *C. ariakensis* from North Carolina, *O. angasi* from Australia, *O. chilensis* from New Zealand and Chile, *B. ostreae* from *O. edulis* from Maine, and from *B. perspora* from *O. equestris* from North Carolina. GenBank-published sequences from *Bonamia* sp. from *O. edulis* from the Adriatic Sea (EU672891), *Bonamia* sp. from *O. chilensis* from Chile (AY539840), *B. exitiosa* (DQ312295), and *B. ostreae* (AF162097, AF262995) were too short; these were not used so as to not affect subsequent phylogenetic and distance analyses. All sequences were bidirectional except the archival *Bonamia* sp. sequences from *O. angasi*, and some of these sequences were missing part of the 5.8S rDNA gene. The alignment was done using the automatic setting in MAFFT v. 6 (Katoh and Toh 2008).

Parsimony analysis of SSU rDNA sequence data was conducted using PAUP* 4.0b10 (Swofford 2002). One thousand bootstrap replicates with 100 random additions were performed. Gaps were treated as missing. Parsimony analysis of ITS region sequence data was done using TNT v. 1.1 (Tree analysis using New Technology; Goloboff, Farris, and Nixon 2008) made available with the sponsorship of the Willi Hennig Society. A new technology search was completed with 100 bootstrap replicates and 10 random additions. A 50% majority rule unrooted consensus tree was generated using PAUP* 4.0b10.

Bayesian inference analyses of SSU and ITS region rDNA sequence data were conducted using MrBayes v. 3.1.2 (Ronquist and Huesenbeck 2003). MrModeltest v. 2.3 was used to determine the best model for each dataset. Are We There Yet? (AWTY; Wilgenbusch et al. 2004) was used to determine if stationarity had been reached for the

SSU rDNA dataset. Fifty percent majority rule consensus trees were generated using PAUP*4.0b10 (Swofford 2002).

Distance Analyses

Distance analyses were done using MEGA v. 4 (Tamura et al. 2007). Incomplete sequences were removed from the above alignments so that all sequences spanned identical gene regions. For the ITS region rDNA alignment, the SSU rDNA portion of the amplicon produced by the HaploITSf + ITS-B primer set was removed. The datasets were then realigned using MAFFT v. 6 (Katoh and Toh 2008). For the *Bonamia* spp. SSU rDNA dataset, uncorrected p-distances were calculated between the different *Bonamia* clades that were indicated by the parsimony and Bayesian analyses. Gaps and missing data were only eliminated in pairwise sequence comparisons (pairwise deletion option). Standard error estimates were determined by a bootstrap procedure (1000 replicates). For the ITS region rDNA dataset, uncorrected p-distances were calculated between and within each *Bonamia* clade, again as indicated by the parsimony and Bayesian analyses of the ITS region rDNA sequences. Again, the pairwise deletion option was used and standard error estimates were determined by a bootstrap procedure (1000 replicates).

In Situ Hybridization

Standard, chromogenic *in situ* hybridization (ISH) assays were performed on tissue sections of *C. ariakensis* from Florida, *O. auporia* from New Zealand, and *O. conchaphila* and *O. edulis* from California that were PCR-positive for a *Bonamia* sp. The assays relied on a cocktail of three digoxigenin-labeled, *B. exitiosa*-specific anti-sense

probes, CaBon461, CaBon166, and CaBon1704 (Hill et al. 2010; Table 1.2), and a newly designed *B. ostreae*-specific probe, Bost171 (Table 1.2). For each sample, experiments included the following treatments applied to serial histological sections: a no probe control (25 µl hybridization buffer only), a positive control, and a standard experimental treatment using CaBon probes (Hill et al. 2010) and/or Bost171 (Table 1.2). The protocol was modified from Stokes and Burreson (2001). Paraffin-embedded tissue sections (6 µm) were de-paraffinized in xylene for 10 minutes, re-hydrated in a series of ethanol washes (100% ethanol x 2 for 10 min; then 95%, 80%, 70% ethanol for 1 min each), and then placed in tap water for 1 min. Slides were then equilibrated in phosphate-buffered saline (PBS, 2 x 5 min) and subsequently treated with proteinase K (50 µg/ml in PBS, 18 min at 37°C). In order to stop proteolysis, the slides were then washed with 0.2% glycine in PBS (5 min at room temperature) followed by a 10-min incubation in 2x SSC (3M NaCl, 0.3M sodium-citrate, pH 7.0) at room temperature.

Slides were subsequently placed in a slide mailer box filled with 16 ml pre-warmed prehybridization solution (4x SSC, 50% formamide, 5x Denhardt's solution, 0.5 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA, distilled water) for 1 h at 42°C. The slides were removed from the boxes and carefully wiped to remove any excess solution. A PAP pen was used to encircle the tissue, creating a reservoir for the digoxigenin-labeled oligonucleotide probe cocktail (50 µl total volume; 2 ng/µl each of CaBon461, CaBon166, and CaBon1704 for the *B. exitiosa*-specific ISH, and 3 ng/µl of Bost171 for the *B. ostreae*-specific ISH), which was subsequently pipetted onto the tissue section and covered with a plastic cover slip. The slides were incubated in a thermocycler equipped with a slide holder at 90°C for 12 min and then cooled on ice for 1-2 min. A humid

chamber was prepared by using a plastic, airtight container and placing 4x SSC + 50% formamide solution-saturated 3MM paper in the bottom. The slides were then placed on wooden applicator sticks inside the humid chamber and sealed in the chamber for incubation overnight at 42°C.

The next morning, cover slips were removed, and then the slides were treated with a descending series of SSC washes (2x SSC, 2 x 5 min at room temperature; 1x SSC, 2 x 5 min at room temperature; 0.5x SSC, 2 x 10 min at 42°C or 45°C for the Bost171 probe and CaBon probe cocktail, respectively). The slides were then placed in buffer 1 (100mM Tris-HCl, 150mM NaCl, pH 7.5; 1 min at room temperature) and then in a slide mailer box filled with 16 ml of blocking solution (buffer 1, 0.3% Triton, 2% normal sheep serum; 30 min at room temperature). The anti-digoxigenin alkaline phosphatase conjugate (Roche Applied Science, Indianapolis, IN) was then diluted 1:500 with antibody solution (buffer 1, 0.3% Triton, 1% normal sheep serum), and 50 µl of the diluted antibody was placed on each slide and coverslipped with a plastic cover. The slides were placed in the humid chamber (with fresh 3MM paper saturated with water and new wooden applicator sticks) for 3 h at room temperature.

After the 3-h incubation, the cover slips were removed, and slides were washed in buffer 1 (2 x 5 min), then in buffer 2 (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5; 2 x 5 min). Then the slides were added to a slide mailer box filled with 16 ml of color solution (buffer 2, 74.2 mg/ml nitroblue tetrazolium (NBT), 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution, 24 mg/ml levamisole). The slide box was covered with foil for a 2-h incubation at room temperature. Slides were placed in TE (10mM Tris-HCl, 1mM EDTA, pH 8; 5 min at room temperature) in order to stop the

color reaction, and then carefully rinsed in distilled water. After staining in 0.05% Bismarck Brown Y (filtered with Whatman 4 filter paper) for 2 min, the slides were rinsed with distilled water (3 x). An aqueous-based mounting medium was used for coverslipping. After the slides were dry, they were examined on an Olympus BX51 light microscope.

A fluorescent *in situ* hybridization (FISH) assay was used to detect the *Bonamia* sp. found in *O. sandvicensis*. Anti-sense probes HIBon-167 (5'-CTAATATGCACAGCCGCCAG-3') and HIBon-634 (5'-CGATTATGGCCTCTCTCCAC-3') tagged with Alexa Fluor 488 labels (purchased from Invitrogen) were designed in MacVector 8.0 to specifically target this parasite's SSU rRNA. The assay was optimized and tested for specificity against known *Bonamia* species (*B. exitiosa*, *B. perspora*, and *B. ostreae*). Slides were treated with HIBon-167+HIBon-634 (each at 10 ng/µl), and a no probe control (hybridization buffer only) and a positive control for ensuring general probe accessibility to oyster tissue (Oe-309 at 10 ng/µl, 5'-TCATGCTCCCTCTCCGG-3') were also used.

The FISH assay was performed as in Carnegie et al. (2006) except a descending ethanol series was used for rehydration instead of a descending isopropanol series. Tissue sections were deparaffinized in xylene (3 x for 2 min) and subsequently rehydrated in the descending ethanol series (100% ethanol, 3 x 30 s; then 80%, 50%, 30% for 30 s each) into tap water (1 min). The tissue was then equilibrated in PBS (1 min) and digested with Proteinase K (100 µg/ml in PBS) for 10 min at 37°C. This was followed by a wash in PBS plus 0.2% (w/v) glycine (5 min) and acetylation using acetic anhydride (5% [v/v] in 0.1M triethanolamine-HCl, pH 8.0; 10 min at room temperature), a wash in PBS (5 min),

and equilibration in 5x SET (750mM NaCl, 6.4mM EDTA, 100mM Tris pH 8; 5 min at room temperature). Slides were flooded with hybridization buffer (5x SET, 0.02 mg/ml BSA, 0.025% [w/v] SDS; 10 min at 42°C) and drained of excess buffer. Then, 25 µl of hybridization buffer containing HIBon-167 and HIBon-634 (each at 10 ng/µl) was added to each slide. After the slides were covered with plastic or paraffin coverslips, they were incubated overnight at 42°C in a humid chamber. The next day, they were washed with 0.2x SET (3 x at 42°C for 2.5 min total), air dried, mounted with glycerol-in-PBS medium, and coverslipped. Slides were then evaluated on an Olympus Provis epifluorescence microscope equipped with a red-green dual bandpass filter.

RESULTS

Bonamia-generic PCR

Putative *Bonamia* spp. were detected in 11 out of 14 oyster species examined, with *Isognomon* sp. from Florida, *O. denselamellosa* from Japan, and *Saccostrea cucullata* from New Zealand being exceptions (Table 1.1). A putative *Bonamia* sp. was also not found in *S. glomerata* from Whangarei Harbour, New Zealand or in *O. conchaphila* from Drakes Estero, California or British Columbia, Canada. However, these oyster species were positive for a *Bonamia* sp. at other locations: *S. glomerata* in New South Wales, Australia and in *O. conchaphila* from Elkhorn Slough, California (Table 1.1).

PCR prevalence of *Bonamia* spp. parasitism ranged from 0.5-88.3% overall (Table 1.1). *Bonamia* spp. were detected in experimentally deployed *C. ariakensis* in North Carolina and Florida (PCR prevalence range 35.3-42.1%), wild *O. equestris*

collected in North and South Carolina (2.0-4.0%), *O. conchaphila* from Elkhorn Slough, California in 2004 (21.7-86.7%) and 2009 samples (88.3%), *O. edulis* from California (20.7-82.0%), *O. sandvicensis* from Hawaii in 2006 (65.8%) and 2007 samples (70.0%), *O. angasi* from Australia (2.4%), *S. glomerata* from Australia (0.5%), *O. auporia* from New Zealand in 2007 (9.1%) and 2009 samples (5.6%), *O. chilensis* from New Zealand (5.0%), *O. chilensis* from Chile (18.8%), *O. puelchana* from Argentina (10.5%), and *O. stentina* from Tunisia (10.6%). Other samples were sent to our laboratory as confirmed *Bonamia* sp. positive as determined by histopathology: *O. equestris* from Argentina, *O. edulis* from the Netherlands and Maine, and *O. chilensis* from Chile and New Zealand (indicated by a single * in Table 1.1).

Alignment Results

For the SSU rDNA alignment, MAFFT v.6 chose L-INS-i, which is an iterative refinement method that aligns a set of sequences containing one alignable domain, and in the pair-wise alignment, flanking sequences are ignored by the Smith-Waterman algorithm. For the ITS rDNA alignment, MAFFT v. 6 chose the standard strategy, FFT-NS-i iterative refinement method (max. 2 iterations).

SSU rDNA Sequencing Results

Complete SSU rDNA sequences of putative *Bonamia* sp. parasites were found in *C. ariakensis* from Florida (GenBank accession number JF831807), *O. auporia* from New Zealand (JF831806), *O. edulis* and *O. conchaphila* from California (JF831804 and JF831805, respectively), *O. equestris* from Argentina (JF831801), *S. glomerata* from

Australia (JF831802), and *O. sandvicensis* from Hawaii (JF831803). One to three PCR-positive oysters, two to three primer sets, and three to 10 clones per primer set were used to obtain complete SSU rDNA sequences (Table 1.3). Putative *Bonamia* spp. SSU rDNA consensus sequences ranged from 1749 to 1766 bp in length. The putative *Bonamia* sp. SSU rDNA sequences found in *C. ariakensis* from Florida, *O. auporia* from New Zealand, *S. glomerata* from Australia, *O. conchaphila* from California, and *O. equestris* from Argentina were 99-100% identical to the SSU rDNA sequences of *B. exitiosa* from Australia (DQ312295) and New Zealand (AF337563), *B. roughleyi* (AF337563), *Bonamia* sp. in *O. stentina* from Tunisia (GQ385242), and *Bonamia* sp. in *C. ariakensis* from North Carolina (AY542903).

Two putative *Bonamia* spp. were detected in *O. edulis* from California. The SSU rDNA sequence of one was 99% identical to *B. exitiosa* (DQ312295) and *B. roughleyi* (AF337563). Sequence from the other was 99% identical to *B. ostreae* SSU rDNA (AF262995 and AF192759).

The putative *Bonamia* sp. SSU rDNA sequence found in *O. sandvicensis* from Hawaii was 90-91% similar to that of *B. exitiosa* (DQ312295), *B. roughleyi* (AF337563), the *Bonamia* sp. from *O. stentina* (GQ385242), the *Bonamia* sp. from *C. ariakensis* from North Carolina (AY542903), *B. ostreae* (AF262995), and *B. perspora* (DQ356000).

ITS Region rDNA Sequencing Results

Putative *Bonamia* sp. ITS region rDNA sequences were found in all hosts from which putative *Bonamia* sp. SSU rDNA sequences were characterized, including *C. ariakensis* from Florida (JF712867 – JF712871), *O. equestris* from North and South

Carolina (JF831575 – JF831602), *O. equestris* from Argentina (JF831556 – JF831574), *O. puelchana* from Argentina (JF831603 – JF831638), *O. stentina* from Tunisia (JF831684 – JF831718), *O. auporia* from New Zealand, (JF831658 – JF831677), *O. chilensis* from New Zealand (JF831639 – JF831657), *O. angasi* from Australia (JF831678 – JF831680), *S. glomerata* from Australia (JF831681 – JF831684), *O. conchaphila* from California (JF831719 – JF831800), *O. chilensis* from Chile (JF831849 – JF831856), *O. edulis* from California (JF831808 – JF831848), *O. sandvicensis* from Hawaii (JF831863 – JF831879), and *O. edulis* from the Netherlands (JF831857 – JF831862). GenBank accession numbers for each unique sequence found per oyster host species and used in subsequent ITS region rDNA distance and phylogenetic analyses are listed in Table 1.4. One to 29 clones per individual were sequenced (with one to seven *Bonamia* spp. PCR-positive individuals sequenced per oyster host) (Table 1.4).

Most *Bonamia* sequences were 98-100% similar to *B. exitiosa* ITS region rDNA sequences (Table 1.5). "Type" *B. exitiosa* ITS region rDNA sequences were designated as such since they came from the type host, *O. chilensis*, and type location, New Zealand. The two most commonly found sequences matched those of accession numbers EU709070 and EU709073. Two sets of putative *Bonamia* sp. ITS region rDNA sequences, *Bonamia* sp. sequences from Chile and *Bonamia* sp. sequences in *O. edulis* from California, were only 83-85% similar to *B. exitiosa*; although 99-100% similarity to *B. exitiosa* SSU rDNA was observed. These sequences were only 83-86% similar to each other.

Both sets of putative *B. ostreae* ITS region rDNA sequences found in *O. edulis* from California and the Netherlands showed similarity to *B. ostreae* from Maine

(EU709108 and EU709110): 90-99% and 95-98%, respectively. When a BLAST search was performed on putative *Bonamia* sp. ITS region rDNA sequences from *O. sandvicensis* from Hawaii, *Bonamia* spp., *Haplosporidium costale*, and *Haplosporidium nelsoni* sequences were the only ones to produce significant alignments. However, these were aligning only in the SSU rDNA portion of the amplicon; there were no identical or close matches to the ITS region rDNA.

Phylogenetic Analyses of Bonamia spp. SSU rDNA

For the Bayesian analyses, MrModeltest v. 2.3 chose GTR + I + Γ (General Time Reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites) was chosen for the SSU rDNA data. Ten thousand trees were generated (10,000,000 generations; sample frequency = 1,000), and the first 25% were removed as burnin.

Parsimony and Bayesian analyses of the SSU rDNA sequences yielded trees with similar topologies (Figures 1.2 and 1.3). The *Bonamia* sp. found in *O. sandvicensis* (GenBank accession number JF831803) was basal to other *Bonamia* lineages, with 100% bootstrap support in the parsimony analysis and a posterior probability of 100 in the Bayesian analysis for its inclusion in the *Bonamia* spp. clade. All of the following sequences formed a monophyletic clade with *B. exitiosa* (AF337563 and DQ312295) and *B. roughleyi* (AF508801) in both analyses (bootstrap support = 78%, posterior probability = 99): the *Bonamia* spp. from *O. edulis* and *O. conchaphila* from California (JF831804 and JF831805, respectively), *O. chilensis* from Chile (GQ366703 and AY860060), *O. puelchana* and *O. equestris* from Argentina (JF495409 and JF831801, respectively), *O.*

stentina from Tunisia (GQ385242), *O. edulis* from Spain (EU016528), *O. auporia* from New Zealand (JF831806), *O. angasi* and *S. glomerata* from Australia (JF495408 and JF831802, respectively), *C. ariakensis* from North Carolina and Florida (AY542903 and JF831807, respectively), *O. chilensis* from New Zealand (JF495410), and two partial *Bonamia* sp. SSU rDNA sequences from *O. edulis* from Italy (EU598800 and EU598801). Parsimony analysis portrayed this large clade as a strongly supported sister clade to the *B. ostreae-B. perspora* clade (bootstrap support = 100%), whereas Bayesian analysis indicated a less clear relationship between these two clades (posterior probability = 76). A *B. perspora-B. ostreae* clade was only weakly supported (bootstrap support = 51, posterior probability = 79).

Phylogenetic Analyses of Bonamia spp. ITS Region rDNA

Parsimony analysis of the ITS region rDNA data produced six well-supported clades in an unrooted 50% majority rule consensus tree (Figure 1.4). All of the *Bonamia* sp. sequences found in *O. sandvicensis* from Hawaii formed a monophyletic clade (bootstrap support = 100) and in the analysis appeared sister to the monophyletic clade of *B. perspora* (bootstrap support = 100). The *B. ostreae* sequences from Maine, California, France, and the Netherlands also formed a monophyletic clade (bootstrap support = 96) and appeared sister to the clade containing the Hawaiian *Bonamia* sp. sequences and *B. perspora* (bootstrap support =100). Sister to the *B. ostreae* clade (bootstrap support = 100) was a monophyletic clade containing all sequences that grouped with *B. exitiosa*/*B. roughleyi* in the SSU analyses (bootstrap support =100). Within this clade, the *Bonamia* sp. ITS region rDNA sequences found in *O. edulis* from California formed a distinct

clade separate from the *Bonamia* sp. sequences found in *O. chilensis* from Chile. Each of these formed monophyletic clades (bootstrap support = 100) and a sister relationship between these sequences was weakly supported (bootstrap support = 77). This entire clade was sister to a monophyletic clade (bootstrap support = 88) of "type" *B. exitiosa* ITS region rDNA sequences and *Bonamia* sp. sequences found in *C. ariakensis* from North Carolina and Florida, *O. equestris* from North Carolina and South Carolina, *O. equestris* and *O. puelchana* from Argentina, *O. conchaphila* from California, *O. angasi* and *S. glomerata* from Australia, *O. auporia* and *O. chilensis* from New Zealand, and *O. stentina* from Tunisia. Within the *B. exitiosa* clade, a weakly supported subclade (bootstrap = 70) contained sequences found only in *O. conchaphila* from California.

For the Bayesian analysis, MrModeltest v. 2.3 chose GTR + I + Γ (General Time Reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites) for the ITS region rDNA data. Forty million generations were performed (sample frequency = 1000), resulting in forty thousand trees with the first 34,999 trees removed in order to compute the consensus. A 50% majority rule Bayesian consensus tree was generated based on the remaining 5,001 trees (Figure 1.5), and as in the parsimony analysis, there were six well-supported, monophyletic clades (posterior probabilities = 100). The topology, however, differed somewhat: the *B. exitiosa* clade appeared sister to the clade containing *Bonamia* sp. sequences found in *O. edulis* from California, and together appeared sister to the clade containing *Bonamia* sp. sequences found in *O. chilensis* from Chile (though this sister relationship was not supported, posterior probability = 69); and all of the *Bonamia* sp. sequences found in *O.*

sandvicensis from Hawaii appeared sister to the monophyletic clade of *B. ostreae* sequences (posterior probability of sister relationship = 100).

Distance Analyses

Uncorrected p-distances, or the total number of base differences per site averaged over all sequence pairs, between *Bonamia* spp. clades as indicated by the SSU rDNA phylogenetic analyses are shown in Table 1.6. 1670 positions were analyzed in the final dataset. Uncorrected p-distances were comparable, ranging from 0.025 – 0.031, except between all described *Bonamia* spp. and the Hawaiian *Bonamia* sp. (0.084 – 0.086).

Table 1.7 shows the mean uncorrected p-distances, or number of base substitutions per site averaged over all sequence pairs, *within* each *Bonamia* clade as indicated by the ITS rDNA phylogenetic analyses. The greatest uncorrected p-distance was among *B. ostreae* sequences and among the Hawaiian *Bonamia* sp. sequences. Mean uncorrected p-distances *between* each *Bonamia* clade as indicated by the ITS rDNA phylogenetic analyses is shown in Table 1.8. Estimates of divergence range from 0.094 to 0.324. 717 total positions were analyzed in the ITS region rDNA final dataset containing 242 *B. exitiosa* sequences, 27 *Bonamia* sp. sequences from Chilean *O. chilensis*, 17 Hawaiian *Bonamia* sp. sequences, 19 *Bonamia* sp. from Californian *O. edulis*, 17 *B. perspora* sequences, and 31 *B. ostreae* sequences.

Host Species Confirmation

Mitochondrial 16S rDNA sequencing of infected host species confirmed the morphological identifications of oysters. BLAST searches revealed that the *O. edulis*

mt16S rDNA sequence from California was identical to the *O. edulis* 16S rDNA sequences in GenBank (DQ093488 and DQ280032). The mt16S rDNA sequence of *O. auporia* was 99% similar to *O. equestris* (AY376603) and *O. auporia* (AF052064). The *O. conchaphila* mt16S rDNA sequence from California was identical to that of *Ostrea lurida* from British Columbia, Canada (FJ768589), and molecular analyses suggest these may be synonymous (Polson et al. 2009). The *O. sandvicensis* mt16S region rDNA was 99% similar to the fingerprint oyster, *Dendostrea crenulifera* (syn. to *Pustulostrea tuberculata* Lamarck 1804 as reported in Carriker and Gaffney 1996; EU815984 and EU815985) and 94-95% similar to *Alectryonella plicatula* Gmelin 1790 (AF052072). There were no *O. sandvicensis* sequences in GenBank. Dr. Ami Wilbur at the University of North Carolina Wilmington confirmed the identity of infected and *Bonamia* spp. PCR-positive *O. equestris* from Argentina, and the identity of *O. stentina* was confirmed in Hill et al. (2010).

Histopathology and In Situ Hybridization

Microcells were observed in at least one individual from each oyster host that was PCR-positive for *Bonamia* spp. except *O. auporia* from New Zealand. The histological presentation of the parasite in each host was typical: microcells characteristic of *Bonamia* spp. were found both extracellularly and within hemocytes, with no observation of more conventionally haplosporidian forms such as spores or large multinucleate plasmodia. Infections were light to moderate in intensity except in *C. ariakensis*, which can be heavily infected (Burreson et al. 2004), and light to moderate hemocytosis was typically observed as a host response. Disruption of tissue was only modest (Figures 1.6 – 1.8).

In situ hybridization using digoxigenin-labeled probes was also performed on four *C. ariakensis* from Florida, four *O. conchaphila* from California (three individuals from the 2004 sample and one individual from the 2009 sample), four *O. edulis* from California, and three *O. auporia* from New Zealand. Hybridization to the *B. exitiosa*/*B. exitiosa*-like specific probes, which target SSU rRNA, was observed in at least one of the oysters examined (Figures 1.9 and 1.10) from each species except for in *O. auporia*. Most infections were light. The *O. conchaphila* and *O. edulis* samples from California showed no hybridization to the *B. ostreae*-specific probe. All positive and negative controls performed as expected.

Fluorescent *in situ* hybridization was performed on *O. sandvicensis* from Hawaii using anti-sense probes HIBon-167 and HIBon-634 (Table 1.2) specific to this particular species. Hybridization was observed in one oyster that was diagnosed as a moderate, systemic infection by histopathology (Figure 1.10). The no-probe control showed no hybridization, and hybridization did not occur in samples infected with other known *Bonamia* species.

DISCUSSION

From this study, it is apparent that *Bonamia* species have a wider geographic and host distribution and are more diverse than first appreciated. *Bonamia* spp. were detected by PCR, histopathology, and ISH in almost every oyster host and location examined (Table 1.1). *Bonamia exitiosa* appears to be particularly widespread, infecting a variety of oyster hosts around the world; while *B. ostreae* and *B. perspora* seem to be host specialists maintaining well-defined geographic ranges. Cryptic *Bonamia* species

diversity was also uncovered in Hawaiian *O. sandvicensis* and California *O. edulis* hosts, while *B. roughleyi* was not detected in any samples.

Bonamia exitiosa and Bonamia exitiosa-like species

BLAST searches (Altschul et al. 1997) and phylogenetic analyses of SSU and ITS region rDNA revealed the affinity of most *Bonamia* sp. sequences, found in hosts around the world, to *B. exitiosa*. In all phylogenetic analyses (Figures 1.2 – 1.4), *Bonamia* sequences found in *C. ariakensis* from Florida and North Carolina, *O. equestris* from North Carolina, South Carolina, and Argentina, *O. puelchana* from Argentina, *O. conchaphila* from California, *O. angasi* and *S. glomerata* from Australia, *O. auporia* from New Zealand, *O. chilensis* from New Zealand, and *O. stentina* from Tunisia appeared in the same clade as *B. exitiosa* (and *B. roughleyi* in the SSU rDNA phylogenetic analyses). *Bonamia* sequences from *O. edulis* from California and *O. chilensis* from Chile appeared in the *B. exitiosa/B. roughleyi* clade in SSU rDNA phylogenetic analyses (Figures 1.2 and 1.3), but had divergent ITS region rDNA sequences (Figures 1.4 and 1.5). Histological presentation of these divergent parasites was similar in all cases, as expected given the lack of morphological differentiation even between described species, so histopathology was not a useful for delineating these cryptic species. However, molecular data such as ITS region rDNA was useful for revealing diversity, and provide a guideline for what should be considered *B. exitiosa* and what is divergent. All the *Bonamia* sp. sequences appearing in the same clade as *B. exitiosa* in both the SSU and ITS region rDNA phylogenetic analyses should thus be considered *B. exitiosa*.

Because the *Bonamia* sp. in *O. edulis* from California and the *Bonamia* sp. in *O. chilensis* from Chile were only divergent from *B. exitiosa* in the ITS region rDNA (83-85% similarity, Table 1.5; 83-86% similarity to each other), but not in the SSU rDNA, I feel that it is appropriate for these species, and any other *B. exitiosa* species that have been identified based solely on SSU rDNA, to be considered "*B. exitiosa*-like" until additional loci have been characterized. A monophyletic clade with 100% bootstrap support in the parsimony analysis and a posterior probability of 100 in the Bayesian analysis unrooted consensus trees (Figures 1.4 and 1.5) contained *B. exitiosa* sequences as well as sequences from these two samples. Within this clade, there appear to be three distinct clades: the *Bonamia* sp. in *O. edulis* from California (bootstrap support = 100, posterior probability = 100), the *Bonamia* sp. in *O. chilensis* from Chile (bootstrap support = 100, posterior probability = 100), and *B. exitiosa* (bootstrap support = 88, posterior probability = 100). In the uncorrected p-distances analysis between *Bonamia* species in the ITS region rDNA, these three clades are more divergent from other *Bonamia* clades than from one another (Table 1.8). Because of this affinity, it is possible that these may be strains of *B. exitiosa*. However, it is possible that these species may be novel. Given the variability of ITS region rDNA, sequence alignments are not easy to verify. So, while the alignments and subsequent analyses are the best they can be, I would caution the use of this marker for phylogenetic analyses, especially since the parsimony and Bayesian analyses resulted in differing topologies. The usefulness of this marker lies in confirmation of species identity. As mentioned above, it is imperative that additional loci be developed for phylogenetic and distance analyses. The *B. exitiosa* clade also contains a weakly supported subclade in the parsimony analysis (bootstrap support = 70)

that is composed entirely of sequences found in *O. conchaphila* from California (Figure 1.4), which may represent incipient speciation of a lineage within *B. exitiosa*. This clade does not appear in the Bayesian analysis consensus tree (Figure 1.5); instead, *B. exitiosa* sequences from *O. conchaphila* are distributed throughout the clade.

A *B. exitiosa*-like parasite has never been documented in California prior to this study, but preliminary ISH data from our laboratory suggested that a *B. exitiosa*-like parasite was present in archival *O. edulis* tissue from 1968 (unpublished data). Experimental *O. edulis* were "fed" tissues of moribund *O. edulis* from Pigeon Point, California, and a microcell parasite was detected by histopathology and identified based on its morphology as *B. ostreae* (Farley et al. 1988). Reexamination of this same material using ISH found these microcells to instead be a *B. exitiosa*-like parasite. Based on the results from the archival material, it was not surprising to find a parasite whose morphology and SSU rDNA sequence was similar to *B. exitiosa* in contemporary *O. edulis*. This archival material, though, was the same material upon which the hypothesized introduction of *B. ostreae* from California to Europe was based (Katkansky et al. 1969). Because *Bonamia* species are nearly impossible to differentiate morphologically, we must question the validity of identifications that have been done previously based on morphology alone. Furthermore, we must question established hypotheses that have possible misidentifications as their basis.

Bonamia ostreae sequences, along with these *B. exitiosa*-like sequences, were detected in *O. edulis* sampled in 2005. These data provide molecular confirmation of a contemporary presence of *B. ostreae* in California as documented morphologically by Friedman et al. (1989). However, there is no molecular evidence to date of the presence

of *B. ostreae* prior to the 1970s when the introduction to Europe was proposed to occur (Elston et al. 1986). In addition, the current study found *B. exitiosa*, and not *B. ostreae* infecting the native California oyster, *O. conchaphila*, in Elkhorn Slough—the proposed location of origin of *B. ostreae*. Finding no indication of *B. ostreae* in Elkhorn Slough in these samples gives further cause to question the origins of this parasite and the source for its introduction to Europe—expecting that the native oyster would harbor the parasite if, in fact, it were endemic.

Histopathology and ISH provided visual confirmation of infections for all *B. exitiosa* and *B. exitiosa*-like species (Figures 1.6 and 1.7), except for the *Bonamia* sp. found in *O. auporia* from New Zealand. For these particular samples, there was considerable tissue loss during the slide washing process, so it is possible that *Bonamia* cells were lost along with the tissue. Because of this, the experiment was repeated several times and multiple sections/slides had to be cut from a block where *Bonamia* cells were detected by PCR. Thus, a localized infection could have been missed when subsequent ISH experiments were performed. *Ostrea auporia,* however, appears to be conspecific to *O. equestris* and *O. stentina* (Shilts et al. 2007), and the specific probes did exhibit hybridization to the *Bonamia* sp. within these species' tissues. Therefore, it is quite plausible that *B. exitiosa* also infects *O. auporia* in New Zealand.

Furthermore, the conspecificity of these three hosts present in nonadjacent geographic locations—*O. equestris* along the western Atlantic coast, *O. auporia* in New Zealand, and *O. stentina* in the Mediterranean—may help explain the near cosmopolitan distribution of the parasite. However, it also appears that *B. exitiosa* has developed a host generalist strategy and perhaps this has also aided its dispersal around the world.

Bonamia sp. in Ostrea sandvicensis from Hawaii

As mentioned above, SSU rDNA sequences do not provide sufficient resolution to distinguish some *Bonamia* species, but sequence similarity and phylogenetic analyses based on this region do suggest that the species found in *O. sandvicensis* from Hawaii is a novel species.

It was expected that the putative *Bonamia* sp. SSU rDNA sequence found in *O. sandvicensis* would be yet another observation of a *B. exitiosa*-like species, since a *B. exitiosa*-like parasite was detected by ISH in archival *C. gigas* tissue sections taken in 1972 from Hawaii (unpublished data). However, when contemporary sequences were compared to those from other described *Bonamia* spp., they were only 90-91% similar, when the identity between currently described *Bonamia* species only varies between 94 and 98%, and it was 85% similar to SSU rDNA of *M. tapetis* (AY449710) and *M. mercenariae* (FJ518816), species within the sister genus to *Bonamia* in the haplosporidian phylogeny (Reece et al. 2004). The variability in SSU rDNA between *Bonamia* species is minimal when compared to other closely related taxa, such as *Minchinia* species, where similarity ranges from 86 to 91% between SSU rDNA sequences of differing species within the genus (from BLAST results). Based on SSU rDNA sequencing data alone, then, it is unlikely that the *Bonamia* sp. from Hawaii is *B. exitiosa* or any other described species, but appears to be a novel *Bonamia* or *Minchinia* species. The morphology of the parasite, though, fits the description of a typical *Bonamia* species: a 2-3-μm, uninucleate cell predominantly associated with hemocytes (Figure 1.8), while the morphology of *Minchinia* species is distinct. Therefore, while this

Bonamia sp. is more unlike other *Bonamia* species than currently described *Bonamia* species are to one another, the morphology supports its inclusion in the genus *Bonamia.*

Furthermore, in SSU rDNA sequence phylogenetic analyses, the Hawaiian *Bonamia* sp. appears at the base of the clade containing the other *Bonamia* species sequences, with strong support for its inclusion in the *Bonamia* clade (Figures 1.2 and 1.3). The ITS region rDNA analyses give further support for this being a distinct species as the *Bonamia* sp. sequences obtained from *O. sandvicensis* form a distinct monophyletic clade in unrooted trees (Figures 1.4 and 1.5). The basal position of this novel species in the SSU rDNA analyses poses the question: did *Bonamia* parasites, known for inhabiting oysters in more temperate climates, evolve from more tropical origins?

The oyster host itself, *O. sandvicensis*, may provide further support for a tropical origin of *Bonamia* parasites as well. Sequencing partial 16S host mtDNA produced a surprising result: *O. sandvicensis* appears to have a closer affinity to Lophinae genera (*Lopha*, *Alectryonella*, and *Dendostrea*) than to Ostreinae genera (*Ostrea*, *Ostreola*, *Cryptostrea*, and *Teskeyostrea*). Members of the subfamily Lophinae are thought to be older evolutionarily than those comprising Ostreinae based on paleontological (Stenzel 1971) and molecular evidence (partial 28S rDNA in Littlewood 1994 and 16S mtDNA in Jozefowicz and Ó Foighil 1998). Therefore, the SSU rDNA phylogenetic analyses' placement of the Hawaiian *Bonamia* sp. at the base of the entire *Bonamia* clade might be correct, and a tropical origin of *Bonamia* species may be plausible. Additional loci, such as COI or actin, need to be developed for *Bonamia* species and additional tropical oyster species need to be examined in order to determine if this hypothesis is supported at

multiple loci. However, these results give some insight as to where to direct future research efforts in order to further explore the origins of *Bonamia*.

Bonamia ostreae and Bonamia perspora

Based on the locations surveyed and previous studies, it appears that both *B. ostreae* and *B. perspora* are host specialists and maintain limited geographic ranges compared to that of *B. exitiosa*. *Bonamia ostreae* has only been detected in *O. edulis*, and this study proved no different. Putative *Bonamia* sp. SSU and ITS region rDNA sequences found in *O. edulis* from the Netherlands and some sequences found in *O. edulis* from California are within the same clade as *B. ostreae* from Maine and France (Figures 1.2 – 1.5). This study did not examine southern hemispheric *O. edulis*, which have been reported to occur in Australia (Morton et al. 2003) and in South Africa (Haupt et al. 2010; FAO 2007). Therefore, it is possible that *B. ostreae* is present within its type host in the Southern Hemisphere. A host specialist strategy and a potential inability to survive variable environmental conditions (i.e., requiring cool, temperate climates) may provide barriers to dispersal for this particular species.

The data collected to date suggest that *B. perspora,* like *B. ostreae*, is also a host specialist, but has an even more limited geographic distribution. *Bonamia perspora* has only been detected in *O. equestris* from North Carolina (Carnegie et al. 2006). Despite the conspecificity of *O. auporia* and *O. stentina* to *O. equestris* (Shilts et al. 2007) as mentioned earlier, *B. perspora* was not found within these hosts in New Zealand or Tunisia, respectively. One explanation could be that *B. perspora* has only been found at very low prevalence (<5.6% prevalence; Carnegie et al. 2006), so perhaps larger sample

sizes will be needed in order to find this parasite elsewhere. Alternatively, *B. perspora* could have been established in North Carolina *O. equestris* after the geographic disjunction of the conspecific hosts. More sampling would need to be done in order to test either hypothesis.

The phylogenetic relationship between these two species, however, is as yet unresolved. In the SSU rDNA analyses, the clade containing *B. ostreae* and *B. perspora* is sister to the entire *B. exitiosa/B. roughleyi/B. exitiosa*-like clade (Figures 1.2 and 1.3). However, neither the Bayesian or parsimony analyses of the SSU rDNA sequences resolve the relationship between *B. ostreae* and *B. perspora* sequences (bootstrap support = 51, Figure 1.2; posterior probability = 79; Figure 1.3). The unrooted parsimony consensus tree of ITS region rDNA analysis shows an alternative hypothesis with *B. ostreae* sister to a clade containing both *B. perspora* and the Hawaiian *Bonamia* sp. (bootstrap support = 100; Figure 1.4), while the Bayesian analysis suggests that *B. perspora* is sister to a clade containing both *B. ostreae* and the Hawaiian *Bonamia* sp. (posterior probability = 100; Figure 1.5). Again, additional loci should be developed in order to determine this interspecific relationship. The phylogenetic relationships between species in the ITS region DNA analysis should especially be regarded cautiously given the variability in this region.

No Detection of Bonamia Species

There were some hosts and locations where *Bonamia* spp. were not detected by PCR (Table 1.1). *Saccostrea glomerata* from New Zealand were all PCR negative for *Bonamia* spp., and this was somewhat surprising since *B. exitiosa* was detected in *O.*

auporia in the Tamaki Estuary, just 150-200 km south of Whangarei Harbour where the *S. glomerata* samples screened for this study were collected. Furthermore, *B. exitiosa* was found in other *S. glomerata* samples from Australia (Table 1.1). *Bonamia* spp. were not detected in *O. conchaphila* from Drakes Estero, California or from Lemmens Inlet, British Columbia, while *B. exitiosa* was detected in *O. conchaphila* at other locations in this study. Because *Bonamia* spp. are known to infect both *S. glomerata* and *O. conchaphila*, it is possible that the parasite is simply not present at these specific locations. However, the sample sizes may have been too small to detect the parasite(s) if the prevalence was low.

Bonamia spp. were also not detected by PCR in an *Isognomon* sp. from Florida, *Ostrea denselamellosa* from Japan, or *S. cucullata* from New Zealand. *Bonamia* spp. have never been documented in these hosts, so perhaps *Bonamia* parasites do not infect these oyster species, the parasite(s) are not present at these locations, and/or the sample sizes were too small to capture a parasite at low prevalence, particularly since PCR prevalence of some samples was very low (e.g., 0.5% in a sample of 200 *S. glomerata* from Australia).

The type *B. roughleyi* SSU rDNA sequence was also not found in any sample at any location. As mentioned earlier, Hill et al. (2010) questioned the validity of this species, and not finding it, even after screening 200 *S. glomerata* in Australia, its type host and locality, during the peak of disease (August 2007), makes the legitimacy of *B. roughleyi* even more suspect.

Conclusions

The diversity of *Bonamia* species is far greater than previous studies indicated. By examining little-studied oyster hosts from around the world, we revealed one new species in Hawaii, and perhaps two new species or subspecies in Chile and California (based on the ITS region rDNA parsimony analysis). The Hawaiian *Bonamia* sp. appears basal to all other *Bonamia* sp. in SSU rDNA analyses, suggesting that *Bonamia* parasites may have evolved from more tropical locations. SSU rDNA, however, does not provide sufficient resolution of all *Bonamia* species diversity, and therefore should not be used for absolute identification of species. Instead, the ITS region rDNA, which seems to provide a means of providing distinction between some currently defined species, like *B. exitiosa*, would be useful for species delineation. Developing ISH probes that target ITS region rDNA—knowing that there is potential variation within *B. exitiosa* or even distinct species—may be part of the solution. Additional loci need to be developed as well in order to confirm or refute the hypotheses suggested by the SSU and ITS region rDNA phylogenetic analyses in this study.

Region	Oyster Species	Location	Collection Date	Sample Size	*Bonamia* spp. PCR Prevalence (%)
North Atlantic	*Crassostrea ariakensis*	Fort Pierce, Florida, USA	4/16/2007	17	6/17 (35.3%)
	Crassostrea ariakensis	Fort Pierce, Florida, USA	6/14/2007	19	8/19 (42.1%)
	Isognomon sp.	Long Key, Florida, USA	7/10/2009	30	0/30 (0%)
	Ostrea equestris	Old House Creek, South Carolina, USA	4/12/2006	55	2/55 (3.6%)†
	Ostrea equestris	Wilmington, North Carolina, USA	6/22/2005	200	8/200 (4.0%)††
	Ostrea equestris	Morehead City Port, North Carolina, USA	8/1/2005	150	3/150 (2.0%)†††
South Atlantic	*Ostrea puelchana*	San Antonio Bay, Argentina	3/22/2005	57	6/57 (10.5%)†
	Ostrea equestris	San Antonio Bay, Argentina	04/2007	3	3/3 (n/a*)
North Pacific	*Ostrea conchaphila*	Lemmens Inlet, British Columbia, Canada	4/01/2005	25	0/25 (0%)
	Ostrea conchaphila	Drakes Estero, California, USA	7/7/2004	15**	0/15** (0%)
	Ostrea conchaphila	Elkhorn Slough, California, USA	5/27/2004	15**	13/15** (21.7 - 86.7%)
	Ostrea conchaphila	Elkhorn Slough, California, USA	09/17/2009	60	53/60 (88.3%)
	Ostrea edulis	Hog Island, California, USA	10/7/2005	32**	21/32** (20.7 - 82.0%)
	Ostrea sandvicensis	Kaneohe Bay, Hawaii, USA	06/2006	120	79/120 (65.8%)
	Ostrea sandvicensis	Kaneohe Bay, Hawaii, USA	10/18-20/2007	60	42/60 (70.0%)
	Ostrea denselamellosa	Seto Inland Sea, Okayama Prefecture, Japan	7/13/2009	40	0/40 (0%)
South Pacific	*Ostrea angasi*	Pambula River, New South Wales, Australia	11/2006	42	1/42 (2.4%)
	Saccostrea glomerata	Georges River, New South Wales, Australia	08/2007	200	1/200 (0.5%)
	Ostrea auporia	Tamaki Estuary, Glendowie, New Zealand	8/14/2007	44	4/44 (9.1%)
	Ostrea auporia	Karaka Bay, Tamaki Estuary, Auckland, New Zealand	3/13/2009	90	5/90 (5.6%)
	Ostrea chilensis	Foveaux Strait, New Zealand	01 & 03/2005	5	4/5 (n/a*)
	Ostrea chilensis	Foveaux Strait, New Zealand	06/13/2004	40	2/40 (5.0%)
	Saccostrea cucullata	Whangarei Harbour, Northland, New Zealand	3/2/2008	50	0/50 (0%)
	Saccostrea glomerata	Whangarei Harbour, Northland, New Zealand	5/1/09	60	0/60 (0%)
	Ostrea chilensis	Chiloe Island, Chile	12/2003	1	1/1 (n/a*)
	Ostrea chilensis	Chiloe Island, Chile	10/2003	32	6/32 (18.8%)
Mediterranean Sea	*Ostrea stentina*	Hammamet, Tunisia	06/2007	85	8/85 (10.6%)
North Sea	*Ostrea edulis*	Lake Grevelingen, The Netherlands	05/2008	1	1/1 (n/a*)

*Samples sent were known to have *Bonamia* spp. infections as detected by histology.
**More than one individual in a single DNA extraction, and therefore PCR reaction.
† C_f+C_r primers used instead of Bon-319F + Bon-524R.
†† Cf +Cr primers used instead of Bon-319F + Bon-524R. Five of these were *B. perspora* only, one was *B. exitiosa* only, and two were co-infections.
††† Cf +Cr primers used instead of Bon-319F + Bon-524R. One of three were *B. perspora*.

TABLE 1.1. Sample collection data information *Bonamia* spp. PCR prevalence data using primers Bon-319F + Bon-524R (unless otherwise noted).

Primer/Probe	Sequence (5' – 3')	Use	Reference
C_F	CGGGGGCATAATTCAGGAAC	*Bonamia*-generic PCR; SSU sequencing	Carnegie et al. 2000
C_R	CCATCTGCTGGAGACACAG	*Bonamia*-generic PCR; SSU sequencing	Carnegie et al. 2000
Bon-319F	TTTGACGGGTAACGGGGAATGCG	*Bonamia*-generic PCR; SSU sequencing	Hill et al. 2010
Bon-524R	CTTGCCCTCCGCTGGAATTC	*Bonamia*-generic PCR	Hill et al. 2010
Bon-745R	CTAATGCATTCAGGCGCGAG	*Bonamia*-generic PCR; SSU sequencing	Carnegie et al. 2006
Bon-925F	ATTCCGGTGAGACTAACTTATG	*Bonamia*-generic PCR; SSU sequencing	White 2008
Bon-927R	CATAAGTTAGTCTCACCGGAATT	*Bonamia*-generic PCR; SSU sequencing	This study
Bon-990R	CTTAGTCGACATCGTTTATGGTTGGG	*Bonamia*-generic PCR; SSU sequencing	White 2008
Bon-1110R	CCTTTAAGTTTCACTCTTGCGAG	*Bonamia*-generic PCR; SSU sequencing	White 2008
Bon-1310F	GAGACCCCACCCATCTAAC	*Bonamia*-generic PCR; SSU sequencing	Carnegie et al. 2006
HIBon-620F	CGGCACGCACGTAAGTGGAG	Hawaiian *Bonamia*-specific PCR; SSU sequencing	This study
16S-A	AACCTGGTTGATCCTGCCAGT	Universal SSU PCR; sequencing	Medlin et al. 1988
16S-B	GATCCTTCCGCAGGTTCACCTAC	Universal SSU PCR; sequencing	Medlin et al. 1988
haplo-ITSf	GGGATAGATGATTGCAATTRTTC	Haplosporidia*-generic PCR; ITS sequencing	Hill et al. 2010
ITS-B	TATGCTTAAATTCAGCGGGT	Universal ITS PCR; ITS sequencing	Goggin 1994 (=reverse primer D)
16Sar	CGCCTGTTTATCAAAAACAT	Universal mt16S PCR; sequencing of host mtDNA	Kessing et al. 1989
16Sbr	CCGGTCTGAACTCAGATCACGT	Universal mt16S PCR; sequencing of host mtDNA	Kessing et al. 1989
CaBon166	CGAGCAGGGTTTGTCACGTAT	*B. exitiosa*-specific dig-ISH	Hill et al. 2010
CaBon461	TTCCGAATAGGCAACCGAAG	*B. exitiosa*-specific dig-ISH	Hill et al. 2010
CaBon1704	CAAAGCTTCTAAGAACGCGCC	*B. exitiosa*-specific dig-ISH	Hill et al. 2010
Bost171	CCGCCGAGGCAGGGTTTGT	*B. ostreae*-specific dig-ISH	This study
HIBon-167	CTAATATGCACAGCCGCCAG	Hawaiian *Bonamia*-specific FISH	This study
HIBon-634	CGATTATGGCCTCTCTCCAC	Hawaiian *Bonamia*-specific FISH	This study
Oe-309	TCATGCTCCCTCTCCGG	Universal SSU probe	This study

*Primer was designed to anneal to most haplosporidian genera.

TABLE 1.2. PCR primer and *in situ* hybridization probe sequences used in this study.

Host Species and Sampling Location	No. of Host Individuals (from which parasite sequence was obtained)	Primers Used to Generate Complete *Bonamia* sp. SSU rDNA fragment	SSU rDNA Region Amplified (bps)	No. of Clones Sequenced
C. ariakensis (Florida)	1	16S-A + Bon-745R	1-734	5
	1	$C_F + C_R$	658-1382	6
	1	Bon-1310F + 16S-B	1307-1749	5
O. auporia (New Zealand)	3	16S-A + Bon-927R	1-883	4
	3	Bon-319F + Bon-990R	324-485	4
	3	Bon-925F + 16S-B	907-1749	3
O. conchaphila (California)	3	16S-A + Bon-927R	1-883	5
	3	Bon-319F + Bon-990R	324-485	4
	3	Bon-925F + 16S-B	907-1749	4
O. edulis (California)	1	16SA + Bon-1050R	1-1026	6
	1	Bon-925F + 16S-B	907-1749	5
O. equestris (Argentina)	2	16S-A+Bon-1110R	1-1079	5
	3	Bon-925F+16S-B	907-1749	9
O. sandvicensis (Hawaii)	2	16S-A + Bon-927R	1-901	6
	2	HIBon-620F + Bon-1110R	640-1098	6
	2	Bon-925F + 16S-B	925-1766	7
S. glomerata (Australia)	1	16S-A+Bon-990r	1-985	4
	1	Bon925f+16S-B	907-1749	7

TABLE 1.3. *Bonamia* spp. SSU rDNA sequencing information. "SSU rDNA region amplified" lists the nucleotide range that the primer set amplifies within the complete *Bonamia* spp. SSU rDNA fragment, excluding the primer sequences.

TABLE 1.4

Bonamia species	Sampling Location	Host species	GenBank Accession Nos.
B. exitiosa	FL, USA	*C. ariakensis*	JF712867 - JF712869
B. exitiosa	NC, USA	*C. ariakensis*	EU709024 - EU709031, EU709037, EU709038, EU709043 - EU709045, EU709051, EU709053
B. exitiosa	NC & SC, USA	*O. equestris*	JF831575 - JF831580, JF831585 - JF831590, JF831592 - JF831594, JF831596 - JF831599, JF831601, JF831602
B. exitiosa	ARG	*O. equestris*	JF831556, JF831557, JF831559 - JF831563, JF831565 - JF831574
B. exitiosa	ARG	*O. puelchana*	EU709055, EU709057, EU709058, EU709060, EU709061, EU709063, EU709064, EU709067, EU709068, JF831603 - JF831615, JF831617 - JF831624, JF831627 - JF831638
B. exitiosa	NZL	*O. chilensis*	EU709069, EU709071, EU709073 - EU709075, JF831639 - JF831657
B. exitiosa	NZL	*O. auporia*	JF831658 - JF831669, JF831671 - JF831677
B. exitiosa	AUS	*O. angasi*	EU780688 - EU780692, JF831678 - JF831680
B. exitiosa	AUS	*S. glomerata*	JF831681 - JF831684
B. exitiosa	TUN	*O. stentina*	GU356032 - GU356035, JF831685 - JF831689, JF831691 - JF831713, JF831715 - JF831718
B. exitiosa	CA, USA	*O. conchaphila*	JF831719 - JF831738, JF831740, JF831741, JF831743 - JF831761, JF831763 - JF831767, JF831769 - JF831774, JF831776, JF831778 - JF831788, JF831790, JF831792 - JF831800
B. ostreae	CA, USA	*O. edulis*	JF831830 - JF831840, JF831842 - JF831848
B. ostreae	ME, USA	*O. edulis*	EU709105 - EU709111
B. ostreae	NLD	*O. edulis*	JF831857 - JF831862
B. perspora	NC, USA	*O. equestris*	EU709112, EU709115 - EU709121, EU709123 - EU709129
Bonamia sp.	HI, USA	*O. sandvicensis*	JF831863 - JF831879
Bonamia sp.	CA, USA	*O. edulis*	JF831808 - JF831818, JF831820, JF831821, JF831823 - JF831825, JF831827 - JF831829
Bonamia sp.	CHL	*O. chilensis*	EU709079, EU709080. EU709082, EU709084, EU709086, EU709087, EU709091, EU709093, EU709095, EU709098, EU709102, JF831849 - JF831856

GenBank accession numbers of *Bonamia* spp. ITS region rDNA sequences.

TABLE 1.5

***Bonamia* sp. host and location**	**Percent Similarity to *B. exitiosa* ITS region rDNA**
C. ariakensis (Florida)	98%
O. equestris (North & South Carolina)	98-99%
O. equestris (Argentina)	98-100%
O. puelchana (Argentina)	98-100%
O. auporia (New Zealand)	98-100%
O. angasi (Australia)	98-99%
S. glomerata (Australia)	99-100%
O. conchaphila (California)	98-99%
O. chilensis (Chile)	83-85%
O. edulis (California)	84-85%

Percent sequence similarity in the ITS region rDNA among *Bonamia exitiosa* and *B. exitiosa*-like species as determined by SSU rDNA sequencing and phylogenetic analyses.

TABLE 1.6

Group	*Bonamia exitiosa*	Hawaiian *Bonamia sp.*	*Bonamia ostreae*	*Bonamia perspora*	*Minchinia* spp.
Bonamia exitiosa	-	0.007	0.004	0.004	0.006
Hawaiian *Bonamia* sp.	0.086	-	0.007	0.007	0.008
Bonamia ostreae	0.031	0.086	-	0.005	0.006
Bonamia perspora	0.025	0.084	0.029	-	0.006
***Minchinia* spp.**	0.125	0.153	0.131	0.128	-

Mean uncorrected p-distances among *Bonamia* spp. means and the outgroup *Minchinia* spp. for the SSU rDNA dataset. Numbers in gray indicate the standard error estimates obtained by 1000 bootstrap replicates.

TABLE 1.7

Bonamia clade	*p-distance*	No. of sequences analyzed
B. exitiosa	0.005	242
Chilean *Bonamia* sp. in *O. chilensis*	0.007	27
Hawaiian *Bonamia* sp. in *O. sandvicensis*	0.020	17
Californian *Bonamia* sp. from *O. edulis*	0.004	19
B. perspora	0.005	17
B. ostreae	0.020	31

Mean uncorrected p-distances within *Bonamia* clades for the ITS region rDNA dataset.

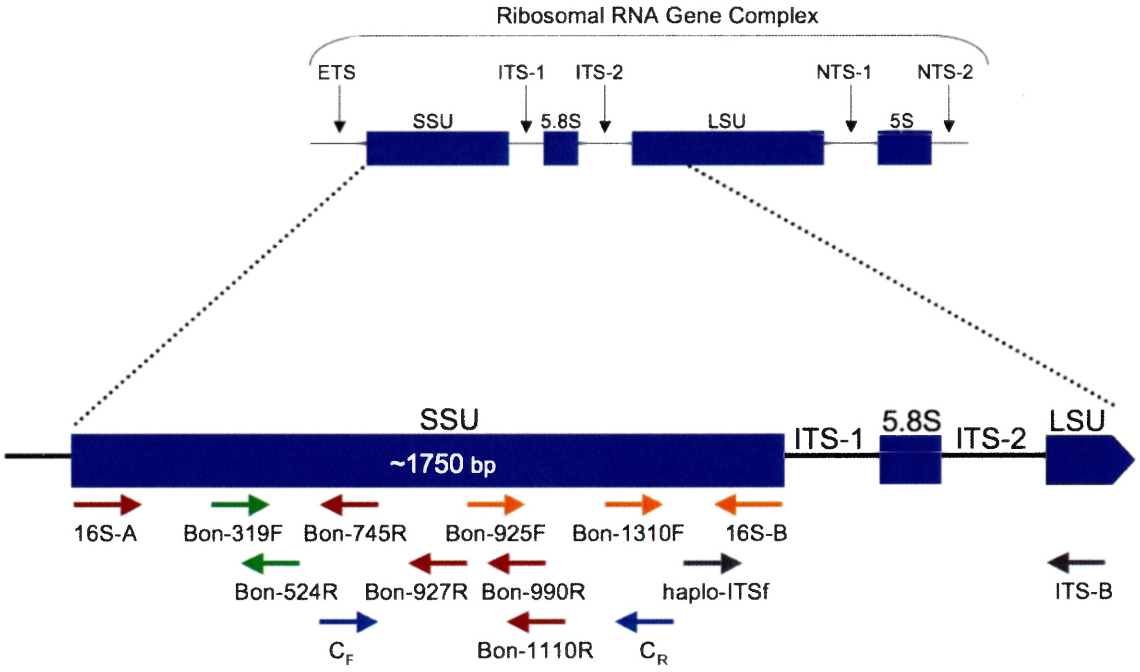
TABLE 1.8

Group	*Bonamia exitiosa*	*Bonamia* sp. (HI, USA)	*Bonamia* sp. (CHI)	*Bonamia* sp. (CA, USA)	*Bonamia perspora*	*Bonamia ostreae*
Bonamia exitiosa	-	0.022	0.012	0.013	0.023	0.021
***Bonamia* sp. (HI, USA)**	0.298	-	0.022	0.023	0.020	0.021
***Bonamia* sp. (CHI)**	0.094	0.284	-	0.016	0.022	0.023
***Bonamia* sp. (CA, USA)**	0.104	0.324	0.169	-	0.022	0.023
Bonamia perspora	0.275	0.259	0.295	0.292	-	0.023
Bonamia ostreae	0.263	0.313	0.292	0.286	0.281	

Mean uncorrected p-distances among *Bonamia* spp. for the ITS region rDNA dataset.

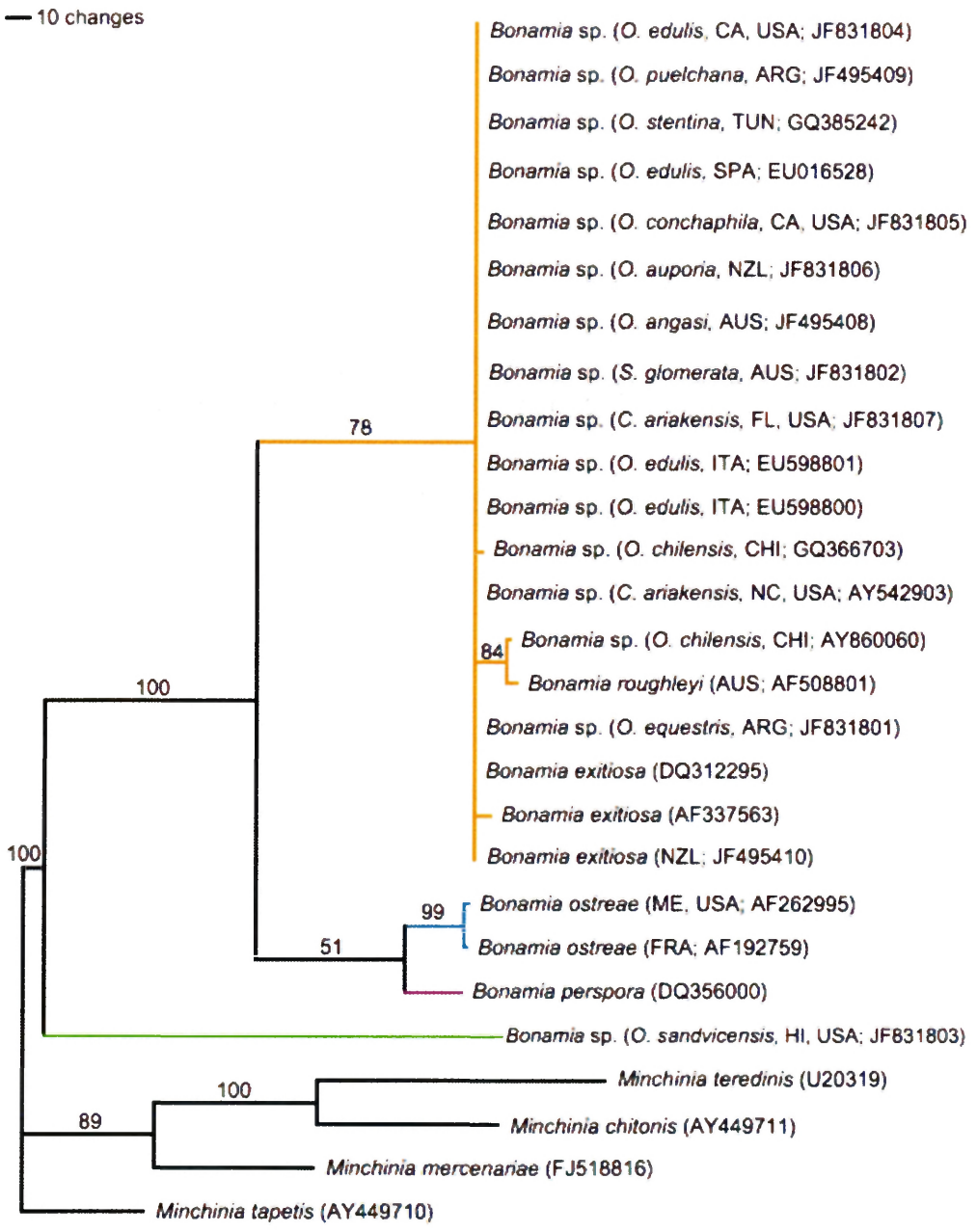
Numbers in gray indicate the standard error estimates obtained by 1000 bootstrap replicates.

FIGURE 1.1



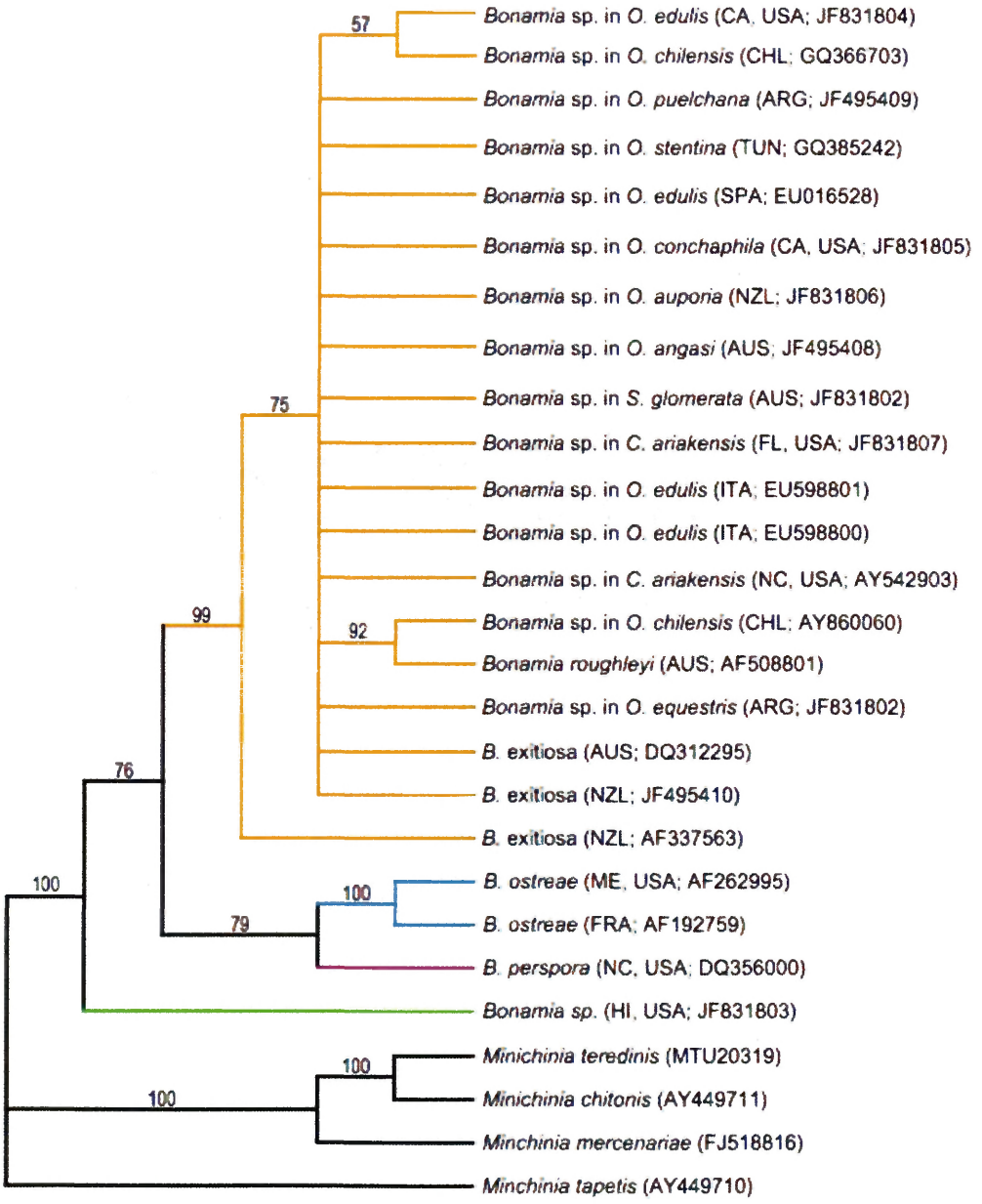
Hypothetical, schematic depiction of the ribosomal RNA gene complex of *Bonamia* species. Arrows indicate PCR primer binding sites. Forward primers (F) were matched with reverse primers (R), and colors indicate which primers were used in combination.

FIGURE 1.2



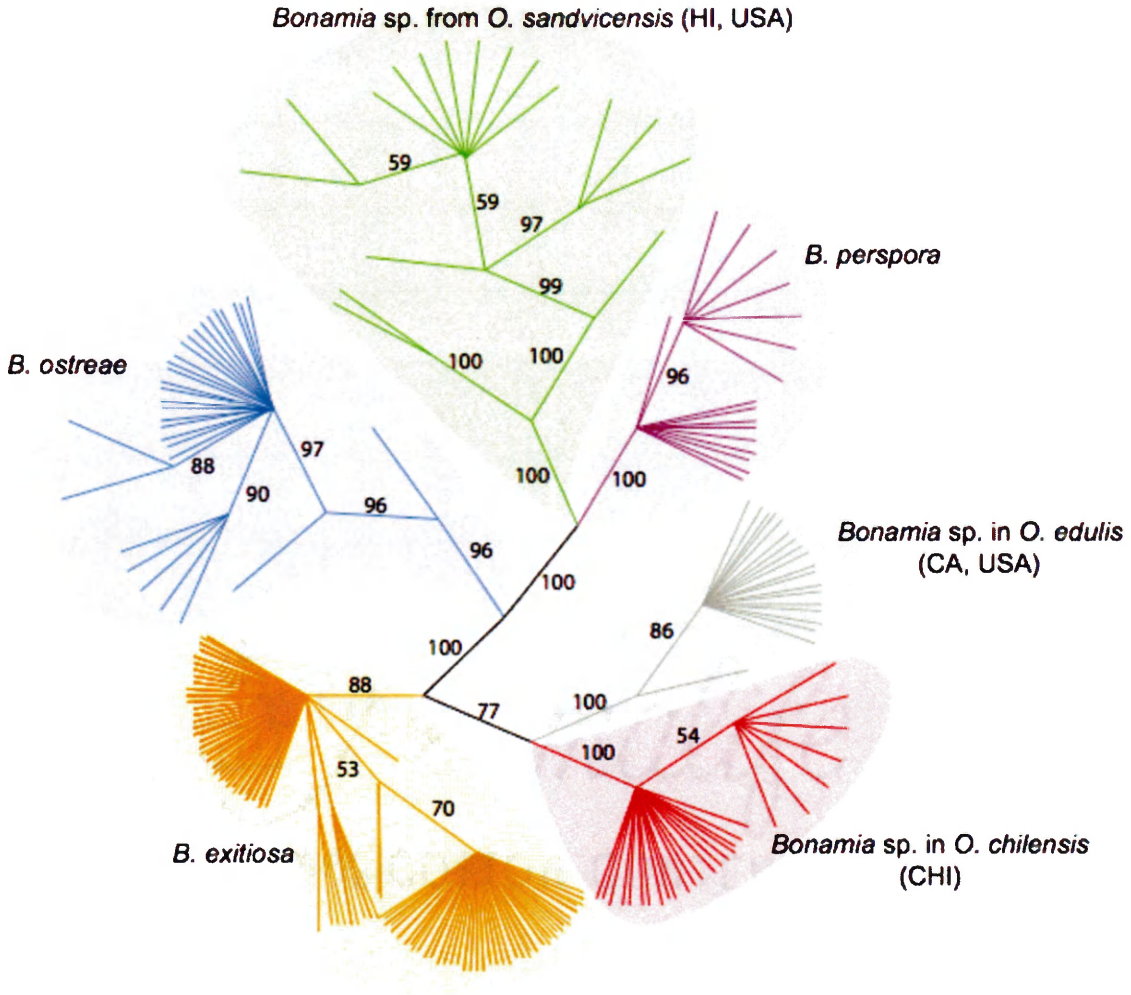
Bonamia spp. SSU rDNA consensus phylogram. Parsimony analysis (1000 bootstrap replicates with 100 random additions) based on 285 informative characters performed in PAUP* 4.0b10 (Swofford 2002) following alignment of *Bonamia* spp. SSU rDNA sequences using MAFFT v. 6 (Katoh and Toh 2008). Gaps were treated as missing. *Minchinia* species were chosen as the outgroup. Colors indicate distinct *Bonamia* lineages.

FIGURE 1.3



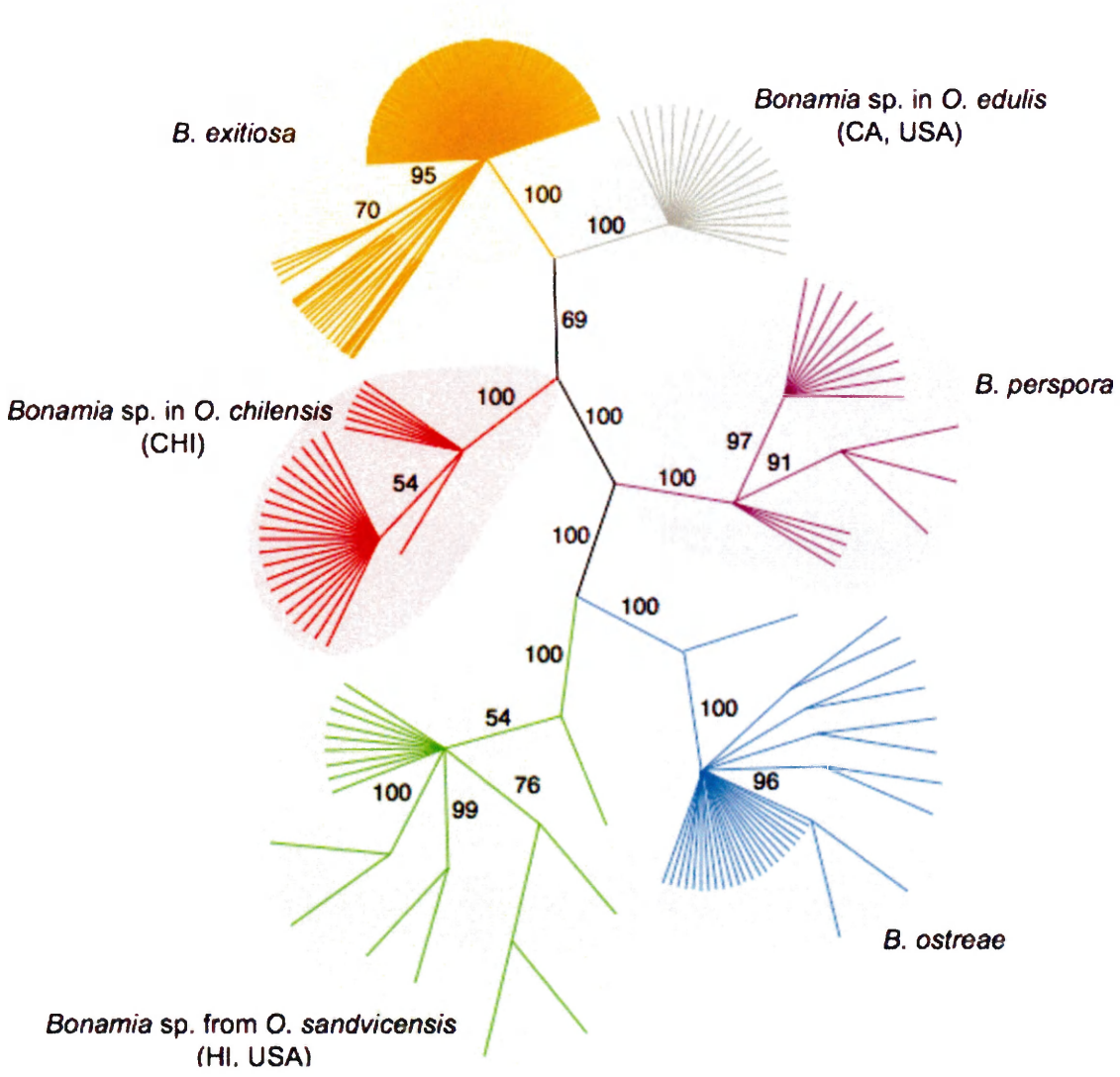
Bonamia spp. SSU rDNA Bayesian 50% majority rule consensus tree. Bayesian inference analysis conducted in MrBayes v. 3.1.2 (Ronquist and Huesenbeck 2003) using model GTR + I + Γ, as determined by MrModeltest v. 2.3, following alignment using MAFFT v. 6 (Katoh and Toh 2008) of *Bonamia* spp. SSU rDNA sequences. Ten thousand trees were produced (10,000,000 generations, sample freq. = 1000), with 25% removed as burnin. The consensus tree was generated using PAUP *4.0b10 (Swofford 2002) using *Minchinia* species as the outgroup. Colors indicate distinct *Bonamia* lineages.

FIGURE 1.4



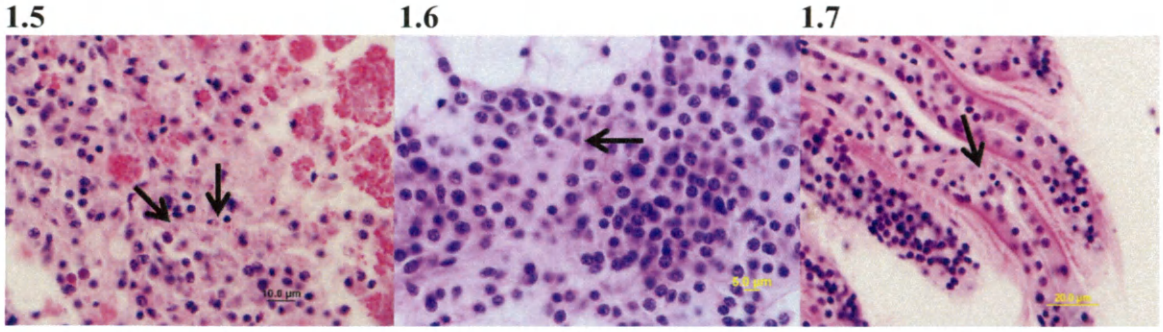
Bonamia spp. ITS region rDNA unrooted parsimony consensus tree (50% majority rule). Parsimony analysis (100 bootstrap replicates with 10 random additions) conducted using a new technology search in TNT v. 1.1 (Goloboff, Farris, and Nixon 2008) following alignment in MAFFT v. 6 (Katoh and Toh 2008) of *Bonamia* spp. ITS region rDNA sequences. The consensus tree was generated using PAUP*b10 (Swofford 2002). Colors indicate distinct *Bonamia* lineages.

FIGURE 1.5



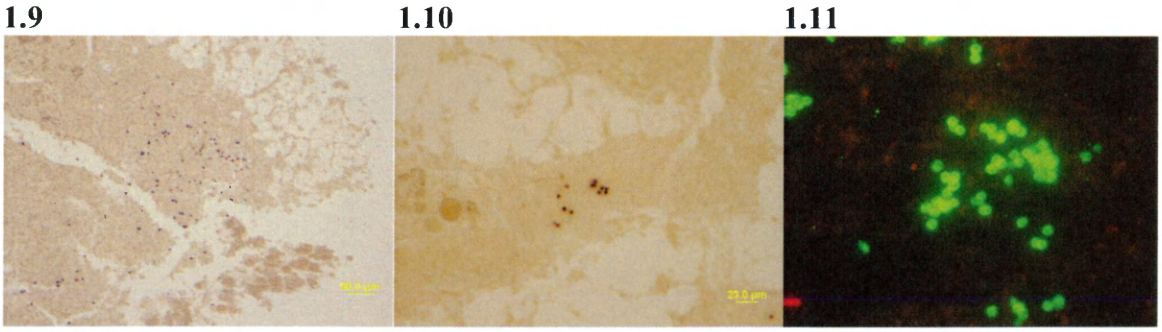
Bonamia spp. ITS region rDNA unrooted Bayesian consensus tree (50% majority rule). Bayesian inference analysis conducted in MrBayes v. 3.1.2 (Ronquist and Huesenbeck 2003) using model GTR + I + Γ, as determined by MrModeltest v. 2.3, following alignment using MAFFT v. 6 (Katoh and Toh 2008) of *Bonamia* spp. ITS region rDNA sequences. Forty thousand trees were produced (40,000,000 generations, sample freq. = 1000), with the first 34,999 trees removed in order to compute a consensus tree. A consensus of the remaining 5,001 trees was generated using PAUP*b10 (Swofford 2002). Colors indicate distinct *Bonamia* lineages.

FIGURES 1.6 – 1.8



Light microscopy of *Bonamia* spp. in H & E-stained oyster tissue sections. **1.6.** *Bonamia exitiosa* microcells (arrows) in an *Ostrea conchaphila* section from California. **1.7.** *Bonamia exitiosa*-like sp. microcell (arrow) in an *Ostrea edulis* section from California. **1.8.** *Bonamia* sp. microcell (arrow) in the gills of an *Ostrea sandvicensis* section from Hawaii.

FIGURES 1.9 – 1.11



In situ hybridization (ISH) images for molecular confirmation of histopathological diagnoses. **1.9.** Hybridization of digoxigenin-labeled probes to *Bonamia exitiosa* in *Ostrea conchaphila* from California. **1.10.** Hybridization of digoxigenin-labeled probes to a *Bonamia exitiosa*-like sp. in *O. edulis* from California. **1.11.** Fluorescent *in situ* hybridization to the *Bonamia* sp. in *O. sandvicensis* from Hawaii.

CHAPTER TWO: Observation of a Novel *Bonamia* sp. (Haplosporidia) Infecting *Ostrea sandvicensis* Sowerby, 1871 in Hawaii, USA

INTRODUCTION

Protistan parasites of oysters within the genus *Bonamia* (Haplosporidia) are being observed in an increasing number of locations and hosts around the world (Chapter One). *Bonamia exitiosa* appears to have a near-global distribution, while *Bonamia ostreae* and *Bonamia perspora* seem to be confined to their type hosts, *Ostrea edulis* and *Ostrea equestris*, respectively, within the Northern Hemisphere. The relationships between these described species have yet to be fully resolved, however, and the evolutionary origins of *Bonamia* species are still unknown.

Most disease studies focus on commercially important oyster species, and thus our knowledge revolves around what we know about parasites and disease in these few species. Non-commercial oyster species are little studied, but could play an important role in the evolution of oyster parasites, such as *Bonamia* species, and help fill in phylogenetic gaps.

Ostrea sandvicensis Sowerby, 1871 is a small, non-commercial oyster that inhabits brackish to mixoeuhaline waters of the Hawaiian Islands and is commonly found on reefs in Kaneohe Bay and Ala Moana, Oahu, Hawaii (Carriker and Gaffney 1996). In an effort to better understand the host and geographic distribution of the genus *Bonamia* (Chapter One), this little-studied oyster species was examined. Histopathology revealed the presence of a microcell parasite that is identical morphologically to *B. exitiosa*. However, molecular techniques (PCR, sequencing of the SSU rDNA gene, and *in situ* hybridization) revealed the presence of a novel *Bonamia* species. Results of phylogenetic analyses that include this Hawaiian *Bonamia* sp. shed light on the origins of *Bonamia* parasites.

MATERIALS AND METHODS

Sample Collection

Ostrea sandvicensis were collected by snorkeling in June 2006 (n=120) and October 2007 (n=60) from coral reefs in Kaneohe Bay, Hawaii—just a few meters from the shore of Coconut Island where the Hawaiian Institute of Marine Biology is located (Figure 1). Shell heights were measured, and oysters were shucked, with small pieces of gill and mantle tissue (~3-5 mm^3) preserved in 95% ethanol for molecular analyses. For the 2007 sample, sections of remaining gill, mantle and digestive gland tissues were fixed for standard histopathology in Davidson's fixative (Shaw and Battle 1957), and then transferred to 70% ethanol. All instruments used for dissection were sterilized with 95% ethanol and flamed between each sample. Samples were brought back to the Shellfish Pathology Laboratory at the Virginia Institute of Marine Science for further processing.

Histology, DNA extraction and *Bonamia*-generic PCR, *Bonamia* sp. SSU and ITS region rDNA sequencing, sequence alignments, distance analyses, molecular phylogenetics, fluorescent ISH, and oyster host mitochondrial 16S sequencing were done as in Chapter One.

RESULTS

Bonamia-generic PCR

Evaluation of 120 *O. sandvicensis* from Kaneohe Bay, Hawaii, sampled June 2006, revealed 79 oysters (65.8%) PCR-positive for a *Bonamia* sp. using the Bon-319F/Bon-524R assay. Evaluation of 60 *O. sandvicensis* from the same location sampled in October 2007 revealed similar results, with 42 oysters (70.0%) PCR-positive for a *Bonamia* sp. using the same *Bonamia*-generic assay.

SSU rDNA Sequencing and Phylogenetic Results

PCR-cloning and DNA sequencing of the SSU rDNA from the *Bonamia* sp. infecting *O. sandvicensis* produced a 1766-bp sequence: a consensus of one to seven clones from two individual oysters. This sequence was submitted to GenBank and given accession number JF831803, and has an ambiguity at base 114 where four clones had a T and four had an A. This sequence was aligned with SSU rDNA sequences from GenBank and other sequences generated in Chapter One, and then analyzed by parsimony and Bayesian methods using species from the genus *Minchinia*, which is sister to *Bonamia* in the haplosporidian phylogeny, as an outgroup. Both trees converged on similar topologies (Figures 1.2 and 1.3), where the *Bonamia* sp. from Hawaii appeared basal to the entire *Bonamia* clade (bootstrap support = 100; posterior probability = 100).

ITS Region rDNA Sequencing and Phylogenetic Results

PCR-cloning and DNA sequencing of the parasite's ITS rDNA region produced 17 unique sequences from 17 clones that differed greatly, ranging from 79.2 to 99.7% similarity to one another. Length varied from 615-616 to 712-720 bps (GenBank Accession numbers JF831863 – JF831879). Parsimony and Bayesian analyses showed the sequences from this *Bonamia* sp. to be monophyletic (Figures 1.4 and 1.5).

Histopathology and Fluorescent ISH

Subsequent histopathological examination of 25 oysters from the October 2007 PCR-positive samples confirmed the presence of small numbers of uninucleate *Bonamia* sp. cells (three oysters had rare, multifocal infections; three oysters had rare, local

infections; one oyster had a moderate, systemic infection; and no *Bonamia* sp. microcells were found in 18 oysters). Heavy hemocyte infiltration was observed in the oyster with a moderate, systemic infection (Figure 1.8) as well as the disintegration of gill connective tissue. Digestive tubules appeared atrophied, and the epithelium seemed to be sloughing off into the lumen. Microcells were located within the digestive gland and in the gills. Bi-nucleate, tri-nucleate, and multinucleate cell forms were observed, and most microcells were found within hemocytes, though a few were found in the connective tissue. Some microcells also had a vacuole in the center with marginalized chromatin.

Fluorescent *in situ* hybridization on the *Bonamia* sp.-infected *O. sandvicensis* section was positive, indicating the presence of the unique SSU rDNA of this *Bonamia* sp. (Figure 1.11).

Host Species Confirmation

DNA sequencing of the mt16S region of the oyster host generated a 439-486 bp product. Performing a BLAST (Altschul et al. 1990) search of these sequences showed 99% maximum identity to *Dendostrea crenulifera* (syn. to *Pustulostrea tuberculata* (Lamarck 1804) (Carriker and Gaffney 1996)) (89-93% query coverage; GenBank accession numbers EU815984 and EU815985) and 94-95% maximum identity to *Alectryonella plicatula* (Gmelin 1790), the fingerprint oyster (91-94% query coverage, GenBank accession number AF052072). There are no *O. sandvicensis* sequences in GenBank.

Genetic Distance

Uncorrected p-distances, or the total number of base differences per site averaged over all sequence pairs, between the Hawaiian *Bonamia* sp. clade and other *Bonamia* species as indicated by the SSU rDNA phylogenetic analyses is shown in Table 1.6. 1670 positions were analyzed in the final dataset. Uncorrected p-distances among all described *Bonamia* spp. and the Hawaiian *Bonamia* sp. ranged from 0.084 – 0.086, while uncorrected p-distances among all described species ranged from 0.25 – 0.031.

DISCUSSION

Bonamia sp. in *Ostrea sandvicensis*

Diagnosis. Infections were found mainly within hemocytes, though some cells were found in connective tissue. Uninucleate and binucleate cells were common, and some multinucleate plasmodia were present. The cells were almost exclusively contained inside hemocytes, in all tissues and organs (i.e., there was no particular tissue tropism). Nuclei were central to slightly eccentric—not strongly eccentric, like *B. ostreae*. Cell diameters were measured (n = 30) in the one host in which the parasite was abundant (moderate infection by histopathology) and the mean diameter (+/- 1 SEM) was 2.83 ± 0.07 μm), ranging from 2.19-3.91 μm. This one individual displayed some general hemocytosis, but this could have related partly to co-infection by cestodes.

DNA nucleotide sequences. In phylogenetic analyses, nucleotide sequences from the SSU rRNA gene and ITS region rDNA form distinct monophyletic clades, separate from those of other known *Bonamia* species including *B. exitiosa*/*B. roughleyi*, *B. ostreae*, and *B. perspora.*

Reference material deposited. Replicate H&E-stained histological sections from infected *O. sandvicensis* oysters that were confirmed by PCR and ISH assays to be infected with *Bonamia* sp. will be deposited as reference materials with the United States Department of Agriculture National Parasite Collection (USNPC) and with the Office Internationaldes Epizooties (OIE) genus *Bonamia* reference collection at IFREMER, Laboratoire de Génétique Aquaculture et Pathologie. Nucleotide sequences of the SSU rRNA gene and ITS region rDNA were deposited in GenBank under accession numbers JF831803 and JF831863 – JF831879, respectively.

Type host. *Ostrea sandvicensis.*

Type locality. Kaneohe Bay, Hawaii, USA (19°43′ 46″ N, 155°4′ 7″ W).

A novel *Bonamia* species was detected in *O. sandvicensis* in Hawaii based on SSU and ITS region rDNA sequencing data. Morphologically, the predominant cell form is identical to the small, uninucleate microcell typical of *B. exitiosa* infections. However, when SSU rDNA data is examined, this parasite clearly forms a monophyletic clade—separate from other *Bonamia* species, but clearly within the genus. *Bonamia ostreae*, *B. perspora*, *B. exitiosa,* and now this novel species vary sufficiently in the SSU rDNA to produce separate, monophyletic clades (Figures 1.2 and 1.3). Furthermore, the Hawaiian *Bonamia* sp. forms a distinct, monophyletic clade in the ITS region rDNA phylogenetic analysis (Figures 1.4 and 1.5), and thereby provides further justification for the designation of the Hawaiian *Bonamia* sp. as a new species. While morphology remains quintessential to taxonomical classification, its usefulness is limited for the genus *Bonamia* as morphological distinctions at the species level are not evident using light

microscopy or transmission electron microscopy. Therefore, molecular methods of divergence must be employed.

By definition *Bonamia* parasites are small (2-3 μm), uninucleate "microcells" (Pichot et al. 1980). Because these cell forms are indistinguishable at the species and even genus level (*Bonamia* species vs. *Mikrocytos* species), misidentifications have occurred—emphasizing and exposing the limitations of histopathological methods for discriminating species of microcell parasites, *Bonamia* specifically. A microcell parasite was detected in *C. gigas* from Hawaii in 1972 (Farley et al. 1988), and reexamination of the tissue using *in situ* hybridization revealed that it was a *Bonamia exitiosa*-like species based on SSU rDNA probes, not *Mikrocytos mackini*, another microcell parasite, as originally identified using histopathology. It was surprising, then, to find a cryptic *Bonamia* sp. in *O. sandvicensis*, though more sampling is needed to determine if the *B. exitiosa*-like species is contemporarily present.

The presence/absence of certain cell forms also cannot serve as morphological indicators of *Bonamia* species since production of certain cell forms, such as spores, could vary due to stress or other environmental conditions. Until the observation of spores in *B. perspora* (Carnegie et al. 2006), it was thought that *Bonamia* was a genus of non-spore formers within a phylum of spore-forming protists. Using characters that may be influenced by processes other than evolution have the potential to confound phylogenetic analyses. So, ultrastructural characters were often used to define species of *Bonamia*, such as diameter of cells, nuclei, or haplosporosomes. However, these dimensions overlap for all species (see Table 4 in Carnegie et al. 2006), rendering

ultrastructure problematic for species designation as well. Less confounding methods for determining species boundaries and relatedness must therefore be considered.

Molecular analyses have been increasingly used to characterize and classify closely related taxa. DNA analyses have limitations as well, but when taxa are indistinguishable morphologically such as *Bonamia* species, great strides can be made in the clarification of taxonomy and phylogenic relationships using these techniques: including "safeguarding against duplicate taxonomic descriptions" (Tautz et al. 2002). Genomic DNA sequences also do not vary with life stage or developmental phase or with varying hosts and/or tissue locations (Berthe et al. 1999). These analyses are also less subjective and would therefore decrease the chances of misidentification.

The rRNA gene complex has been used most commonly in examining the relationships of members within the phylum Haplosporidia (Flores et al. 1996; Carnegie et al. 2000, Reece and Stokes 2003; Cochennec-Laureau et al. 2003; Reece et al. 2004; Burreson and Reece 2006; Carnegie et al. 2006). SSU rDNA is also used in barcoding (e.g., Chantangsi and Leander 2010). Here, the SSU rRNA gene is used to reconstruct the phylogeny of *Bonamia* parasites, and allows for identification of a novel *Bonamia* species when paired with histopathological confirmation.

Further support for the Hawaiian *Bonamia* sp. being novel lies in the identity of the host, which most likely belongs to the subfamily Lophinae. Partial *O. sandvicensis* 16S mtDNA was sequenced, and a BLAST search revealed similarity to *Dendostrea* and *Alectryonella* species, which belong to the subfamily Lophinae within the family Ostreidae. This molecular evidence suggests that *O. sandvicensis* has a closer affinity to

lophine genera (*Lopha*, *Alectryonella*, and *Dendostrea*) than to ostreine genera (*Ostrea*, *Ostreola*, *Cryptostrea*, and *Teskeyostrea*). *Bonamia* species are typically associated with members of the ostreid oysters (Farley et al. 1988), but *Bonamia* sp. have since been detected in crassostreid species: *Crassostrea angulata* (Katkansky et al. 1969), *Crassostrea rivularis* (syn. *ariakensis*) (Cochennec et al. 1998), and *Crassostrea ariakensis* (Burreson et al. 2004); and saccostreid species, *Saccostrea glomerata* (Cochennec-Laureau et al. 2003; Chapter One). However, no association with lophine species has been documented to date.

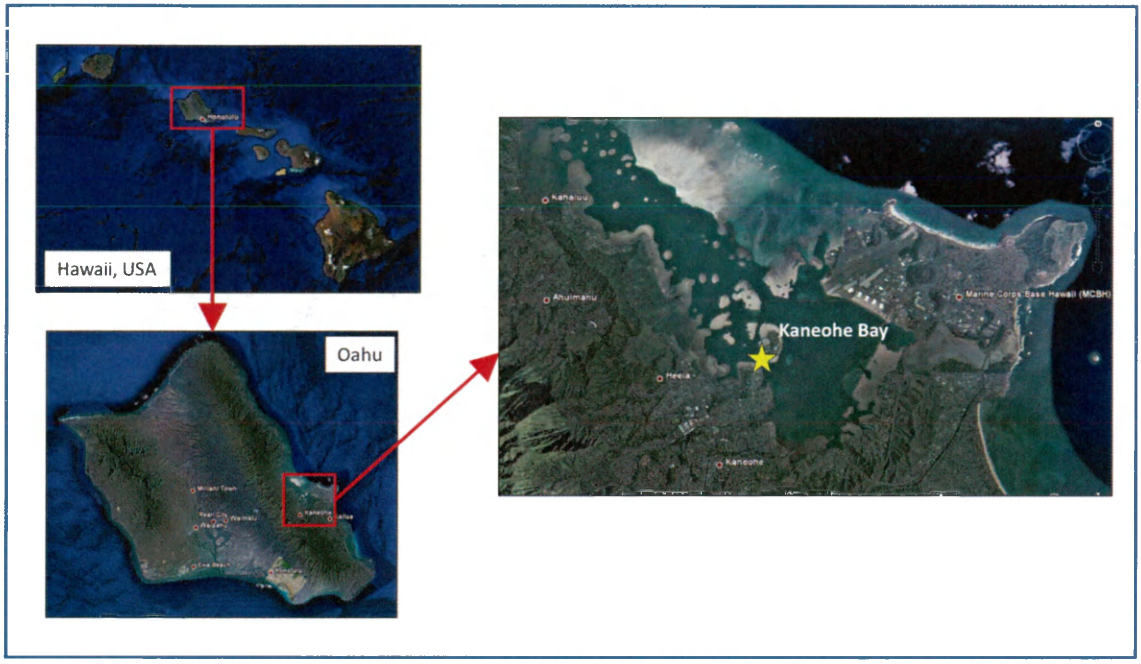
Members of the subfamily Lophinae are thought to be older evolutionarily than those comprising Ostreinae based on paleontological (Stenzel 1971) and molecular evidence (partial 28S rDNA in Littlewood 1994 and 16S mtDNA in Jozefowicz and Ó Foighil 1998). A lophine species, then, should appear basal in an oyster phylogeny containing Ostreinae and Lophinae species. Both phylogenetic analyses of SSU rDNA produced topologies where the Hawaiian *Bonamia* sp. is placed basal to the entire *Bonamia* clade—suggesting that this *Bonamia* sp. is ancestral to other described *Bonamia* species. Additional genetic loci need to be examined, such as actin or COI mtDNA, to determine if this topology is constant across more than one locus. However, if Farenholz' rule, which states that "parasite phylogeny mirrors host phylogeny" (Brooks 1979) is regarded, then the placement of this *Bonamia* sp. at the base of the *Bonamia* tree may be correct. Subsequent investigators should address the placement of *O. sandvicensis* in the genus *Ostrea*, and a co-evolutionary study involving both host and parasite, could further illuminate the origin of *Bonamia* species.

If the Hawaiian *Bonamia* sp. is indeed ancestral, we might expect to see a spore stage since this is a character that defines all haplosporidians, and *B. perspora*, which is known to produce spores, appears to evolve from the Hawaiian *Bonamia* sp. according to the SSU rDNA tree topology. No evidence of spores was observed in the Hawaiian *Bonamia* sp., though. If the SSU rDNA phylogeny is correct, and the Hawaiian *Bonamia* sp. does not produce spores, then perhaps sporulation was abandoned twice during the evolution of *Bonamia* species—once with the Hawaiian *Bonamia* and again with species derived after *B. perspora.* Alternatively, all or some *Bonamia* species may have the ability to sporulate under certain conditions (perhaps due to environmental stressors) or switch reproductive strategies, and we have only been able to capture this in *B. perspora.* Additional histopathology will need to be done in order to determine if either hypothesis is correct.

Did *Bonamia* species, which are best known as inhabiting oysters of more temperate and cold-water climates, originate from tropical waters? Or will the examination of other loci support an alternative and more parsimonious hypothesis with respect to spore evolution: showing *B. perspora* as ancestral to all other *Bonamia* species based on the observation of sporulation (Carnegie et al. 2006) and its more typical haplosporidian cell cycle? As mentioned above, additional loci need to be examined and more samples from tropical regions need to be obtained in order to address these questions. The impact of this parasite on oysters in Hawaii is also unknown, as *O. sandvicensis* are not harvested commercially. However, the knowledge of the parasite's presence is important when considering importation of species into Hawaii for aquaculture. As has been made evident in Europe and in Australia and New Zealand,

other *Bonamia* species have the potential to cause disease and devastating mortality in oyster populations.

FIGURE 2.1



Google Earth images of the collection site (indicated by the star) in Kaneohe Bay, Hawaii, USA near the Hawaii Institute of Marine Biology.

CHAPTER THREE: Phylogeography of *Bonamia exitiosa* Based on Internal Transcribed Spacer (ITS) Region Ribosomal DNA (rDNA) Sequence Data

OBJECTIVES

Objective 1: Evaluate sequence variation in the ITS region rDNA of *B. exitiosa* among all known hosts and locations to infer differences in population structure.

$\mathbf{H_0}$: ITS region rDNA variation of *B. exitiosa* is homogenous among populations, and therefore, this particular marker is not suitable to distinguish populations of *B. exitiosa.*

$\mathbf{H_A}$: ITS region rDNA variation of *B. exitiosa* is heterogeneous among populations, and thus produces a geographic signal upon which dispersal hypotheses can be based.

Objective 2: Based on network analyses of the ITS region rDNA (Objective 1), develop dispersal hypotheses regarding how *B. exitiosa* came to achieve its wide distribution.

$\mathbf{H_1}$: *Bonamia exitiosa* achieved its current distribution through multiple anthropogenic introductions of infected host species such as transplantation of oysters for aquaculture or fisheries restoration or via ship hulls or ballast water.

$\mathbf{H_2}$: *Bonamia exitiosa* was dispersed with its oyster hosts naturally via rafting or other non-anthropogenic means.

$\mathbf{H_3}$: Dispersal of the parasite occurred via vicariance, i.e. the separation of continuous ancestral populations or taxa by environmental events such as the breakup of continents (Avise 2000).

$\mathbf{H_4}$: A combination of the above events led to the current distribution of *B. exitiosa.*

INTRODUCTION

Bonamia exitiosa (Hine et al. 2001) is a protistan parasite of oysters within the phylum Haplosporidia (Sprague 1979). It was originally observed in *Ostrea chilensis* in New Zealand (Dinamani et al. 1987), but since its description (Hine et al. 2001), it has been observed in several oyster hosts in various locations around the world as found in Chapter One (Table 3.1). It infects *O. chilensis* from New Zealand, and also potentially *Ostrea auporia* from New Zealand based on PCR results. However, there is no histological evidence for the latter (Chapter One). It also infects *Ostrea angasi* and *Saccostrea glomerata* in Australia; *Ostrea puelchana* and *Ostrea equestris* from Argentina; wild *O. equestris* and experimental *C. ariakensis* along the southeastern USA; *Ostrea conchaphila* in California; and *Ostrea stentina* in Tunisia (Chapter One). Even though it is not yet clear how bonamiasis impacts some populations, especially with respect to non-commercial hosts, understanding how it came to achieve its wide distribution is important since this parasite has caused severe mortality in some oyster populations (Doonan et al. 1994, Burreson et al. 2004, Cranfield et al. 2005). If the most recent observations of *B. exitiosa* are the results of contemporary introductions, and not of long, established presences that have gone unnoticed, it is imperative that preventative measures be taken to obviate similar economic and ecological losses due to accidental introductions elsewhere.

Phylogeography explores the principles and processes involved in the geographical distributions of genealogical lineages, especially those within and among closely related species (Avise 2000). The internal transcribed spacer (ITS) region ribosomal DNA (rDNA), defined as ITS-1 and ITS-2 region rDNA and the intervening

5.8S rRNA gene, has been useful in distinguishing *Bonamia* at the species level with the discovery of cryptic *Bonamia* species diversity in *O. edulis* from California and *O. chilensis* from Chile (Chapter One). Is there enough variability in the ITS region rDNA, then, to determine the population structure of a single species like *B. exitiosa*? Furthermore, can the diversity of sequences provide information regarding approximate timing of introduction (i.e., recent vs. not-recent) in order to better understand when, and perhaps lead to hypotheses of how, current populations were established?

Some dispersal hypotheses have already been put forth. For example, *B. exitiosa* purportedly reached Australia from its presumed origins in New Zealand through shipment of live, commercial-sized oysters, which were held in Victorian and Tasmanian waters in the early 1990s (Hine and Jones 1994, Hine 1996). Additionally, Abollo et al. (2008) detected a *B. exitiosa*-like species in *O. edulis* in Galicia, NW Spain, and they hypothesize that the parasite could have been inadvertently introduced through the legal or illegal importation of oysters from *B. exitiosa*-endemic areas. The authors also suggest the possibility of an introduction via the ballast water and outer hulls of ships, which was a hypothesis proposed by Bishop et al. (2006) regarding the presence of *B. exitiosa* in North Carolina. I aimed to test the validity of these standing hypotheses and develop additional hypotheses regarding the dispersal of *B. exitiosa* using network analyses to examine ITS region rDNA sequences of *B. exitiosa* found in New Zealand, Australia, Argentina, Tunisia, and along the east and west coasts of the United States.

MATERIAL AND METHODS

Sequence Alignment

Bonamia exitiosa sequences obtained in Chapter One and from the VIMS Shellfish Pathology Laboratory archives were aligned using the automatic setting in MAFFT v. 6 (Katoh and Toh 2008). Two alignments were performed: one containing complete *B. exitiosa* ITS region rDNA sequences (defined as complete ITS-1, the 5.8S rRNA gene, ITS-2, and ~20 bp of large subunit (LSU) rDNA at the 3' end of each sequence) and an expanded alignment containing complete ITS region rDNA sequences and partial SSU rDNA (~220 bp at the 5' end of each sequence); the amplicon generated in PCR by the HaploITSf + ITS-B primers (Hill et al. 2010). To date, the identified SSU rDNA sequences of *B. exitiosa* vary by less than 1%, but there are some polymorphisms present. Network analyses were done with the inclusion and exclusion of this portion to see if the polymorphisms present affected the resulting topologies.

Before the alignments were performed, clones from a single individual were compared. When there were identical clones from a single individual, a consensus sequence was used in the alignment. The GenBank accession numbers of the sequences used for each alignment are listed in the Appendix. Once each alignment was produced, the ends were trimmed so that all sequences were of equal length.

The total number of unique sequences per total number of clones with respect to geographic location was calculated (Table 3.2). Because the number of clones per region varied, the number of unique clones was standardized to 12 clones per individual oyster in order to compare diversity at each region on a more even scale (Table 3.3 and Figure 3.3).

Network Analyses

Both alignments of *B. exitiosa* sequences described above were analyzed using TCS (Clement et al. 2000), which estimates gene genealogies and is also known as statistical parsimony. Gaps were treated as a fifth state and the program calculated maximum connection steps at 95%.

RESULTS

Sequence Alignment

For the ITS region rDNA alignment, 265 *B. exitiosa* sequences, representing two to 19 unique sequences obtained from an individual host, found among a total of 447 PCR fragments (503 bp) whose sequences were determined from a total of 9 oyster host species were aligned. Two hundred ninety *B. exitiosa* sequences representing two to 13 unique sequences obtained from an individual host were found among a total of 410 cloned PCR fragments (720 bp) whose sequences were again determined from a total of 9 oyster host species. These 292 sequences were included in the expanded alignment (See Appendix). Note that the additional sequences represented in the expanded dataset only had polymorphisms in the SSU rDNA portion.

Information regarding the number of individual oysters per geographic region from which *B. exitiosa* sequences were obtained and other information regarding clone number and averages per geographic location are presented in Table 3.2. *Bonamia exitiosa* sequences were obtained from two to 12 and two to 11 oysters in the ITS region rDNA analysis and the expanded dataset analysis, respectively. The number of clones per individual varied from three to 28, and the number of unique sequences per individual

ranged from two to 20 (Table 3.2). The total number of clones per region ranged from 24 (Australia) to 106 (California) in the ITS region rDNA analysis (Table 3.2a) and from 14 (Australia) to 104 (California) in the expanded dataset (Table 3.2b), with the total number of unique sequences ranging from 13 (Australia) to 62 (California) and seven (Australia) to 82 (California), respectively. The ratio of unique sequences per oyster host to the total number of clones obtained per region ranged from 0.33 (North Carolina, South Carolina, Florida) to 0.58 (California) for the ITS region rDNA analysis and 0.5 (Australia) to 0.79 (California) for the expanded dataset.

TCS Analysis

The resulting networks from the TCS analyses are shown in Figures 3.1 and 3.2. Individual sequences are represented by either solid ovals or pie charts, if the specific sequence was found in more than one location, which are described in more detail below. Colors represent sampling locations from which sequences were obtained.

TCS Analysis of ITS Region rDNA. Of the 265 *B. exitiosa* ITS region rDNA sequences analyzed, 195 unique sequences were found overall among all hosts and locations. The resulting network is in Figure 3.1. There appear to be four reasonably well-defined clusters: (1) the "Cosmopolitan Group," which includes *B. exitiosa* sequences from almost all sampling locations except California; (2) the "Atlantic Coast" group, which represents sequences from North and South Carolina, Florida, Argentina, and one sequence from Tunisia and is the least distinct of the clusters; (3) the "Southern Hemisphere" group, which is composed mostly of sequences from Argentina, New

Zealand, Australia; and (4) the "California" Group, which includes sequences found only in California.

The most common sequence (represented in Figure 3.1 by the large pie chart within the "Cosmopolitan Group") was found at every sampling location where *B. exitiosa* ITS region rDNA sequences were obtained except in California, and in every oyster host species except *O. conchaphila* from California, *S. glomerata* from Australia, and *O. chilensis* from New Zealand. This sequence was found in a total of 25 individuals: four *O. stentina* from Tunisia (four oysters from Tunisia of 25 total oysters from which this sequence was obtained or 16% of the pie chart), four *O. puelchana* and three *O. equestris* from Argentina (28%), one *O. auporia* from New Zealand (4%), one *O. angasi* from Australia (4%), five *O. equestris* and seven *C. ariakensis* from North Carolina, South Carolina, and/or Florida (48%). The pie chart seen extending down in the "Cosmopolitan Group" region of the network (Figure 3.1) represents a sequence found in five individuals: one *O. stentina* from Tunisia (20%), two *O. puelchana* from Argentina (40%), and two *C. ariakensis* from North Carolina (40%).

The second most common sequence (represented in Figure 3.1 by the large pie chart at the bottom of the "Southern Hemisphere" group) was found in nine individuals: two *O. puelchana* from Argentina (22%), three *O. chilensis* and three *O. auporia* from New Zealand (66.7%), and one *S. glomerata* from Australia (11.1%). The small, orange and yellow pie chart extending from this larger pie chart represents a sequence found in two individuals: one *O. chilensis* from New Zealand (50%) and one *O. puelchana* from Argentina (50%). The other large pie chart found near the top of the "Southern Hemisphere" group in Figure 3.1, represents a sequence found in four individuals: three

O. chilensis from New Zealand (75%) and one *O. equestris* from Argentina (25%). A smaller, orange and red pie chart extends from this and represents a sequence found in two individuals: one *O. equestris* from Argentina (50%) and one *O. stentina* from Tunisia (50%).

The third most common sequence (represented in Figure 3.1 as the large blue oval at the top of the "Atlantic Coast" group, marked with an asterisk) was only found in North and South Carolina in a total of eight individuals: four *O. equestris* and four *C. ariakensis*. The next most common sequence (represented in Figure 3.1 by the large blue oval in the "Atlantic Coast" group marked with two asterisks) was only found in North Carolinian oysters. This sequence was found in three *O. equestris* and three *C. ariakensis* (a total of six individual oysters). Also within the "Atlantic Coast" group (Figure 3.1), the larger oval extending from the single-asterisked blue oval represents a sequence found in two *O. equestris* and one *C. ariakensis* from North and South Carolina (a total of three individual oysters, as indicated by a "3" in Figure 3.1). At the center of the small network extending from the double-asterisked blue oval (found at the bottom of the "Atlantic Coast" group, Figure 3.1) is a blue oval representing a sequence found in one *O. equestris* and one *C. ariakensis* from North Carolina. The blue oval above a "2" in the "Atlantic Coast" group (Figure 3.1) represents two *O. equestris* from North Carolina.

Each of the two, large green ovals seen in the "California" group (Figure 3.1) represents a different sequence (1 bp difference). Both sequences were found in a total of five individuals each and were only found in *O. conchaphila* from California. The yellow and green pie chart that is seen between the "California" and "Southern Hemisphere" groups (Figure 3.1) represents a sequence found in four individuals: two *O. auporia* and

one *O. chilensis* from New Zealand (75%) and one *O. conchaphila* from California (25%).

TCS Analysis of the Expanded Dataset. Of the 290 *B. exitiosa* SSU-ITS region rDNA sequences analyzed, 234 unique sequences were present. The resulting network can be found in Figure 3.2. Again, four reasonably well-defined clusters emerged, as above in the ITS region rDNA analysis.

The most common sequence (represented in Figure 3.2 by the large pie chart within the "Cosmopolitan Group") was found at every sampling location where *B. exitiosa* was detected by PCR, again except in California, and in every oyster host species except *O. conchaphila* from California, *S. glomerata* from Australia, and *O. chilensis* from New Zealand. This sequence was found in a total of 23 individuals: four *O. stentina* from Tunisia (four oysters from Tunisia of 23 total oysters from which this sequence was obtained or 17.4% of the pie chart), four *O. puelchana* and three *O. equestris* from Argentina (40.4%), one *O. auporia* from New Zealand (4.3%), one *O. angasi* from Australia (4.3%), and five *O. equestris* and five *C. ariakensis* from North Carolina, South Carolina, and/or Florida (43.4%). The smaller pie chart within the "Cosmopolitan" grouping in Figure 3.2 represents a sequence found in four individuals: one *O. stentina* from Tunisia (25%), two *O. puelchana* from Argentina (50%), and one *C. ariakensis* from North Carolina (25%).

The second most common sequence (represented in Figure 3.2 by the large pie chart at the bottom of the "Southern Hemisphere" group) was found in seven individuals: one *O. puelchana* from Argentina (14.2%), two *O. chilensis* and three *O. auporia* from New Zealand (71.4%), and one *S. glomerata* from Australia (14.2%). The smaller, yellow

and orange pie chart in the center of the small network within the "Southern Hemisphere" group (Figure 3.2) represents three individuals: two *O. chilensis* from New Zealand (66.7%) and one *O. equestris* from Argentina (33.3%). Two pie charts branch from this one: the orange and red pie graph represents one *O. stentina* from Tunisia (50%) and one *O. equestris* (50%) from Argentina; the yellow and green pie chart represents one *O. chilensis* and one *O. auporia* from New Zealand (66.7%) and one *O. conchaphila* from California (33.3%). The slightly larger yellow oval (marked with a "2" in the "Southern Hemisphere" group in Figure 3.2) represents a sequence that came from two *O. chilensis* from New Zealand.

The third most common sequence was found only in North and South Carolina (as represented by the largest blue oval marked by an asterisk in the "Atlantic Coast" group in Figure 3.2). This sequence was found in six individuals: four *O. equestris* and two *C. ariakensis*. The blue oval with two asterisks (Figure 3.2, "Atlantic Coast" group) represents a sequence found in two *O. equestris* and two *C. ariakensis* from North Carolina. The blue oval marked with a "3" represents a sequence found in two *O. equestris* and one *C. ariakensis* from North and South Carolina, and the blue oval marked with a "2" is a sequence that was found in two *O. equestris*—one from North Carolina and one from South Carolina.

As in Figure 3.1, each of the two large green ovals seen in the "California" grouping in Figure 2 represents a different sequence (1 bp different from one another, as above). Again, both sequences were found in five individuals each and were only found in *O. conchaphila* from California. All other sequences throughout the networks are

represented by small ovals of the same size in Figures 3.1 and 3.2 and were found only once in one individual oyster.

DISCUSSION

TCS network analysis (Clement et al. 2000) was performed using an alignment containing *B. exitiosa* ITS region rDNA and an expanded alignment, containing ITS region rDNA and partial SSU rDNA. Both alignments produced similar networks, though the analysis of the expanded dataset had more unique sequences as expected given the point mutations found in the SSU rDNA region. However, it appears that including or excluding the ~220-bp SSU rDNA portion did not fundamentally change the topography of the network.

The results of the analyses reveal that there is population structure among *B. exitiosa* ITS region rDNA sequences, demonstrating that while some sequences appear to be confined to particular geographic areas, others are cosmopolitan in their distribution. Both networks display a strong geographic signal in the distribution of *B. exitiosa* sequences. Each comprises four reasonably well-defined clusters: (1) the Cosmopolitan Group, which represents *B. exitiosa* sequences from almost all sampling locations except California, (2) the Atlantic Coast group, which represents sequences from North and South Carolina, Florida, and Argentina (and one from Tunisia), (3) the Southern Hemisphere group, which is composed mostly of sequences from Argentina, New Zealand, Australia, and (4) the California Group, which only includes sequences found in California. Phylogeographic patterns such as these likely indicate that historical as well as contemporary factors shaped the current distribution of *B. exitiosa*.

The predominant cell form of *B. exitiosa* in host tissue is a naked, uninucleate microcell less than 5 μm in size, but unfortunately it is unknown what form the parasite takes when released from the host and into the environment. Spores or other long-lived life stages, however, have never been observed in *B. exitiosa* infections. The duration of *B. exitiosa* survival outside of the host is also unknown, but Arzul et al. (2009) found that purified *B. ostreae* cells from host tissue, which are similar morphologically to *B. exitiosa*, had a clear preference for specific environmental conditions such as temperature (<25°), salinity (euhaline), and perhaps pH. Additionally, the percentage of *B. ostreae* cells producing esterase activity (a measure of cell viability) decreased, significantly at most salinities, after being in suspension for 48 h (Arzul et al. 2009). Therefore, it seems unlikely that the parasite could disperse great distances, traveling through inconsistent environments, on its own. Without evidence of spores or other long-lived life stages of the parasite or other apparent physical barriers of protection from varying environmental conditions, then, co-dispersal of the parasite and host(s) seems most likely.

In general, dispersal and distribution of oysters can be placed into two categories: anthropogenic or non-anthropogenic/natural. At least since Roman times, oyster species have been intentionally transplanted to novel locations worldwide (Andrews 1980), usually in an effort to supplement a fishery locally diminished by overfishing and/or disease (for summary see Ruesink et al. 2005). As a result, many pathogens have been introduced to new locales and to naïve, native hosts (Bishop et al. 2006)—with translocation of aquatic organisms being a major underlying cause of molluscan disease outbreaks (Berthe et al. 1999). Whether intentional, as with introduction for aquaculture or fisheries restoration, or not, such as with hitchhiker species—human intervention has

shaped the current geographic distribution of oysters, and likely the distribution of infecting parasites.

Of course, natural mechanisms have also influenced the current distribution of oysters and their parasites. Beyond range expansion via transmission from one oyster to the next in juxtaposing populations, other mechanisms facilitate dispersal of hosts and their parasites. Such mechanisms include rafting on substrata such as plants, wood, and volcanic pumice along oceanic currents (Barber et al. 1959, Ó Foighil et al. 1999, Thiel and Gutow 2005), as well as vicariance, the separation of continuous ancestral populations or taxa by environmental events, e.g. the breakup of a continental landmass severing populations (Avise 2000). In some cases it is impossible to distinguish between anthropogenic and natural means of dispersal. In others, resulting data and historical context provide a clearer indication of one or the other. Based on the geographic patterns resulting from the TCS network analyses, as well as the diversity of the sequences at each location, I discuss potential distribution hypotheses of *B. exitiosa*. A summary of these hypotheses can be found in Table 3.4.

Southern Hemisphere Sequences

Some *B. exitiosa* sequences seem to be restricted to the Southern Hemisphere, as depicted in the "Southern Hemisphere" network in Figures 3.1 and 3.2. Thirty-eight sequences for the ITS region rDNA analysis and 44 sequences for the expanded dataset analysis were found only in Argentina, New Zealand, and Australia, with two exceptions: one sequence from California that is identical to a sequence found in New Zealand, and another sequence found in Tunisia that is identical to a sequence also found in Argentina.

Because of the geographic disjunction between these particular sequences, it is possible that these are evidence of convergent evolution/homoplasy. However, it is also possible that the identical sequences originated from one locale or the other and were subsequently distributed via an unknown mechanism (perhaps anthropogenic facilitation given the geographic distance) at an unknown time. Unfortunately, there is no way to distinguish between these two possibilities given these data.

With respect to all other sequences found in this network, it appears that some mechanism is allowing for gene flow to occur between populations of *B. exitiosa* in the Southern Hemisphere. A likely mechanism is the rafting of oysters infected with *B. exitiosa* in surface currents such as the Antarctic Circumpolar Current. While the surface currents are complex and do vary depending on season (especially in the Indian and western Pacific Ocean) the predominant flow is eastward (Wright 1989), linking all the southern hemispheric sampling sites. Volcanic pumice is one particular substrate that follows along this trajectory—from the South Sandwich Islands (off the coast of Argentina) on to Australia and New Zealand (Coombs and Landis 1966), and from New Zealand to Chile (referenced in Ó Foighil et al. 1999) and can stay afloat for months to several years (Thiel and Gutow 2005). Furthermore, Ó Foighil et al. (1999) discuss that rafting on volcanic pumice most likely facilitated the trans-Pacific dispersal of host, *O. chilensis*, from New Zealand to Chile, ruling out vicariance and human introduction. Bull kelp (*Durvillaea antarctica*) rafts are also abundant in the Southern Ocean (Smith 2002), and Donald et al. (2005) argued that perhaps this substratum is what transported *O. chilensis* from New Zealand to Chile instead of pumice since this species commonly lives in bull kelp holdfasts (Powell 1979 in Donald et al. 2005). Either substratum certainly

provides a means of transport for the host, which could conceivably transport the parasite *B. exitiosa.*

Bonamia exitiosa proper has yet to be found infecting *O. chilensis* in Chile. However, a cryptic *B. exitiosa*-like species that has an identical SSU rDNA sequence, but divergent ITS region rDNA sequence has been found in Chilean *O. chilensis* (White 2008, Chapter One). Perhaps *B. exitiosa* was not found in Chile because the sample size was too low (n = 32), especially if the parasite occurs in low prevalence. Nevertheless, overall the data support the connectivity of the southern hemispheric *B. exitiosa* populations. To further validate this hypothesis additional samples from Chile and from the African coasts would need to be collected. Of course, other dispersal factors may be involved as well, but the resulting geographic signal from the ITS region rDNA network analyses, along with indication of natural dispersal of a oyster host species, supports, at least in part, natural dispersal of *B. exitiosa* in the Southern Hemisphere via rafting.

Californian Sequences

Fifty-nine *B. exitiosa* sequences from the ITS region rDNA analysis and 71 sequences from the expanded dataset from *O. conchaphila* from California were not found at any other sampling location, with the exception of two (Figure 3.1) or three identical clones (Figure 3.2) from New Zealand. Additionally, sequences from other regions were not found in the California cluster. Based on this analysis, it appears that there is little or no connectivity between California *B. exitiosa* populations and those of other regions. It is difficult to speculate as to what makes California unique compared to other sampling locations in this study, but one host that appears to be present at all

locations, but not in California, is *O. stentina.* A recent phylogenetic study of *Ostrea* species using nuclear ITS-1 and mitochondrial 16S and cytochrome c oxidase subunit I (COI) loci found *O. stentina*, *O. equestris*, and *O. auporia* to be conspecific (Shilts et al. 2007). All are known hosts of *B. exitiosa*, and occur in nearly every geographic region from which *B. exitiosa* has been detected: New Zealand/Australia, the southeastern USA, and the Mediterranean Sea, with the exception of California (unless it is present cryptically). Therefore, it is possible that *B. exitiosa* co-dispersed with this single host, providing a possible explanation for connectivity among sequences found at all sampling sites, except California, in the Cosmopolitan Group (Figures 3.1 and 3.2). For California, though, I hypothesize that the presence of *B. exitiosa* may reflect a limited invasion event, perhaps from the Southern Hemisphere or the Atlantic Coast (Figures 3.1 and 3.2). It could be that *B. exitiosa*-infected *O. stentina*/*O. auporia*/*O. equestris* were introduced and subsequently transmitted the parasite to the native oyster, *O. conchaphila*, but did not establish populations, or again, perhaps this oyster species is present cryptically.

In Elkhorn Slough, California alone, 38 of 58 known marine invasive species were likely introduced through oyster culture (referenced in Ruesink et al. 2005), so if not *O. stentina*, another host could have introduced the parasite. *Crassostrea gigas*, for instance, is one of the most cosmopolitan macroscopic marine invertebrates (Ruesink et al. 2005) and has been hypothesized to be a carrier or reservoir for *B. exitiosa* (Lynch et al. 2010). *Bonamia exitiosa* ITS region rDNA sequences from *C. gigas* were not obtained for this study, but should be considered in future efforts to better understand the patterns seen here.

The presence of *B. exitiosa* in California does not appear to be a recent invasion based on the high amount of sequence diversity compared to the standardized number of unique sequences from the other regions examined, especially when compared to Australian and Argentinean sequences (Table 3.3 and Figure 3.3). Evidence of a recent introduction would likely show a network dominated by a single sequence. Unfortunately, because there is no molecular clock, timing of introduction cannot be addressed in a more systematic and specific manner.

Additionally, California *B. exitiosa* sequences were unique and not found elsewhere. Because *O. conchaphila/O. lurida* populations have been so depleted due to overfishing of natural beds in the last half of the 19th century with continued decline in the mid-1920s (Andrews 1980), there is limited movement of this host species. Assuming a tight dispersal association with host and parasite, limited movement of the host would thereby limit the dispersal of the parasite.

As discussed in Chapter One, *Bonamia* species diversity appears to be great in California relative to other sampling regions—with observations of a cryptic *B. exitiosa*-like species in *O. edulis*, *B. ostreae*, and *B. exitiosa.* Could this diversity somehow be related to the isolation of these parasites as indicated by the lack of connectivity of *B. exitiosa* ITS region rDNA sequences from California?

Cosmopolitan Sequences

The "Cosmopolitan Group" in Figures 3.1 and 3.2 represents sequences found in all sampling locations except in California. Perhaps this is representative of dispersal over some unknown time period of a lineage particularly adaptable to new hosts and

environments, or it may reflect recent extensive anthropogenic dispersal. Mechanisms of distribution probably vary, but anthropogenic means (e.g. intentional and unintentional introduction or transplantation of oysters for aquaculture or fisheries restoration, or via ship hulls or ballast) seem most likely given the geographic disjunction of these sequences.

As mentioned above, Shilts et al. (2007) recently synonymized *O. stentina*, *O. equestris*, and *O. auporia*. Each of these oyster species were described in the 1800s: *O. stentina* Payraudeau in 1826, *O. equestris* Say 1834, and while *O. auporia* was described in 1981 by Dinamani and Beu, it is synonymous with *Ostreola virescens* Angas 1868 (Cook 2010). Thus, this oyster species has been established in its various locales for over 142 years, making it possible for *B. exitiosa* to have been established for at least this long. Therefore, introduction via natural or anthropogenic means in recent decades cannot fully explain the distribution of this single host and this parasite. Without a molecular clock and more genetic data of the hosts themselves, it is difficult to say exactly when this distribution occurred. Conspecificity of other oyster hosts may have led to the current distribution of parasites, but there is still some discrepancy as to the phylogeny of the oysters themselves. As Carriker and Gaffney (1996) report: the taxonomy and systematics of oysters are far from being resolved.

Wooden ships during the Age of Exploration traveling from the Mediterranean and the Antipodes on to west and east coasts of North and South America fouled with a small oyster, such as *O. stentina*, could have provided transport of *B. exitiosa*. Wooden ships and vessels had little or no antifouling treatment during the Age of Exploration (referenced in Carlton and Hodder 1995), so shipping traffic during this time could have

played an important role in influencing gene flow of *B. exitiosa* among these regions. This impact could be historically important as ships and vessels inadvertently provided recurrent and perhaps continuous transport between isolated environments (Carlton and Hodder 1995)—perhaps extending the range and facilitating the establishment of new populations of oyster hosts when physical barriers might prohibit natural dispersal, and meanwhile, helping to expand the range of *B. exitiosa* as well. While this could be influencing gene flow currently (as of the 20th century), it probably is doing so to a lesser degree: faster boats allow for decreased retention of fouling organisms, decreased port residency times allow for decreased fouling accumulations, the use and efficacy of antifouling treatments has increased, and vessels are better maintained (Carlton and Scanlon 1985; Carlton 1992). The building material of ships themselves also effect fouling: wood is more favorable to settle upon than steel (Allen 1953).

Bishop et al. (2006) suggested that recent anthropogenic dispersal via ballast waters may explain how *B. exitiosa* came to be in North Carolina. However, the ITS region sequence diversity that we see in North Carolina, South Carolina, and Florida, is not indicative of a recent introduction. Again, if it were recent, I would expect for the data to demonstrate a founder effect.

Atlantic Coast Sequences

Network analyses of *B. exitiosa* ITS region rDNA also reveals a more loosely defined cluster of sequences that are closely related to the "Cosmopolitan group," but appear to be restricted to the western Atlantic Coast (North Carolina, South Carolina, Florida, and Argentina; Figures 3.1 and 3.2), found in wild *O. puelchana* and *O.*

equestris, and experimental *C. ariakensis*. *Ostrea equestris* (syn. *O. stentina* (Shilts et al. 2007)) is thought to extend from North Carolina south to Argentina (Harry 1985), and if, in fact, a continuous population of this host exists, it is reasonable to assume that the "Atlantic Coast" *B. exitiosa* dispersed naturally through direct transmission. With oyster populations in close proximity, other non-anthropogenic facilitation mechanisms such as hydrodynamics and topographical features may also affect the distribution of the parasite through the water column (Cranfield et al. 2005). In this case, it is possible that the distribution of *B. exitiosa* along the Atlantic Coast may have little to do with the transport of hosts. Though, natural co-dispersal with a host or hosts is also a possibility and difficult to distinguish from the former.

Potential anthropogenic impacts also cannot be discounted. Being a small, non-commercial species (Harry 1985), it is unlikely that *O. equestris* were intentionally moved for aquaculture or restoration purposes. However, it is possible that these small oysters were one of the many culprits of ship fouling as mentioned above, or they could have been hitchhikers, attached to commercially important species. Meanwhile, oyster transplantations could have allowed for transport of *B. exitiosa* along the Atlantic Coasts of North and South America as well.

Future work should include determining if there is a continuous range of susceptible hosts along the North and South American Atlantic coastlines and if most *B. exitiosa* sequences found from North Carolina to Argentina continue to cluster with the Atlantic coast group.

Origins of Bonamia exitiosa

With increasing observations of *B. exitiosa* around the world, the origin of the parasite is becoming less clear. Unfortunately these results do not indicate an origin. Because *B. exitiosa* was first observed histologically in *O. chilensis* from Foveaux Strait New Zealand in 1964 (Hine and Jones 1994), it has been hypothesized that the parasite is enzootic to this region (Corbeil et al. 2006 call it "antipodean;" Hine 1996). Based on the results of this study, however, one could argue that it could have South American origins, as sequences from Argentina are the most widely distributed throughout the networks. Could this be an indication of origin? More clones were available for analysis from Argentina (106) than from Australia/New Zealand (60), but the standardized number of unique sequences per individual oyster is smallest in Australia compared to other regions (Table 3.2, Figure 3.3), which could be an indication of less diversity and therefore, a more recent introduction to Australia.

The SSU rDNA phylogeny from Chapter One suggests that *Bonamia* species may have evolved from tropical regions. Exploration of more tropical locations from additional non-commercial host species may provide further insight to the derivation of *B. exitiosa.*

Future Work

In order to better understand the current distribution and historical dispersal of *Bonamia* species, more samples need to be obtained to fill geographic gaps: South Africa, Asia, and the Caribbean, in particular. These hypotheses are based on a limited number of data obtained, so undersampling may have occurred. Sample sizes were small in

Australia and New Zealand, where *B. exitiosa* is thought to have originated, as stated above. Therefore, the results may be biased.

Also, in order to better understand how *B. exitiosa* came to achieve its current distribution, it is essential to understand how its hosts were distributed. The most likely hypothesis is that parasite distribution is heavily influenced by the distribution of hosts, and perhaps that of one widely distributed host, *O. stentina.* Subsequent investigators should test this hypothesis by developing multiple genetic loci of oyster hosts and *B. exitiosa,* and further, develop a molecular clock. This could help elucidate phylogeographic patterns and dispersal timing of the various hosts and the parasite and perhaps lend to insight into the question of origin.

Region	Oyster Species	Location	Collection Date	Sample Size	*Bonamia* spp. PCR Prevalence (%)
West Atlantic Coast	*Crassostrea ariakensis*	Fort Pierce, Florida, USA	4/16/2007	17	6/17 (35.3%)
	Crassostrea ariakensis	Fort Pierce, Florida, USA	6/14/2007	19	8/19 (42.1%)
	Ostrea equestris	Old House Creek, South Carolina, USA	4/12/2006	55	2/55 (3.6%)†
	Ostrea equestris	Wilmington, North Carolina, USA	6/22/2005	200	8/200 (4.0%)††
	Ostrea equestris	Morehead City Port, North Carolina, USA	8/1/2005	150	3/150 (2.0%)†††
	Ostrea puelchana	San Antonio Bay, Argentina	3/22/2005	57	6/57 (10.5%)†
	Ostrea equestris	San Antonio Bay, Argentina	04/2007	3	3/3 (n/a*)
East Pacfic Coast	*Ostrea conchaphila*	Elkhorn Slough, California, USA	5/27/2004	15**	13/15** (21.7 - 86.7%)
	Ostrea conchaphila	Elkhorn Slough, California, USA	09/17/2009	60	53/60 (88.3%)
South Pacific	*Ostrea angasi*	Pambula River, New South Wales, Australia	11/2006	42	1/42 (2.4%)
	Saccostrea glomerata	Georges River, New South Wales, Australia	08/2007	200	1/200 (0.5%)
	Ostrea auporia	Tamaki Estuary, Glendowie, New Zealand	8/14/2007	44	4/44 (9.1%)
	Ostrea auporia	Karaka Bay, Tamaki Estuary, Auckland, New Zealand	3/13/2009	90	5/90 (5.6%)
	Ostrea chilensis	Foveaux Strait, New Zealand	01 & 03/2005	5	4/5 (n/a*)
	Ostrea chilensis	Foveaux Strait, New Zealand	06/13/2004	40	2/40 (5.0%)
Mediterranean Sea	*Ostrea stentina*	Hammamet, Tunisia	06/2007	85	8/85 (10.6%)

*Samples sent were known to have *Bonamia* sp. infections as detected by histology.
**More than one individual in a single DNA extraction, and therefore PCR reaction.
† C_f+C_r primers used instead of Bon-319F + Bon-524R.
†† Cf +Cr primers used instead of Bon-319F + Bon-524R. Five of these were *B. perspora* only, one was *B. exitiosa* only, and two were co-infections.
††† Cf +Cr primers used instead of Bon-319F + Bon-524R. Two of the three were *B. exitiosa*; one of three was *B. perspora*,

TABLE 3.1. Sample collection data for *B. exitiosa,* and PCR prevalence data using *Bonamia* generic primers Bon-319F + Bon-524R (unless otherwise noted).

a.

Region	No. Individual Oysters Sequenced	Number of clones/individual	No. of Unique Clones/Individual	Total No. of Clones	Total No. of Unique Clones	Total No. Unique Clones/Total No. of Clones
NC/SC/FL	12	3 - 11	2 - 8	97	32	0.33
ARG	8	7 - 21	2 - 12	105	58	0.55
TUN	4	11 - 20	7 - 10	55	31	0.56
NZL	7	7 - 10	2 - 8	59	23	0.39
AUS	2	11 & 13	8 & 3, respectively	24	13	0.54
CA	5	16 - 28	9 - 19	106	62	0.58

b.

Region	No. Individual Oysters Sequenced	No. of Clones/Individual	No. of Unique Clones/Individual	Total No. of Clones	Total No. of Unique Clones	Total No. Unique Clones/Total No. of Clones
NC/SC/FL	11	4 - 11	2 - 9	82	54	0.65
ARG	8	8 - 20	2 - 13	94	65	0.69
TUN	4	11 - 20	7 - 11	55	39	0.69
NZL	7	7 - 10	2 - 10	60	44	0.73
AUS	2	4 & 10	3 & 4, respectively	14	7	0.5
CA	5	16 - 26	12 - 20	104	82	0.79

TABLE 3.2. a. Sequence data for the ITS region rDNA dataset per geographic region. **b.** Sequence data for the expanded dataset per geographic region. Number of individual oysters sequenced indicates the number of individual oysters from which *B. exitiosa* sequences were obtained.

TABLE 3.3

Region	Standardized Mean No. of Unique Sequences per Oyster (± SD)
NC/SC/FL	7.17 ± 2.20
ARG	7.00 ± 2.45
TUN	8.00 ± 2.69
NZL	6.56 ± 2.51
AUS	5.33 ± 2.91
CA	7.98 ± 1.53

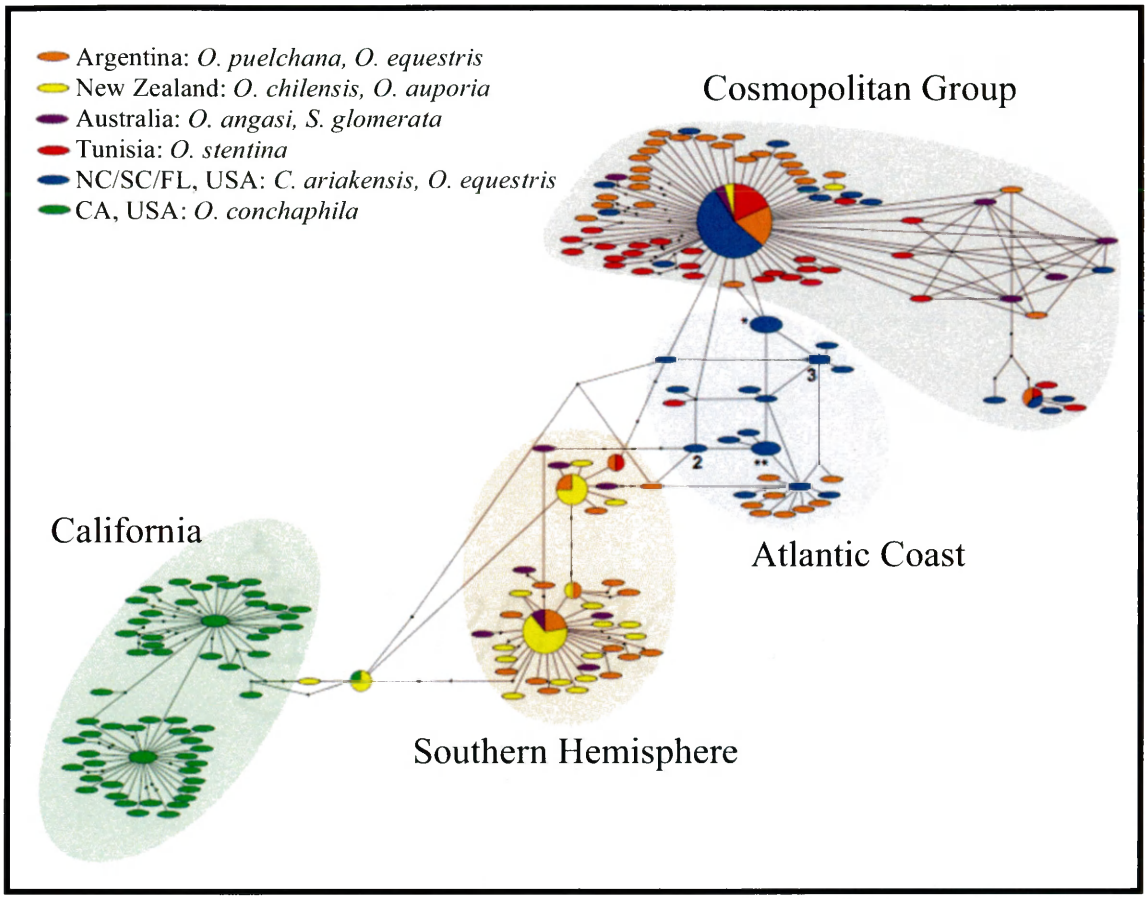
Standardized mean number of unique sequences per oyster with respect to region. Standardized to 12 clones per oyster to compensate for differences in number of clones per region (for the ITS region rDNA dataset). Corresponds to Figure 3.3.

TABLE 3.4

Geographical Grouping	Introduction Hypothesis	Proposed Mechanism
Cosmopolitan	anthropogenic & natural	Dispersal of *O. stentina*
Western Atlantic Coast	anthropogenic & natural	Ship fouling, ballast water, oyster transplantation; natural transmission from host to host
Southern Hemisphere	likely natural	Rafting with host(s) on substrata via surface currents such as the Antarctic Circumpolar Current
California	likely anthropogenic	Transplantation of oysters?

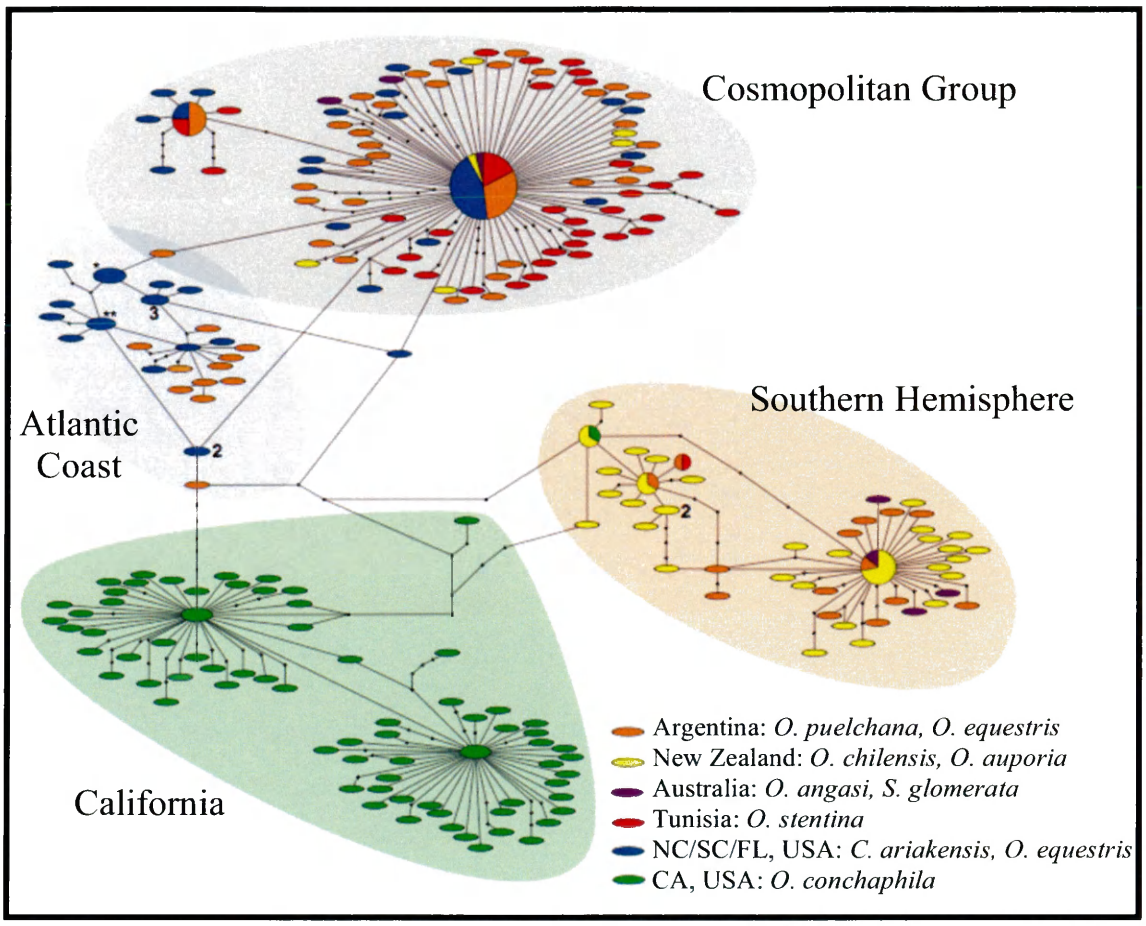
Summary of dispersal hypotheses based on the results of the TCS network analyses.

FIGURE 3.1



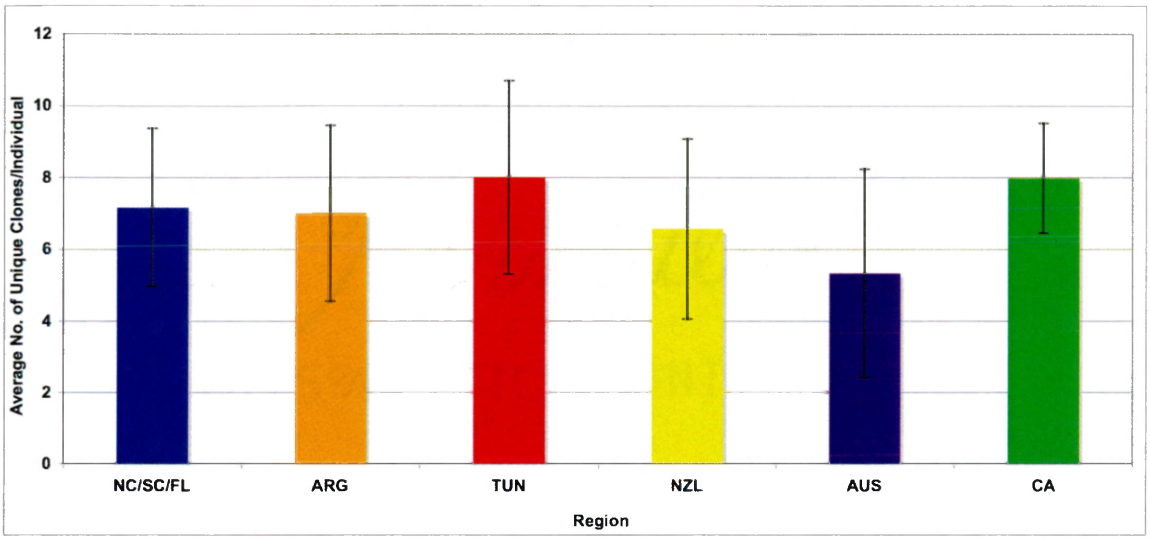
TCS network of the *B. exitiosa* ITS region rDNA dataset. Generated using TCS v. 1.21 (Clement et al. 2000) where gaps were treated as a fifth state and maximum connection steps were calculated at 95%. The network was modified using Adobe Illustrator.

FIGURE 3.2



TCS network of the *B. exitiosa* expanded dataset (partial SSU + ITS region rDNA). Generated using TCS v. 1.21 (Clement et al. 2000) where gaps were treated as a fifth state and maximum connection steps were calculated at 95%. Network was modified using Adobe Illustrator.

FIGURE 3.3



Average number of unique sequences observed per individual in each sampling region from the ITS region rDNA dataset. Standardized to 12 clones/individual given unequal mean sequence numbers/per individual (Table 3.3). Error bars indicate standard deviation.

APPENDIX A.

GenBank Accession Numbers for the ITS region rDNA Alignment (Chapter Three)

Sampling Location	Host species	Individual/Case No.	Total No. of Clones Represented	GenBank Accession No.
FL, USA	*C. ariakensis*	FLHB416-16	3	JF712868
			1	JF712867
	C. ariakensis	FLHB614Ca-10	1	JF712871
			2	JF712870
			1	JF712869
NC, USA	*C. ariakensis*	2828-6	5	EU709054
			1	EU709038
			1	EU709051
			1	EU709053
			1	pending
	C. ariakensis	2927-5	1	EU709045
			2	EU709026
			1	EU709044
	C. ariakensis	3114-2	1	EU709025
			2	EU709037
			1	EU709028
			1	EU709029
			1	EU709024
			2	EU709031
			1	EU709030
			1	EU709027
	C. ariakensis	3139-17	1	EU709034
			3	EU709039
			2	EU709033
			1	EU709036
			1	EU709035
			2	EU709032
	C. ariakensis	3218-22	3	EU709040
			5	pending
			1	EU709041
			1	EU709042
			1	pending
SC, USA	*O. equestris*	3526-49	1	JF831601
			7	JF831594
	O. equestris	3526-53	3	JF831595
			1	JF831585
			2	JF831596
			1	JF831597
			1	JF831599

Sampling Location	Host species	Individual/Case No.	Total No. of Clones Represented	GenBank Accession No.
NC, USA	*O. equestris*	2810-21	1	JF831575
			1	JF831592
			1	JF831577
			2	JF831578
			1	JF831576
			1	JF831579
			1	JF831580
	O. equestris	2845-40	1	JF831581
			8	JF831582
			1	JF831583
			1	JF831584
	O. equestris	2845-41	1	JF831586
			1	JF831587
			1	JF831588
			1	JF831589
			1	JF831590
			2	JF831591
			3	JF831600
			1	JF831602
ARG	*O. equestris*	L119-1	5	JF831574
			1	JF831573
			1	JF831572
			1	JF831570
	O. equestris	L119-2	1	JF831569
			2	JF831568
			1	JF831567
			1	JF831566
			1	JF831565
			1	JF831563
	O. equestris	L119-3	1	JF831562
			1	JF831561
			1	JF831560
			1	JF831559
			2	JF831558
			1	JF831557
			2	JF831556
ARG	*O. puelchana*	L99-12	4	EU709064
			5	pending
			1	EU709067
			1	EU709068
			4	JF831614
			1	JF831619
			1	pending

Sampling Location	Host species	Individual/Case No.	Total No. of Clones Represented	GenBank Accession No.
			1	pending
			1	pending
			1	JF831612
			1	JF831613
	O. puelchana	L99-31	1	JF831617
			3	JF831638
			1	JF831622
			2	JF831607
			1	JF831633
			2	JF831615
			1	JF831610
			1	JF831628
			1	JF831608
			1	JF831606
			1	JF831609
	O. puelchana	L99-37	1	EU709058
			9	EU709056
			1	EU709057
			1	EU709055
			1	EU709061
			1	EU709060
			1	JF831605
			1	JF831632
	O. puelchana	L99-53	9	JF831616
			1	JF831603
			1	JF831635
			1	JF831634
			1	JF831623
			1	JF831631
			1	JF831630
			1	JF831620
			1	JF831636
			1	JF831629
			1	JF831625
			1	JF831618
	O. puelchana	L101-31	8	EU709059
			1	EU709063
NZL	*O. chilensis*	Hine	4	JF831656
			1	EU709074
			1	EU709071
			2	EU709073
			1	EU709075
	O. chilensis	Ochil7	1	JF831651

Sampling Location	Host species	Individual/Case No.	Total No. of Clones Represented	GenBank Accession No.
			3	pending
			1	pending
			1	JF831646
			1	JF831652
			1	JF831647
			1	JF831640
			1	JF831653
	O. chilensis	Ochil 37	1	JF831648
			4	JF831643
			1	JF831657
			1	JF831642
			1	JF831649
			1	JF831655
	O. auporia	Oa9	7	JF831658
			1	JF831659
	O. auporia	Oa18	7	JF831664
			1	JF831665
	O. auporia	Oa39	1	JF831666
			1	JF831667
			1	JF831669
			3	JF831670
			1	JF831671
	O. auporia	5393-65	4	JF831672
			1	JF831673
			1	JF831674
			1	JF831675
			1	JF831676
			1	JF831677
AUS	*O. angasi*	O. angasi	1	EU780688
			1	EU780690
			3	EU780689
			1	EU780687
			1	EU780692
			1	EU780691
			1	EU780686
			2	JF831678
			1	JF831679
			1	JF831680
AUS	*S. glomerata*	GR-176	9	JF831681
			1	JF831682
			1	JF831683
TUN	*O. stentina*	Os/4890-58	13	GU356032
			2	GU356033

Sampling Location	Host species	Individual/Case No.	Total No. of Clones Represented	GenBank Accession No.
			1	GU356034
			1	JF831685
			1	JF831686
			1	JF831687
			1	GU356035
	O. stentina	Os/4890-73	1	JF831688
			1	JF831689
			2	JF831690
			1	JF831691
			1	JF831692
			2	JF831693
			1	JF831694
			1	JF831695
			1	JF831696
			1	JF831698
	O. stentina	Os/4890-78	1	JF831699
			1	JF831701
			2	JF831702
			1	JF831703
			1	JF831705
			1	JF831706
			1	JF831707
			1	JF831708
			1	JF831709
	O. stentina	Os/4890-79	2	JF831710
			1	JF831712
			1	JF831713
			4	JF831714
			1	JF831715
			1	JF831716
			1	JF831717
			1	JF831718
CA, USA	*O. conchaphila*	OconES-6	9	JF831719
			1	JF831720
			1	JF831721
			1	pending
			1	pending
			1	JF831722
			1	JF831723
			1	JF831724
			1	JF831725
			2	JF831726
			1	JF831728

Sampling Location	Host species	Individual/Case No.	Total No. of Clones Represented	GenBank Accession No.
			1	JF831729
			1	JF831730
			1	JF831731
			1	JF831733
			1	JF831735
			1	JF831736
			1	JF831734
			1	JF831738
	O. conchaphila	OconES-12	8	JF831739
			1	JF831740
			1	JF831741
			3	JF831742
			1	JF831743
			1	JF831744
			1	JF831746
			1	JF831748
			1	JF831749
			1	JF831750
			1	JF831751
			1	JF831752
			1	JF831753
			1	JF831755
			1	JF831756
	O. conchaphila	OconES-14	1	JF831757
			1	JF831758
			1	JF831759
			1	JF831760
			3	JF831761
			2	JF831762
			1	JF831763
			1	JF831765
			1	JF831766
			1	JF831767
			1	JF831769
			1	JF831771
			1	JF831772
	O. conchaphila	SF09-33-16	1	JF831773
			4	JF831774
			3	JF831777
			1	JF831778
			1	JF831779
			1	JF831780
			1	JF831781

Sampling Location	Host species	Individual/Case No.	Total No. of Clones Represented	GenBank Accession No.
			1	JF831782
			1	JF831783
			1	JF831784
			1	JF831785
			1	JF831786
			1	JF831787
			1	JF831788
	O. conchaphila	SF09-33-39	6	JF831789
			1	JF831790
			6	JF831791
			1	JF831794
			1	JF831795
			1	JF831796
			1	JF831797
			1	JF831799
			1	JF831800

APPENDIX B.

GenBank Accession Numbers for the Expanded dataset (Chapter Three)

Sampling Location	Host species	Individual/Case No.	Total No. Clones Represented	GenBank Accession No.
FL, USA	*C. ariakensis*	FLHB416-16	3	JF712868
			1	JF712867
	C. ariakensis	FLHB614Ca-10	1	JF712871
			2	JF712870
			1	JF712869
NC, USA	*C. ariakensis*	2927-5	1	EU709045
			1	EU709043
			1	EU709026
			1	EU709044
	C. ariakensis	3114-2	1	EU709025
			2	EU709037
			1	EU709028
			1	EU709029
			1	EU709024
			2	EU709031
			1	EU709030
			1	EU709027
	C. ariakensis	3139-17	1	EU709034
			3	EU709039
			2	EU709033
			1	EU709036
			1	EU709035
			2	EU709032
	C. ariakensis	3218-22	3	EU709040
			1	EU709041
			1	EU709042
SC, USA	*O. equestris*	3526-49	1	JF831601
			6	JF831594
			1	JF831593
	O. equestris	3526-53	2	JF831595
			1	JF831585
			2	JF831596
			1	JF831597
			1	JF831598
			1	JF831599
NC, USA	*O. equestris*	2810-21	1	JF831575
			1	JF831592
			1	JF831577
			1	JF831576
			1	JF831578
			1	JF831579

Sampling Location	Host species	Individual/Case No.	Total No. Clones Represented	GenBank Accession No.
			1	JF831580
	O. equestris	2845-40	1	JF831581
			8	JF831582
			1	JF831583
			1	JF831584
	O. equestris	2845-41	1	JF831586
			1	JF831587
			1	JF831588
			1	JF831589
			1	JF831590
			2	JF831591
			3	JF831600
			1	JF831602
ARG	*O. equestris*	L119-1	4	JF831574
			1	JF831573
			1	JF831572
			1	JF831571
			1	JF831570
	O. equestris	L119-2	1	JF831569
			1	JF831568
			1	JF831567
			1	JF831566
			1	JF831565
			1	JF831564
			1	JF831563
	O. equestris	L119-3	1	JF831562
			1	JF831561
			1	JF831560
			1	JF831559
			2	JF831558
			1	JF831557
			2	JF831556
ARG	*O. puelchana*	L99-12	2	EU709064
			1	JF831611
			1	EU709067
			1	JF831604
			1	JF831626
			2	JF831614
			1	JF831619
			1	JF831612
			1	JF831613
	O. puelchana	L99-31	1	JF831617
			1	JF831637
			3	JF831638
			1	JF831622
			2	JF831607

Sampling Location	Host species	Individual/Case No.	Total No. Clones Represented	GenBank Accession No.
			1	JF831633
			1	JF831615
			1	JF831610
			1	JF831628
			1	JF831608
			1	JF831606
			1	JF831609
	O. puelchana	L99-37	1	EU709058
			6	EU709056
			1	EU709057
			1	EU709055
			1	EU709061
			1	EU709060
			1	JF831627
			1	JF831605
			1	JF831624
			1	JF831632
	O. puelchana	L99-53	8	JF831616
			1	JF831603
			1	JF831635
			1	JF831621
			1	JF831634
			1	JF831623
			1	JF831631
			1	JF831630
			1	JF831620
			1	JF831636
			1	JF831629
			1	JF831625
			1	JF831618
	O. puelchana	L101-31	8	EU709059
			1	EU709063
NZL	*O. chilensis*	Hine	1	JF831656
			1	EU709074
			1	JF831641
			2	EU709069
			1	EU709071
			2	EU709073
			1	EU709075
	O. chilensis	Ochil7	1	JF831651
			1	JF831650
			1	JF831639
			1	JF831645
			1	JF831644
			1	JF831646
			1	JF831652

Sampling Location	Host species	Individual/Case No.	Total No. Clones Represented	GenBank Accession No.
			1	JF831647
			1	JF831640
			1	JF831653
	O. chilensis	Ochil 37	1	JF831648
			3	JF831643
			1	JF831654
			1	JF831657
			1	JF831642
			1	JF831649
			1	JF831655
NZL	*O. auporia*	Oa9	1	JF831658
			1	JF831659
			3	JF831660
			1	JF831661
			1	JF831662
			1	JF831663
	O. auporia	Oa18	7	JF831664
			1	JF831665
	O. auporia	Oa39	1	JF831666
			1	JF831667
			1	JF831668
			1	JF831669
			2	JF831670
			1	JF831671
	O. auporia	5393-65	4	JF831672
			1	JF831673
			1	JF831674
			1	JF831675
			1	JF831676
			1	JF831677
AUS	*O. angasi*	O. angasi	2	JF831678
			1	JF831679
			1	JF831680
AUS	*S. glomerata*	GR-176	8	JF831681
			1	JF831682
			1	JF831683
			1	JF831684
TUN	*O. stentina*	Os/4890-58	13	GU356032
			2	GU356033
			1	GU356034
			1	JF831685
			1	JF831686
			1	JF831687
			1	GU356035
	O. stentina	Os/4890-73	1	JF831688

Sampling Location	Host species	Individual/Case No.	Total No. Clones Represented	GenBank Accession No.
			1	JF831689
			2	JF831690
			1	JF831691
			1	JF831692
			1	JF831693
			1	JF831694
			1	JF831695
			1	JF831696
			1	JF831697
			1	JF831698
	O. stentina	Os/4890-78	1	JF831699
			1	JF831700
			1	JF831701
			1	JF831702
			1	JF831703
			1	JF831704
			1	JF831705
			1	JF831706
			1	JF831707
			1	JF831708
			1	JF831709
	O. stentina	Os/4890-79	1	JF831710
			1	JF831711
			1	JF831712
			1	JF831713
			4	JF831714
			1	JF831715
			1	JF831716
			1	JF831717
			1	JF831718
CA, USA	*O. conchaphila*	OconES-6	7	JF831719
			1	JF831720
			1	JF831721
			1	JF831722
			1	JF831723
			1	JF831724
			1	JF831725
			1	JF831726
			1	JF831727
			1	JF831728
			1	JF831729
			1	JF831730
			1	JF831731
			1	JF831732
			1	JF831733
			1	JF831735

Sampling Location	Host species	Individual/Case No.	Total No. Clones Represented	GenBank Accession No.
			1	JF831736
			1	JF831734
			1	JF831737
			1	JF831738
	O. conchaphila	OconES-12	5	JF831739
			1	JF831740
			1	JF831741
			3	JF831742
			1	JF831743
			1	JF831744
			1	JF831745
			1	JF831746
			1	JF831747
			1	JF831748
			1	JF831749
			1	JF831750
			1	JF831751
			1	JF831752
			1	JF831753
			1	JF831754
			1	JF831755
			1	JF831756
	O. conchaphila	OconES-14	1	JF831757
			1	JF831758
			1	JF831759
			1	JF831760
			1	JF831761
			1	JF831762
			1	JF831763
			1	JF831764
			1	JF831765
			1	JF831766
			1	JF831767
			1	JF831768
			1	JF831769
			1	JF831770
			1	JF831771
			1	JF831772
	O. conchaphila	SF09-33-16	1	JF831773
			1	JF831774
			2	JF831775
			1	JF831776
			3	JF831777
			1	JF831778
			1	JF831779
			1	JF831780

Sampling Location	Host species	Individual/Case No.	Total No. Clones Represented	GenBank Accession No.
			1	JF831781
			1	JF831782
			1	JF831783
			1	JF831784
			1	JF831785
			1	JF831786
			1	JF831787
			1	JF831788
	O. conchaphila	SF09-33-39	6	JF831789
			1	JF831790
			3	JF831791
			1	JF831792
			1	JF831793
			1	JF831794
			1	JF831795
			1	JF831796
			1	JF831797
			1	JF831798
			1	JF831799
			1	JF831800

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VITA

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