GENETIC AND MORPHOLOGICAL DISCRIMINATION OF SPECIES WITHIN THE NOMINAL *Brachidontes exustus* (MOLLUSCA: BIVALVIA) CRYPTIC SPECIES COMPLEX FROM THE FLORIDA KEYS

by

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ABSTRACT OF THE DISSERTATION

Genetic and Morphological Discrimination of Species

Within the Nominal *Brachidontes exustus* (Mollusca: Bivalvia)

Cryptic Species Complex from the Florida Keys

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The discovery of sibling and cryptic species complexes in the oceans has dramatically increased the estimated number of extant marine species. However, most cryptic species complexes remain taxonomically obscure, lacking descriptions of the morphological or ecological differences defining the species. The distributions and morphologies of species in the nominal *Brachidontes exustus* complex in the Florida Keys were investigated utilizing molecular and multivariate statistical techniques.

DNA barcoding, a method of comparing newly generated sequences of the mitochondrial cytocrome *c* oxidase I gene (COI) from specimens of unknown species to a database of known sequences from voucher specimens, identified two cryptic species on Long Key, Florida Keys. Two differing habitats, which were <5 km apart, had single-species populations, even though both locations were within the dispersal range of larval recruits from the other location. This was the first record from the Florida Keys for these species to be encountered as single species populations.

Tests for pseudo-crypsis among three species of the *B. exustus* morphospecies complex collected throughout the Florida Keys were performed with multivariate morphometics. Specimens were assigned to species using RFLP-based molecular methods. A discriminant function was constructed that, based on shell morphology, assigns individual mussels to a certain species with a high confidence (95%). Morphological differences among the species were sufficient to create robust statistical methods of resolving species using shell morphology alone. The suite of functions will facilitate future manipulation experiments with live specimens. The morphologies of the two most common species, provisionally called Bahamian and Gulf, were more similar in locations of coexistence than in locations of exclusivity.

An improved molecular-based technique for determining species, a multiplex PCR with species-specific forward oligonucleotides, was designed and tested. The method discriminates species by visualization of PCR products after electrophoresis on an agarose gel stained with ethidium brominde. This is a low cost, high throughput method that can effectively screen large numbers of specimens from the entire geographic range of the nominal species. This method can be used to identify species using larvae or juveniles which are unlikely to have the shell differences that can be used in the multivariate morphometric approach.

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Chapter 1

Cryptic Species in Aquatic and Marine Environments and the Scorched Mussel, *Brachidontes exustus,* Species Complex

Biodiversity, sibling species, and cryptic species complexes

A central unknown of biology is the number of extant species on Earth (May 1988). Putting aside the difficulties in adopting a species concept suitable to all branches of biology (Mayden 1997; Hey 2006), the biological species concept, in which species are defined as groups of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1963), is accepted by most biologists, zoologists, and ecologists. The most cited estimate of global species diversity is based on scaling the trophic relationships of one well-studied genus of beetle, at a single tropical rainforest location, to a worldwide estimate (Erwin 1983). Although current named species only approach 1.5 million, Wilson (1988) estimated, in consultation with other taxonomic specialists, that the actual number is most likely in the range of 5 to 30 million. Sibling and cryptic species were generally not considered in the above global species estimates. Contemporarily with the above estimates, polymerase chain reaction (PCR) and DNA sequencing techniques were being developed and applied to population genetics questions. Instances of hidden and unexpected diversity in the form of sibling species and cryptic species complexes within what had been previously presumed to be single species would clearly affect the global species estimates. The costs associated with molecular tools have subsequently dropped corresponding to an explosion of published research investigating population genetics and species boundaries.

In the few locations where cryptic species in multiple taxa have been thoroughly investigated, an order of magnitude increase in invertebrate diversity was found (Knowlton 1993). If cryptic species are ubiquitous across taxa at rates similar to what have been found at the few intensely studied locations, there should be a corresponding order of magnitude increase in global estimates of invertebrate biodiversity. Beyond the implications for counting and cataloging species, morphologically indistinguishable, but ecologically distinct, species have important implications for understanding comparative biology (Caputi et al. 2007), community ecology (Knowlton and Jackson 1994; Bortolus 2008), ecosystem function, and conservation (Knowlton and Jackson 1994; Hey et al. 2003; Agapow et al. 2004).

Cryptic species are collections of two to many closely-related species under the biological species model (Mayr 1963) that have been erroneously classified as one nominal species. The existence of cryptic species has been known for nearly 300 years (Winker 2005), and their expected widespread existence formed an important pillar in Mayr's (1963) arguments for a biologically-based species definition. The relatively recent availability and application of molecular techniques, including allozyme polymorphisms and DNA sequencing techniques, have greatly increased the number of taxa where cryptic species complexes have been uncovered (Knowlton 1993; Knowlton 2000; Bickford et al. 2006). Cryptic and sibling species are known from nominal species in many invertebrate (Knowlton 1993) and vertebrate families (Jones and Vanparijs 1993; Highton 1995; Omland et al. 2000; Mayer and von Helversen 2001; Comstock et al. 2002; Jockusch and Wake 2002) and are presumably common, as would be expected

if sibling species represent the recent branching of independent irreversible evolutionary lineages. Studying cryptic and sibling species and their ecological interactions is essential to understanding interspecific divergence and biological evolution.

A discussion of the terms cryptic and sibling species is necessary to avoid potential confusion. Most authors currently differentiate between sibling and cryptic species (Bickford et al. 2006) using Knowlton's (1993) description where 'sibling' denotes a species pair of recent cladogenesis. In such cases a phylogeny created from a suite of mitochondrial and nuclear genes will resolve the species pair in question as a clearly monophyletic unit within the fuller phylogeny of the genus (Moore 1995). 'Cryptic' species, on the other hand, may not be strictly sister in the phylogenetic sense, but rather only need be indistinguishable by morphology. Two or more species may be considered cryptic if they have been classified as a single species because they are, at least superficially, indistinguishable. The term sibling species was coined by Mayr (1942), and his definition clearly stated that 'sibling', in this case, did not necessarily imply a close genetic relationship. Most early studies used Mayr's original term (Grassle and Grassle 1976) even though multiple species may be present. 'Cryptic species complex' is the more descriptive term for multiple indistinguishable species and has been adopted by most authors. The terms and definitions employed by Knowlton's (1993) clarification will be used for sibling and cryptic species in this dissertation.

Freshwater systems can act as islands of discrete suitable habitats where cryptic species may develop because of terrestrial barriers to gene flow. In desert springs of the

southwest United States the common *Pyrgulopsis* snail may include as many as nine cryptic species (Liu et al. 2003). The barriers to gene flow that give rise to cryptic species in freshwater need not be the result of current basin geography. In the freshwater cladocerans, cryptic species are common (Forro et al. 2008) and in temperate North America at least one sympatric cryptic complex is thought to represent re-contact from separate glacial refugia (Penton et al. 2004).

Once genotypes are known by molecular means, sibling pairs or species within a complex can often be distinguished by morphology (Kamermans et al. 1999; Simison and Lindberg 1999; Leite et al. 2008; Morard et al. 2009), habitat type (Tarjuelo et al. 2001; Crummett and Eernisse 2007), resource use (Knowlton and Jackson 1994; Wellborn and Cothran 2007), mode of development (Veliz et al. 2003), reproduction, geography (de Vargas et al. 1999), or multivariate statistical approaches (Baylac et al. 2003; Sáez et al. 2003; Sáez and Lozano 2005). The species can then be referred to as pseudo-cryptic or pseudo-sibling species. Pseudo-cryptic species often appear in broad ranging nominal species that have been assumed to be morphologically plastic (Knowlton and Jackson 1994; de Vargas et al. 1999; Sáez et al. 2003; Sáez and Lozano 2005; Morard et al. 2009). The observed genotype-dependent morphological differences are then attributed to individual species within the complex and not local ecotypic influences. Morphological and ecological differences often become important in discriminating cryptic species and reinforce the need for taxonomic and ecological expertise (Schlick-Steiner et al. 2007) to complement the use of DNA barcoding for cataloging species diversity (Hebert et al. 2003).

Finding evidence for reproductive isolation in the field is often intractable but may be inferred by adopting strict reciprocal monophyly of multiple loci as the sole criterion for distinguishing species by molecular methods (Avise 1994; Moore 1995). Investigations of potential hybridization or mechanisms of reproductive incompatibility are complementary to genetic investigations. It is important to note that gene trees and species phylogenies are not the same thing. Species phylogenies can be inferred from gene trees. However, gene trees can be complicated by hybridization and introgression or differences in rates of mutation in different parts of a chromosome (Moore 1995). Only a robust multi-locus set of congruent gene trees approaches the topology of the phylogeny (Avise 1994).

The distribution of cryptic species across taxa, habitats, and geographies has been proposed to be non-random (Bickford et al. 2006) which would have significant consequences for conservation management as well as for understanding ecological interactions and evolutionary theory. Conservation efforts may be impacted if some groups of animals have a higher incidence of cryptic species. What may be prescribed for a nominal species may only pertain to one cryptic species within a complex (Bickford et al. 2006). A meta-analysis of a literature database on cryptic species found the phenomenon to be ubiquitous across all metazoan taxa and across all biomes (Pfenninger and Schwenk 2007), suggesting that there is a predictable proportion of cryptic diversity in each metazoan group. Sampling bias could create this artifact because all metazoan groups have not had the same level of scrutiny, but as the population genetics of more taxa is investigated a more detailed understanding of the distribution of cryptic species

within animal groups will be revealed.

Cryptic species in light of ecological competition theory

The existence of sympatric cryptic species poses ecological questions about the coexistence of seemingly identical species. Traditional competition theory, expressed in the competitive exclusion principle (Hardin 1960), suggests that true species analogs sharing an identical niche, with equivalent competitiveness, cannot have stable co-existence. The concept of the niche is that of an n-dimensional hyper-volume defined by axes of resource use and environmental conditions within which populations of a species reproduce and are able to maintain a long-term average net reproductive rate greater than or equal to one (Hutchinson 1957). Much of the argument that species analogs cannot share a niche comes from the outcomes of Lotka-Volterra models where two or more species share the same resource. Over time, small fluctuations in the populations will drive the less abundant species to extinction, leaving only a single competitor. If there is no competitive advantage of one species over the others, the species have the same capacity to become the sole survivor, determined by stochastic population fluctuations in the earliest moments of the model run. Alternatively, if a model is created where one species is defined as a more efficient user of resources, that species will consistently drive the less efficient species to extinction because their resource needs are lower than the threshold of the lesser competitor. Tilman expressed this as the R* rule (Tilman 1977), where the species with the lowest resource needed to maintain equilibrium (R*) will avoid exclusion by the other competitor. If predictions of competition theory manifest in patterns of sympatric cryptic species, there should be habitat partitioning,

interspecific resource allocation, character displacement, or some other mechanism to mitigate direct competition between the species.

Chesson (1991) suggests that only social behavior, discriminately directed towards conspecifics and heterospecifics, could maintain full niche sharing. Some cases of cryptic species with seemingly identical niches have proven to have mechanisms that mitigate the apparent niche competition, such as larval resource partitioning in a neotropical skipper complex (Bickford et al. 2006) or local mate competition and population size-dependant sex ratio adjustment in some cryptic species of fig-pollination wasps (Zhang et al. 2004).

In the freshwater amphipod species complex of *Hyalella azteca,* three morphologically very similar sympatric species appear to have been driven to morphological convergence by size-selective predation by fish (Wellborn and Cothran 2004), while at the same time the species partition the available habitat by location within the water column (Wellborn and Cothran 2007). The differences in the species' mitochondrial COI sequences imply nearly 9 million years of separation and the coexistence of seemingly identical morphologies appears the result of predator-mediated natural selection of species with ecologically distinct resource utilization.

Some sibling species pairs appear to occupy overlapping niches. The sibling pair of seed-eating Mexican bean beetles, *Acanthoscelides obtectus* and *A. obvelatus,* can sometimes be found completing development within the same seed. However, upon closer inspection

of the species distributions, direct competition is rarely observed and the available resources are partitioned by wild vs. cultivated bean strains and altitude (Alvarez et al. 2006). The cultivated bean-eating species has become a global pest of bean crops while the sibling species is restricted to locations with wild bean populations.

Not all cryptic species pairs have clearly defined habitat partitioning or morphological differences. The echinoderm genus *Ophiothrix* has been a long-standing taxonomic problem because of high degrees of morphological variability. Traditionally the intertidal North Atlantic and Mediterranean specimens were assigned as *O. fragilis,* while individuals from >20 m depth in the Mediterranean were assigned as *O. quinquemaculata.* A gene tree created from sequences of a portion of the 16S rDNA yielded two clades with genetic distances of 9-12% in the Mediterranean that matched neither the geography nor morphology used for separating the nominal species (Baric and Sturmbauer 1999). Instead, individual of both clades were present in samples assigned to both species by morphology and habitat.

Nominal species with apparently broad ecological requirements and distributions have proven to be cryptic species (Knowlton 1993; Knowlton and Jackson 1994; Knowlton 2000; Hebert et al. 2004). On tropical reefs, the *Montastraea annularis* cryptic complex of corals has been found to partition available habitat by depth and larval recruitment to the substrate (Knowlton and Jackson 1994). In a cryptic species pair of marine snails in the genus *Lottia* along the California coast, habitat is partitioned at microscales. At locations where their ranges overlap, one species is found on rocks in the high-intertidal

while the second species is restricted to the mid-intertidal, living on stalked barnacles (Crummett and Eernisse 2007).

Several species that have been used as pollution indicators have been found to be cryptic species complexes (Grassle and Grassle 1976) or sibling pairs with differing tolerances for the target pollutant (Sturmbauer 1999; Warwick and Robinson 2000). The potential for striking differences in toxicity tolerances presents difficulties in interpreting past use of the indicator species where the actual members of the complex are unknown, while at the same time presenting an opportunity for finer biological discrimination of pollution levels.

Cryptic species in marine environments

Cryptic species in marine systems often remained undiscovered because high dispersal potential and few obvious geographic barriers in species with planktotrophic larvae suggest full genetic mixing over broad geographic ranges (Palumbi 1994). Morphological variation is usually attributed to environmental phenotypic plasticity. Low-dispersing marine species with direct development, brooding, or crawling larvae have a higher potential for cryptic species because populations theoretically can become genetically isolated more easily than those with high-dispersal, planktonic stages and the associated high gene flow (Palumbi 1994; Nichols and Barnes 2005). In marine systems it is difficult to observe reproductive isolation when it occurs and normal intraspecific geographic and phenotypic variation can hide any species-specific morphological differences. As a result, many broadly-distributed marine species were considered

cosmopolitan by most taxonomists (Klautau et al. 1999). In the first striking example of cryptic species complexes in the marine realm, Grassle and Grassle (1976) examined allozymes of the supposed cosmopolitan species of polychaete, *Capitella capitata,* and discovered that, even in a geographical sampling restricted to Massachusetts, this nominal species was in fact a complex of six species.

Sibling and cryptic species complexes are now known for many marine taxa (Knowlton 1993; Palumbi 1994; Knowlton 2000; Blanquer and Uriz 2007; Boissin et al. 2008), including fish (Colborn et al. 2001; Hyde et al. 2008), nematodes (Warwick and Robinson 2000), bryozoans (Gomez et al. 2007), cnidarians (Dawson and Jacobs 2001; Schama et al. 2005), poriferans (Klautau et al. 1999; Nichols and Barnes 2005; Blanquer and Uriz 2007), echinoderms (Solé-Cava and Thorpe 1992; Baric and Sturmbauer 1999; Benzie 1999; Boissin et al. 2008), annelids (Grassle and Grassle 1976; Bastrop 1998; Westheide and Schmidt 2003; Rice et al. 2008), brachiopods (King and Hanner 1998), and crustaceans, including copepods (Lee 2000; Chen and Hare 2008), decapods (Knowlton 1986; Asakura and Watanabe 2005; Mathews 2006), and amphipods (Kelly et al. 2006). Groups that are supposed cosmopolitan members of the plankton such as coccolithophores (Sáez et al. 2003), foraminiferans (de Vargas et al. 1999; Morard et al. 2009), and marine cladocerans (Durbin et al. 2008), have unexpectedly yielded cryptic species complexes. Cryptic species of mollusks will be treated separately in the next section.

Estuarine species may have a higher proportion of cryptic diversity because adults and

larvae can be limited to specific brackish or low-salinity habitats with intervening open ocean acting as a physiological or physical barrier to dispersal and genetic mixing. Some authors suggest that many of the broad-ranging species with populations that span open ocean and estuarine habitats may harbor morphological crypsis and are, in fact, habitat-adapted species (Bilton et al. 2002). At least one estuarine amphipod common to the east coast of North America consists of a north and south cryptic species pair (Kelly et al. 2006). The southern African estuarine sandprawn, *Callianassa kraussi,* is a tropical and subtropical sibling species pair (Teske et al. 2009).

The ascidian *Ciona intestinalis,* a powerful model system in chordate biology that has had the whole genome sequenced, was recently found to be a cryptic species pair, calling into question the results of comparative biology experiments using specimens from different geographic locations (Caputi et al. 2007).

Cryptic species of marine mollusks

Many examples of cryptic species have been found in mollusks (Knowlton 1993; Knowlton 2000) and more continue to be discovered. The review here is not exhaustive, but touches on some of the potential relationships that molluscan cryptic species can display.

Shell morphology was often the main criterion used for early species descriptions of gastropods and bivalves. Depending on the level of grouping or splitting by a particular taxonomist, highly variable shell morphology could lead to either multiple species names or a single species name for a whole range of morphologies. Highly variable gastropods,

such as *Crepidula*, were described based on shell morphology, even though shell shape is strongly influenced by substrate. For example, in northern Chile, several morphologically indistinguishable *Crepidula* species inhabit shells used by hermit crabs. Previously, the mode of larval development distinguished two species. Allozyme data revealed that one hermit crab shell-inhabiting *Crepidula* species is a sibling species pair where the species have distinct differences in how the larvae feed on nutritional embryos (Veliz et al. 2003). The family Calyptraeidae, which includes *Crepidula*, is likely to yield more cryptic species complexes because of their troublesome taxonomy. The variable shells yield few good morphological characters with which to compare individuals and nominal species may have near global ranges. In instances where a single broad-ranging nominal species has been investigated, the species diversity found is often sizeable. The nominal *Crepidula aculeata* is made up of at least eight species, each occurring in a broad hydrogeographic province (Collin 2005). Nominal gastropod species with more restricted ranges, but a wide variety of habitats and shell variability, may also be strong candidates for cryptic diversity. The small intertidal limpet *Notoacmea helmsi* is known from exposed and sheltered rocky shores, mud flats, and eelgrass beds throughout New Zealand. The nominal species is a complex of at least five cryptic species with each species predominantly found in only one of the known habitats (Nakano and Spencer 2007). However, divergent shell shapes in variable habitats are sometimes instances of ecotypic responses, even if there is genetic divergence among geographically-separated populations as within the Pacific rocky shore *Littorina subrotundata* (Kyle and Boulding 1998).

The common and wide-ranging bivalve species *Macoma balthica* was suggested to be a northern and southern species pair along the Atlantic coast of North America (Meehan 1985) and later the interpretation was bolstered by a transoceanic study that included samples of northern European populations (Kamermans et al. 1999). Subsequent studies across the north Atlantic, north Pacific, and Arctic Oceans bolstered the north-south cryptic species interpretation and the temperate Atlantic Ocean species was given the name *M. petalum*. The northern boreal species, *M. balthica*, is a genetically complicated amalgamation of Pacific and Atlantic Ocean types that hybridized after the opening of the Bering Straight. Multiple introgression events have established localized populations with varying degrees of Atlantic or Pacific heritage (Väinölä 2003; Strelkov et al. 2007).

The Lucinidae are a family of bivalves with chemosynthetic gill symbionts. A recent revision, including molecular data for the genus *Anodontia,* nearly doubled the number of species when a single nominal species, *Anodontia edentula,* was discovered to have been a taxon that included eight species. The phylogeny was used to group the eight new species into two new genera because they were not monophyletic with other species in the genus (Taylor and Glover 2005).

The abyssal plains of the deep sea have been assumed to constitute a relatively uniform habitat where species exist over wide ranges within ocean basins. There are few studies of population genetics of abyssal mollusks because of the difficulty in obtaining specimens, the rarified nature of museum collections compared to the vastness of the oceans, and the low likelihood that quality DNA can be extracted from formalin-fixed or

dried material. Of the few nominal species that have been examined, unexpected diversity is often present. The gastropod *Frigidoalvania brychia* was collected from 500 m to 1100 m depth at a series of stations off the coast of Massachusetts in the 1960s. The shallower depths contained extremely divergent 16S rDNA haplotypes, suggestive of three species, while the greater depths had only one lineage (Quattro et al. 2001). A strong role of depth as a factor in the speciation process in the deep sea is an intriguing possibility and has been suggested by other researchers (Yeatman and Benzie 1994).

Deep-sea hydrothermal vent systems afford the potential for cryptic speciation because the ridge system forms a linear archipelago of discrete suitable habitats where geologic factors can limit gene flow. There are many mollusks with a similar genetic structure of allopatric sibling species along ridges separated by transform faults, including limpets (Johnson et al. 2008; Matabos et al. 2008), mussels (Won et al. 2003), and clams (Goffredi et al. 2003).

Intriguing ecological phenomena have been explained by the discovery of cryptic species. Poecilogony, the ability of one species to posess multiple larval strategies, is considered rare in marine environments. An accepted example was the opisthobranch *Alderia modesta* from the California coast. Genetic comparisons of the planktotrophic and lecithotrophic larval morphs revealed that the divergent larval strategies reflect cryptic species (Ellingson and Krug 2006) and not a true example of poecilogony. Other gastropod mollusks lacking shells have been found to have highly divergent genetic structure suggestive of cryptic species. The direct-developing nudibranch, *Doris*

kerguelenensis, with a range that spans the Drake Passage between Antarctica and South America, has a mitochondrial phylogeny that is suggestive of a recent explosion of new species radiating from glacial refugia (Wilson et al. 2009).

Cephalopods may also harbor cryptic species. Recently, a mid-sized octopus from the northeast coast of Brazil and nearby offshore islands that had been grouped with *Octopus vulgaris* from southern Brazil and the Mediterranean was found to be a distinct species. Variation in the large mitochondrial subunit ribosomal RNA gene (mt 16s rDNA) revealed species level differences that were subsequently verified morphologically (Leite et al. 2008). *Photololigo* squid along the north Australian coast were classified as one broadly ranging species, but were shown by allozymes to be four species that appear to have speciated allopatrically with depth (Yeatman and Benzie 1994).

Cryptic species have also been discovered in broad-ranging nominal species when distinct genetic breaks are discovered within a continuous near-shore distribution. The American oyster, *Crassostrea virginica,* was surveyed along its entire distribution from the St. Lawrence Seaway to the Gulf of Mexico and found to have a deep genetic discontinuity on the Atlantic coast of Florida, splitting the species into primarily Gulf and Atlantic-based clades (Reeb and Avise 1990). A broad suite of near-shore intertidal, estuarine and terrestrial coastal species have this population structure (Avise 1990) and may share a common biogeographic history. In the oyster, it appears that the genetic break is the outcome of past climate and sea level changes which produced formerly discontinuous distributions that have subsequently come back into contact (Reeb and

Avise 1990).

The Brachidontes exustus species complex

The nominal scorched mussel morphospecies, *Brachidontes exustus* (Linnaeus 1758), is a small bivalve (maximum length ≈ 25mm) in the family Mytilidae which commonly inhabits rock pilings, seawalls, and wharf pilings in the intertidal zone and is most abundant in the lower intertidal (Seed 1980a). Abbott (1974) considered *B. exustus* and *B. domingensis* separate species with the range of *B. exustus* from North Carolina to Texas and the West Indies and *B. domingensis* from Bermuda, the Bahamas, southeast Florida, and throughout the Caribbean. Later authors (Rios 1985; Jensen and Harasewych 1986) introduced taxonomic confusion by reciprocally synonymizing the two names. Rios (1985), working in Brazil, considered *B. exustus* the primary name and *B. domingensis* a synonym. Jensen and Harasewych (1986), publishing in a volume on the fauna of Bermuda, mistakenly used the more recent *B. domingensis* as the primary name. Although these authors were working at opposite ends of the potential range and designated a different primary name, the combined conclusions suggest that a single intertidal *Brachidontes* species spans the entire range from North Carolina to Argentina.

As with other intertidal mussels, *B. exustus* was assumed to display variable morphology in response to environmental conditions encountered during development (Seed 1968; Nalesso et al. 1992; Seed 1992; Reimer and Tedengren 1996) and the supposed morphological differences noted by Abbot (1974) were attributed to phenotypic variation. Other intertidal mytilids have been found to have broad geographic distributions (Siddall

1980; Wood et al. 2007) and may have hybrid zones, most notably species in the genus *Mytilus* (Seed 1992; Hilbish et al. 2000; Gardner and Thompson 2009). The taxonomic treatment of variable intertidal mytilids likely influenced the treatment of the *Brachidontes* genus in the western Atlantic.

Reciprocal monophyly and congruence of multiple gene trees can be used as a metric for identifying species boundaries (Avise 1994; Palumbi 1994). A phylogeny of the species would be topologically similar with multiple congruent gene trees. *Brachidontes exustus*, upon having nuclear and mitochondrial genes sequenced and gene trees constructed, was found to have five cryptic species within the nominal species in the western Atlantic (Lee and Ó Foighil 2004; Lee and Ó Foighil 2005) (Figure 1.1). In the majority of sampled locations throughout the Caribbean, Gulf of Mexico, and Atlantic coast, only one species was encountered. The five cryptic species have been given informal names that correspond to their core geographic ranges, Gulf, Atlantic, Bahamas, Antilles, and Western Caribbean (Figure 1.2). The Florida Keys appear to be unique among all of the sampled locations because four of the five species have been collected there (Figure 1.3). In no other region have more than two species been encountered and in most collections throughout the western Atlantic there was only one species. Some locations of coexistence do not appear to be secondary contact of allopatric ranges. For example, the Atlantic species was found along with the Western Caribbean species at Boca del Toro on the Panamanian coast. Far to the east, on the island of Trinidad, an Atlantic population was found very near (<20 km) to an Antilles species population (Lee and Ó Foighil 2005). The Panamanian and Trinidadian locations are nearly 2200 km apart and equally

far from the nearest known Atlantic species population on Florida's Atlantic coast. This suggests that the Gulf and Atlantic species, while distributed on both coasts of the Floridian Peninsula like other continuous near-shore fauna (Avise 1990; Reeb and Avise 1990), represent just one of multiple genetic breaks in the complex. Other near-shore Floridian species have a single genetic break between the Atlantic and Gulf-side populations that is the result of a single vicariance event. The genetic structure of the *Brachidontes exustus* group is the outcome of a more complex evolutionary history.

The four species collected from the Florida Keys came from three sites: Boca Chica Key, Spanish Harbor/West Summerland Key (the Keys Highway is constructed upon fill that connected many smaller islands and at this location it is unclear on which historical island the collection site would be located), and Key Biscayne (Lee and Ó Foighil 2004) (Figure 1.3). At each of the three sites two species were encountered, although at each location the two species were different. The Boca Chica Key collection had a single Atlantic species individual together with the Bahamian species. The Spanish Harbor/West Summerland Key location had a mixed population of Gulf and Bahamian species specimens. The Key Biscayne location had Bahamian and Antillean species in very low abundances.

Brachidontes exustus is common in the Florida Keys. The morphospecies has been used as a proxy for water quality in at least one study tracking environmental changes in Florida Bay. An increase in *B. exustus* shell abundance in the upper portions of sediment cores was given as evidence of lowered water quality brought about by the Everglades'

water diversion (Brewster-Wingard et al. 2001). Shifts in abundance could also accompany changes in species composition. The water quality inference could be confounded by differences in the cryptic species' tolerances to other environmental factors, particularly salinity. Differences in environmental tolerances of individual species within the complex have not been investigated and there are many more questions than answers.

For the *Brachidontes exustus* species complex to be useful as study organisms, reliable methods for determining species within the complex must be developed. Investigations of mating compatibility, adult and larval environmental tolerances, or community dynamics among the complex's species are impossible without the ability to determine the species identities of living specimens. Multivariate methods based on external morphometrics have the most advantages for these types of experiments and for ecological studies. Larvae and juveniles however, are unlikely to have morphometric characters that can easily distinguish among species. A method based on molecular variation is the most useful for investigations of larval ecology, recruitment, and distributions of indistinct juveniles. Combined molecular and morphology-based methods of species determination will allow the *Brachidontes exustus* species complex to be useful study organisms for asking many interesting questions in marine ecology and evolution. The objective of this dissertation was to develop molecular and morphological methods to accurately discriminate among the species in the complex in order to gain a better understanding of the species' distributions and morphological variation in the Florida Keys.

Figure 1.1 Gene trees created from mitochondrial cytochrome *c* oxidase subunit I (COI) and nuclear internal transcribed spacer 1 (ITS-1) from all *Brachidontes exustus* COI and ITS-1 sequences available in NCBI GenBank database. Trees were constructed using neighbor-joining method from a similarity matrix based on Jukes-Cantor distance model. Boxes represent five species in *Brachidontes exustus* complex with arrows pointing to congruent gene tree topology. Informal name of each species appears in box. Names based on core geographic region of species' known distribution.

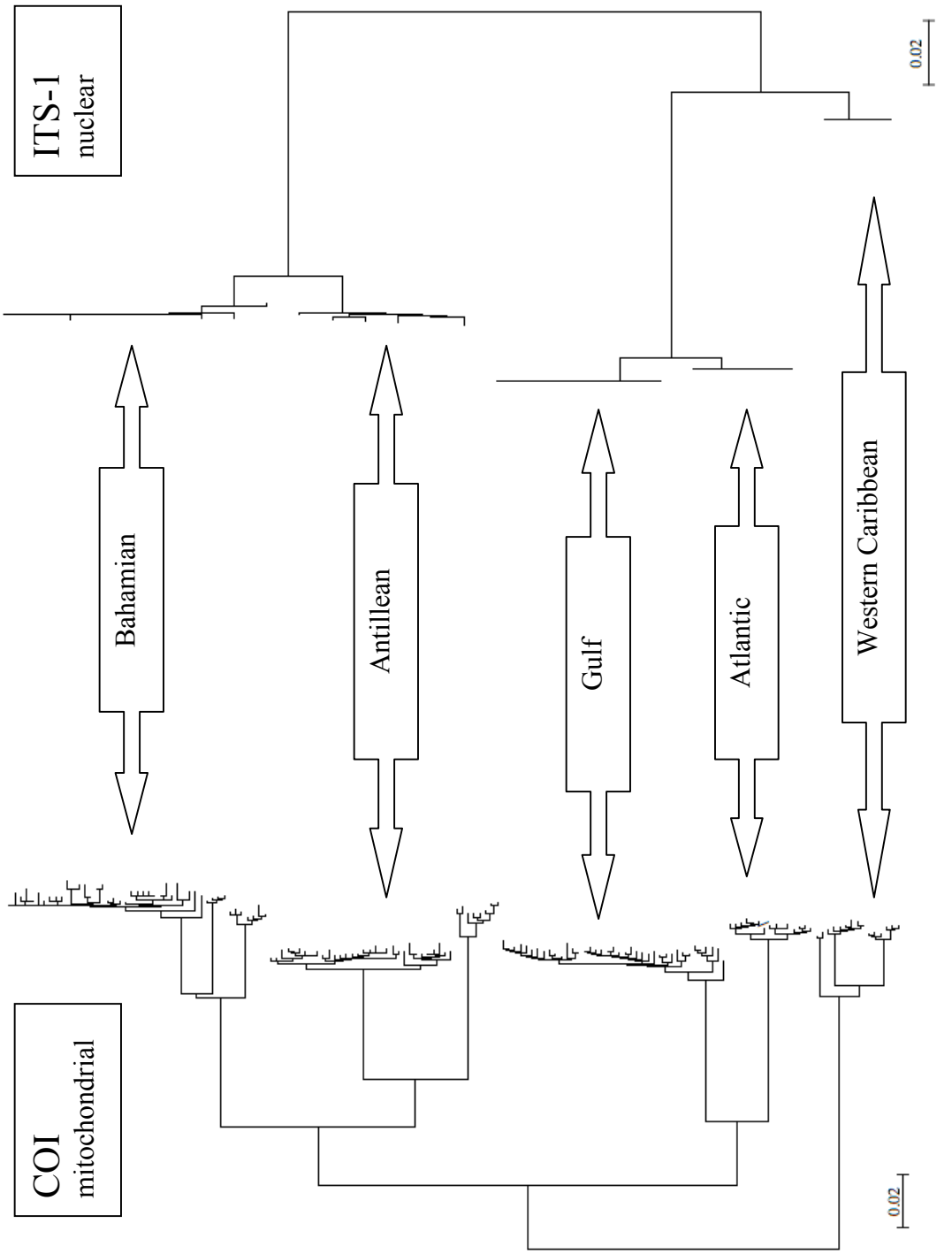

ITS-1
nuclear
0.02
Bahamian
Antillean
Gulf
Atlantic
Western Caribbean
COI
mitochondrial
0.02

Figure 1.1

Figure 1.2

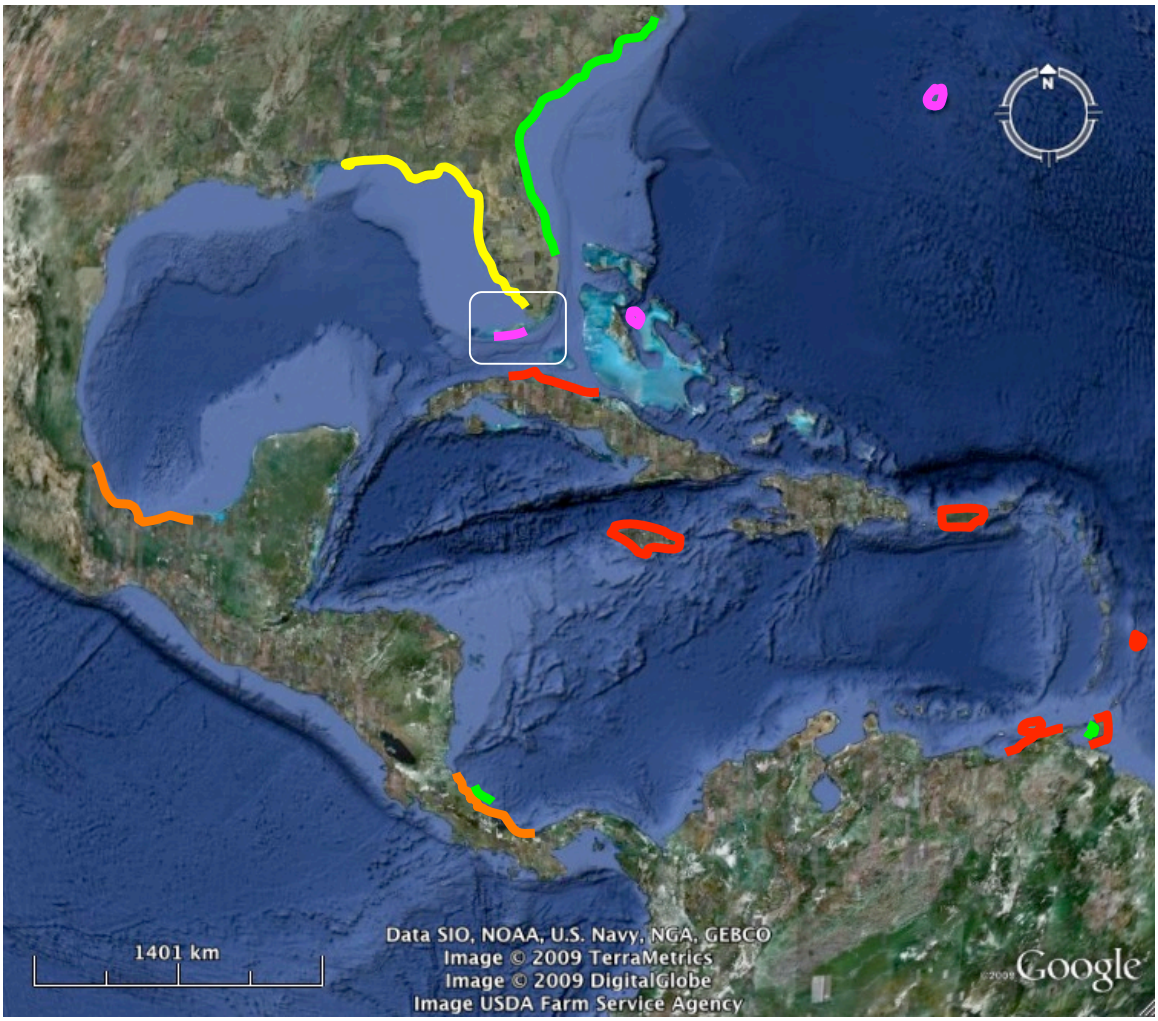


Figure 1.2 Image of western Atlantic showing collection locations for five species in the *B. exustus* complex (from Lee and Ó Foighil 2004 and 2005). Map image created from Google Earth. White box bounds the Florida Keys, location of this research and focus of figure 1.3.

Figure 1.3

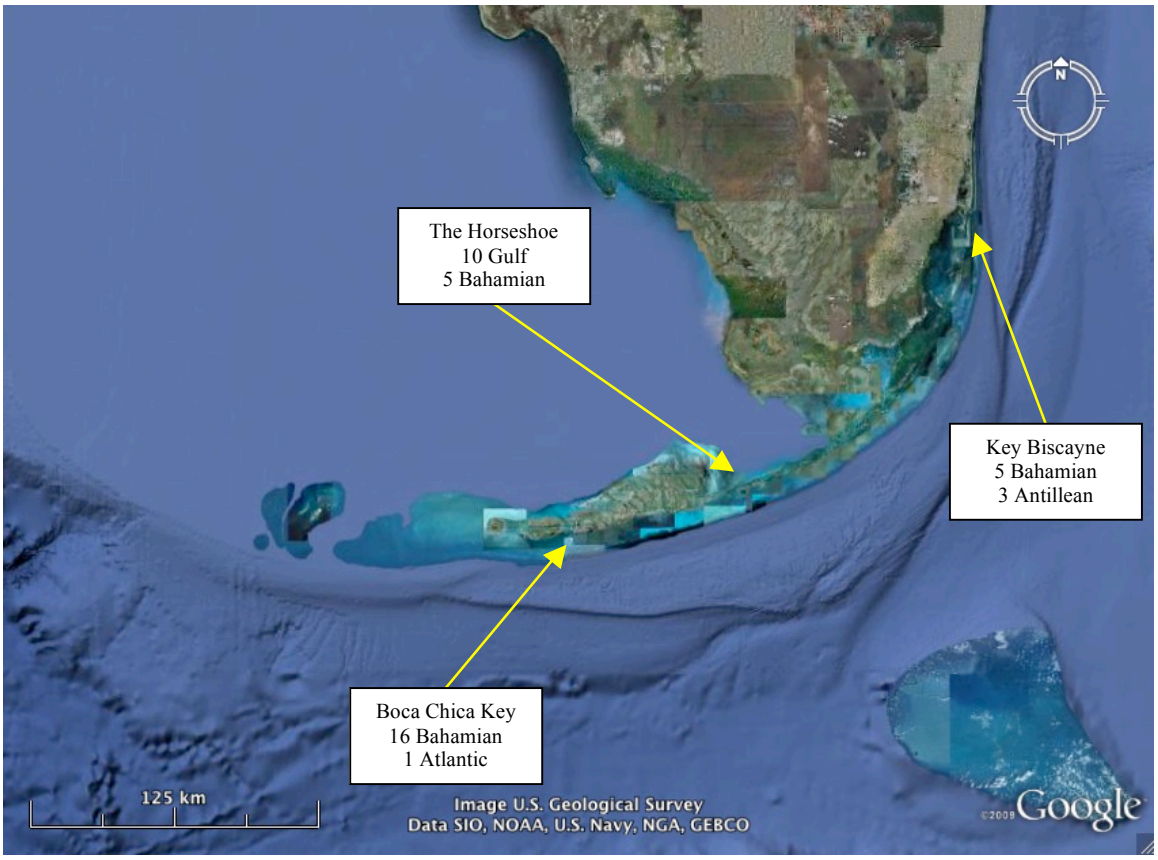


Figure 1.3 Image of Florida Keys showing three collection locations of Lee and Ó Foighil (2004): Boca Chica Key, The Horseshoe site on Spanish Harbor/West Summerland Key, and Key Biscayne. Number of specimens of each species genotyped by mtDNA from each location indicated in box. Image created from Google Earth.

Chapter 2

DNA Barcoding Reveals *Brachidontes* Mussels from Two Distinct Intertidal Habitats on Long Key, Florida Keys, Are Cryptic Species and Not Ecotypes

Abstract

The nominal morphospecies *Brachidontes exustus* represents a cryptic species complex with multiple genetic disjunctions resulting in regionally-dominant, but range-restricted, species throughout the western Atlantic, Caribbean, and Gulf of Mexico. In the Florida Keys, four species were previously identified using molecular techniques. Specimens were collected January 2005 from two distinct habitats, a seawall and a mangrove, on Long Key, Florida Keys. The locations are separated by <5 km. Eight specimens from the mangrove and four from the seawall were sequenced for the mitochondrial COI gene. Two seawall specimens were sequenced at the internal transcribed spacer 2 (ITS-2), in the nuclear ribosomal gene cluster, after the COI sequences appeared to be from the male mitochondrial line. The COI and ITS-2 sequences indicate that the two locations on Long Key, Florida Keys, have different single-species populations. The four seawall specimens were the Antillean species while the eight mangrove specimens were the Gulf species. Given that these mussels broadcast spawn, with subsequent planktotrophic larval development, the sites likely share a common pool of potential larval recruits. Single-species populations at each location are suggestive of habitat partitioning, ecological filters, or differential recruitment.

Introduction

The morphospecies *Brachidontes exustus* (Linnaeus 1758) is a small intertidal mytilid that has been shown to be a cryptic species complex throughout its western Atlantic, Gulf of Mexico, and Caribbean Basin range. Five molecular taxonomic units (MTU) within the nominal species have been identified using nuclear and mitochondrial gene trees. Each MTU has been given an informal name corresponding to its core geographical distribution; Antilles, Atlantic, Bahamas, Gulf, and Western Caribbean (Lee and Ó Foighil 2005). Four of these cryptic species (Antilles, Atlantic, Bahamas, and Gulf) have been found in the Florida Keys (Lee and Ó Foighil 2004). The four species are nested, two sister-species apiece, within two of the three main branches revealed by a ribosomal 28S gene tree (Lee and Ó Foighil 2005). The five cryptic species have not yet been formally described and currently remain under the single morphospecies taxon, *Brachidontes exustus*.

DNA barcodes have been proposed as a method for genetically cataloging the world's biological diversity (Hebert et al. 2003). The proposed "barcode" is a short section of the mitochondrial genome that can identify unknown specimens when compared to an existing database of sequences, and are particularly useful in identifying cryptic species (Hebert et al. 2004; Gomez et al. 2007) or other closely-related species (Packer et al. 2009). The 5′ region of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene, amplified by the well-known "universal" COI primer pair LCO 1490 and HCO 2198 of Folmer et al. (1994), has emerged as the agreed upon sequence for DNA barcoding for most species. Some bivalves have a unique mitochondrial inheritance system that may

complicate the effectiveness of DNA barcoding. Doubly uniparental inheritance (DUI) of sex-linked mitochondrial lineages (Zouros et al. 1994; Mizi et al. 2005) results in maternally and paternally-inherited mitochondrial genomes coexisting in males. DNA barcodes that do not match the known sequences of the species may result if tissues used for DNA extraction are enriched with the male line of mitochondria.

Lee and Ó Foighil (2004) collected nominal *Brachidontes exustus* from three rocky intertidal sites from the Florida Keys (Figure 1.3). The Bahamian and Gulf species were most commonly encountered. The Bahamian species dominated their most southerly collection site at Boca Chica Key, while the Gulf species was most common at the Horseshoe site on Spanish Harbor/West Summerland Key, though intermingled with individuals of the Bahamian species. The Atlantic and Antillean species were much less common and were identified from very few individuals. The only genetically verified records of these two presumably rarer species include a single Atlantic species specimen found with an otherwise Bahamian population at Boca Chica Key, and three sub-adults of the Antillean species found along with five Bahamian species individuals at Key Biscayne (Lee and Ó Foighil 2004). Lee and Ó Foighil (2004) did not collect specimens from mangroves, a habitat for *B. exustus* encountered by Bennett and Wilan (2003) in the Florida Keys and subsequently by Lee and Ó Foighil in Panama (Lee and Ó Foighil 2005), where they found the Western Caribbean and Atlantic species in a mixed population.

Sibling species and cryptic species complexes in what were previously believed to be

single species are well known for many marine taxa (Knowlton 1993). In the *Mytilus* complex of *M. californianus*, *M. galloprovincialis,* and *M. trossulus* on the western coast of North America, the distributions appear to be maintained by subtle habitat differences that influence local post-recruitment dominance (Heath et al. 1995; Johnson and Geller 2006). Likewise, local habitat differences may influence *Brachidontes* cryptic species distributions in the Florida Keys. The goal of the present study was to determine the species distribution in the two distinct habitat types on Long Key, where *B. exustus* occurs. A previous study of Floridian *Brachidontes* spp. (Lee and Ó Foighil 2004) did not include specimens from seawall or mangrove habitats, nor were multiple samples taken from different locations on a single island.

Materials and Methods

Study area

Long Key, a 6-km long Y-shaped island in approximately the middle of the Florida Keys archipelago, has an interior lagoon fringed by mangroves with a connection to the bayside of the island (Figure 2.1). The nearest mile-marker on US Route 1 serves as a useful universal landmark for the Florida Keys and the number of the nearest mile-marker is included with the collection locations. Mile marker 0 corresponds to the western edge of Key West, and the mile number increases as one moves east and north towards mainland Florida.

The first collection location (latitude 24°48.10 N, longitude 80°50.58 W) is an ocean-side vertical concrete seawall near mile marker 66 at the west end of Long Key, adjacent to

the Long Key Viaduct (Figure 2.1). The lowest intertidal zone of the seawall is approximately 1 m above a sandy bottom. Wave exposure at this location is high and directly impacts the exposed substrate. The second collection location (latitude 24°49.22 N, longitude 80°48.45 W), near mile marker 68 and approximately 4 km to the northeast, is a mangrove-lined boating channel that leads from the bay-side of the island to the interior lagoon and is protected from direct wave action.

Sample collection

Living specimens were collected in January 2005. Four small individuals approximately 11 mm in length were found in the lower intertidal on the seawall and all were collected. A total of 46 individuals ranging in size from 6 mm to 22 mm were collected from an abundant population in the mangrove habitat. All specimens were immediately transferred to a seawater flow-through system and maintained alive for several days before being frozen at -40ºC. The samples were subsequently transported on dry ice and stored at -80ºC prior to dissection and molecular characterization.

Specimens were thawed to room temperature, dissected, and the soft tissues separated from the shell. The disarticulated left valve of each subsequently typed specimen was photographed using a Nikon digital SLR camera mounted on a Zeiss Stemi 2000-C dissecting scope.

DNA extraction and molecular characterization

Total genomic DNA was isolated from approximately 20 mg of tissue from either the

posterior adductor muscle in larger specimens or mantle tissue in smaller specimens. The extraction was accomplished with a DNeasy Tissue Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. Extracts were stored at -20ºC until used as PCR templates.

The target fragment for DNA barcodes, the 5′ end of the cytochrome *c* oxidase subunit I (COI) gene from the mitochondrial genome, was amplified by polymerase chain reactions (PCR) using Taq PCR Mastermix (Qiagen, Valencia, California) and the well known "universal" COI primer pair (LOC 1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HOC 2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) from Folmer et al. (1994) with a thermal cycler protocol of 3 min initial denaturing at 95ºC followed by 35 cycles of 95ºC for 1 min, 40ºC for 1 min, 72ºC for 1.5 min, with a final 7 min extension at 72ºC.

A second target fragment, the second internal transcribed spacer (ITS-2) region from the nuclear ribosomal gene cluster, was sequenced for two specimens from the seawall for reasons discussed below. The ITS-2 was amplified with the forward primer (5′-CATCGATATCTTGAACGC-3′) from Lopez-Piñión et al. (2002) initially designed for European scallops and the reverse primer (5′-GCTCTTCCCGCTTCACTCG-3′) from (Xu et al. 2001) initially designed for various species of *Crassostrea.* The thermal cycler protocol was 3 min initial denaturing at 94ºC followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 60 s with a final extension of 5 min at 72ºC. All reactions were prepared in 50-µl volumes and a negative control containing all reagents and the

primer pair, without the DNA template, was included with each amplification series. The resulting PCR products, controls, and a 100-bp ladder were run on a 2% agarose gel, stained with ethidium bromide, and photographed under UV transillumination.

PCR products were checked for the appropriate size, purified with a MinElute kit (Qiagen, Valencia, California) according to the manufacturer's directions and subsequently directly cycle-sequenced in both directions with the above amplification primers utilizing the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Forester City, California) and an automated DNA sequencer (310 Avant Genetic Analyzer, Applied Biosystems, Forester City, California).

Chromatograms of sequences were edited manually by comparing both strands in 4Peaks (by Griekspoor and Groothuis, Mekentosj.com). The edited sequences were compared to an existing database of sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) of the National Center for Biotechnology Information (NCBI). The most significant alignment with the highest percent congruence to published sequences was treated as the species identification.

Gene tree

A subsample of COI sequences representing each of the five cryptic species and their within-species diversity from their total geographic range were obtained from GenBank (accession numbers AY621879, AY621909, AY621911, AY621913, AY621914, AY825105, AY825201, AY825202, AY825204 and AY825216) (Lee and Ó Foighil

2004; Lee and Ó Foighil 2005) and combined with nine of the ten newly-generated maternal COI sequences. The sequences (see Appendix) were aligned in ClustalX and a gene tree was created using the neighbor-joining method.

Results

Species assignment from sequences

The gene tree created from the seven mangrove and two seawall specimen COI sequences and select COI sequences obtained from GenBank show the relationships of the specimens to the five cryptic species within the complex (Figure 2.2). Two specimens from the seawall location were excluded as explained below. Table 2.1 shows species identifications based on comparisons to the NCBI database for all sequences generated in this study.

Seawall location

The resulting COI sequences, when compared to the NCBI database, had closest matches to an Antilles species sequence and a Biscayne clade species sequence (later named Antilles after gene trees from the full western Atlantic and Caribbean range showed that the clade collected from Key Biscayne in the initial Florida Keys sampling was the dominant species of the Antilles). The match was 100% for two of the four specimens (Lee and Ó Foighil 2004; Lee and Ó Foighil 2005). The two other sequences were hard to clean and edit because of many instances of unclear peaks in both the forward and reverse sequences. The two cleaned and edited sequences had sections at both ends that were ambiguous, which were cropped from the full sequence before being compared to

the NCBI database. The two sequences were 543 and 621 nucleotides long and returned respectively an 82% and 83% closest match to GenBank accession number AY621945, a male mitochondrial line COI sequence of a Bahamian specimen collected at Boca Chica Key (Lee and Ó Foighil 2004). The most congruent non-male COI sequence match for both specimens was an Antillean species sequence (AY825208) (Lee and Ó Foighil 2005) with 80% congruence.

The specimens that yielded the presumptive male COI sequence were subsequently sequenced at the ITS-2 locus to determine their species identity. The resulting sequences were 355 nucleotides long and when compared to the NCBI database returned either 100% or 99% congruence with AY621970. Sequence AY621970 is the complete sequence of the internal transcribed spacer 2, and flanking portions of the 5.8S and 28S ribosomal RNA genes, obtained from voucher specimen 300123.6 of the University of Michigan Museum of Zoology. The specimen was collected from Key Biscayne, Florida (Lee and Ó Foighil 2004). Both of the newly-generated ITS-2 sequences contained a 4-nucleotide deletion relative to the Bahamian sequences at aligned location 130, a polymorphism consistent with the single Antillean species ITS-2 sequences in GenBank, and lacking in any Bahamian species ITS-2 sequence. The four seawall specimens are thus grouped with the Antilles species, by either mitochondrial COI or nuclear ITS-2 sequences. The Antilles species is one of the two less common species encountered by Lee and Ó Foighil (2004) in the Florida Keys and this new record more than doubles the confirmed number of individuals of this species in south Florida.

Mangrove location

Eight specimens from the mangrove were chosen for sequencing based on extremes in size and shape revealed by a Principal Components Analysis based on morphometrics (see Chapter 3). The expectation is that if more than one species is present at this location the extremes of size and shape would likely include representatives of the cryptic species. COI sequences of seven specimens were 660 nucleotides long and when compared to the NCBI database returned in all cases a Gulf species closest match with 100% or 99% congruence (Table 2.1). The COI sequence of one individual, M68-5, had many ambiguous peaks at both the 5′ and 3′ ends and a clean sequence of 474 nucleotides was excised from the longer sequence. This 474-nucleotide-long fragment was compared to the NCBI database and returned 99% congruence to a Gulf species COI sequence. The shorter COI sequence was not included in the gene tree, but nonetheless serves as a positive identification of this specimen as the Gulf species.

Discussion

One major criticism of DNA barcodes as a method of species designation is the possibility of misidentifications based on errors in the database of linked sequences. This is a problem with using GenBank data, where a submitter of the sequence names the taxon to which the sequence is linked. There is no outside review of the validity of the taxonomy and only the original submitter can change the name attached to the sequence. *Brachidontes exustus* appears to suffer from just such a problem. The linked taxon in the NCBI database is *Hormomya exustus*, a generic name long ago synonymized to *Brachidontes exustus* (Soot-Ryen 1969). This problem in taxonomy now extends to the

Barcode of Life Database (BOLD) by virtue of its linking to the NCBI database. BOLD utilizes only a standardized 648-nucleotide fragment from voucher specimens whose origin and current status are recorded (Hebert and Gregory 2005). Additionally, without the proper taxonomic treatment of the cryptic species, there is no way to know that cryptic species are involved without a detailed knowledge of the clade information included in the information for each species in the GenBank database. While the BOLD attempts to be more rigorous than the GenBank database because voucher specimens are required for each sequence, the lack of taxonomic treatment for each of the cryptic species within the *Brachidontes exustus* complex likely dooms them to obscurity until a proper taxonomic treatment is undertaken (Schlick-Steiner et al. 2007).

Lee and Ó Foighil (2005), remarking on the distributions of the five *Brachidontes* species throughout the entire known Atlantic, Gulf of Mexico, Bahamas, and Caribbean range, note that the Gulf/Atlantic clade appears mostly on continental margins, while the Bahamas/Antilles clade is found predominately on oceanic islands. The abiotic aspects of the mangrove habitat may be more similar to continental margins, while the seawall habitat may be more similar to the conditions found on oceanic islands. Bieler and Mikkelsen (2004) note that there are distinct differences between the bivalve species assemblages on the bay and ocean-sides of the Florida Keys archipelago. The observed distribution of *B. exustus* species on Long Key may be representative of the same environmental factors that influence other bivalve species' distributions in the Florida Keys. Furthermore, the environmental conditions of the core distributions of the individual *Brachidontes* species in the western Atlantic, Gulf of Mexico, Bahamas, and

Caribbean Sea observed by Lee and Ó Foighil (2005) potentially relate to the conditions coinciding with the species' distribution on Long Key. High salinity and low productivity associated with ocean islands are present at the seawall, while lower salinity and high productivity conditions associated with continental margins are present at the mangrove site.

Only four individuals were found on the approximately 250 m of seawall after considerable effort and all individuals were collected and used for genetic typing. Based on the very low abundance and small size of the collected specimens, this location likely represents a marginal habitat for *Brachidontes* spp.*,* survivable by only one of the species within the complex. The four seawall individuals were only half the maximum length of individuals found in the mangrove habitat yet they are likely sexually mature. Mytilids have doubly uniparental inheritance of sex-linked mitochondrial lineages (DUI) (Zouros et al. 1994) and the initial extraction appears to have been contaminated by sperm from a portion of gonadal tissue extracted with the mantle tissue. Only sexually mature males would have sperm in sufficient quantities to make a significant contribution of the paternally-inherited COI sequence to the PCR amplification. The smallest reproductive individuals encountered during a study of the gametogenic cycle of *Brachidontes exustus* at Wassaw Island, Georgia were about 10 mm long (Sweeney and Walker 1998). This is slightly smaller than the specimens collected from the seawall on Long Key. If the threshold for reproductive size is similar for the Atlantic and Antillean species, then the seawall specimens are within the limits of size for reproductive individuals.

The presumptive male sequences are slightly less congruent with the Antillean species COI (80%) than they are with the known male COI sequences (82%) from Bahamian specimens. An alignment of the presumptive male sequence fragments in ClustalX showed the sequences to be very close to each other with few single nucleotide substitutions and one section of over 100 nucleotides identical between the two sequences. The close congruence suggests that the sequences are not amalgamations of amplified maternal and paternal COI PCR products but are the sequences for the male COI of the Antillean species. In other bivalves that display DUI the male mitochondrial genome has been hypothesized to have been replaced and reset to the female mitochondrial line (Mizi et al. 2005). The similar 20% differences between the Bahamian male COI and the maternal Antillean species COI may be a result of a resetting of the male COI line to the maternal mitochondrial line after speciation. A fuller gene tree comprising the male COI sequences of each species within the complex would answer the questions of male mitochondrial genome origins. Contamination by mitochondrial DNA from sperm would not affect the amplification of the nuclear ITS-2 used for the species designation of the two specimens that yielded a presumptive male COI sequence.

Ideally, a range of size classes from the seawall location would have been used for species identifications, but all four of the specimens found on the seawall were approximately 11 mm in length. Wave exposure at the seawall is a prominent environmental feature and could be a factor in limiting abundance and survival at the seawall. At Moss Landing, California and along the central coast of California, *Mytilus* spp. occur in mixed populations in which the dominant species differs among age classes.

When wave exposure is more intense, one species of *Mytilus* comprises a greater proportion of adults than juveniles, suggesting that the adults of this one species are more resistant to dislodgement by wave action (Heath et al. 1996; Johnson and Geller 2006). Wave exposure may potentially play a role in the *Brachidontes* spp. population composition at the seawall location, although wave exposure alone cannot explain the observed pattern in the mangrove habitat. Antillean species individuals should be present in the mangrove, a habitat with low wave exposure, if wave-induced dislodgement were the only environmental attribute influencing the distribution.

The harsh conditions under high wave exposure may limit the growth or set a maximum attainable size for the few individuals that persist at the seawall. Alternatively, larger individuals may still be susceptible to dislodgement once over a certain size threshold. While all of the seawall specimens were about the same length, they may be of varying ages and not representative of a single settlement cohort because growth may slow to near zero under the stress of intense wave exposure.

Unfortunately, the small sample size at the seawall location and limited number of collection locations from Long Key truncate the explanatory power of this study. The inclusion of the apparently marginal habitat at the seawall for a study of species distributions in the Florida Keys became a necessity because many locations that seemed suitable for *Brachidontes* spp. did not have populations and specimens were collected whenever they were encountered. Nonetheless, the stark contrast of species-specific populations of cryptic species at such proximal locations and differing habitats warranted

including such a marginal habitat for comparison. The only other record of the Antillean species in the Florida Keys is from a habitat at Key Biscayne with very low abundances of *Brachidontes* spp. mussels (Lee and Ó Foighil 2004). The Antillean species may be more common in the Florida Keys than the records to date suggest because marginal habitats have not been actively searched in the few existing studies on this species complex.

At the mangrove location, specimens were found as dense clusters of individuals within the byssal threads of another bivalve, *Isognomon* sp., which were, in turn, attached to the mangrove roots. It was not uncommon to find 20 or more individuals within the threads of a single *Isognomon sp*. Specimens were collected from the same mangrove location during the summer of 2002 but were not used in this study. Shell morphology over the 2.5-year period remained consistent and the largest shells from the two collection times were about the same length (≈ 25 mm). Unlike the individuals collected from the seawall, the mangrove specimens identified by COI sequences spanned the entire range of sizes collected and the specimens chosen for sequencing were at the extremes of size and shape variation. If individual settlement events were dominated by a single species, there should be a change in the species composition at the various size classes and between the two collections times. Based on the consistent species designation by sequence data over the entire range of size classes, combined with the consistent shell morphology, it appears that the exclusivity of the Gulf species at the mangrove location was maintained over the 2.5 years between collections. This would be unlikely if the observed distribution were the result of stochastic larval settlement.

In contrast to the Long Key locations, some other sites in the Florida Keys have more than one cryptic species of *B. exustus* coexisting on the same substrate (Lee and Ó Foighil 2004) (see Chapter 3). Neither of the Long Key collection locations was the rocky shore habitat sampled by Lee and Ó Foighil (2004). Mixed-species populations may reflect an overlap of suitable habitat that only exists at some rocky shore locations. Species may be contemporaneous on the same rocky shore by habitat partitioning, by depth, for example. Abiotic factors or biotic interactions could limit one or both species' distribution on micro-scales in such a way to facilitate coexistence. Further investigation of the species' distributions, at spatial scales of individuals to islands, is warranted to elucidate the mechanisms of coexistence or exclusion among the species within the complex.

Differential larval transport from single-clade source populations could potentially impact adult distributions. If the larvae of each species are not transported to all potential locations, the differences noted in the species' distributions could be an artifact of larval transport processes and not ecological filtering. This seems unlikely given that the closely-related species *B. granulata, B. modiolus,* and *B. variabilis* broadcast spawn with planktotrophic larvae that can stay in the water column for up to 40 days before settlement (Campos and Ramorino 1980; Fields and Moore 1983; Morton 1988). The species within the *B. exustus* complex likely have a comparable planktotrophic larval duration. The long planktonic phase would provide ample opportunity for tidal fluxes and currents to overwhelm any local larval source cohesiveness.

A study of connectivity using biophysical modeling in Caribbean fish populations suggests that long-range dispersal may be much rarer than simple assumptions of pelagic larval duration and ocean currents would indicate (Cowen et al. 2006). The four kilometers between the Long Key collection locations are well within the ecologically-relevant magnitudes of dispersal of 10 to 100 km found by the Cowen et al. (2006) model. Within the dispersal distances advanced by their model, larvae from both Long Key populations would have a very high likelihood of being delivered to the nearby location. Additionally, their model implies that larvae of all the cryptic *Brachidontes* species present in the Florida Keys would be delivered to all suitable Florida Keys locations, suggesting that species-specific populations must reflect recruitment or post-recruitment processes and not clade-limited settlement events.

Previous authors (Lee and Ó Foighil 2005) were unable to determine the species of a given individual by morphology alone. However, representative shell shapes are included in their nuclear and mitochondrial gene trees. A previous investigation of plasticity of *Brachidontes* spp. in the Florida Keys found large overlaps in the shell morphologies of specimens sampled from habitats with different wave exposures (Bennett and Wilan 2003). Their conclusion of environmentally-induced plasticity is severely undermined by the discovery of the cryptic species complex in the Florida Keys. Two of Bennett and Wilan's collection locations, the Horseshoe on Spanish Harbor/West Summerland Key and the mangrove on Long Key, do not have the same species distributions. The Horseshoe site has a mixture of Gulf and Bahamian species (Lee and Ó Foighil 2004) (see Chapter 3), while the mangrove location population is shown here to be limited to

the Gulf species. The broad morphological variation that was observed at the sites sampled by Bennett and Wilan (2003) is probably the result of morphological differences between the cryptic species. The overlap in shell morphology between locations is likely the result of similarity of the morphology of the Gulf species in both locations. The morphological variation observed does not reflect ecotypic differences within a single species.

Multivariate statistical methods to determine species by shell morphometics may be constructed with a large number of specimens that are unequivocally assigned to species by molecular methods. However, a statistical method is only possible if there are consistent genotype-dependent morphological differences that overwhelm any phenotypically-variable morphology. Sampling individuals from locations where the species coexist is the obvious starting point for examining this question because mussels from these locations should presumably be exposed to identical phenotype-inducing environmental cues and would be expected to display similar phenotypic responses. Any consistent morphological differences between the species at locations of coexistence would only be those whose expression is more dependent on genotype than phenotype. Morphological discrimination of cryptic species would make manipulation experiments possible because the mussels would not have to be killed in order to determine species. Reciprocal transplant or common-brood experiments could then be used to investigate the precise mechanisms regulating species survivability at single and multiple-species locations.

Notwithstanding Lee and Ó Foighil's (2005) observations on morphology, there were some obvious qualitative differences in gross shell morphology between the specimens from the two Long Key locations. Shells collected from the seawall were thicker with more robust ribbing and were wider across both valves compared to shells of similar length collected from the mangrove habitat. When viewed laterally, the overall shell outline of the seawall specimens was more modioliform, while the mangrove specimens were more mytiliform (Figure 2.3). For the seawall specimens, the anterior margin was more rounded, the umbo in a more dorsal orientation, and the overall ventral-dorsal margin angle less than in specimens of similar size from the mangrove. These morphological differences match the known phenotypic plasticity of mytilids in habitats with different wave exposures (Seed 1968), salinities (Nalesso et al. 1992), or shorelines (Morton 1991). However, in light of the existence of cryptic species, it is unclear whether the observed morphological variation arises from phenotypic expression, genotypic constraints, or a combination of the two.

The discovery of cryptic species in such close proximity in the Florida Keys opens the possibility that hidden biodiversity within *Brachidontes* spp. populations may be found in other locations throughout the Caribbean if sampling efforts were increased. Most of the Caribbean island locations sampled by Lee and Ó Foighil (2005) were from a single collection site and yielded a single species. The two locations sampled on Trinidad, which yielded different species, provide an exception. While the two Trinidadian locations are about 20 km apart, the Atlantic species was collected at the town of Chaguaramas and the Antillean species was collected at Maracas Bay. Although the

habitat types are not listed, habitat specificity similar to what was observed on Long Key may exist at these Trinidadian collection locations. Maracas Bay is on the north side of the island with an exposure to the Atlantic Ocean, while Chaguaramas is to the west, on the Gulf of Paria. The species distributions on Trinidad may be influenced by habitats with oceanic island and continental margin-like environments, as with the distributions observed at Long Key.

The Atlantic species was collected in eastern Panama, along with the Western Caribbean species, from the same microhabitat, the byssal threads of *Isogonomon* sp. attached to mangrove roots (Lee and Ó Foighil 2005). The Gulf species was encountered in this same habitat in the Florida Keys. It is unclear if Lee and Ó Foighil sampled mangrove root habitats in locations other than Panama. Most of their sampling throughout the Antilles was a single location on each island. The habitat types for the collections were not specifically described, except for the one mangrove location in Panama. This suggests that most collections were made in the more commonly recognized rocky-intertidal habitat and that mangrove habitats were excluded from the sampling locations, other than the one collection location that specifically mentions the mangrove habitat.

Many intriguing questions remain regarding the ecological mechanisms that affect the distribution of this cryptic species complex in the Florida Keys and the western Atlantic. More records of the species in close proximity may be discovered if sampling efforts are increased. Mangrove habitats in the Caribbean may support populations that are not the dominant regional species. Understanding the mechanisms in specific habitats that

maintain or exclude each species within the complex offers the potential to ask questions regarding interactions of very closely related species in the oceans. This little-studied complex may become a good model to investigate these types of difficult to answer questions.

Table 2.1 Specimen, habitat type, sequenced gene, fragment length in nucleotides used for database comparisons, NCBI accession number of sequence with most significant alignment, percentage congruence of *de novo* sequence to NCBI sequence, and species.

Specimen	Habitat	Gene	Length	Significant alignment	Congruence	Species
M66-1	seawall	COI	660	AY825154	100%	Antillean
M66-2	seawall	ITS-2	355	AY621970	99%	Antillean
M66-3	seawall	COI	660	AY621849	99%	Antillean
M66-4	seawall	ITS-2	355	AY621970	99%	Antillean
M68-1	mangrove	COI	660	AY621885	99%	Gulf
M68-2	mangrove	COI	660	AY621915	99%	Gulf
M68-3	mangrove	COI	660	AY621915	100%	Gulf
M68-4	mangrove	COI	660	AY621897	99%	Gulf
M68-5	mangrove	COI	474	AY621915	99%	Gulf
M68-6	mangrove	COI	660	AY621913	99%	Gulf
M68-8	mangrove	COI	660	AY621913	99%	Gulf
M68-11	mangrove	COI	660	AY621915	99%	Gulf

Figure 2.1

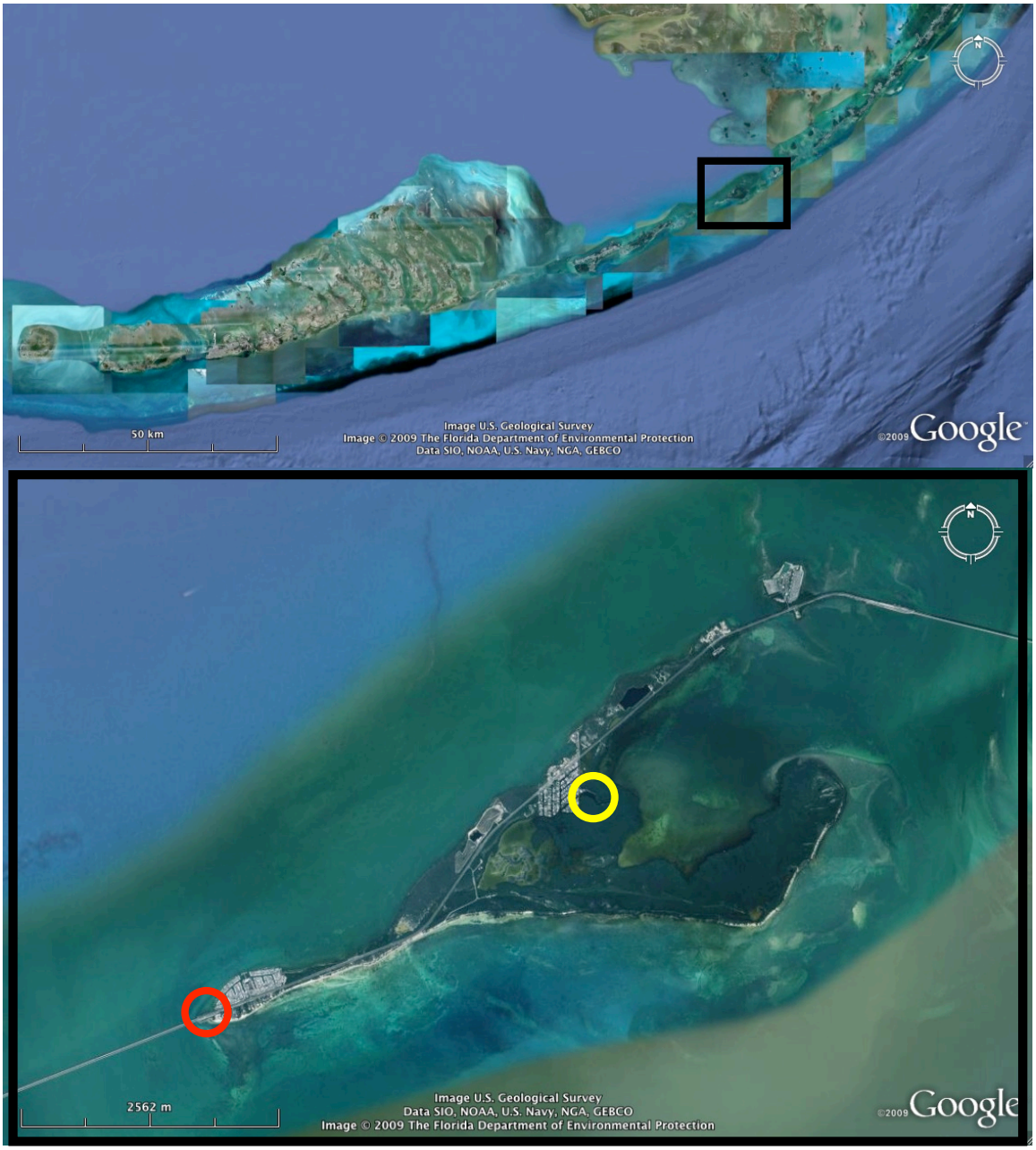


Figure 2.1 Image of Florida Keys with box highlighting Long Key. Lower image of Long Key with collection locations: red circle is M66, seawall habitat location, yellow circle is M68, mangrove habitat location. Images from Google Earth.

Figure 2.2 Phylogram generated from COI sequences of representative cryptic species in the *Brachidontes exustus* complex. GenBank sequence numbers and corresponding cryptic species are labeled. The *de novo* sequences in bold are represented by mile location and specimen number.

Figure 2.2

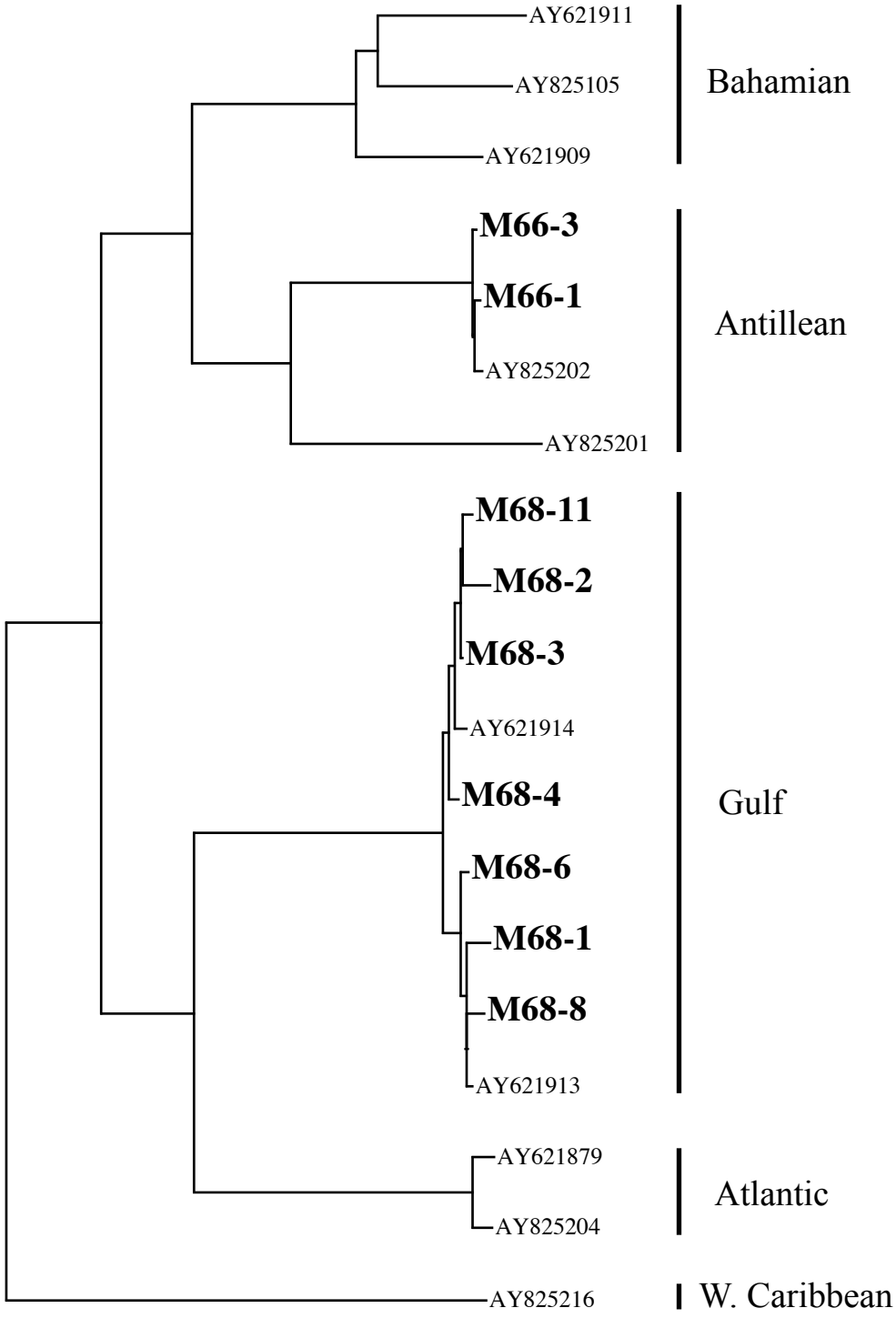

AY621911
AY825105
AY621909
Bahamian
M66-3
M66-1
AY825202
AY825201
Antillean
M68-11
M68-2
M68-3
AY621914
M68-4
M68-6
M68-1
M68-8
AY621913
Gulf
AY621879
AY825204
Atlantic
AY825216
W. Caribbean

Figure 2.3 Disarticulated left valves of 12 specimens from two sample locations on Long Key. Shells a to d were collected from the seawall, M66, and are Antillean species. Shells e to l were collected from the mangrove habitat, M68, and are Gulf species. Specimen codes for each individual: a) M66-1 b) M66-2 c) M66-3 d) M66-4 e) M68-1 f) M68-2 g) M68-3 h) M68-4 i) M68-5 j) M68-6 k) M68-8 l) M68-11. Scale bar = 10 mm.

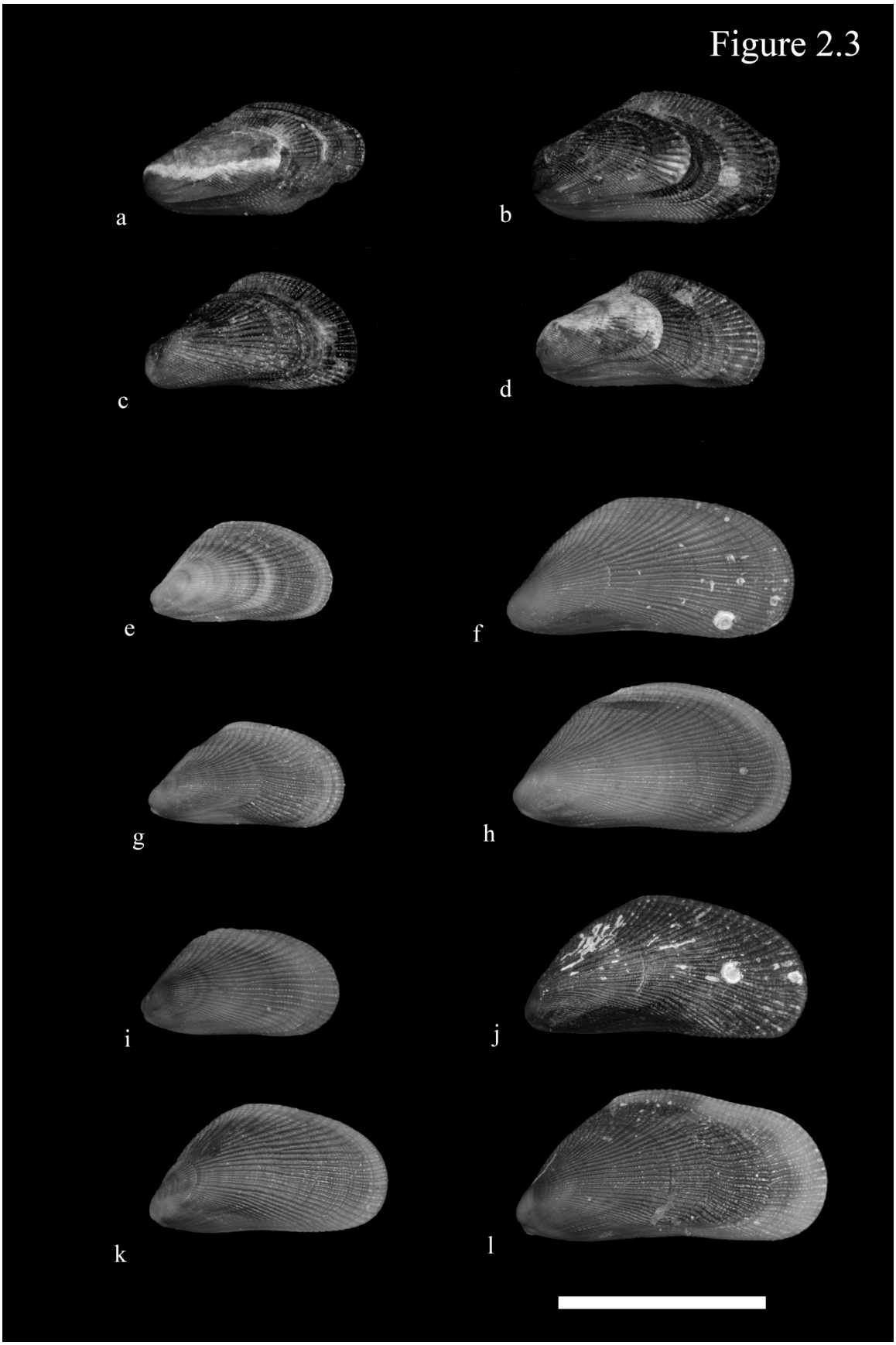


Figure 2.3
a
b
c
d
e
f
g
h
i
j
k
l

Chapter 3

Molecular Identification of Three Cryptic Species of *Brachidontes* from the Florida Keys is Paralleled by Differences in Shell Morphology

Abstract

The small intertidal bivalve morphospecies *Brachidontes exustus* was recently found to be a cryptic species complex in its western Atlantic range. Specimens of *Brachidontes exustus* were collected in July 2002 and January 2005, from 7 distinct locations on 6 different islands in the lower and middle Florida Keys. Species characterizations of 107 individuals using RFLP of the ITS region from the nuclear ribosomal cluster and COI from mitochondrial DNA found more than one species at some sites and a single species at other locations. Multivariate morphometrics distinguished between the two most common species of the *Brachidontes* complex and successfully discriminated one of the rarer species from the two common species. Principal component and discriminant analyses showed that, while there was some variation in the species morphologies in different habitats, between species morphological differences were more dependent on genetic identity than habitat type. The shells of the Gulf and Bahamian species collected from locations of coexistence were more similar morphologically than specimens collected from single-species locations. The morphological differences between individuals from these locations was not the result of phenotypic variation of a single species, but rather demonstrate another example of assumed plasticity masking underlying genetic differentiation.

Introduction

Sibling species and cryptic species complexes in what were previously believed to be highly morphologically plastic species are now well known for many marine taxa (Reeb and Avise 1990; Kamermans et al. 1999; Dawson and Jacobs 2001; Lee and Ó Foighil 2004; Terranova et al. 2007). Occasionally, when species are identified using molecular techniques and gene trees, multivariate methods can be developed to distinguish among the cryptic species (Veliz et al. 2003). However, the complex will usually remain under a single taxon if no morphological characteristics can be identified to distinguish among the species of the complex (Schlick-Steiner et al. 2007). When diagnostic morphological features are found the species may then be referred to as pseudo-cryptic or pseudo-sibling species (Sáez et al. 2003; Blanquer and Uriz 2008).

The broadly distributed western Atlantic morphospecies, *Brachidontes exustus* (Linnaeus 1758), has recently been shown, with nuclear and mitochondrial gene trees, to be a cryptic species complex with five regionally dominant and generally allopatric species (Lee and Ó Foighil 2004; Lee and Ó Foighil 2005). Populations generally limited to a single species within the complex were found in five core geographic areas: 1) southeast Atlantic coast of North America 2) Gulf of Mexico 3) Bahamas and Bermuda 4) Antilles Islands to the north coast of South America 5) Caribbean coast of Central America. Each regionally dominant species has been given an informal name corresponding to their core geographic distribution. The names are, in relation to the above geographic regions, Atlantic, Gulf, Bahamas, Antilles, and Western Caribbean (Lee and Ó Foighil 2005).

Nearly all sampled locations within each of the core geographic locations contained only one species. However, more than one species was encountered in at least one location within a core geographic range: the Atlantic species was collected from a mixed population with the Western Caribbean species at Boca del Toro, Panama. The Atlantic species was also found in the eastern Caribbean, on the island of Trinidad, where two sampled locations had single-species populations of the Atlantic and Antillean species exclusive to each collection site. The Atlantic species was collected on the island's west side, while the Antillean species was found on the east side (Lee and Ó Foighil 2005).

The Florida Keys appear to be unique among all locations sampled because four of the five species known from the Western Atlantic species complex have been identified from the archipelago using molecular techniques (Lee and Ó Foighil 2004). Even more intriguing, all three locations sampled in the Florida Keys by Lee and Ó Foighil had two species identified and the combination of the two species was different for each collection location; Bahamian and Atlantic species from Boca Chica Key, Bahamian and Gulf species from Spanish Harbor/West Summerland Key, and Bahamian and Antillean species from Key Biscayne (Lee and Ó Foighil 2004). The Florida Keys samples of Lee and Ó Foighil (2004) were dominated by two apparently common species, while the two other species appeared to be very rare. A single Atlantic species specimen was collected from the otherwise Bahamian population at Boca Chica Key and three Antillean specimens were encountered, along with a very low density of Bahamian species individuals, in a marginal habitat at Key Biscayne (Lee and Ó Foighil 2004). At the third Florida Keys collection location of Lee and Ó Foighil (2004), Gulf and Bahamian species

were both common, with the Gulf species comprising a slightly higher percentage of the sample.

The internal transcribed spacers (ITS-1 and ITS-2, collectively referred to as the ITS), within the nuclear ribosomal transcription unit, are polymorphic regions which have been used to differentiate closely related species of bivalves (White et al. 1994; Dixon et al. 1995; Yu et al. 2000; Fernández et al. 2001), sub-populations (Yu et al. 2000), indistinct juveniles (Heath et al. 1996; Johnson and Geller 2006), and sibling species and their hybrids (Heath et al. 1995). Restriction fragment length polymorphism (RFLP) analysis of the ITS region has successfully identifed unknown individual scallops to a corresponding set of four species from the coast of Spain (Lopez-Piñión et al. 2002). The ITS region, along with a portion of the 28S ribosomal gene, was used to construct nuclear gene trees for *Brachidontes exustus* (Lee and Ó Foighil 2004; Lee and Ó Foighil 2005). The published ITS sequences show the sister clades in the Florida Keys have ITS-2 lengths that differ by about 75 bp. This length difference of the ITS was targeted to assign specimens to sister-clades. One sister clade, the Bahamian/Antillean, was assayed for species-specific restriction fragment length polymorphisms (RFLP) of the ITS to assign specimens to species. The second clade, the Gulf/Atlantic was assayed for species-specific restriction fragment length polymorphism at the COI gene from mtDNA because informative polymorphisms in the second species pair proved problematic for diagnostic RFLP. The mitochondrial COI gene is commonly used for molecular phylogenies because of its relatively fast mutation rate, but conserved protein coding, single strand, and high number of copies per cell. A 648-bp fragment has become the standard for DNA

barcoding in many species and is easily amplified with a universal primer set (Folmer et al. 1994).

Pseudo-crypsis in this nominal species is likely because the taxonomic history suggests that two species names based on shell morphology persisted until the mid 1980s. Phenotypic variation observed in other intertidal mytilids suggested that the observed morphological differences of scorched mussels were within the expected variability of mytilid species (Seed 1968). Taxonomic revisions of some mytilid genera greatly reduced the number of species names (Siddall 1980) and have been confirmed molecularly (Wood et al. 2007). However, the distribution of species attached to the historical taxonomic nomenclature (Abbott 1974) prior to the most recent efforts to synonymize names (Rios 1985; Jensen and Harasewych 1986) matches closely with the core distributions of the sister clades as revealed by gene trees. The pooled Gulf/Atlantic species are one clade possibly corresponding to the historical name *B. exustus,* and the Bahamas/Antilles species that form the second clade are likely connected to the historical name *B. domingensis*. Morphological differences between the clades corresponding to those noted for the two historical species names in the western Atlantic are investigated based on species designations determined from molecular diagnostics.

The region of overlap for these species displayed in the Florida Keys is the ideal location to investigate morphological differences among the species within this complex because four species are present throughout the general area and, in some cases, are present as mixed populations at the same collection location (Lee and Ó Foighil 2004). In these

locations of co-existence, individuals would be under presumably identical phenotype-influencing environmental conditions and any morphological differences between species should be a function of genotype and not environmentally-induced variation.

Materials and Methods

Study area

The Florida Keys are a curved archipelago of Pleistocene limestone islands, mud islands, and reefs extending southwest from the southern tip of the Floridian peninsula running approximately 190 km from Key Largo to Key West. The nearest mile-marker on US Route 1 serves as a useful universal landmark for the Florida Keys and this study includes the number of the nearest mile-marker along with the collection location. Mile 0 corresponds to the start of US Route 1 at the western end of Key West and the mile number increases as one moves east and north along the archipelago towards mainland Florida. Collections from a total of six islands were made in intertidal and subtidal locations ranging from Key West (mile 2) to Long Key (mile 68) (Table 3.1). Habitats differed in ocean or bay-side orientation, wave exposures, and habitat types, but generally were limestone rubble rocky shores or coral rubble and limestone on coarse sandy bottoms. The two most northeasterly collection locations on Long Key differed from all others in that one was a vertical concrete seawall adjacent to the Long Key Viaduct, while the second was a mangrove-lined boating channel.

Sample collection

Living specimens were collected in July 2002 and February 2005. All specimens

collected in 2002 were dissected and measured within hours of collection and the soft tissues and shells subsequently preserved in 95% ethanol. Most of these specimens dried while in storage. Specimens collected in 2005 were transferred to a seawater flow-though system and were maintained alive for several days before being frozen at -40ºC. Frozen samples were transported on dry ice and stored at -80ºC prior to shell measurement, dissection, and molecular characterization.

Frozen specimens were thawed at room temperature, measured, dissected, and the soft tissues separated from the shell. The disarticulated left valve of each subsequently typed specimen was photographed using a Nikon digital SLR camera mounted on a Zeiss Stemi 2000-C dissecting microscope.

DNA extraction and molecular characterization

Total genomic DNA was isolated from the frozen specimens collected in 2005 from approximately 20 mg of tissue excised from the posterior adductor muscle in larger specimens or mantle tissue in smaller specimens. The specimens collected in 2002 had dried in transit and the DNA had to be extracted from approximately 1 mm^2 of dried tissue. In all cases the extraction was accomplished with a DNeasy Tissue Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. Extracts were stored at -20ºC until used as PCR templates.

Two target fragments, one from the nuclear ribosomal gene cluster that encompassed the internal transcribed spacer region, comprising the ITS-1, 5.8s ribosomal gene, and the

ITS-2, and a second, the cytochrome *c* oxidase subunit I (COI), were amplified by polymerase chain reactions (PCR) using Taq PCR Mastermix (Qiagen, Valencia, California). The ITS was amplified with the primer pair (5'-GTTTCCGTAGGAGAACCTG-3' and 5'-CTCGTCTGATCTGAGGTC-3') from Heath et al. (1995) initially designed for *Mytilus* spp. The COI was amplified with the well known "universal" COI primer pair (LCO 1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO 2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') from Folmer et al. (1994). The ITS primers anneal to the flanking regions of the 18S and 28S ribosomal genes, while the COI primers anneal to highly conserved sequences within the COI that amplify a 660 bp fragment of the 5′ end of the COI gene. The ITS fragment was amplified with a thermal cycler protocol of 3 min initial denaturing at 94ºC followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min with a final 5 min extension at 72ºC. The COI was amplified using the protocol of Folmer et al. (1994). Each reaction was prepared in 25-μl volumes and a negative control containing all reagents and the primer pair, without the DNA template, was included with each amplification series. The resulting PCR products and negative controls were run alongside a 100-bp ladder on a 1% agarose gel stained with ethidium bromide and visualized by UV transillumination.

All ITS-1, ITS-2, and COI sequences of the *Brachidontes exustus* cryptic species complex available from GenBank were retrieved and examined using the program EnzymeX (Mekentosj.com) for endonuclease restriction sites that would yield species-specific patterns. Restriction Fragment Length Polymorphism (RFLP) digests were

performed with 10 µl of the resulting PCR product in 20-µl volumes. The length of the ITS amplicon, which includes the ITS-1, 5.8s, and ITS-2, distinguishes the two sister clades because of a 75-nucleotide deletion in the Bahamian/Antillean clade relative to the sister Gulf/Atlantic clade. A double digest with the restriction endonucleases *Hae*III and *Mnl*I was used for discriminating between the Bahamian and Antillean species with the ITS PCR product. A single digest with the restriction endonuclease *Mnl*I was used with the COI PCR product for discriminating between the Gulf and Atlantic species. In both cases the entire digestion volume was run on a 2.5% agarose gel, stained with ethidium bromide and visualized under UV transillumination.

Morphological analysis

A suite of morphological measurements (Figure 3.1) were made with calipers to the nearest 0.1 mm for each specimen: length (*L*) measured as the maximum distance from umbo to posterior margin, height (*H*) measured as the maximum distance from the ventral line to dorsal margin, maximum width (*W*) across both valves, anterior dorsal margin length (*ADM*), measured from the umbo to the most posterior dysodont tooth, the length of the ligament (*LIG*), the number of primary (*PT*) and dysodont teeth (*DT*), and the number of ribs (*RIB*) crossed by a line perpendicular to the ventral margin line at half the total shell length on the left valve were counted under a dissecting microscope. The angle (*ANG*) of the umbo between the anterior dorsal margin line and ventral line on the left valve was marked on paper using two straight edges and measured with a protractor. Specimens across the range of sizes collected in each location were used for the morphological analysis with only specimens <5 mm in shell length excluded.

Specimens were collected in 2002 from three locations with differing habitats, a mangrove-lined boating channel that leads from the bay-side of the island to an interior lagoon on Long Key (mile 68) and two bay-side rocky shores with differing levels of wave exposure. A wave-protected location (the interior of the Horseshoe, Spanish Harbor/West Summerland Key, mile 35) and a relatively less protected site at Burnt Point Island, mile 56 (Table 3.1). Two of these locations, the Long Key mangrove, mile 68 and the Horseshoe, mile 35, were re-sampled during the 2005 collections. A test of morphological differences between habitat types, prior to molecular characterization, was done with 20 specimens from each of the three locations collected in 2002. All morphological values were log transformed prior to multivariate statistical treatments. Principal Component analyses (PCA), t-tests of PC 2, and discriminant function analyses (DA) were performed using the statistical package STATISTICA (Statsoft Inc).

The specimens collected in 2005 were used in morphological comparisons between species with a sub-sample of specimens from the 2002 collections from which DNA could successfully be extracted.

Results

Genetic typing

Each primer pair successfully amplified a single product in all samples. The initial amplification of the ITS revealed that more than one sister-clade was present in the Florida Keys samples. The differences in amplicon length were clearly discernable under

UV transillumination after electrophoresis. The longer fragment was approximately 1050 nucleotides long while the shorter fragment was approximately 975 nucleotides long. These fragment lengths correspond, respectively, to the pooled Gulf and Atlantic sister species and the pooled Bahamian and Antillean sister species. Florida Keys locations were found to have either a single sister-clade (the Bahamian/Antillean clade at M2 Key West, M6 Boca Chica Key, M11 Shark Key, and M66 the seawall location at Long Key and the Gulf/Atlantic clade at M68, the mangrove location at Long Key) or both sister-clades (M56, Burnt Point Island and M35, Spanish Harbor/West Summerland Key). Long Key was the only island sampled at two distinct locations and the two differing habitats at the Long Key locations had single, albeit different, clades present.

There were two banding patterns observed from the RFLP analysis of Bahamian/Antillean clade specimens. The endonucleases chosen cleaved in numerous locations and produced multiple small fragments less than 150 bp long. The species designations are based upon the three longest fragments visible in the RFLP because fragments smaller than 100 bp are difficult to clearly discern on the gel after electrophoresis. The three longest fragments matched the expected lengths predicted from the simulated restriction digests performed in the program EnzymeX. The fragments of the Bahamian species were consistent across all specimens with three clear bands at about 340, 220, and 130. The Antillean species had two bands visible above the100-bp cut-off, one at 340 bp and one at 130 bp. There was one banding pattern observed in all digests of the COI from all Florida Keys specimens typed to the Gulf/Atlantic clade by ITS length. That pattern had three bands, one each at approximately 350 bp, 160 bp, and

110 bp. This matched the expected pattern for the Gulf species from the simulated digestion in EnzymeX. The expected banding pattern for the Atlantic species, (two bands, one at 350 bp and one at 310 bp) was not observed in any Florida Keys specimen. Two specimens collected from Foley Beach, South Carolina, near Charleston, displayed the expected Atlantic species RFLP pattern.

The Gulf and Antillean species designations were confirmed by sequencing the COI gene for eight Gulf and two Antillean individuals which, when compared to the NCBI database, matched the species designations by RFLP (see Chapter 2). All Bahamian/Antillean clade specimens encountered at all locations other than four small Antilles species specimens collected from M66, the seawall, and the two small Antilles specimens from M11, Shark Key, were Bahamas species. All Gulf/Atlantic clade specimens were typed to the Gulf species (Table 3.1).

Principal components analysis

Covariates of size (length, width, height, anterior dorsal margin length, and ligament length) generally factored strongly on PC 1, while shape and sculpture measurements (ribs, primary and dysodont teeth, and umbo angle) factored with PC 2. This was the general pattern in all subsequent PC analyses with minor differences in the percent variance explained by each component except in the PCA of the Gulf and Bahamian species in locations of exclusivity, where the angle of the umbo weighted heavily with size covariates in PC 1. The factor loadings for each PCA are displayed in Table 3.2.

A PCA based on the Bahamian, Antillean, and Gulf species specimens from all locations showed marked clustering. The Gulf species was generally lower in value for both PC 1 and PC 2 than the Bahamas and Antilles specimens. However, the species did not cluster in species exclusive groups (Figure 3.2 and Table 3.2). A PCA limited to all specimens of Bahamian and Antillean species from all locations (Figure 3.3) did not show species clustering. A PCA of the Gulf and Antillean species (Figure 3.4) found the Antillean species to have lower PC 2 scores than the Gulf species. The PCA of Bahamian and Gulf species from all locations (Figure 3.5) showed clustering but there was still some overlap between the species. The PCA of Bahamian and Gulf species in locations of coexistence (Figure 3.6) showed that most of the overlap in the species' PC scores were from the specimens that were collected in locations of coexistence, while the PCA of these two species in locations of exclusivity (Figure 3.7) showed a very clear separation of the two species clusters.

The Bahamian and Gulf species were encountered in both single species and mixed species populations. To test if the morphology of each species differs between locations of exclusivity and coexistence, PCAs for each species comparing specimens from mixed and single-species locations were performed (Figures 3.8 and 3.9). In both species' cases, a t-test of the PC 2 scores from locations of exclusivity and coexistence showed the morphology of both the Gulf and Bahamian species were not significantly different ($p=0.21$ and $p=0.084$ respectively) in the two types of populations. However, in both cases the species in exclusivity had relatively higher PC 1 scores showing that each species grew to a larger size in the locations of exclusivity than in locations of

coexistence.

Discriminant function analysis

A series of forward stepwise discriminant functions were constructed with various Gulf, Bahamian, and Antillean species groupings. Root 1 vs. root 2 is shown in Figure 3.10 for the pair of functions created with the Gulf and Bahamian species specimens from locations of coexistence and all Antillean species specimens. The six Antillean species specimens cluster together with no intervening specimen from the other two species. There is some overlap in the Bahamian and Gulf species root 1 scores. The two root functions correctly assigned to species the Gulf, Bahamian, and Antillean specimens (respectively, 91.7%, 97% and 83.3%) for an overall correct discrimination rate of 93.7% (Table 3.3).

Pair-wise discriminant functions were created for each species combination. In all cases, Gulf and Bahamian specimens from locations of coexistence and all six Antillean specimens were used for the creation of the function. The Gulf and Bahamian species that were collected from locations exclusive to that species were excluded from the construction of the function and subsequently used as the validation set. Seven variables, when used in the canonical function, effectively discriminated between the Gulf and Bahamian species. The canonical score, C, is assigned to individual specimens based on these values: *ADM*=anterior dorsal margin length, *RIB*=number of ribs at half the length, *ANG*=the angle of the umbo, *H*=height, *PT*=number of primary teeth, *LIG*=length of ligament, and the final term is a constant. The pairwise discriminant function to

discriminate the Gulf and Bahamian species is:

$$C=-4.83 \ln ADM+4.75 \ln RIB+6.25 \ln ANG-7.34 \ln H+.8 \ln PT+1.57 \ln LIG-25.49.$$

There is some overlap of the canonical scores of the two species (Figure 3.11, Table 3.3) and no single number of the C value makes a clear distinction for all specimens. All but one specimen of the Bahamian species has a value of $C<0.3$. Likewise, for the Gulf species, all cases but one have a value of $C>0.3$. For classification purposes of unknown individuals, $C=0.3$ can be used as the demarcation between Gulf and Bahamian species designations with this function. To validate the function, the 28 Bahamian and 16 Gulf specimens collected from locations exclusive to each species and excluded from the construction of the function had canonical scores that were used to assign species identifications. All 44 cases were correctly identified with respect to species.

A second stepwise discriminant function utilizing the same two sets of specimens for construction and validation, but limited to measurements that can be taken from the exterior shell of live specimens, was nearly as successful in properly identifying to species individuals from the validation set. The second function of only external measurements,

$$C=- 4.83 \ln ADM+5.25 \ln RIB+5.93 \ln L+2.9 \ln ANG-4.67 \ln H-24.13,$$

produced a best species demarcation value of $C=0.45$ and all 44 scores from the

validation set correctly identified the species of each specimen.

Discriminant functions were created with the Bahamian species specimens from locations of coexistence and the Antillean species specimens from all locations which resulted in a demarcation value of C=-1.3. (Figure 3.12, Table 3.3). The function for discriminating specimens between the Bahamian and Antillean species is:

$$C=8.5\ \ln ADM-5.3\ \ln W-3.84\ \ln RIB+3.88\ \ln ANG-11.29.$$

Gulf species specimens from locations of coexistence and the Antillean species specimens from all locations created a function with a demarcation value of C=-1.6 (Figure 3.13, Table 3.3). The function for discriminating between the Gulf and Antillean species is:

$$C=8.01\ \ln RIB+2.82\ \ln DT-5.92\ \ln W+10.52\ \ln L$$
$$-10.31\ \ln H+1.24\ \ln PT+3.24\ \ln ADM-41.06.$$

Morphology between habitat types

A Principal Component Analysis (PCA) failed to exclusively cluster specimens from the same habitat type (Figure 3.14). The first two extracted principal components (PC 1 and PC 2) encapsulated 72.3 % of the total variance. Covariates of overall size (length, height, width, anterior dorsal margin length, and ligament length) heavily weighted to PC 1 explained 51.2% of the variance (Table 3.2). PC 1 scores for all locations ranged nearly

across the full range of values from -2 to 2 because specimens from the full range of sizes >5 mm collected at each location were used in the analysis. PC 2 encompassed 21.1% of the total variation and was most heavily weighted to number of ribs at half the total length, number of primary and dysodont teeth, and the angle of the umbo (Table 3.2). PC 2 is a proxy for shell shape and sculpture and displays some clustering by habitat types with more specimens collected from rocky shore habitats generally having PC 2 scores less than about 0.5 and specimens collected from the mangrove habitat having scores greater than -0.5.

A Discriminant Function Analysis was performed to statistically maximize separation of the locations based upon specimen morphology. The three locations create two root functions that compare each location to the other two locations. A graphical representation of the function's scores, plotted as root 1 vs. root 2 of the functions, (Figure 3.15) with 95% confidence ellipses, shows considerable overlap of the three collection locations. At least one individual from each of the three locations falls in a region bound by the confidence ellipses of all three locations. Twelve of the 40 rocky shore specimens fall within the area shared by the 95% confidence ellipses from the two rocky shore locations, while only 6 specimens are within the confidence ellipses of the mangrove location and each of the rocky shore locations. The differences by habitat locations are significant, even though the created function can assign only 75% the specimens to the proper location (Table 3.3). The function was more successful at properly assigning specimens from M68, the mangrove location (95%), than from either of the rocky shore locations, M35 and M56, (70% and 65% respectively).

Discussion

Ideally, a single method would have been employed to determine species in a single RFLP, but the differing levels of variation in each sister-clade made each of the methods employed suitable for only one clade. RFLP analysis of the ITS was problematic for the Gulf/Atlantic species pair because there are far fewer potential restriction site differences between these species than with the Bahamian/Antillean sibling pair. In all restrictions attempted with the Gulf/Atlantic species pair, the expected restriction site based on the published sequences turned out to have allelic differences that yielded an undigested band from an allele lacking the restriction site. In the Bahamian/Antillean sibling species pair, there were many informative restriction sites of the ITS that could be targeted for species designation and the banding patterns produced in the RFLP were unambiguous. In this species pair, the COI gene is very diverse with a gene tree topology that has three tip clades for the Bahamian species and two tip clades for the Antillean species (Lee and Ó Foighil 2005). No endonuclease was identified that could discriminate among all four species and few restriction sites were identified that were unambiguous for the Bahamian/Antillean pair. The two methods that were employed, while not ideal, did give unambiguous species designations.

The Bahamian and Gulf species dominated the distribution of species in the Florida Keys. The two most southwestern sample locations, mile 2 and 6, were exclusively the Bahamian species. The location at mile 11, Shark Key, had very few specimens, but there were both Bahamian and Antillean species. The location at mile 35, the Horseshoe site, Spanish Harbor/West Summerland Key, was sampled in 2002 and 2005. At both

sampling times the population at this location was a mixture of Bahamian and Gulf individuals. Burnt Point Island, mile 56, was sampled only in 2002. At this location, a mix of Bahamian and Gulf species individuals was found. The Antillean species was encountered at the seawall on Long Key, mile 66, where the population was exclusively this species in very low abundance (see Chapter 2). Only 4 km to the northeast, at the mangrove location, mile 68, all the typed specimens were Gulf species (see Chapter 2). This distribution is very similar to that observed by Lee and Ó Foighil (2004). The major difference is that the Gulf species was encountered in an exclusive and abundant population in the mangrove habitat on Long Key. Additionally, the Antillean species was encountered more often and further southwest than previously recorded by Lee and Ó Foighil (2004).

Lee and Ó Foighil (2004) suggested that the Antillean species might be more common than the few individuals they collected from Key Biscayne. At the Key Biscayne location they found very few specimens and included juveniles from this location in their phylogeny. None of their other collections in the Florida Keys included sub-adults. They speculated that the Antillean species may not survive to adulthood in the Florida Keys and that a more thorough sampling of small individuals from other locations in the Florida Keys might show the Antillean species to be common in the Florida Keys as juveniles. Many specimens were in the 5 to 10 mm length range and no Antillean species individuals were encountered intermingled with other populations in locations with abundant *Brachidontes* spp. The record of this species is limited to four specimens from the seawall on Long Key, mile 66, and two small specimens collected at the Shark Key

boat ramp, mile 11. The habitat description of the Key Biscayne collection location of Lee and Ó Foighil (2004) is much like the Shark Key location, with rocks scattered about the shallows with the mussels in crevices or within the byssal threads of *Isognomon* spp. on the under sides of rocks. Lee and Ó Foighil's (2004) supposition that the Antillean species is more common in the Florida Keys mixed in as juveniles in other populations was not bolstered by the species distribution found in this study. However, the species may be more common in marginal habitats and would likely be found more often if marginal habitats were searched actively.

Locations sampled for this study included two of the collection sites of Lee and Ó Foighil (2004). As with their collection at the Horseshoe site in Spanish Harbor/West Summerland Key, a mixed Gulf and Bahamas species population was encountered in both the 2002 and 2005 collections. A total of 42 specimens were typed at this location and were found to be an equal mix with 21 individuals of each species (Table 3.1). An exclusive Bahamian population was found at Boca Chica Key and we did not encounter the Atlantic species, as had Lee and Ó Foighil (2004).

The distributions of dominant species were maintained over the three years between the 2002 and 2005 collections at the Horseshoe site in Spanish Harbor/West Summerland Key. Likewise, the species distribution found in 2001 by Lee and Ó Foighil (2004) and the 2005 collection from the same island had the same dominant species. The maintenance of single and mixed-species locations over multiple years suggests that the distributions are not the result of species-specific larval settlement events or other

stochastic processes. As broadcast spawners, with an extended pelagic larval phase and planktotrophic development (Campos and Ramorino 1980; Fields and Moore 1983; Sweeney and Walker 1998), it would be expected that all of the species known from the Florida Keys could potentially disperse throughout the archipelago in just a few generations (Grantham et al. 2003) and that mixed populations would be the norm. Locations with mixed-species and single-species populations persisting over several years suggest that there are subtle environmental tolerances not shared by all species within the complex. These environmental differences influence survivorship of recruits among the potential species in a consistent manner over several years.

Other researchers working on bivalve distributions in the Florida Keys have noted long standing differences between bayside and oceanside species compositions (Bieler and Mikkelsen 2004; Mikkelsen and Bieler 2007) and offer salinity as a potential driver. Salinity differences in the Florida Keys between bayside and oceanside locations would be consistent with the salinities encountered by the species in their core geographical range, where the Bahamas and Antilles clade species are found on oceanic islands and the Gulf and Atlantic species are found on continental margins. Co-evolved biotic interactions from the core geographic range, which are not currently acting in the Florida Keys, are an alternative possibility. What those interactions might be remain open questions.

The morphology of these *Brachidontes* species is indeed variable. The PCA scatter plots for both the habitat locations and species affiliations show that there is a range of scores

for each habitat location or species. There was no single diagnostic morphological character that could be used to determine species. However, there are some species-specific trends in morphology that were used to develop discriminatory statistics. Multivariate morphometrics of shell attributes yielded a robust method for assigning specimens to species.

In the comparison of morphology by locations (Figure 3.14), the mangrove location specimens grouped together, having higher PC 2 scores, while specimens from the two rocky shores generally had low PC 2 scores, but there was considerable overlap. It had initially been interpreted as plasticity of a single species, but, in light of the presence of cryptic species, that conclusion is obviously false. Both rocky shore locations (M35 and M56) are locations of species coexistence between the Bahamian and Gulf species. Of the 20 specimens from each rocky shore habitat that were used in this analysis, 12 specimens from M56, Burnt Point Island, and 13 from M35, the Horseshoe on Spanish Harbor/West Summerland Key, have been typed to species by RFLP. The M56 location had 9 Bahamian and 3 Gulf specimens, while the M35 location had 8 Bahamian and 5 Gulf species specimens. The mangrove habitat (M35) was a location of Gulf species exclusivity. The overlap in morphologies from the different habitats (Figure 3.2) is consistent with the direct comparison of Gulf and Bahamian morphologies (Figure 3.4) if the Gulf species specimens from the rocky shore locations have higher PC 2 scores than Bahamian species specimens from the same location. The Gulf specimens would group together in the PCA regardless of the habitat type from which the specimen was collected.

There are some notable differences between specimens collected from locations of species coexistence and exclusivity. The means of the PC 2 scores for the Bahamian and Gulf species are more divergent in locations of exclusivity than in locations of coexistence. The two species appear to have more similar shell morphologies in locations of coexistence. A model of plasticity where closely related species share similar phenotypic responses is the best fit with the data. However, even in the locations of coexistence, where individuals from both species are subject to an identical environment, there is a significant difference between the PC 2 scores of the Gulf and Bahamian species ($p=0.046$).

The growth of mussel shells is not strictly allometric. Much of the growth of the largest individuals is limited to the posterior margin (Seed 1980b). This growth characteristic skews the PC 2 scores to make the morphologies seem more similar than they actually are when comparing PC 2 scores alone. The effect on the distribution of PC 2 scores is that the two species have similar PC 2 scores only at the size extremes of Gulf and Bahamian species. The PC 2 scores of the smallest Gulf species specimens are nearly the same as the PC 2 scores of the largest Bahamas species specimens. This is mostly a consequence of the number of ribs at half the length influencing the PC 2 score of the largest specimens. One striking observation is in the factor loadings of the PCA of Gulf and Bahamian species in locations of exclusivity. In all other PCA factor loadings, the covariates of size group together in the first factor. Conversely, in the PCA of Gulf and Bahamian species from locations of exclusivity, the loading for the angle of the umbo loads to factor 1 with variables of size. This shift in loadings made it impossible to

directly compare PC 2 scores as a proxy for shape between this PCA and the PCA of Gulf and Bahamian species in locations of coexistence. Instead, the PC 2 scores from the single PCA that included all Gulf and Bahamian specimens was grouped by individuals from locations of exclusivity and coexistence so that the shell morphologies could be compared based on the same factor loadings.

To test the variation within each species, a PCA comparing specimens of each species from locations of exclusivity to specimens of the same species from locations of coexistence was performed. In both cases there was no statistical difference between PC 2 scores from the two locations. The differences between the mangrove and rocky shore individuals of the Gulf species were surprisingly little. Wave exposure has a strong influence on the morphology of mussels (Seed 1968). Wave action at the mangrove location is much less than at the two exposed rocky shore locations but appears to have little effect on shell morphology in this species. The comparison of Bahamian specimens from locations of exclusivity and coexistence had a similar result. The PC 2 scores were not significantly different between the locations of exclusivity and coexistence. This was a less surprising result than for the Gulf species because both exclusive and coexisting Bahamian populations were found on rocky shores with similar environmental conditions. While this morphospecies has traditionally been considered to be highly variable, it appears that the morphology is more dependent on the genetic affiliation of the specimen than on the environmental conditions under which it develops.

Cryptic complexes without morphological cues to distinguish species will likely remain

under one taxon (Schlick-Steiner et al. 2007). In the *B. exustus* complex, the discriminant analysis correctly assigned specimens to species with near 95% confidence, showing that there are species-specific morphological differences that can be used to determine the likely species in the Florida Keys. The discriminant function between the Antillean specimens and the Gulf and Bahamian species, while not particularly robust because of the small number of specimens, will nonetheless be useful for workers in the Florida Keys classifying specimens to species. This is the first step in helping to address the taxonomy of this group and resurrecting species names that have been synomomized. The type specimens and their collection locations, in light of the core geographic regions occupied by each of the species in the complex, and the historical names that have been attached to mussels from this genus, may reveal that all five species in the complex have at one point been named. There is a pool of potential species names from which to resurrect the valid status of these species.

In the Florida Keys, the three species (the Bahamian, Antillean, and Gulf) are pseudo-cryptic. The consistent morphology of the species in all habitats can distinguish Gulf and Bahamian species using the canonical discriminant function. The sample size of the Antillean species was small and the morphological differences between the species may be an artifact of small sample size. Alternatively, the morphologies reflect species level differences and will become better quantified with a larger sample size. The Gulf and Atlantic sibling species pair could not be compared because no Atlantic species specimens were recovered.

Mussels in the genus *Brachidontes* from regions beyond the scope of this study have also been assumed to be phenotypically plastic in different habitats (Morton 1991; Tanaka and Alveres de Magalhaes 1999). Some of these nominal morphospecies are now known to be cryptic species complexes and these include: *Brachidontes adamsianus* from the Panamanian Pacific coast; *Brachidontes variabilis* from Hong Kong Harbor; and an unnamed *Brachidontes* from Darwin Harbor, Australia, (Lee and Ó Foighil 2005; Terranova et al. 2007). A clear species level difference between the Indian Ocean and Pacific Ocean populations of *B. variabilis* was found using 16S mitochondrial gene sequences (Terranova et al. 2007), but their small sample size from Hong Kong did not resolve the highly divergent cryptic species in Hong Kong observed by Lee and O Foighil (2005) in their 28S nuclear phylogeny. Morton (1988) notes that *B. variabilis* is common on mangrove roots in the estuaries of Hong Kong but Taylor (1971) recorded the species on coral beachrock at Aldabra Atoll in the Indian Ocean. These likely represent the Indian and Pacific Ocean allopatric species of nominal *B. variabilis*. Terranova et al. (2007) did not examine nuclear 28S sequences leaving unresolved how the species they identified by mitochondrial 16S sequences correspond to the nuclear 28S gene tree. The Lee and Ó Foighil (2005) 28S gene tree included two individuals from Hong Kong populations that showed *B. variabilis* to be a cryptic species complex. Under closer examination, the cryptic species of Hong Kong may represent a location of coexistence between the Indian Ocean and Pacific Ocean species of the nominal *B. variabilis*. Additionally, two individuals from Darwin Harbor, Australia incorporated into the Lee and Ó Foighil (2005) 28S gene tree showed that the un-named *Brachidontes* species at this location is also a cryptic species complex and does not resolve to either of the Hong

Kong *B. variabilis*. Without mitochondrial 16S or COI gene sequences to compare to the other known cryptic species in the genus, the relationship between the Pacific and Indian Ocean clades of *Brachidontes variabilis* remains unresolved. It appears that the *Brachidontes* genus may have a propensity for speciation and many nominal species appear to be cryptic species complexes. This is unlike species in other mussel genera, such as *Mytilus* and *Perna,* which generally have broadly ranging distributions (Wood et al. 2007; Gardner and Thompson 2009). Again, gene trees of nuclear 28S and mitochondrial 16S and COI will resolve these unclear relationships.

The introduction of a cryptic or sibling species can confound conservation efforts when an exotic species replaces a related native species. Along the coast of California, the invading European blue mussel, *M. galloprovincialis* appears to have replaced the native *M. trossulus* unnoticed (Geller 1999). The changeover in species was confirmed with molecular evidence from museum-stored mussels that were collected in the 1870s and 1880s (Geller 1999). Changes in community structure are likely to have taken place but there is no way to know what the changes may have been. The prevalence of allopatric sibling species in many marine groups (Knowlton 1993) and the transfer of larvae and microorganisms via the ballast water of transcontinental ships (Lavoie et al. 1999; Ruiz et al. 2000; Verling et al. 2005) make this type of undocumented shift among cryptic species more likely (Miglietta and Lessios 2009).

Other mussels and bivalves have become invasive after being introduced to new locations. Species in the genus *Brachidontes* share many attributes with other invasive

bivalves, such as planktotrophic larva, gregarious settlement, and dense adult populations. Species from the *B. exustus* complex may potentially be introduced to other parts of the world and become invasive. Understanding the environmental tolerance differences among the species within the complex may help identify the conditions that would support or hinder a potential introduction from becoming established. In some locations, such as the southeast coast of the United States where the Atlantic species is dominant, mussel beds form with densities >10,000 individuals m^2 (Abbott 1974). It appears that the Atlantic species is a relatively new addition to the fauna of the southeast United States. It has the lowest number of cytochrome *c* oxidase subunit I haplotypes and appears, from molecular clock modeling, to have been introduced to this area from a distant southern source population fewer than 10,000 years ago (Lee and Ó Foighil 2005). The high densities on the Atlantic coast may reflect release from predation or disease pressure experienced in the southern parts of its range. The establishment of any one of the species in the complex in other tropical regions of the world is a clear possibility.

Dense bed formation is one of the characteristics of *Dreissena polymorpha* and *Limnoperna fortunei* that contribute to their pest status in freshwater systems (Karatayev et al. 2007). The recently introduced Asian green mussel, *Perna viridis,* has been found in the cooling water intake of a power plant along the west coast of Florida (Benson et al. 2001). The species has established reproductive populations and has spread to other parts of Florida and coastal Georgia (Power et al. 2004). *Mytilus* species have been introduced to many locations around the world and the species composition of mussel populations

often changes with hybridization or displacement (Heath et al. 1995; Heath et al. 1996; Geller 1999; Johnson and Geller 2006). Mussels in the genus *Brachidontes* share many of the attributes, such as planktotrophic larvae, high fecundity, and epi-byssate attachment to hard substrates, with these other successful invaders. While no site encountered in the Florida Keys has the dense mussel beds that are commonly associated with the invasive bivalves mentioned, the Atlantic species has been found as dense mussel beds on jetty rocks in the intertidal zone at Foley Beach, South Carolina, (personal observation). This location is at the northern extreme of the species' distribution and could represent a location of refuge from disease or predation limiting densities in the Florida Keys. If species of *Brachidontes* are introduced to other locations and released from the pressures limiting their densities in their native ranges, they could become invasive. Understanding the differences among the tolerances of the species within the *B. exustus* species complex will help in assessing their invasive potential.

Table 3.1 Mile on Route 1 of collection location, latitude and longitude, island location, years collected, habitat type, ocean or bayside orientation, number of specimens used for genetic analysis from each location, number of each species found in each location, total number of specimens, and total number of specimens of each species.

							Informal Species Names			
Mile	Latitude	Longitude	Location	Collection Years	Habitat	Orientation	Total Typed	Antillean	Bahamian	Gulf	Atlantic
2	24°33.47 N	81°46.52 W	Key West	2005	rocky shore	bayside	12	-	12	-	-
6	24°34.37 N	81°43.25 W	Boca Chica Key	2005	rocky shore	bayside	16	-	16	-	-
11	24°36.04 N	81°38.48 W	Shark Key	2005	rocky shore	oceanside	5	2	3	-	-
35	24°39.21 N	81°18.09 W	Spanish Harbor Key	2002 & 2005	rocky shore	bayside	42	-	21	21	-
56	24°25.35 N	80°59.09 W	Burnt Point Island	2002	rocky shore	bayside	12	-	9	3	-
66	24°48.10 N	80°50.58 W	Long Key seawall	2005	seawall	oceanside	4	4	-	-	-
68	24°49.22 N	80°48.45 W	Long Key mangrove	2005	mangrove	bayside	16	-	-	16	-
						Totals	107	6	61	40	0

Table 3.2 Factor loadings of variables from principal components analyses noting species and locations of specimens included in PCA and corresponding figure of plotted PC scores.

	Bahamian, Gulf, and Antillean species, all locations		Bahamian and Antillean species		Antillean and Gulf species	
	Figure 3.2		Figure 3.3		Figure 3.4	
Variable	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2
Length	0.964	-0.186	-0.977	0.008	0.974	-0.528
Height	0.954	-0.228	-0.975	-0.001	0.973	0.588
Width	0.966	0.03	-0.94	0.038	0.824	-0.365
Dorsal Margin Length	0.978	-0.025	-0.977	0.043	0.925	-0.191
Ligament Length	0.964	0.026	-0.934	0.147	0.881	-0.294
Ribs at Half the Length	-0.017	-0.843	-0.544	-0.272	0.69	0.424
Primary Teeth	-0.18	-0.675	-0.121	-0.683	0.191	0.714
Dysodont Teeth	-0.207	-0.504	-0.112	-0.788	0.063	0.572
Angle of Umbo	-0.228	-0.557	-0.169	0.568	0.419	0.633
Total Variance	0.532	0.202	0.552	0.168	0.544	0.187

Table3.2 Factor loadings of variables from principal components analyses noting species and locations of specimens included in PCA and corresponding figure of plotted PC scores. Continued.

	Bahamian and Gulf species, all locations		Bahamian and Gulf species, locations of coexistence		Bahamian and Gulf species, locations of exclusivity	
	Figure 3.5		Figure 3.6		Figure 3.7	
Variable	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2
Length	0.956	0.214	0.969	0.097	-0.916	0.36
Height	0.947	0.252	0.955	0.208	-0.905	0.311
Width	0.969	0.243	0.964	0.024	-0.969	0.115
Dorsal Margin Length	0.976	0.518	0.974	0.046	-0.969	0.094
Ligament Length	0.963	-0.032	0.972	0.009	-0.934	-0.416
Ribs at Half the Length	-0.104	0.864	-0.11	0.803	0.411	0.742
Primary Teeth	-0.213	0.687	-0.094	0.789	0.411	0.621
Dysodont Teeth	-0.274	0.447	-0.202	0.456	0.178	0.802
Angle of Umbo	-0.335	0.469	-0.357	0.322	0.705	0.1
Total Variance	0.541	0.194	0.54	0.182	0.54	0.182

Table 3.2. Factor loadings of variables from principal components analyses noting species and locations of specimens included in PCA, and corresponding figure of plotted PC scores. Continued.

	Bahamian species, locations of exclusivity and coexistence with Gulf species		Gulf species, locations of exclusivity and coexistence with Bahamian species		M35, M56 and M68 habitat locations	
	Figure 3.8		Figure 3.9		Figure 3.14	
Variable	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2
Length	-0.969	0.196	-0.966	0.247	0.959	0.09
Height	-0.97	0.003	-0.973	-0.323	0.951	0.111
Width	-0.944	0.074	-0.874	0.127	0.934	-0.225
Dorsal Margin Length	-0.974	0.05	-0.922	0.082	0.922	-0.216
Ligament Length	-0.915	0.172	-0.871	0.251	0.941	-0.176
Ribs at Half the Length	-0.602	-0.263	-0.659	-0.34	-0.126	0.731
Primary Teeth	-0.151	-0.69	-0.233	-0.807	-0.297	0.592
Dysodont Teeth	-0.05	-0.805	0.112	-0.642	-0.064	0.638
Angle of Umbo	-0.008	0.59	-0.481	0.088	0.257	0.675
Total Variance	0.541	0.204	0.549	0.175	0.512	0.211

Table 3.3. Discriminant function analysis of shell mophology by locations, species and pairwise species comparisons and coresponding figure.

Mahalanobis distance (*D*) above diagonal, significance level (*P*) below diagonal				% mussels correctly classified	Wilks's λ	F(df)	*P*
Three species from all locations (Figure 3.10)							
	Gulf	Bahamian	Antillean				
Gulf	-	10.94	12.54	91.7			
Bahamian	<0.00001	-	8.82	97			
Antillean	0.00008	0.00129	-	83.3			
Overall				93.7	0.177	7.972	<0.00001
Bahamian and Gulf species from locations of coexistence (Figure 3.11)							
	Gulf	Bahamian					
Gulf	-	10.27		97			
Bahamian	<0.00001	-		91.7			
Overall				94.7	0.2855	17.52	<0.00001
Bahamian and Gulf species from locations of coexistence, external measurements only							
	Gulf	Bahamian					
Gulf	-	9.18		91.7			
Bahamian	<0.00001	-		93.9			
Overall				92.9	0.3089	22.78	<0.00001
Antillean and Bahamian species (Figure 3.12)							
	Antillean	Bahamian					
Antillean	-	10.3		100			
Bahamian	0.00002	-		83.3			
Overall				97.4	0.4271	11.4	<0.00001
Antillean and Gulf species (Figure 3.13)							
	Antillean	Gulf					
Antillean	-	16.887043		100			
Gulf	0.00009	-		100			
Overall				100	0.27	8.492	<0.00001
Three habitat locations (Figure 3.15)							
	M35	M56	M68				
M35	-	2.26	7.38	70			
M56	0.00843	-	7.47	65			
M68	<0.00001	<0.00001	-	90			
Overall				75	0.288	7.492	<0.00001

Figure 3.1

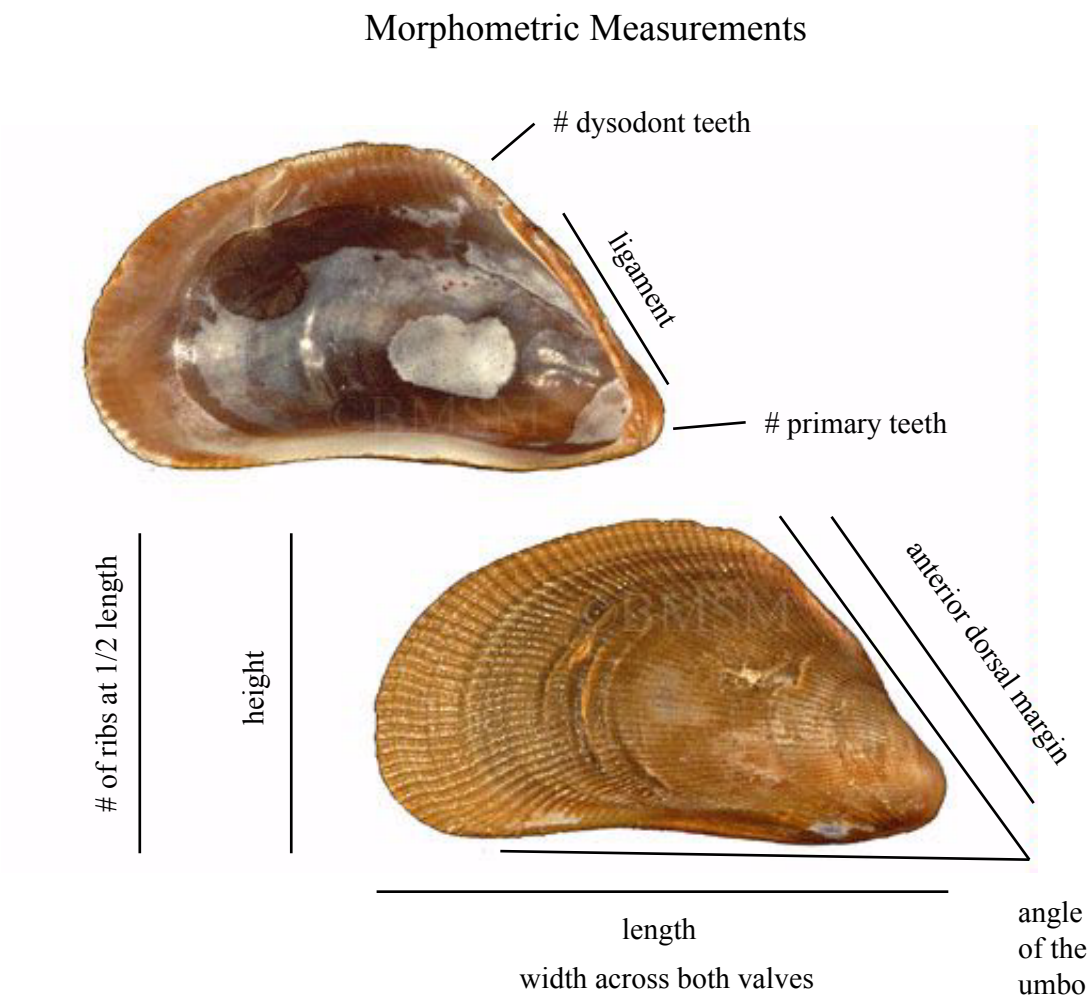


Figure 3.1 Disarticulated shells of sample specimen showing suite of measurements for each specimen. Photo of shell courtesy of Bailey-Matthews Shell Museum.

Figure 3.2

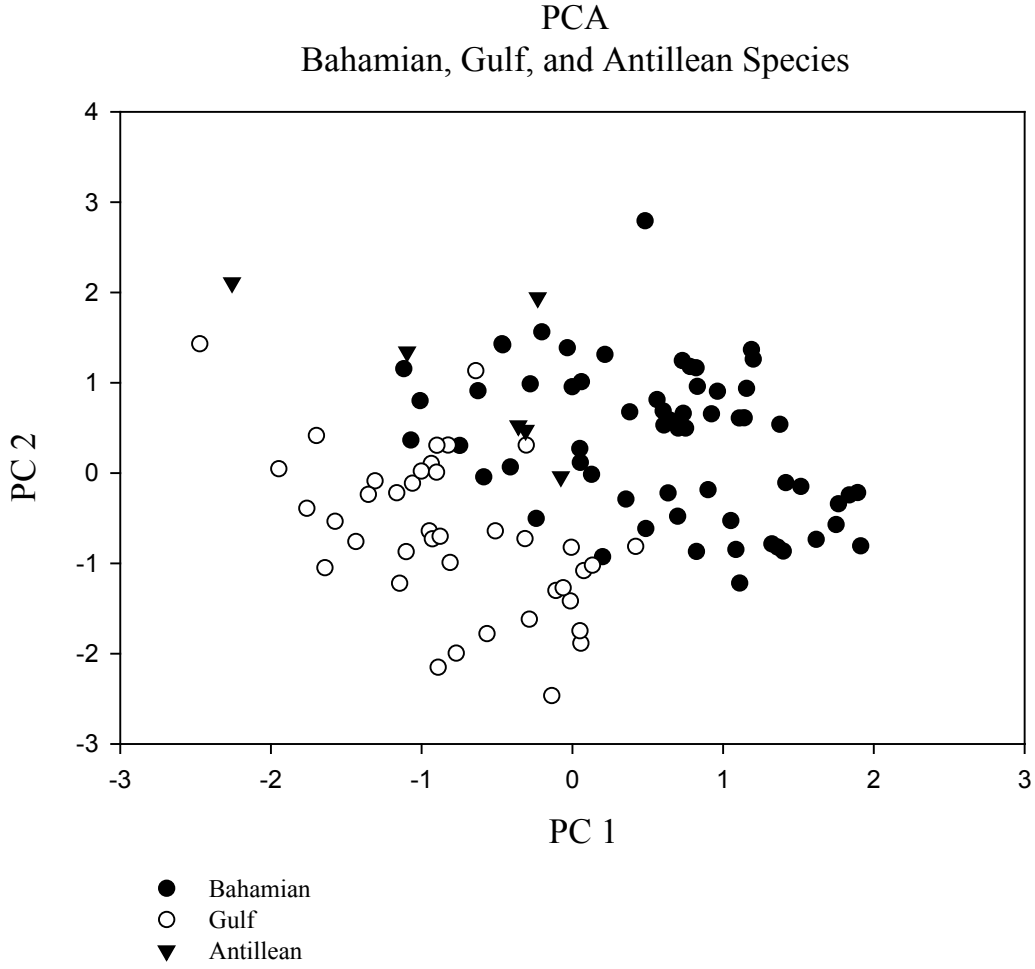


Figure 3.2 Principal components analysis of morphological measurements.

All Bahamian, Gulf, and Antillean specimens from all locations.

Figure 3.3

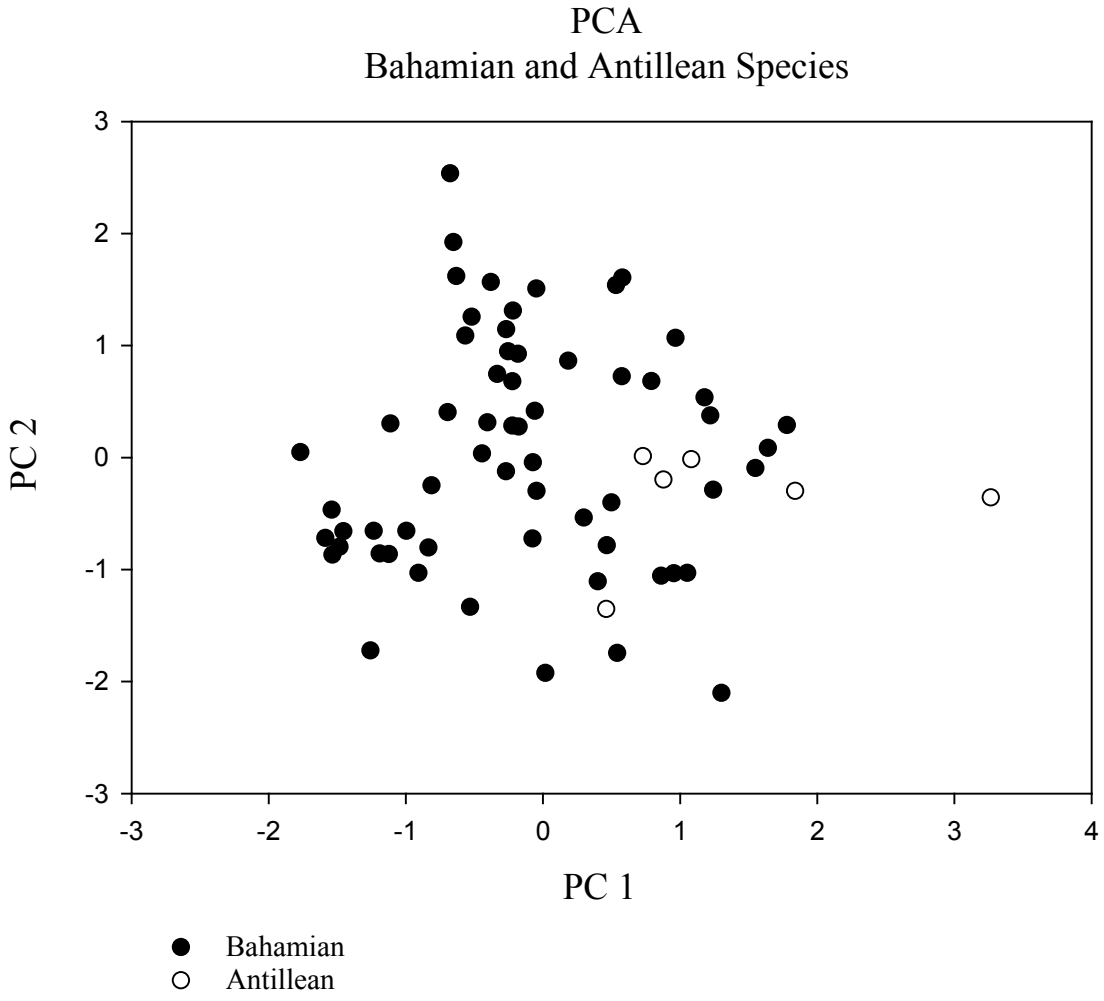


Figure 3.3 Principal components analysis of morphological measurements.

All Bahamian and Antillean species specimens from all locations.

Figure 3.4

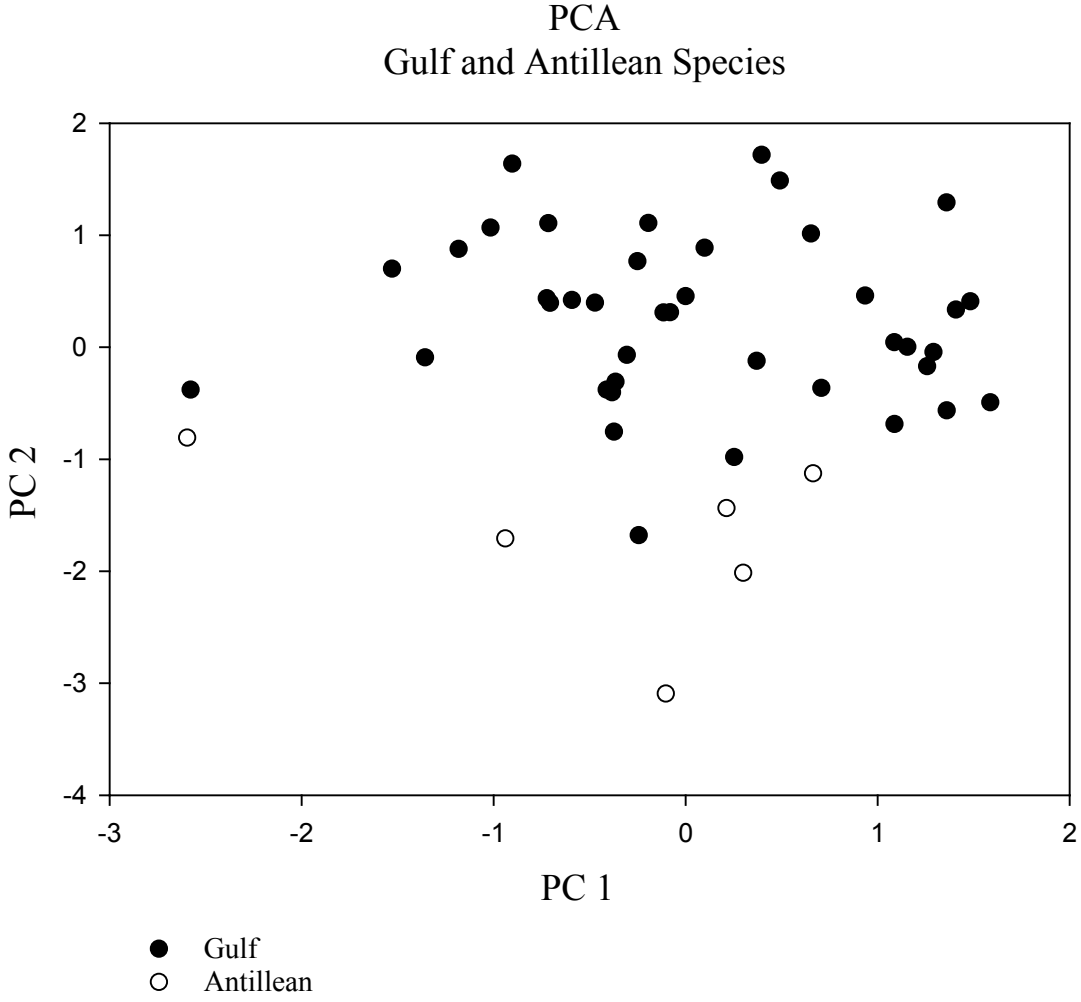


Figure 3.4 Principal components analysis of morphological measurements.

Gulf and Antillean species specimens from all locations

Figure 3.5

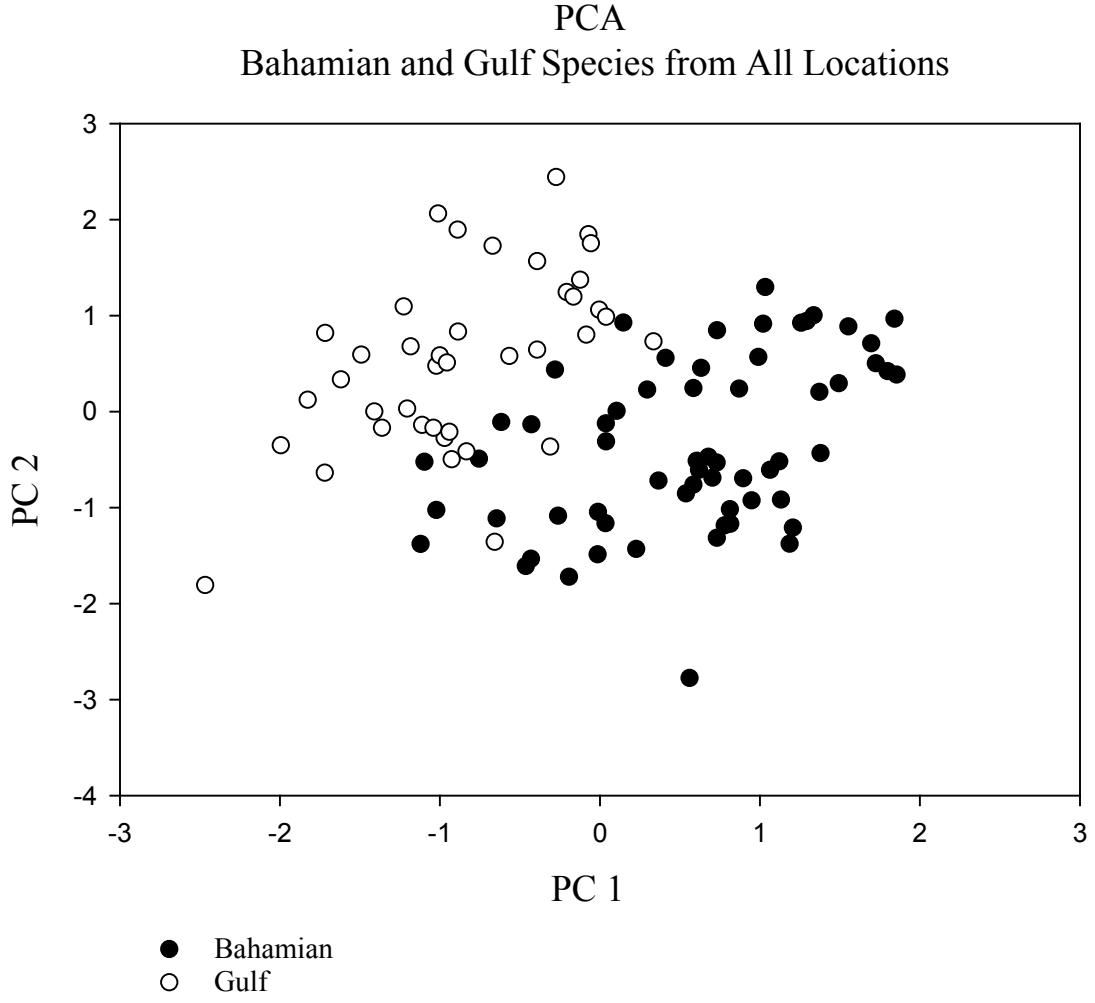


Figure 3.5 Principal components analysis of morphological measurements.

Bahamian and Gulf species specimens from all locations.

Figure 3.6 Principal components analysis of morphological measurements from Bahamian and Gulf species specimens from locations of coexistence. ●=Bahamian species and ○=Gulf species.

Figure 3.7 Principal components analysis of morphological measurements from Bahamian and Gulf species specimens from locations of exclusivity. ●=Bahamian species and ○=Gulf species.

Figure 3.8 Principal components analysis of morphological measurements from Bahamian species specimens from locations of coexistence and exclusivity. ●=locations of coexistence ○=locations of exclusivity.

Figure 3.9 Principal components analysis of morphological measurements from Gulf species specimens from locations of coexistence and exclusivity. ●=locations of coexistence and ○=locations of exclusivity.

Figure 3.6

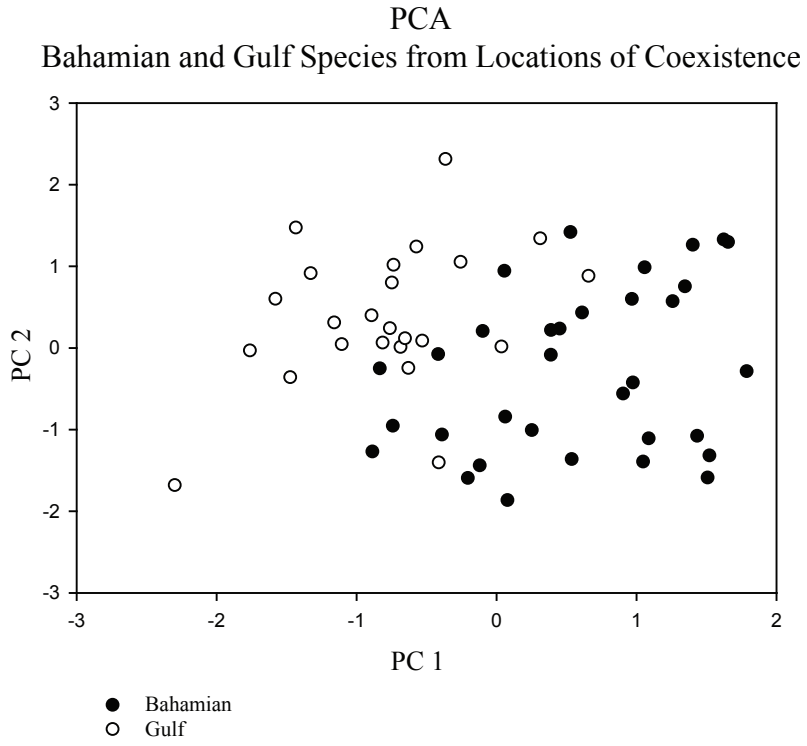


Figure 3.7

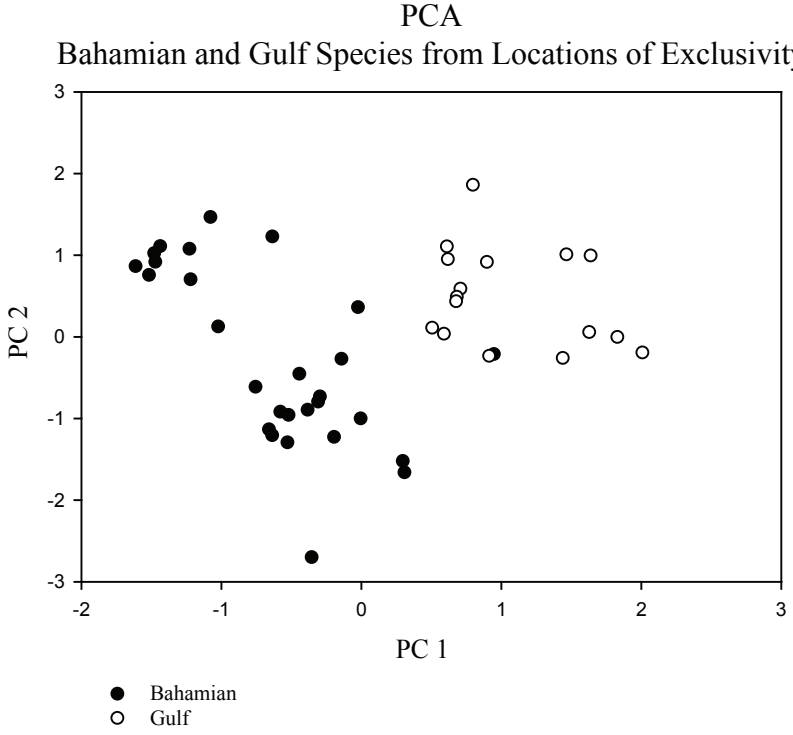
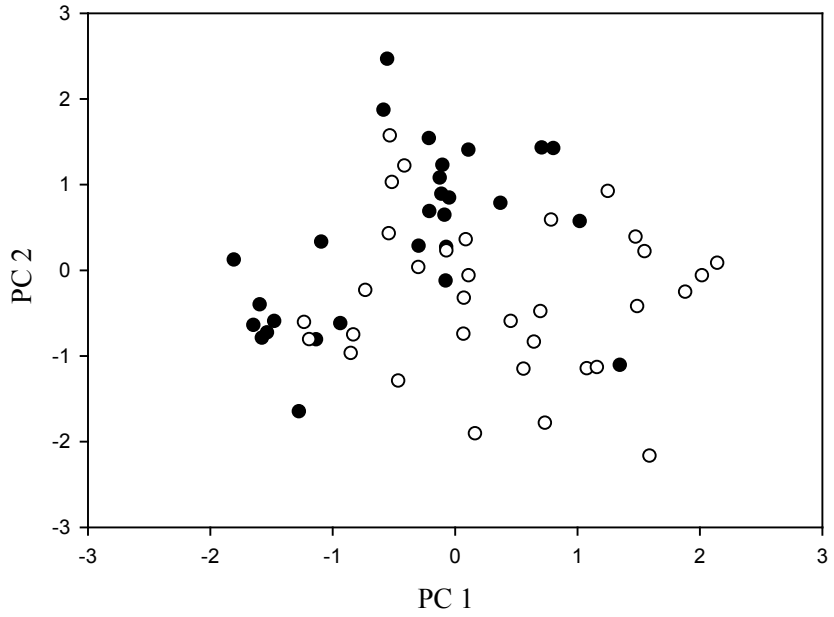


Figure 3.8

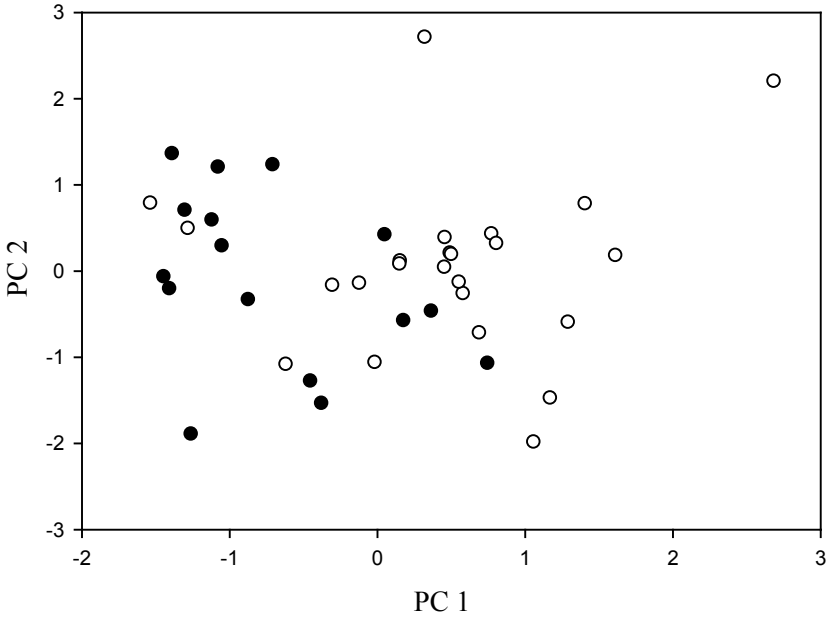
PCA
Bahamian Species from Locations of Coexistence and Exclusivity



● Bahamian species locations of coexistence
○ Bahamian species location of exclusivity

Figure 3.9

PCA
Gulf Species from Locations of Coexistence and Exclusivity



● Gulf species from locations of coexistence
○ Gulf species from locations of exclusivity

Figure 3.10

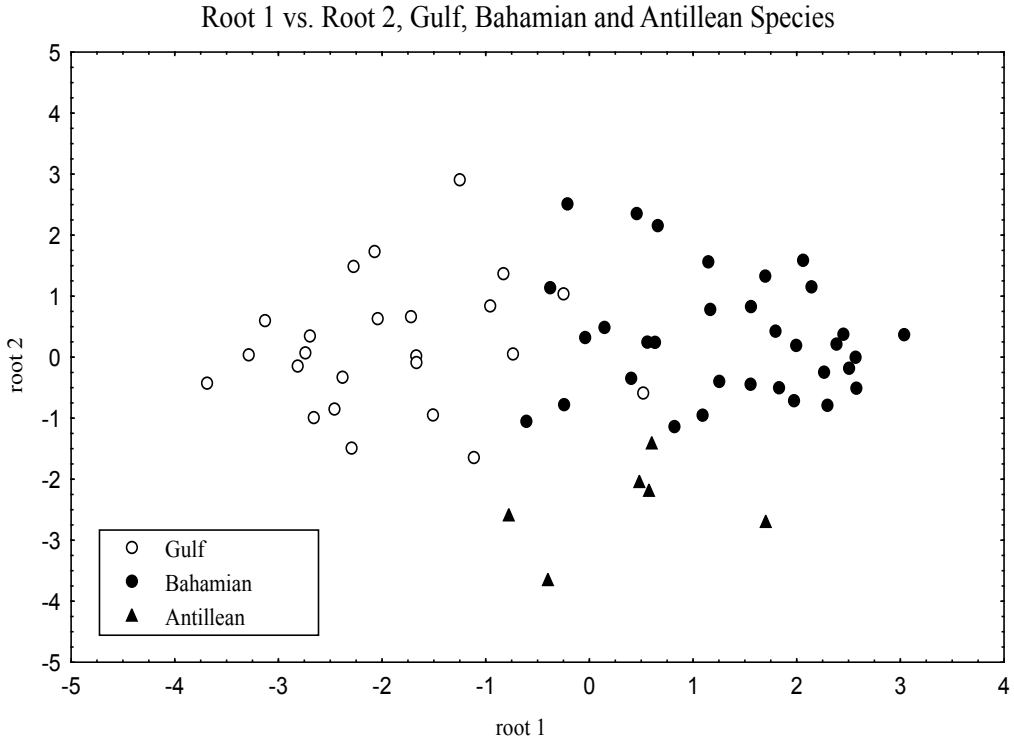


Figure 3.10 Canonical root 1 vs. root 2 from discriminant function analysis of shell morphology of specimens from three species.

Figure 3.11

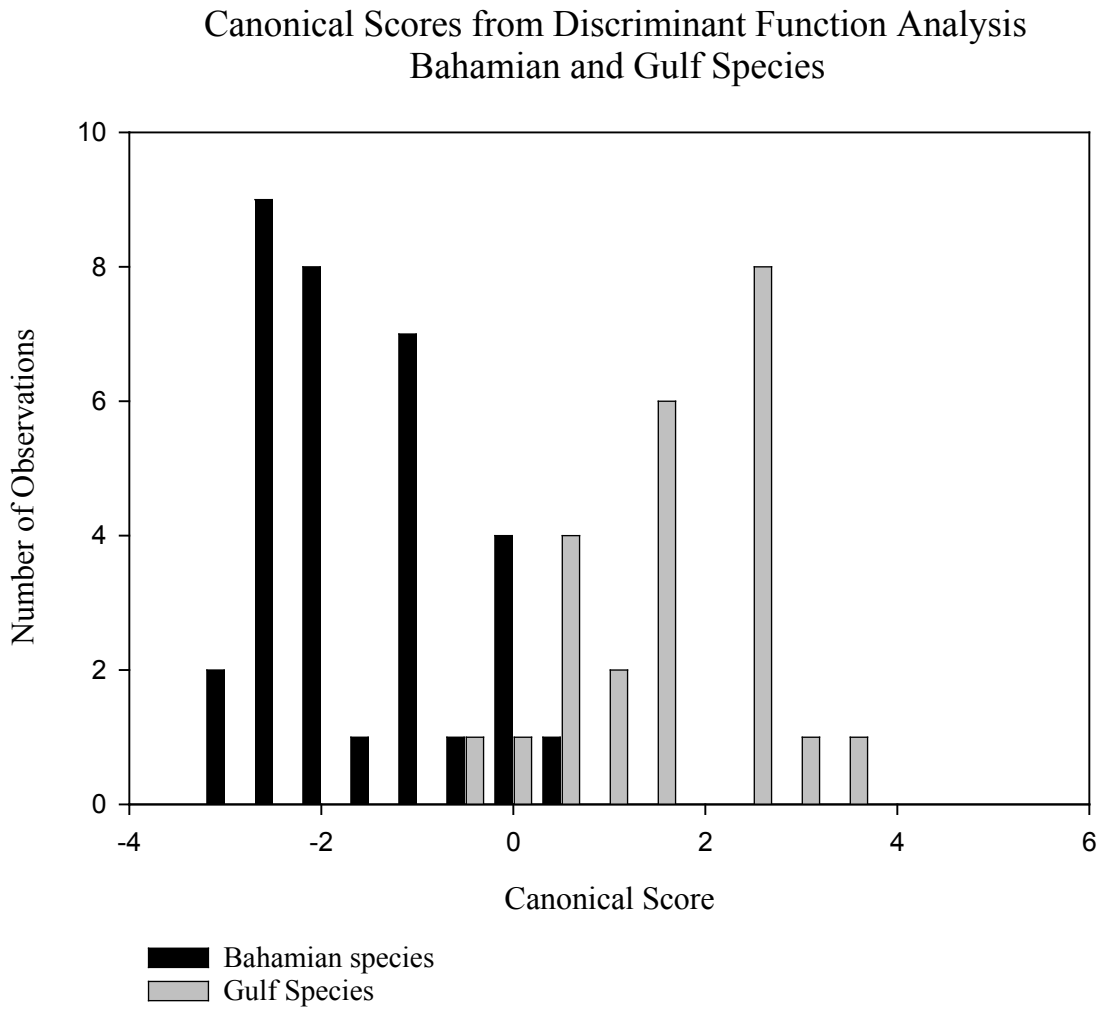


Figure 3.11 Histogram showing number of observations of canonical scores from discriminant function analysis for Bahamian and Gulf species. Black bars are Bahamian species scores and grey bars are Gulf species scores.

Figure 3.12

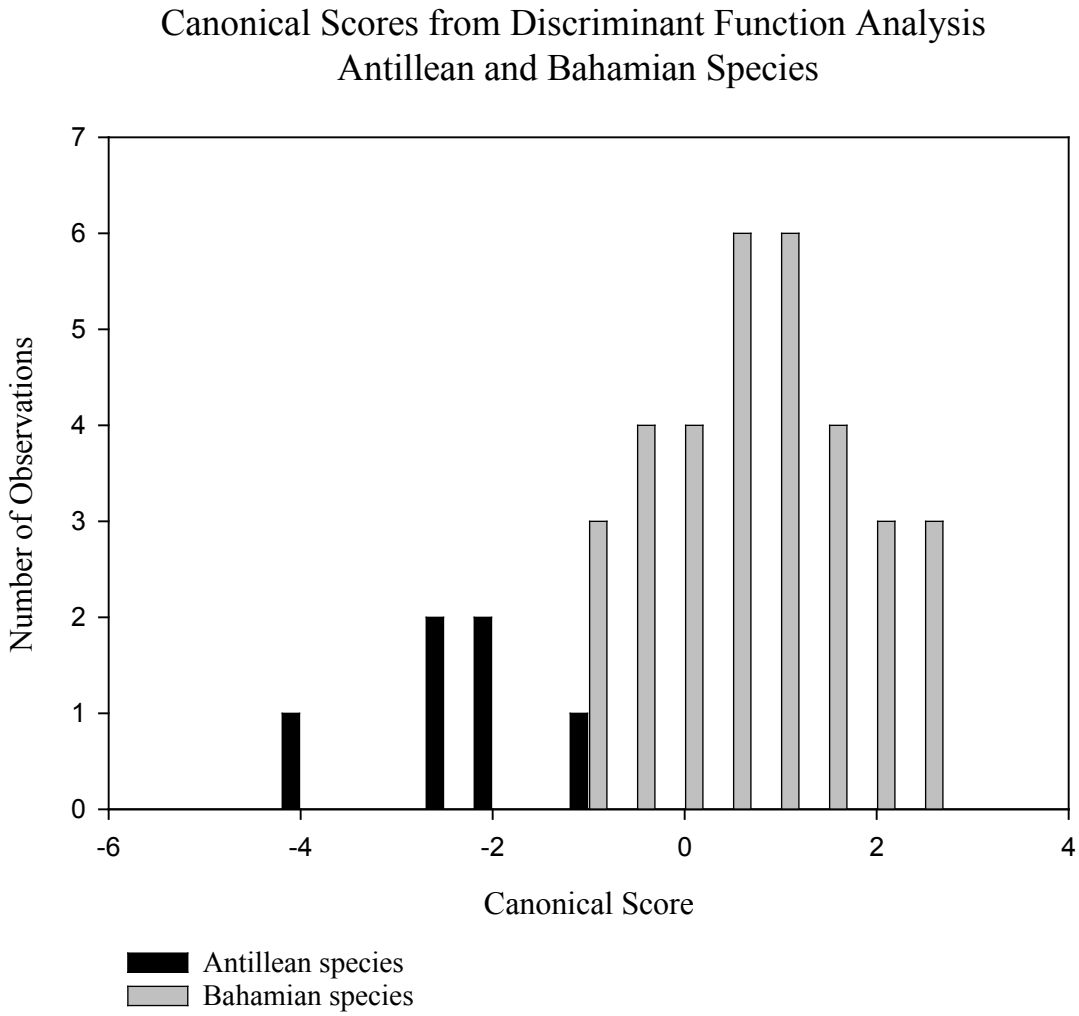


Figure 3.12 Histogram showing number of observations of canonical scores from discriminant function analysis for Antillean and Bahamian species. Black bars are Antillean species scores and grey bars are Bahamian species scores.

Figure 3.13

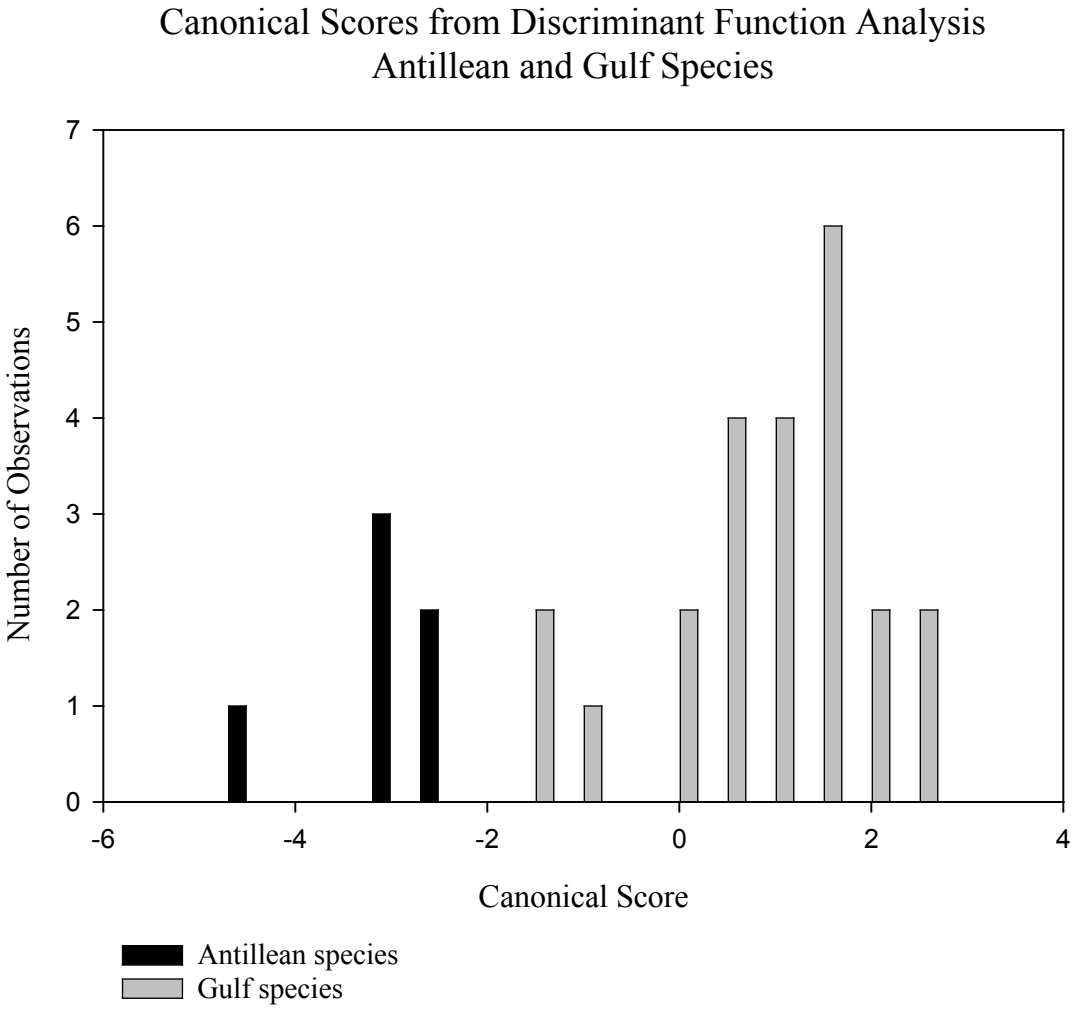


Figure 3.13 Histogram showing number of observations of canonical scores from the discriminant analysis for Antillean and Gulf species. Black bars are Antillean species scores and grey bars are Gulf species scores.

Figure 3.14

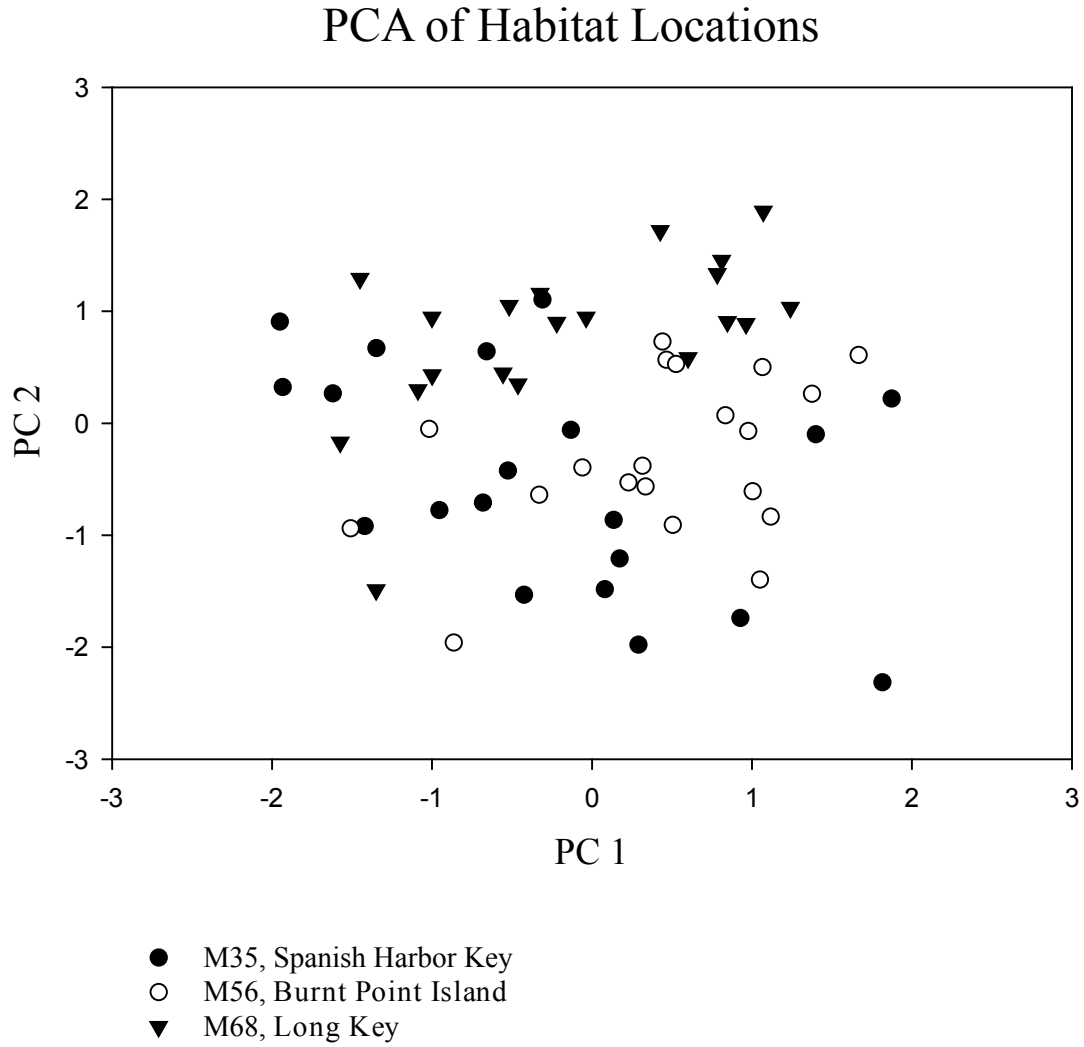


Figure 3.14 Principal components analysis of shell morphology measurements of specimens grouped by collection location.

Figure 3.15

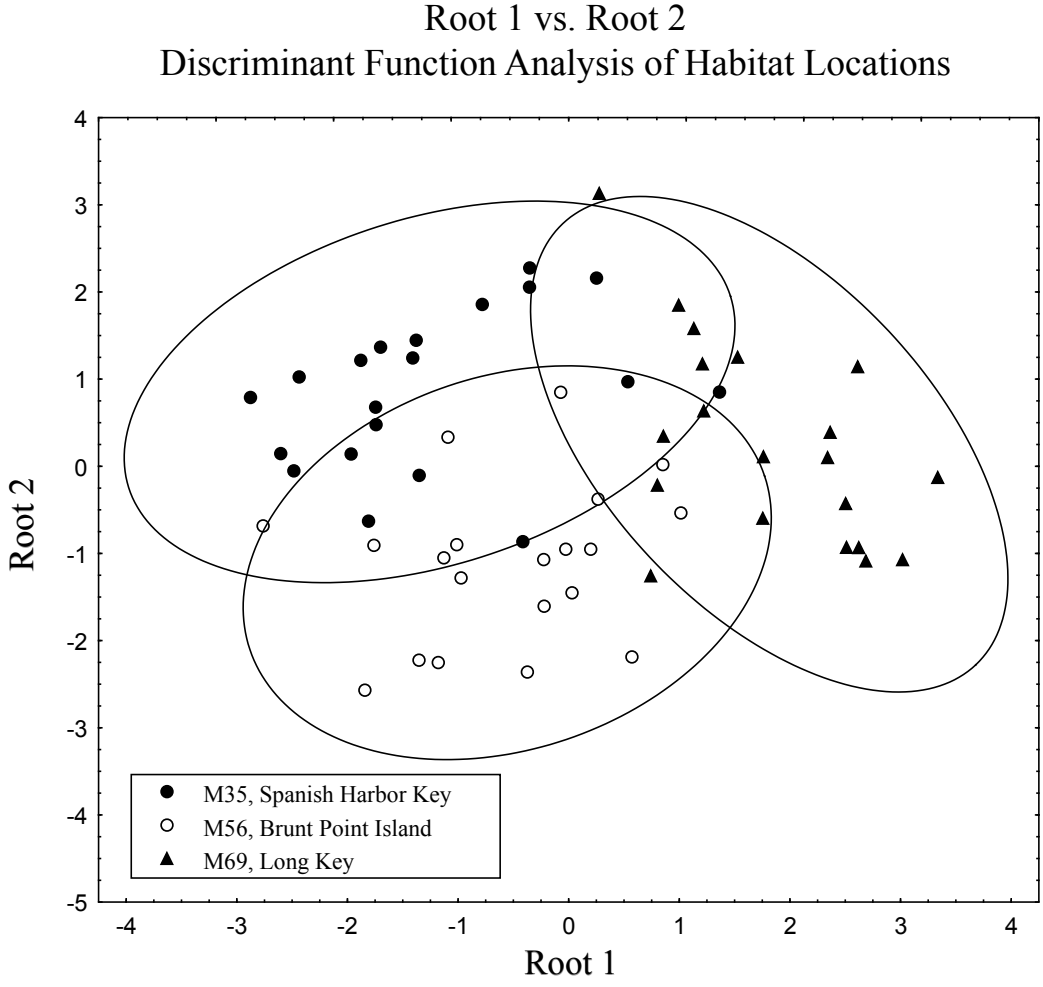


Figure 3.15 Canonical root 1 vs. root 2 from discriminant function analysis of shell morphology of specimens from three collection locations.

Chapter 4

Multiplex (PCR) Identification of Floridian Species in the *Brachidontes exustus* Complex

Abstract

A molecular protocol was developed for the identification of species within the scorched mussel, *Brachidontes exustus,* morphospecies complex. The 5′ end of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene was targeted in a multiplex polymerase chain reaction (PCR) with a set of forward oligonucleotides designed with 3′ terminal ends that each matched one target species with compatible single nucleotide polymorphisms (SNPs). When combined with a universal reverse primer, the set yielded diagnostic fragment lengths for each of the four potential species from southern Florida. The PCR products can be visualized directly under UV-light when run on an agarose gel stained with ethidium bromide, averting the need for secondary treatments like sequencing or restriction fragment length polymorphism (RFLP) analysis to determine species. This new method improves on previous techniques for species identification in the *B. exustus* species complex.

Introduction

The scorched mussel morphospecies, *Brachidontes exustus* (Linnaeus 1758), is a cryptic species complex with five generally allopatric species in the western Atlantic. In some locations, more than one species have been recorded, sometimes coexisting on the same substrate (Lee and Ó Foighil 2004; Lee and Ó Foighil 2005) (see Chapter 3). Other locations appear to have species-specific populations in close proximity (Lee and Ó Foighil 2005) (see Chapter 2). Identification of the species within the complex is hindered by the lack of species-specific morphological diagnostic characteristics. Multivariate morphometrics of shells for species discrimination in the Florida Keys, particularly for the two regionally-dominant species with core geographic ranges in the Gulf of Mexico and the islands of the Bahamas, have been developed and have a high probability of properly assigning specimens to the correct species. However, this multivariate method has not been tested on all combinations of species within the complex nor across the entire geographic range of each species. Additionally, phenotypic variability of mussels is well known (Seed 1968; Nalesso et al. 1992), and excluding the full range of morphological variation in the creation of the multivariate discriminatory methods could diminish the robustness of any morphology-based identification technique.

The difficulty of assigning species to specimens using morphological characteristics alone has left the taxonomy unresolved and currently all five species are collectively referred to as a single taxon, *B. exustus*. Multivariate comparisons of the morphology of large numbers of specimens over the entire geographic range and habitat variation for

each species of the complex would be required to elucidate the species-specific morphological differences needed for full species descriptions.

Ecological investigations of the species' abiotic tolerances, larval settlement events, and biotic interactions will require a reliable method of identifying individuals to species. DNA barcoding (the sequencing and cataloging of a standardized 648-bp long fragment of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene (Hebert et al. 2003), proved useful for this complex because the diversity of COI sequences has been well-documented and is available in the NCBI database (Lee 2000; Lee and Ó Foighil 2004). Unfortunately, the method is time consuming and not well-suited to screening large numbers of samples because a full sequence must be obtained to compare to the known database. The presence of heteroplasmy from doubly uniparental inheritance of sex-linked mitochondrial lineages (DUI) (Zouros et al. 1994; Hoeh et al. 1996; Mizi et al. 2005) can also compromise the ability of DNA barcoding to properly assign specimens to species. The full range of male COI lineages is not as well resolved as the maternal lineages (Lee and Ó Foighil 2004) and, depending on the tissue from which the DNA was extracted, male COI may either become the amplification product or interfere with the reading of peaks in the sequence chromatograms if both maternal and paternal COI products are amplified in equal concentrations.

Restriction fragment length polymorphism analysis (RFLP) techniques have been developed (see Chapter 3), but there are differing levels of genetic variation between the Gulf/Atlantic and Bahamian/Antillean sister clades. The current method requires a two-

step process where the sister clade is first determined by the length of the internal transcribed spacer region (ITS) and the resulting sibling-species pairs are further distinguished by species-specific restriction sites. The Bahamian/Antillean species are identified with a double digest of the ITS nuclear region while the Gulf/Atlantic species pair are determined by a single restriction of the mitochondrial COI gene. The COI-based RFLP approach is still subject to the problems of heteroplasmy if male mitochondrial material is present in the extracted DNA and amplified by PCR. The current method does not address the fifth species of the group, the Western Caribbean species, although being sister to the other two clades, ITS length alone, or the same endonuclease digest used for the Bahamian/Antillean sibling species pair may yield a diagnostic species-specific pattern.

PCR approaches to species identifications have been developed for many marine science applications including identifying species of parasites, bacteria, and disease vectors in ship ballast water (Aridgides et al. 2004; Darling and Blum 2007). In the Bivalvia, PCR-based approaches have been employed for identifying larvae from the plankton (Bell and Grassle 1998; Hare et al. 2000; Bendezu et al. 2005; Larsen et al. 2005; Patil et al. 2005), closely related species with variable morphology (Blair et al. 2006; Pie et al. 2006), and for confirmation of landings in regulated fisheries (Marshall et al. 2007). Generally these approaches identify regions of the target sequences where species-specific primer pairs can be designed so each target species will display species-specific banding patterns that can be visualized on an agarose gel after electrophoresis. Multiplex techniques facilitate species determinations based solely on the lengths of PCR products without the need for

the secondary steps of sequencing or RFLP.

Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variation in most organisms. In very closely related species, such as cryptic complexes and sibling pairs, SNPs may represent most of the inter-species genetic variation making them useful for genotyping. SNPs have been targeted to match the 3´ end of primers in PCR reactions that amplify select alleles and species-specific amplicons. Identifying SNPs is currently a major focus of human genetic research because of the connection of point mutations to human genetic diseases, and the basic technique used in this assay is often used in human genetic research.

The goal of this study was to develop an improved molecular method for determining species of the *B. exustus* complex. A suite of primers was designed and tested for multiplex PCR of the COI mitochondrial gene to generate species-specific fragment lengths that discriminate among all potential species of the *Brachidontes exustus* cryptic species complex in south Florida. The species designation is based on amplicon lengths that are compared to standards.

Materials and methods

Primer design

The cytochrome *c* oxidase I (COI) gene for this species complex has been well characterized. Specimens collected from the Caribbean, Gulf of Mexico, and western Atlantic Ocean, as well as trans-Panamanian congeners and allopatric generic relatives,

are included in phylogenetic studies by Lee and Ó Foighil (2004; 2005). All COI sequences from their study were deposited in GenBank (Accession # AY621835-AY621946, AY825105-AY825216), although under the older taxonomic name of *Hormomya exustus*.

Primers were designed to target SNPs that are conserved in single species. An SNP unique to the target species serves as the anchor for the 3' end of each primer. Generally, the 2nd through the 7th to 10th nucleotides of the primer from the 3' end align with the sequences of all five species of the complex but some designed primers had more pronounced sequence divergences with non-target species. In all cases the designed primer had a final nucleotide that was a mismatch with all non-target species sequences. The mismatch at the 3′ end limited the extension step of the PCR. At least one C or G was included in the final three nucleotides of the primers' 3′ ends to act as the C/G clamp. The three hydrogen bonds of the C/G nucleotide pair provided greater stability for the annealing of the primer and template than would be provided by the two hydrogen bonds of the A/T nucleotide pair (Beasley et al. 1999). Having a C or G at or near the 3′ end ensures extension. Rather than design a primer pair for each species, and to limit the potential for dimerization of additional primers in the reaction, the well known reverse primer HCO 2198 of Folmer et al. (1994) was employed as the sole primer in the reverse direction.

The design of several primers for testing led to a suite of primers from which the most effective amplification and clearest banding patterns could be selected. A design goal was

to identify one primer-annealing location per 100 nucleotides of the sequences of each species. Limiting the final primer location to the 450th nucleotide in each species ensured that the shortest possible fragment would be at least 250-bp long and capable of being visualized easily after electrophoresis. Five primers per species for each of the four species yielded 20 forward primers.

Sister-species pairs were initially treated separately, starting with the Bahamian/Antillean sibling species, the pair with the highest diversity of COI sequence haplotypes. Sequences were aligned in ClustalX (Chenna et al. 2003) and visually inspected for nucleotide substitutions consistent within, and limited to, each of the sibling species. The 85 Antillean and Bahamian species sequences were the most divergent of the sister-species groupings with both species having more than one tip cluster in their COI gene tree topology. The 54 Atlantic and Gulf species sequences were treated next. The Atlantic species had very few intraspecific nucleotide differences making nearly every nucleotide difference from the Gulf species a potential primer anchor site. In the full alignment of 139 sequences from the four species every nucleotide site that had been identified in the previous steps were compared by eye to the other three species. Only 3′ anchor sites with the nucleotide matching the target species, and mismatching all other species, were included for further consideration.

Each potential primer sequence was targeted to be around 25 nucleotides long, and the location of the 5′ end was chosen either by the presence of a run of 5 or more of a single nucleotide or if the oligonucleotide length reached 30 nucleotides. The entire suite of

potential primers was checked for melting temperature using the online tool of Integrated DNA Technologies (http://biotools.idtdna.com/analyzer/Applications/OligoAnalyzer). Only primers with melting temperatures in the 52.5° to 60° C range, or that could be altered by adding or removing nucleotides from the 5' end to come into this range and still remain longer than 18 nucleotides, were included. All potential primers that did not meet the above criteria were discarded from further consideration.

The *Brachidontes exustus* complex has doubly uniparental inheritance of sex-linked mitochondrial lineages where male germ cells are enhanced with a paternal line of mitochondrial DNA (Zouros et al. 1994; Hoeh et al. 1996; Mizi et al. 2005). Male COI in the PCR could cause misidentifications. The suite of primers was checked against all known male COI sequences to ensure that male COI would not be amplified. To confirm that the priming of male COI would be unlikely, the last 5 basepairs of the 3' end of each primer was checked against the 13 published Bahamian male COI sequences and the two known Antillean male COI sequences (see Chapter 2). None of the 5-bp long sequences corresponding to the 3′ ends of the designed primers had matches to the sequences of known male COI.

Lastly, the entire suite of potential primers was checked for hairpins and self-dimerization with the IDT online oligo analyzer. Primers with a hairpin ΔG value below -2.5 or a self-dimerization ΔG value below -8 were removed from consideration. Eight forward and one reverse priming oligonucleotides were manufactured by Integrated DNA Technologies using standard desalting purification. The eight forward primers targeted

one Bahamian, two Antillean, three Gulf, and two Atlantic species-specific sequences. The primers are named for the target species and location on the species' COI sequence that corresponds to the 3′ end of the primer. For example, GF199 is the primer that targets at its 3′ end the 199th nucleotide of the Gulf species COI sequence amplified by the Folmer et al. (1994) universal primer pair.

Primer testing

Each primer was tested with the HCO 2198 reverse primer of Folmer et al. (1994) with genomic DNA of specimens of known species for the expected amplicon length (see Chapters 2 and 3 for genomic DNA extraction protocols). For the amplification tests, a modified cycler protocol from the Folmer et al. (1994) protocol was used which consisted of an initial denaturing at 94° C for 5 min followed by 31 cycles of 94° C for 30 s, 52° C for 60 s, and 72° C for 60 s followed by a final 7 min extension at 72° C. The annealing temperature was raised from the 40° C used in the universal protocol to 52° C because the very low annealing temperature in the universal protocol is required to overcome mismatches between the primers and template DNA that may exist in the many metazoans that the primer pair is capable of amplifying. A low annealing temperature may contribute to mis-priming of the new primer set to locations other than the target COI mitochondrial region. An annealing temperature of 52° C was chosen for the primer pair tests because the suite of primers generally had melting temperatures of about 55° C. Annealing temperatures just below the melting temperature of the primers would have a high probability of priming the target sequences without mis-priming. An annealing temperature of 40° C has a higher potential of mis-priming at non-target locations. All reactions were performed in 25-µl volumes with Qiagen Mastermix Taq. The genomic

DNA was obtained from individuals that had previously been identified to species by either sequencing of COI or RFLP analysis (see Chapters 2 and 3). In the case of the Atlantic species, specimens collected from Foley Beach, South Carolina, a region presumed to be exclusively occupied by the Atlantic species, were used for confirmation of the primers' ability to amplify the Atlantic species. Products were run on a 1.8 % agarose gel stained with ethidium bromide and visualized with UV transillumination.

Each of the eight forward primers was paired with the HOC 2198 reverse primer and tested for amplification with the four species known from the Florida Keys. The cycler protocol was again modified for this group of tests with an initial denaturing at 94° C for 3 min followed by 31 cycles of 94° C for 30 s, 56° C for 30 s, and 72° C for 45 s, followed by a 5 min final extension at 72° C. The annealing temperature was raised to just above the average melting temperature of the primer suite to try to ensure that the primer pairs amplified only the target species. An annealing temperature of 56° C was chosen because most primers' melting temperatures were in the 55° C range and this temperature gave the best potential annealing temperature for the primer pair to amplify the target amplicon and not amplify any species with a mismatch at the primer's 3′ end. The annealing time was also shortened to further limit mis-priming.

A set of four primers was chosen based on the compatibility of the expected fragment lengths for easily discernable visualization and by the specificity revealed from the primer pair tests against the four species. The primer set was tested in a multi-factorial design of each primer pair by the four target species. Each primer test was repeated at a

gradient of annealing temperatures ranging, in 2º C increments, from 50º C to 62º C. The annealing temperature at which the target species was the only clearly visible band was noted.

Multiplex testing

A suite of primers was chosen based on the difference in amplicon size, compatible annealing temperatures, and species specificity revealed by the primer testing and tested in multiplex PCR. A primer mix that contained all four forward primers and the reverse primer was prepared. A series of multiplex reactions was performed over a temperature gradient in single degree increments from 52º C to 58º C. Reactions were performed in 25-µl volumes using Qiagen Mastermix Taq. Optimization was limited to changes in the annealing temperature, annealing time, and concentration of primers.

The suite of primers was adjusted several times to remove primers that amplified non-target species, caused secondary bands, or produced fragments that were not different enough in size to discern after electrophoresis. A final suite of five primers (four forward primers each targeting a single species and a single reverse primer for all species) was selected for determining species using the multiplex reaction.

Species identifications

Once the primer mix and cycler protocol was established, 109 individuals of known species affiliation were tested with the final primer mix. Genomic DNA was obtained from frozen, ethanol-preserved, or ethanol-preserved and subsequently dried material.

Each reaction was performed with extracted genomic DNA that had not been standardized for DNA concentration. Reactions were in total volumes of 25 µl and the product was run on a 3% agarose gel stained with ethidium bromide and visualized under UV transillumination.

Results

Individual primers

All eight of the designed forward primers, in conjunction with the universal reverse primer, amplified a fragment of the expected length in the target species for which the primer was designed. There were no secondary bands in any test of individual primer pairs with the target species. When each primer was tested against the four species over the temperature gradients, there was variation in the specificity of the primer pair. All eight primers amplified some product in the four species at an annealing temperature of 52º C. As the temperature was raised, the brightness of the bands across all non-target species generally diminished. There was generally some product visible, either as an indistinct band or as a smear. The primer pair GF199 and HOC 2198, amplified both Gulf and Atlantic species well at lower annealing temperatures.

Final protocol

The four forward primers with the best results in the multiplex reaction were GF54 for the Gulf species, AC387 for the Atlantic species, BH294 for the Bahamian species, and AN240 for the Antillean species (Figure 4.1). The final protocol, in a 25-µl-reaction volume, had 12.5 µl of 1x Taq PCR Mastermix (Qiagen, Valencia, California), 1 mM of each of the five primers, 1 µl of genomic DNA, and H_2O up to volume. The Taq

Mastermix, in the 25-µl-reaction volume, had a final concentration of 2.5 units Taq DNA Polymerase, 1x Qiagen PCR Buffer, 200 µM of each dNTP, and 1.5 mM $MgCl_2$. The optimum thermal cycler protocol with the best amplification results for the most diverse concentrations of starting DNA was an initial denaturing at 94º C for 5 min followed by 35 cycles of 94º C for 30 s, 54º C for 45 s, and 72º C for 1 min with a final extension at 72º C for 5 minutes. This protocol amplified PCR product from extracted DNA from sample materials stored in a variety of ways. DNA was extracted from specimens that were either frozen at -80° C, stored in ethanol, or ethanol-preserved and subsequently dried. Amplifications were successful without regard to starting DNA concentration or preservation method. The five primers used for species designations are shown in Table 4.1. The matches and mismatches to the template at the 3´ end of the primers are shown in Table 4.2.

Species designations

All 107 specimens typed by this new method are the same as had been determined by RFLP of the ITS or COI (Chapter 3) (Figures 4.2, 4.3, and 4.4). Two specimens collected from Foley Beach, South Carolina were identified as the Atlantic species by this method.

Discussion

Attempts at RFLP analysis of the internal transcribed spacer region (ITS) of the nuclear ribosomal gene cluster consistently revealed what appeared to be undigested product in the Gulf/Atlantic species pair. Later sequencing revealed that in specimens with undigested bands there were two peaks at the associated restriction site, representing two

different copies of the gene at the locus. Cloning into a bacterial plasmid incorporates only one copy of the gene and sequencing multiple transformed colonies of clones would confirm that these are alleles. In the published sequences of GenBank, only one of the two versions of the sequence is recorded hiding the heteroplasmy at this locus. The ITS of the Gulf/Atlantic species pair has few locations of nucleotide polymorphisms between the two species that could be used as restriction sites. The RFLP patterns obtained did not match the expected pattern for either species. The Bahamian and Antillean species pair, on the other hand, did have species-specific differences in the ITS that were targeted for RFLP analysis. The RFLP analysis gave consistent species-specific results. Gulf/Atlantic species discrimination by RFLP analysis of the ITS, however, after many attempts and unexpected failures, had to be abandoned. The mitochondrial genome does not have the problems of allelic differences that the nuclear genome has because the genetic material is present as a single strand with only one copy of each gene. The Gulf/Atlantic species had much less diversity in the COI sequences than the Bahamian/Antillean species pair. An RFLP analysis based on restriction sites in the COI did discriminate between the Gulf and Atlantic species. However, uncertainty remained because different banding patterns could result if male COI was amplified in the PCR.

The new multiplex PCR assay has many applications for elucidating differences in the larval ecology and recruitment of individual species within the complex. The timing of spawning for each cryptic species could be determined by monitoring larval concentrations in the plankton. Monitoring the species of recruits on settlement substrates deployed in different habitats could reveal if all habitats receive recruits of all species or

if there is habitat specificity in recruitment.

This new, simple, timesaving, and cost-effective method can screen a much larger number of specimens than sequencing or RFLP. Previous work on this species complex has a limited number of specimens from any single location (Lee and Ó Foighil 2004; Lee and Ó Foighil 2005). The collections from each location generally had a maximum of 16 specimens. Presumed single-species locations may be an artifact of the small sample size. Other species may be present, but rare. Screening large numbers of specimens with this new method has a stronger chance of finding rare species than the previously employed methods.

The *Brachidontes exustus* complex is a potential experimental model. Questions about the mechanisms by which closely related species with seemingly identical niches maintain coexistence, partition habitat, avoid hybridization, or otherwise mitigate direct competition can be addressed with this complex. The newly described method can be used for investigations of the ecology, morphology or other attributes of this intriguing species complex.

This method has general applicability to any group of closely-related species with sufficient genetic diversity in the COI from which to design species-specific primers. The diversity of haplotypes must be well characterized for species-consistent polymorphisms to be identified. The technique is not limited to the COI gene. Other mitochondrial genes, for example NAD4, cox I or 16s, have mutation rates that are somewhat lower than COI

and could also be the basis for a species-targeted primer scheme. The COI was chosen for this project because the variation was already well described and new sequences were not needed for the assay to be developed. Species with less well characterized genetic diversity compared to the *B. exustus* complex will require sequencing of a sufficient number of specimens from the total range to include most of the diversity of sequences in that gene. Additionally, the primers could be modified for use in real-time PCR applications that could speed the processing of identification further and could potentially be automated.

Table 4.1 All primers designed for this study showing target species, primer name, oligonucliotide sequence, and melting temperature for each primer. An asterisk is next to the name of each primer that was manufactured and tested.

Targert species	Primer name	Primer sequence	Melting temp, ° C
Bahamian			
	BH80	5′-ATTGATTCGGTTGCATTTAATACAC-3′	52.5
*	BH294	5′-TTTGCCTAGTGCGTTATACTTACTTTTG-3′	55.7
	BH429	5′-GCCGGGTCTGGGTCTTTG-3′	58.8
Antillean			
*	AN58	5′-GGGTTAATAGGAGTTGGCTATAGAATG-3′	54.9
*	AN240	5′-GGGCTATGGATTTAGCTTTTCCA-3′	55.2
	AN417	5′-TAGCTATTTTATCTCTTCACCTAGCT-3′	53.1
Gulf			
*	GF54	5′-TGGCGGGAGTTGGCTATAGA-3′	58.3
*	GF199	5′-TTACTTATTGGTGCTTTTGGTAACTGGC-3′	57.5
*	GF237	5′-TAATTGGTGCTA TAG ACCTTGCTTTC-3′	55.1
	GF381	5′-TGTAGAATACCATAGAAGCCCTGCG-3′	58.1
	GF423	5′-GCTATTTTATCACTTCATTTAGCAGGTTCTGGG-3′	59.1
Atlantic			
	AC78	5′-GGCTATAGTATATTGATTCGTTTGCATTTAATG-3′	55.2
	AC150	5′-GTA ATTGTGACTACTCATGCGTTAGTTATG-3′	55.8
*	AC292	5′-GGATTTTACCTAGTGCTTTTTATCTATTGC-3′	54
*	AC387	5′-AGTATCATAGAAGGCCTGCAATAGAC-3′	56.6
	AC450	5′-GGTTCTCTAATAGGTGCTATTAATTTCTTG-3′	54
Western Caribbean			
	WC67	5′-GGTGTTGGTTATAGAATGCTTATTCGTA-3′	55.1
	WC183	5′-CCGTTATGCCACTTCTTATTGGA-3′	55
	WC282	5′-CTGGATCTTGCCTAGAGCATTG-3′	55.5
	WC390	5′-GCAGCCCCGCTATAGACTTA-3′	56.4
	WC420	5′-TATCTCTCCATTTAGCGGGATCA-3′	55.1
Reverse primer			
*	HOC2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	55.3

Table 4.2 Forward primers BH294, AN240, GF54, and AC387 and the nucleotide match of the target species and the nucleotide mismatch at the homologous sequence location for the non-target species.

		Species			
Primer	3' end nucleotide	Bahamian	Antillean	Gulf	Atlantic
BH294	G	G	A	A	A
AN240	A	T	A	G	G
GF54	A	T	T	A	T
AC387	C	T	T	T	C

Figure 4.1

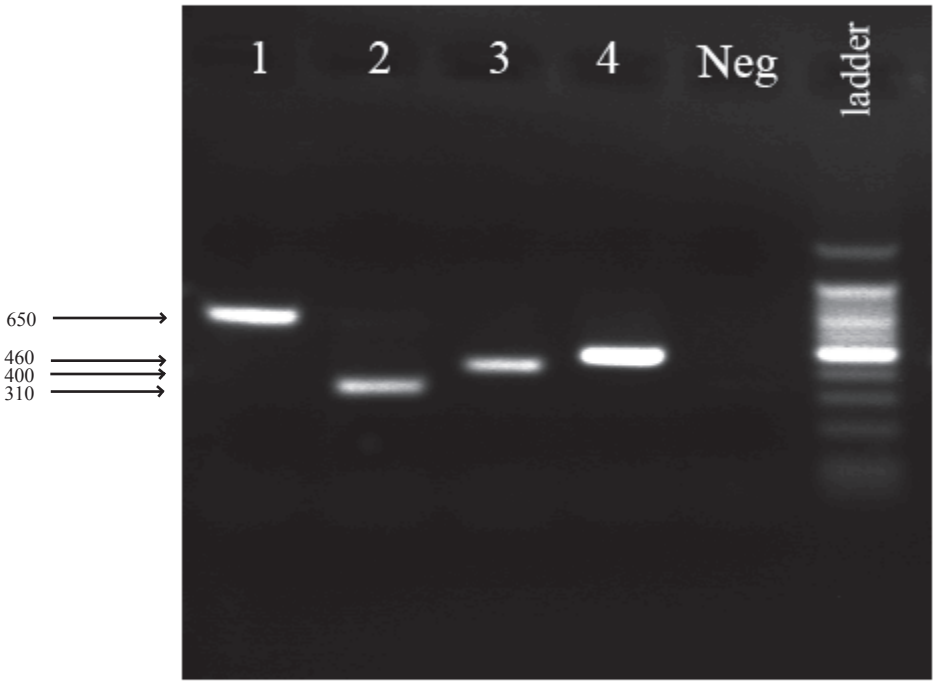


Figure 4.1 Multiplex of four forward and one reverse primers with specimens of known species run on a 2% agarose gel. Numbers correspond to wells. Each well contains multiplex PCR product with known species. Lanes are 1) Gulf, 2) Atlantic, 3) Bahamian, 4) Antillean, 5) negative control, and a 100-bp ladder. Arrows point to amplicon length from multiplex PCR. In all wells amplicon length expected for target species matches actual fragment length, noted by arrows on left side of gel.

Figure 4.2

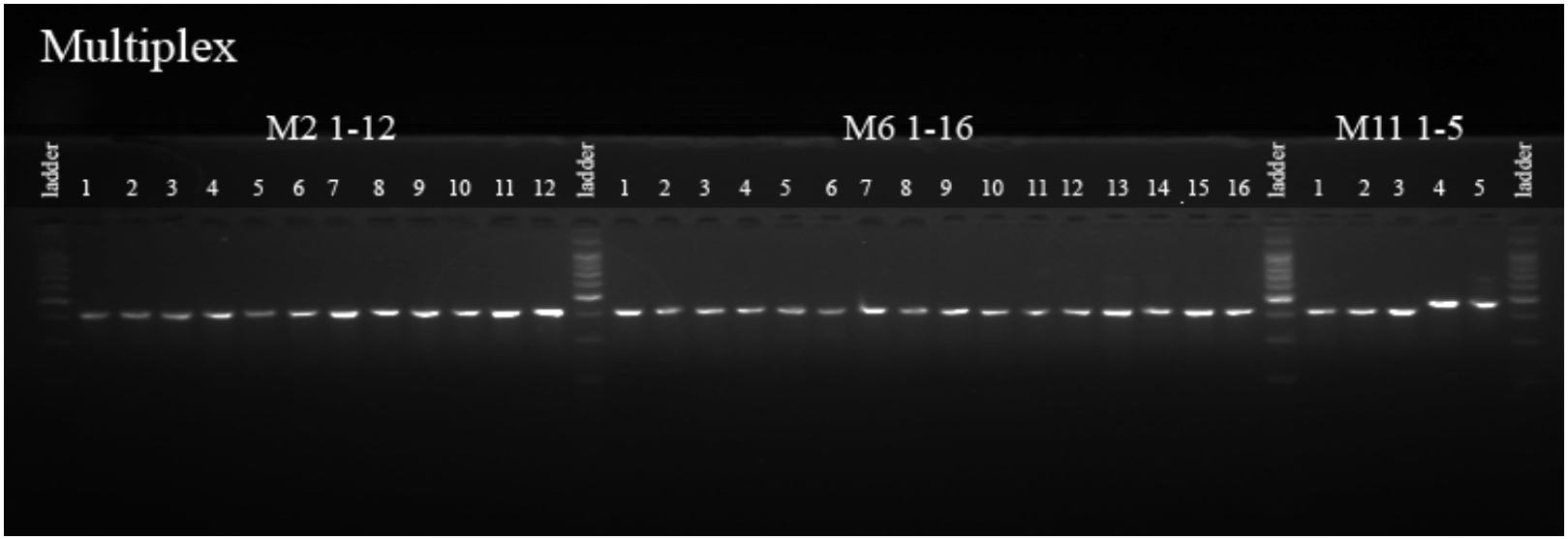


Figure 4.2 Results of multiplex PCR reaction run on a 3% agarose gel showing specimens 1-12 from M2, Key West, specimens1-16 from M6, Boca Chica Key, and specimens1-5 from M11, Shark Key. All specimens, except M11 4-5, are Bahamian species. M11 4-5 specimens are Antillean species.

Figure 4.3

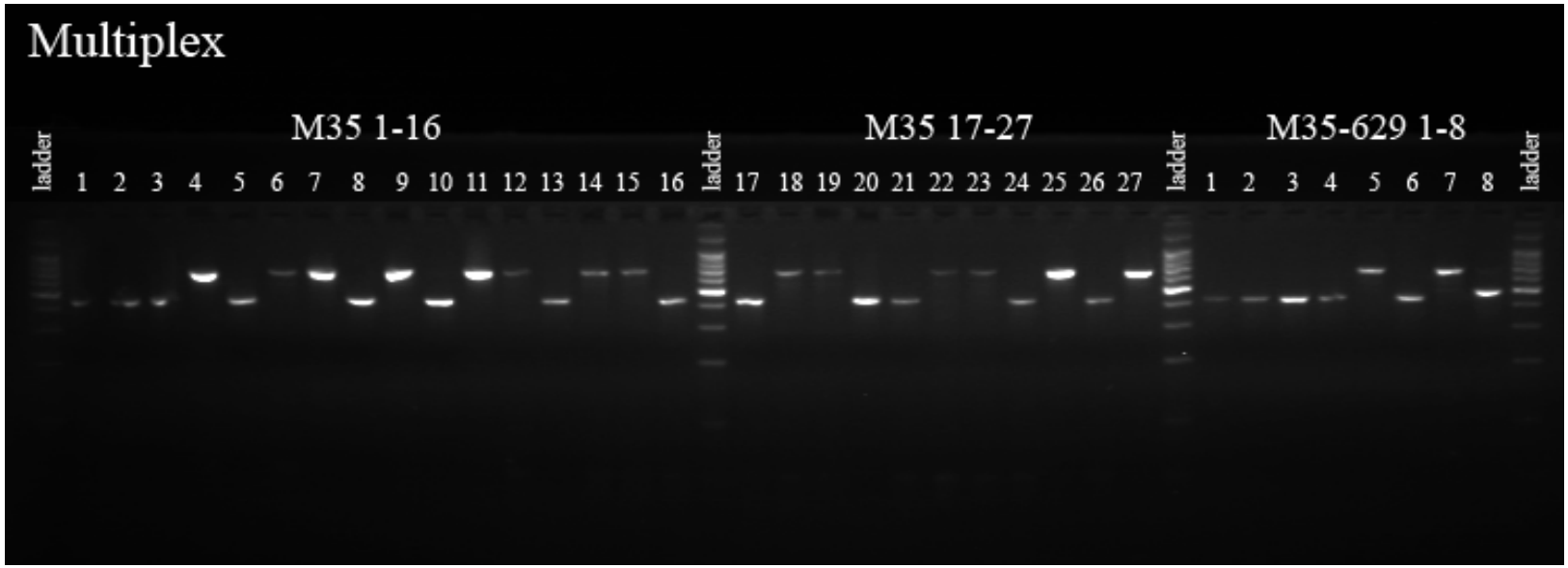


Figure 4.3 Results of multiplex PCR reaction run on a 3% agarose gel. Specimens 1-27 from M35, the Horseshoe site on Spanish Harbor/West Summerland Key and specimens 1-8 from the 2002 collection from the same location (M35-629). There is a mix of Gulf species specimens (bands toward top of the gel), and Bahamian species specimens (bands toward bottom of gel).

Figure, 4.4

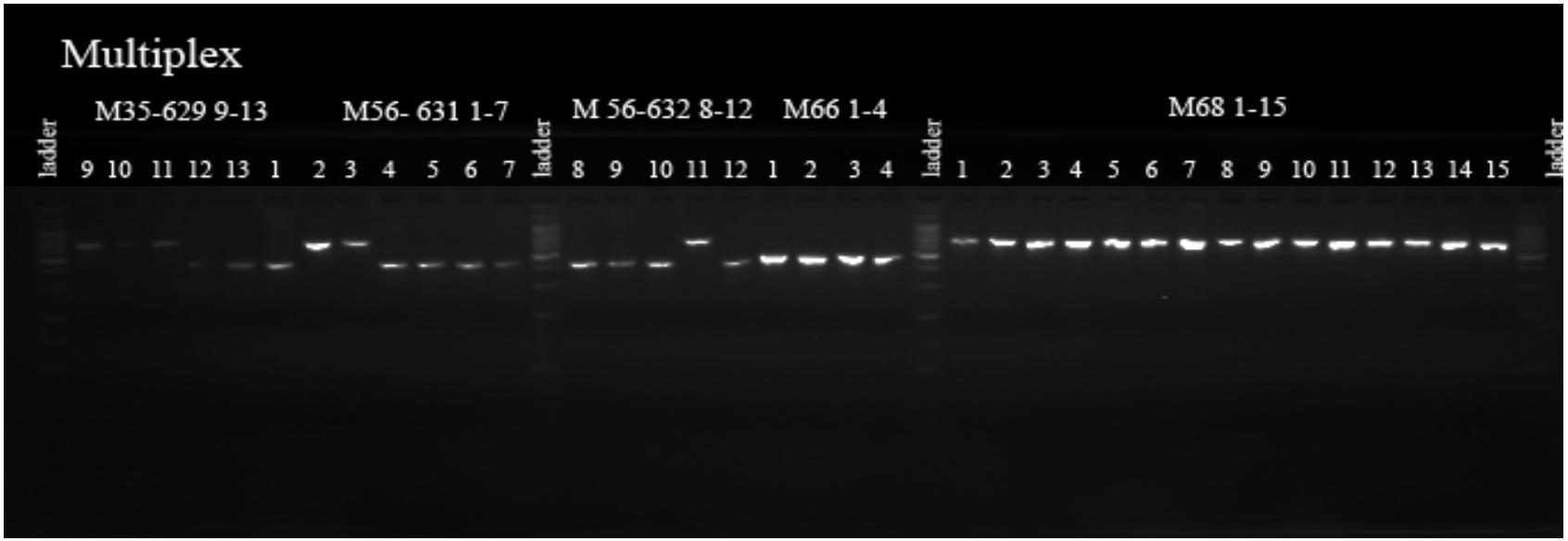


Figure 4.4 Results of multiplex PCR reaction run on a 3% agarose gel. Specimens 9-13 from the Horseshoe site on Spanish Harbor/West Summerland Key, M35-629, and specimens 1-12 from Burnt Point Island, M56-632, from 2002 collections. Specimens 1-4 from M66, seawall on Long Key, and specimens 1-15 from mangrove habitat, M66, Long Point Key. Specimens from the 2002 collections from M35 and M56 are mixed Gulf and Bahamian species individuals. All specimens from Long Key seawall, M66, are Antillean species. All specimens from Long Key mangrove habitat, M68, are Gulf species.

Appendix

All nucleotide sequences generated, Chapter 2. Cytochrome *c* oxidase subunit I (COI) nucleotide sequences for specimens M66-1, M66-3, M68-1, M68-2, M68-3, M68-4, M68-6, M68-8, and M68-11 were used in the construction of Figure 2.2.

M66-1 COI

ACTCTTTATTTAACTTTTGGTATGTGAAGAGGGTTAATAGGAGTTGGTTATAG
AATGTTAATCCGACTTCATTTGATACATCCAGGTAATTTTTTACTAAAATCAG
ATAGATTATATAATGTGATCGTTACTACTCATGCGCTAGTTATAATTTTTTTTG
CTGTGATACCTTTGCTTATTGGTGCGTTTGGTAATTGATTGATTCCTCTATTTT
TAGGGGCTATGGATTTAGCTTTTCCACGAATTAACAATTTTAGGTTTTGAATT
TTGCCCAGTGCTTTATATTTACTTTTATTGTCTGGTTATGTTGAAGAAGGAGTT
GGTACTGGGTGAACTATTTATCCTCCTTTATCTACTGTAGAATTTCATAGAAG
CCCTGCTATGGATTTAGCTATTTTATCTCTTCACCTAGCTGGTTCTGGTTCTTT
AATAGGTGCTATTAATTTTTTAACATCTAACAAAAATTTACCTGTAGATAAAA
TAAAAGGAGAGCGGTCTGTTCTTTATGTGTGGAGAATTACTGTTACAGCATTT
CTTTTACTGCTGTCTTTACCAGTTTTAGCCGGAGGAATTACTATACTGCTTTTT
GACCGTAATTTTAATACTACATTTTTTGATCCTATTGGGGGTGGAGATCCTGT
TCTATTTATGCATTTATTT

M66-2 COI (presumptive male lineage sequence)

NGCATTTCTTACCTTTGGATTGTGAGNGGTTTANTAGGTTTGAGGTATAGAAT
ACCCATTCGCCTACATCTGATACATCCTGGCAACTCCCTTTTACGTTCAGAAA
GACTGTATAATGTTGTTGTCACTACCCATGCGCTTGTTATAATTTTTTTTGCNG

TAATACCACTTCTTATTGGTGCTTTGGGTAATTGATTAATTCCTTTGTTTTTGG
GCGCTATGGATTTAGCGTTTCCGCGAATTAATAATTTTAGATTTTGAATTTTG
CCTAGCGCCCTTTATTTGTTACTATTATCTGGATATATTGAAGAAGGAGTTGG
AACTGGCTGAACAATTTATCCCCCATTGTCTACAATTGAATTTCACAGAAGCC
CAGCCATAGATTTAGCTATTTTATCTTTACATTTAGCTGGATCAGGCTCATTG
ATAGGCGCGANTAATTTTTTAGTTTCTAATAAAAATCTACCAGTTGATAAGAT
AAAAGGTGAGCGGTCCGTCTTGTATGTGTGAAGAATTACTGTGACAGCTTTTC
TTCTCTTAATATCTTTGCCTGTTTTAGCAGGTGGGATACGATNCTGCTAATGG
ANNNGAACNTTAACNCCACTTTCNTNGACCCCGGANGGCANGGGGGGAATC
CCGNTGTTAANTANGCNCCTGGNC

M66-2 ITS-2

ACATTGCGGCTTTGGGTCACTCCCGAAGCCACGCCTGTCTGAGGGTCGGTGA
AACATCAATCGCAAAAAGAAATTTTTGCGCCTTGGGTCGTCGCAGTGACTTTG
TCTCACTTCGTCGCCTTAAATGCAGACCGGTGTCTCCGACCGACTGTCTCTGT
GCGGAGGGACGCATGCCTGGAGAAATACTCTTTCTCGCGATTCCCTTTTTCCG
CAATGCTCTGGTAATGCAGGGACGGGTCGGTGGAGAAACCGTTACGGAGGGT
TTAGCACACAACCAATCTCATCTCCGACCTCAGATCAGACGAGAGTACCCGC
TGAATTTAAGCATATCACTAAGCGGAGGAAAAGAAACTAA

M66-3 COI

ACTCTTTATTTAACTTTTGGTATGTGAAGAGGGTTAATAGGAGTTGGTTATAG
AATGTTAATCCGACTTCATTTGATACATCCAGGTAATTTTTTACTAAAATCAG

ATAGATTATATAATGTGATCGTTACTACTCATGCGCTAGTTATAATTTTTTTTG
CTGTGATACCTTTGCTTATTGGTGCGTTTGGTAATTGATTGATTCCTTTATTTT
TAGGGGCTATGGATTTAGCTTTTCCACGAATTAACAATTTTAGGTTTTGAATT
TTGCCCAGTGCTTTATATTTACTTTTATTGTCTGGTTATGTTGAAGAAGGAGTT
GGTACTGGGTGAACTATTTATCCTCCTTTATCTACTGTAGAATTTCATAGAAG
CCCTGCTATGGATTTAGCTATTTTATCTCTTCACCTAGCTGGTTCTGGTTCTTT
AATAGGTGCTATTAATTTTTTAACATCTAACAAAAATTTACCTGTAGATAAAA
TAAAAGGAGAGCGGTCTGTTCTTTATGTGTGGAGAATTACTGTTACAGCATTT
CTTTTACTGCTGTCTTTACCAGTTTTAGCCGGAGGAATTACTATACTGCTTTTT
GACCGTAATTTTAATACAACATTTTTTGATCCTATTGGGGGTGGAGATCCTGT
TCTATTTATGCATTTATTT

M66-4 COI (presumptive male lineage sequence)

GTACGCTTTACTTAACGTTTGGACTGTGAAGTGGTTTAGTAGGTTTGAGGTAT
AGAATACTCATTCGCCTACATCTGATACATCCTGGCAACTCCCTTTTACGTTC
AGAAAGATTGTATAATGTTGTTGTCACTACCCATGCGCTTGTTATAATTTTTTT
TGCTGTAATACCACTTCTTATTGGTGCTTTTGGTAATTGATTAATTCCTTTGTT
TTTGGGCGCTATGGATTTAGCATTTCCACGAATTAATAATTTTAGATTTTGAA
TTTTGCCTAGCGCCCTTTATTTGTTACTATTATCTGGATATATTGAAGAAGGA
GTTGGAACTGGTTGAACAATTTATCCCCCATTGTCTACAATTGAATTTCACAG
AAGCCCAGCCATAGATTTAGCTATTTTATCCTTACATTTAGCTGGATCAGGCT
CATTGATAGGCGCGATTAATTTTTTAGTTTCTAATAAAAATCTACCAGTTGAT
AAGATAAAAGGCGAGCGGTCTGTCTTGTATGTGTGAAGAATTACTGTGACAG

CTTTTCTTCTCTTAATATCTTTGCCTGTTTTAGCAGGTGGGATTACTATACTGT
TATTTGATCGTAACTTTAACACCACTTTTTTTGACCTGTTGGAGGTGGTGATCC
GGTTTATTAAGGCCCCTGGTC

M66-4 ITS-2

ACATCGATATCTTGAACGCACATTGCGGCTTTGGGTCACTCCCGAAGCCACG
CCTGTCTGAGGGTCGGTGAAACATCAATCGCAAAAAGAATTTTTTGCGCCTTG
GGTCGTCGCAGTGACTTTGTCTCACTTCGTCGCCTTAAATGCAGACCGGTGTC
TCCGACCGACTGTCTCTGTGCGGAGGGACGCATGCCTGGAGAAATACTCTTTC
TCGCGNTTCCCTTTTTCCGCAATGCTCTGGTAATGCAGGGACGGGTCGGTGGA
GAAACCGTTACGGAGGGTTTAGCACACAACCAATCTCATCTCCGACCTCAGA
TCAGACGAGAGTACCCGCTGAATTTAAGCATATCACTAA

M68-1 COI

ACACTTTATTTAGTTTTTGGTATTTGAAGCGGTTTGGCGGGAGTTGGCTATAG
AATATTAATTCGTTTGCATTTGATACATCCTGGTAATTTCTTGTTGAAATCAG
ATAGTCTATACAACGTGATTGTGACTACACATGCTTTAGTTATAATTTTTTTCG
CGGTAATGCCTTTACTTATTGGTGCTTTTGGTAACTGGCTTATTCCTTTAATAA
TTGGTGCTATAGACCTTGCTTTCCCGCGTGTTAATAATTTTAGTTTTTGAATTC
TGCCTAGTGCCTTTTATTTATTATTATTATCAGGTTATGTGGAAGAAGGGGTA
GGGACTGGTTGGACTATTTATCCTCCTTTATCTACTGTAGAATATCATAGAAG
CCCTGCGATGGATCTTGCTATTTTATCACTTCATTTAGCAGGTTCTGGGTCTTT
AATAGGCGCTATTAATTTCTTAACCTCTAATAAAAATCTTCCTGTTAATAAAA

TAAAGGGAGAGCGATCTGTCCTGTATGTATGAAGAATCACGGTTACAGCCTT
CTTGTTGCTGTTATCTTTACCGGTTTTAGCAGGAGCCATCACTATACTGTTGTT
TGATCGTAATTTTAATACCACATTTTACGATCCAATCCAAGGAGGAGATCCTG
TGTTATTTATACATCTTTTT

M68-2 COI

ACACTTTATTTAGTTTTTGGTATTTGAAGCGGTTTGGCGGGAGTCGGCTATAG
AATATTAATTCGTTTGCATTTGATACATCCTGGTAATTTCTTGTTGAAATCAG
ATAGTCTATACAACGTGATTGTGACTACACATGCTTTAGTTATAATTTTTTTCG
CGGTAATGCCTTTACTTATTGGTGCTTTTGGTAACTGGCTTATTCCTTTAATAA
TTGGTGCTATAGACCTTGCTTTCCCGCGTGTTAATAATTTTAGTTTCTGAATTC
TGCCTAGTGCCTTTTATTTATTATTATTATCAGGTTATGTGGAAGAAGGAGTG
GGGACTGGTTGGACTATTTATCCTCCTTTATCTACTGTAGAATACCATAGAAG
CCCTGCGATGGATCTTGCTATTTTATCACTTCATTTAGCAGGTTCTGGGTCTTT
AATAGGCGCTATTAATTTCCTAACCTCTAATAAAAATCTCCCTGTTAATAAAA
TAAAGGGAGAGCGATCTGTCCTGTATGTATGAAGAATCACGGTTACAGCCTT
TTTGTTGCTGTTATCTTTACCGGTTTTAGCAGGAGCCATCACTATACTGGTGGT
TGATCGTAATTTTAATACCACATTTTATGACCCAACCGGAGGAGGAGATCCTG
TGTTATTTATACATCTTTTT

M68-3 COI

ACACTTTATTTAGTTTTTGGTATTTGAAGCGGTTTGGCGGGAGTCGGCTATAG
AATATTAATTCGTTTGCATTTGATACATCCTGGTAATTTCTTGTTGAAATCAG

ATAGTCTATACAACGTGATTGTGACTACACATGCTTTAGTTATAATTTTTTTCG
CGGTAATGCCTTTACTTATTGGTGCTTTTGGTAACTGGCTTATTCCTTTAATAA
TTGGTGCTATAGACCTTGCTTTCCCGCGTGTTAATAATTTTAGTTTCTGAATTC
TGCCTAGTGCCTTTTATTTATTATTATTATCAGGTTATGTGGAAGAAGGAGTG
GGGACTGGTTGGACTATTTATCCTCCTTTATCTACTGTAGAATACCATAGAAG
CCCTGCGATGGATCTTGCTATTTTATCACTTCATTTAGCAGGTTCTGGGTCTTT
AATAGGCGCTATTAATTTCCTAACCTCTAATAAAAATCTCCCTGTTAATAAAA
TAAAGGGAGAGCGATCTGTCCTGTATGTATGAAGAATCACGGTTACAGCCTT
TTTGTTGCTGTTATCTTTACCGGTTTTAGCAGGAGCCATCACTATACTGTTGTT
TGATCGTAATTTTAATACCACATTTTATGATCCAATCGGAGGAGGAGATCCTG
TGTTATTTATACATCTTTTT

M68-4 COI

ACACTTTATTTAGTTTTTGGTATTTGAAGCGGTTTGGCAGGAGTTGGCTATAG
AATATTAATTCGTTTGCATTTGATACATCCTGGTAATTTCTTGTTGAAATCAG
ATAGTCTATACAACGTGATTGTGACTACACATGCTTTAGTTATAATTTTTTTCG
CGGTAATGCCTTTACTTATTGGTGCTTTTGGTAACTGGCTTATTCCTTTAATAA
TTGGTGCTATAGACCTTGCTTTCCCGCGTGTTAATAATTTTAGTTTCTGAATTC
TGCCTAGTGCCTTTTATTTATTATTATTATCAGGTTATGTGGAAGAAGGAGTG
GGGACTGGTTGGACTATTTATCCTCCTTTATCTACTGTAGAATACCATAGAAG
CCCTGCGATGGATCTTGCTATTTTATCACTTCATTTAGCAGGTTCTGGGTCTTT
AATAGGCGCTATTAATTTCTTAACCTCTAATAAAAATCTCCCTGTTAATAAAA
TAAAGGGAGAGCGATCTGTCCTGTATGTATGAAGAATCACGGTTACAGCCTT

TTTGTTGCTGTTATCTTTACCGGTTTTAGCAGGAGCCATCACTATACTGTTGTT
TGATCGTAATTTTAATACCACATTTTATGACCCAATCGGAGGAGGAGATCCTG
TGTTATTTATACATCTTTTT

M68-5 COI

ACACTTTATTTAGTTTTTGGTATTTGAAGCGGTTNGGCGGGAGTTGGCTATAG
AATATTAATTCGTTTGCATTTGATACATCCTGGTAATTTCTTGTTGAAATCAG
ATAGTCTATACAACGTGATTGTGACTACACATGCTTTAGTTATAATTTTTTTCG
CGGTAATGCCTTTACTTATTGGTGCTTTTGGTAACTGGCTTATTCCTTTAATAA
TTGGTGCTATAGACCTTGCTTTCCCGCGTGTTAATAATTTTAGATTCTGAATTC
TGCCTAGTGCCTTTTATTTATTATTATTATCAGGTTATGTGGAAGAAGGAGTG
GGGACTGGTTGGACTATTTATCCTCCTTTATCTACTGTAGAATACCATAGAAG
CCCTGCGATGGATCTTGCTATTTTATCACTTCATTTAGCAGGTTCTGGGTCTTT
AATAGGCGCTATTAATTTCCTAACCTCTAATAAAAATCTCCCTGTTAATAAAA
TAAAGGGAGAGCGATCTGTCCTGTATGTATGAAGAATCACGGTTACAGCCTT
TTTGTTGCTGTTATCTTTACCGGTTTTAGCAGGAGCCATCACTATACTGTTGTT
TGATCGTAATTTTAATACCACATTTTATGATCCAATCGGAGGAGGAGATCCTG
GGGTTATTTATACATCTTTTT

M68-6 COI

ACACTTTATTTAGTTTTTGGTATTTGAAGCGGTTTGGCGGGAGTTGGCTATAG
AATATTAATTCGTTTGCATTTGATACATCCTGGTAATTTCTTGTTGAAATCAG
ATAGTCTATACAACGTGATTGTGACTACACATGCTTTAGTTATAATTTTTTTCG

CGGTAATGCCTTTACTTATTGGTGCTTTTGGTAACTGGCTTATTCCTTTAATAA
TTGGTGCTATAGACCTTGCTTTCCCGCGTGTTAATAATTTTAGTTTTTGAATTC
TGCCTAGTGCCTTTTATTTATTATTATTATCAGGTTATGTGGAAGAAGGAGTA
GGGACTGGTTGGACTATTTATCCTCCTTTATCTACTGTAGAATACCATAGAAG
CCCTGCGATGGATCTTGCTATTTTATCACTTCATTTAGCAGGTTCTGGGTCTTT
AATAGGCGCTATTAATTTTTTAACCTCTAATAAAAATCTCCCTGTTAATAAAA
TAAAGGGAGAGCGATCTGTCCTGTATGTATGAAGAATCACGGTTACAGCCTT
CTTGTTGCTGTTATCTTTACCGGTTTTAGCAGGAGCCATCACTATACTGTTGTT
TGATCGTAATTTTAATACCACATTTTACGATCCAATCGGAGGAGGAGATCCTG
TGTTATTTATACATCTTTTT

M68-8 COI

ACACTTTATTTAGTTTTTGGTATTTGAAGCCGTTTGGCGGGAGTTGGCTATAG
AATATTAATTCGTTTGCATTTGATACATCCTGGTAATTTCTTGTTGAAATCAG
ATAGTCTATACAACGTGATTGTGACTACACATGCTTTAGTTATAATTTTTTTCG
CGGTAATGCCTTTACTTATTGGTGCTTTTGGTAACTGGCTTATTCCTTTAATAA
TTGGTGCCATAGACCTTGCTTTCCCGCGTGTTAATAATTTTAGTTTTTGAATTC
TGCCTAGTGCCTTTTATTTATTATTATTATCAGGTTATGTGGAAGAAGGAGTA
GGGACTGGTTGGACTATTTATCCTCCTTTATCTACTGTAGAATACCATAGAAG
CCCTGCGATGGATCTTGCTATTTTATCACTTCATTTAGCAGGTTCTGGGTCTTT
AATAGGCGCTATTAATTTCTTAACCTCTAATAAAAATCTTCCTGTTAATAAAA
TAAAGGGAGAGCGATCTGTCCTGTATGTATGAAGAATCACGGTTACAGCCTT
CTTGTTGCTGTTATCTTTACCGGTTTTAGCAGGAGCCATCACTATACTGTTGTT

TGATCGTAATTTTAATACCACATTTTACGATCCAATCGGAGGAGGAGATCCTG
TGTTATTTATACATCTTTTT

M68-11 COI

ACACTTTATTTAGTTTTTGGTATTTGAAGCGGTTTGGCGGGAGTCGGCTATAG
AATATTAATTCGTTTGCATTTGATACATCCTGGTAATTTTTTGTTGAAATCAGA
TAGTCTATACAACGTGATTGTGACTACACATGCTTTAGTTATAATTTTTTTCGC
GGTAATGCCTTTACTTATTGGTGCTTTTGGTAACTGGCTTATTCCTTTAATAAT
TGGTGCTATAGACCTTGCTTTCCCGCGTGTTAATAATTTTAGTTTCTGAATTCT
GCCTAGTGCCTTTTATTTATTATTATTATCAGGTTATGTGGAAGAAGGAGTGG
GGACTGGTTGGACTATTTATCCTCCTTTATCTACTGTAGAATACCATAGAAGC
CCTGCGATGGATCTTGCTATTTTATCACTTCATTTAGCAGGTTCTGGGTCTTTA
ATAGGCGCTATTAATTTCCTAACCTCTAATAAAAATCTCCCTGTTAATAAAAT
AAAGGGAGAGCGATCTGTCCTGTATGTATGAAGAATCACGGTTACAGCCTTT
TTGTTGCTGTTATCTTTACCGGTTTTAGCAGGAGCCATCACTATACTGTTGTTT
GATCGTAATTTTAATACCACATTTTATGATCCAATCGGAGGAGGAGATCCTGT
GTTATTTATACATCTTTTT

Literature Cited

Abbott, T. R. 1974. American Seashells. The Marine Mollusks of the Atlantic and Pacific Coasts of North America. Van Nostrand Reinhold, New York.

Agapow, P. M., O. R. P. Bininda-Emonds, K. A. Crandall, J. L. Gittleman, G. M. Mace, J. C. Marshall, and A. Purvis. 2004. The impact of species concept on biodiversity studies. Quarterly Review of Biology 79:161-179.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Alvarez, N., L. Mercier, M. Hossaert-Mckey, J. Contreras-Garduno, G. Kunstler, A. Aebi, and B. Benrey. 2006. Ecological distribution and niche segregation of sibling species: the case of bean beetles, *Acanthoscelides obtectus* Say and *A. obvelatus* Birdwell. Ecological Entomology 31:582-590.

Aridgides, L. J., M. A. Doblin, T. Berke, F. C. Dobbs, D. O. Matson, and L. A. Drake. 2004. Multiplex PCR allows simultaneous detection of pathogens in ship's ballast water. Marine Pollution Bulletin 48:1096-1101.

Asakura, A., and S. Watanabe. 2005. *Hemigrapsus takanoi,* new species, a sibling species of the common Japanese intertidal crab *H. penicillatus* (Decapoda: Brachyura: Grapsoidea). Journal of Crustacean Biology 25:279-292.

Avise, J. C. 1990. Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation. Oikos 63:62-76.

Avise, J. C. 1994. Molecular Markers, Natural History and Evolution. Chapman and Hall, New York.

Baric, S., and C. Sturmbauer. 1999. Ecological parallelism and cryptic species in the genus *Ophiothrix* derived from mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 11:157-162.

Bastrop, R. 1998. Cryptic species in a marine polychaete and their independent introduction from North America to Europe. Molecular Biology and Evolution 15:97-103.

Baylac, M., C. Villemant, and G. Simbolotti. 2003. Combining geometric morphometrics with pattern recognition for the investigation of species complexes. Biological Journal of the Linnean Society 80:89-98.

Beasley, E. M., R. M. Myers, D. R. Cox, and L. C. Lazzeroni. 1999. Statistical refinement of primer design parameters. Pp. 55-72 *in* M. A. Innis, D. H. Gelfand, and J. J. Sninsky, eds. PCR Applications: Protocols for Functional Genomics. Academic Press, San Diego.

Bell, J. L., and J. P. Grassle. 1998. A DNA probe for identification of larvae of the commercial surfclam (*Spisula solidissima*). Molecular Marine Biology and Biotechnology 7:127-137.

Bendezu, I. F., J. W. Slater, and B. F. Carney. 2005. Identification of *Mytilus* spp. and *Pecten maximus* in Irish waters by standard PCR of the 18S rDNA gene and multiplex PCR of the 16S rDNA gene. Marine Biotechnology 7:687-696.

Bennett, K. F., and R. Wilan. 2003. Phenotypic plasticity of *Brachidontes* mussels from differing shorelines and habitats in the Florida Keys. Pp. 4 *in* D. Ó Foighil, and T. Lee, eds. 69th Annual Meeting of the American Malacological Society. American Malacological Society, Ann Arbor, MI.

Benson, A. L., D. C. Marelli, M. E. Frischer, J. M. Danforth, and J. D. Williams. 2001. Establishment of the green mussel, *Perna viridis* (Linnaeus 1758) (Mollusca: Mytilidae) on the west coast of Florida. Journal of Shellfish Research 20:21-29.

Benzie, J. A. H. 1999. Major genetic differences between crown-of-thorns starfish (*Acanthaster planci*) populations in the Indian and Pacific Oceans. Evolution 53:1782-1795.

Bickford, D., D. J. Lohman, N. S. Sodhi, P. K. L. Ng, R. Meier, K. Winker, K. K. Ingram, and I. Das. 2006. Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution 22:148-155.

Bieler, R., and P. M. Mikkelsen. 2004. Marine bivalves of the Florida Keys: a qualitative faunal analysis based on original collections, museum holdings and literature data. Malacologia 46:503-544.

Bilton, D. T., J. Paula, and J. D. D. Bishop. 2002. Dispersal, genetic differentiation and speciation in estuarine organisms. Estuarine, Coastal and Shelf Science 55:937-952.

Blair, D., M. Waycott, L. Byrne, G. Dunshea, C. Smith-Keune, and K. M. Neil. 2006. Molecular discrimination of *Perna* (Mollusca: Bivalvia) species using the polymerase chain reaction and species-specific mitochondrial primers. Marine Biotechnology 8:380-385.

Blanquer, A., and M. Uriz. 2007. Cryptic speciation in marine sponges evidenced by mitochondrial and nuclear genes: a phylogenetic approach. Molecular Phylogenetics and Evolution 45:392-397.

Blanquer, A., and M. J. Uriz. 2008. 'A posteriori' searching for phenotypic characters to describe new cryptic species of sponges revealed by molecular markers (Dictyonellidae: Scopalina). Invertebrate Systematics 22:489-502.

Boissin, E., J. P. Feral, and A. Chenuil. 2008. Defining reproductively isolated units in a cryptic and syntopic species complex using mitochondrial and nuclear markers:

the brooding brittle star, *Amphipholis squamata* (Ophiuroidea). Molecular Ecology 17:1732-1744.

Bortolus, A. 2008. Error cascades in the biological sciences: the unwanted consequences of using bad taxonomy in ecology. Ambio 37:114-118.

Brewster-Wingard, G. L., J. R. Stone, and C. W. Holmes. 2001. Molluscan faunal distribution in Florida Bay, past and present: an integration of down-core and modern data. Bulletins of American Paleontology 361:199-231.

Campos, B. M., and L. Ramorino. 1980. Larval and early benthic stages of *Brachidontes granulata* (Bivalvia: Mytilidae). The Veliger 22:277-281.

Caputi, L., N. Andreakis, F. Mastrototaro, and P. Cirino. 2007. Cryptic speciation in a model invertebrate chordate. Proceedings of the National Academy of Sciences 104:9365-9369.

Chen, G., and M. P. Hare. 2008. Cryptic ecological diversification of a planktonic estuarine copepod, *Acartia tonsa*. Molecular Ecology 17:1451-1468.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research 31:3497-3500.

Chesson, P. 1991. A need for niches? Trends in Ecology and Evolution 6:26-28.

Colborn, J., R. E. Crabtree, J. B. Shaklee, E. Pfeiler, and B. W. Bowen. 2001. The evolutionary enigma of bonefishes (*Albula* spp.): cryptic species and ancient separations in a globally distributed shorefish. Evolution 55:807-820.

Collin, R. 2005. Development, phylogeny, and taxonomy of *Bostrycapulus* (Caenogastropoda: Calyptraeidae), an ancient cryptic radiation. Zoological Journal of the Linnean Society 144:75-101.

Comstock, K. E., N. Georgiadis, J. Pecon-Slattery, A. L. Roca, E. A. Ostrander, S. J. O'Brien, and S. K. Wasser. 2002. Patterns of molecular genetic variation among African elephant populations. Molecular Ecology 11:2489-2498.

Cowen, R. K., C. B. Paris, and A. Srinivasan. 2006. Scaling of connectivity in marine populations. Science 311:522-527.

Crummett, L. T., and D. J. Eernisse. 2007. Genetic evidence for the cryptic species pair, *Lottia digitalis* and *Lottia austrodigitalis* and microhabitat partitioning in sympatry. Marine Biology 152:1-13.

Darling, J. A., and M. J. Blum. 2007. DNA-based methods for monitoring invasive species: a review and prospectus. Biological Invasions 9:1573-1464.

Dawson, M. N., and D. K. Jacobs. 2001. Molecular evidence for cryptic species of *Aurelia aurita* (Cnidaria, Scyphozoa). Biological Bulletin 200:92-96.

de Vargas, C., R. Norris, L. Zaninetti, and S. W. Gibb. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. Proceedings of the National Academy of Sciences 96:2864-2868.

Dixon, D. R., A. M. Sole-Cava, P. P. L., and W. W. H. Holland. 1995. Periostracal adventitious hairs on spat of the mussel *Mytilus edulis*. Journal of the Marine Biological Association of the United Kingdom 75:363-372.

Durbin, A., P. D. N. Hebert, and M. E. A. Cristescu. 2008. Comparative phylogeography of marine cladocerans. Marine Biology 155:1-10.

Ellingson, R. A., and P. J. Krug. 2006. Evolution of poecilogony from planktotrophy: cryptic speciation, phylogeography, and larval development in the gastropod genus *Alderia*. Evolution 60:2293-2310.

Erwin, T. L. 1983. Beetles and other insects of tropical forest canopies at Manus, Brazil, sampled by insecticidal fogging. Pp. 59-75 *in* S. L. Sutton, T. C. Whitmore, and A. C. Chadwick, eds. Tropical Rainforest Ecology and Management. Blackwell, Edinburgh.

Fernández, A., T. García, L. Asensio, M. Á. Rodríguez, I. González, P. E. Hernández, and R. Martín. 2001. PCR-RFLP analysis of the internal transcribed spacer (ITS) region for the identification of three clam species. Journal of Food Science 66:657-661.

Fields, A., and E. Moore. 1983. The larval biology of *Brachidontes modiolus* (Linné, 1767) (Bivalvia: Mytilidae). The Veliger 26:52-61.

Folmer, O., M. Black, W. R. Hoeh, R. A. Lutz, and R. Vrijenhoek. 1994. DNA primers for the amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3:294-299.

Forro, L., N. M. Korovchinsky, A. A. Kotov, and A. Petrusek. 2008. Global diversity of cladocerans (Cladocera; Crustacea) in freshwater. Hydrobiologia 595:177-184.

Gardner, J. P. A., and R. J. Thompson. 2009. Influence of genotype and geography on the shell shape and morphometric trait variation among North Atlantic blue mussel (*Mytilus* spp.) populations. Biological Journal of the Linnean Society 96:875-897.

Geller, J. B. 1999. Decline of a native mussel masked by sibling species invasion. Conservation Biology 13:661-664.

Goffredi, S., L. Hurtado, S. Hallam, and R. Vrijenhoek. 2003. Evolutionary relationships of deep-sea vent and cold seep clams (Mollusca: Vesicomyidae) of the "*pacifica/lepta*" species complex. Marine Biology 142:311-320.

Gomez, A., P. J. Wright, D. H. Lunt, J. M. Cancino, G. R. Carvalho, and R. N. Hughes. 2007. Mating trials validate the use of DNA barcoding to reveal cryptic speciation of a marine bryozoan taxon. Proceedings of the Royal Society of London- B Biological Sciences 274:199-207.

Grantham, B. A., G. L. Eckert, and A. L. Shanks. 2003. Dispersal potential of marine invertebrates in diverse habitats. Ecological Applications 13:S108-S116.

Grassle, J. F., and J. P. Grassle. 1976. Sibling species in the marine pollution indicator *Capitella* (Polychaeta). Science 192:567-569.

Hardin, G. 1960. The competitive exclusion principle. Science 131:1292-1297.

Hare, M. P., S. R. Palumbi, and C. A. Butman. 2000. Single-step species identification of bivalve larvae using multiplex polymerase chain reaction. Marine Biology 137:953-961.

Heath, D. D., D. R. Hatcher, and T. J. Hilbish. 1996. Ecological interactions between sympatric *Mytilus* species on the west coast of Canada investigated using PCR markers. Molecular Ecology 5:443-447.

Heath, D. D., P. D. Rawson, and T. J. Hilbish. 1995. PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. Canadian Journal of Fisheries and Aquatic Sciences 52:2621-2627.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London- B Biological Sciences 270:313-321.

Hebert, P. D. N., and T. R. Gregory. 2005. The promise of DNA barcoding for taxonomy. Systematic Biology 54:852-859.

Hebert, P. D. N., E. H. Penton, J. M. Burns, D. H. Janzen, and W. Hallwachs. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences 101:14812-14817.

Hey, J. 2006. On the failure of modern species concepts. Trends in Ecology and Evolution 21:447-450.

Hey, J., R. S. Waples, M. L. Arnold, and R. K. Butlin. 2003. Understanding and confronting species uncertainty in biology and conservation. Trends in Ecology and Evolution 11:597-603.

Highton, R. 1995. Speciation in eastern North American salamanders of the genus *Plethodon*. Annual Review of Ecology and Systematics 26:579-600.

Hilbish, T. J., A. Mullinax, S. I. Dolven, A. Meyer, R. K. Koehn, and P. D. Rawson. 2000. Origin of the antitropical distribution pattern in marine mussels (*Mytilus* spp.): routes and timing of transequatorial migration. Marine Biology 136:69-77.

Hoeh, W. R., D. T. Stewart, B. W. Sutherland, and E. Zouros. 1996. Multiple origins of gender-associated mitochondrial DNA lineages in bivalves (Mollusca: Bivalvia). Evolution 50:2276-2286.

Hutchinson, G. E. 1957. Concluding remarks. Cold Spring Harbor Symposia on Quantitative Biology 22:415-427.

Hyde, J. R., C. A. Kimbrell, J. E. Budrick, and E. A. Lynn. 2008. Cryptic speciation in the vermilion rockfish (*Sebastes miniatus*) and the role of bathymetry in the speciation process. Molecular Ecology 17:1122-1136.

Jensen, R. H., and M. G. Harasewych. 1986. Class Bivalvia. Pp. 461-491 *in* W. Sterrer, ed. Marine Fauna and Flora of Bermuda. John Wiley and Sons, New York.

Jockusch, E. L., and D. B. Wake. 2002. Falling apart and merging: diversification of slender salamanders (Plethodontidae: Batrachoseps) in the American West. Biological Journal of the Linnean Society 76:361-391.

Johnson, S. B., and J. B. Geller. 2006. Larval settlement can explain the adult distribution of *Mytilus californianus* Conrad but not of *M. galloprovincialis* Lamarck or *M. trossulus* Gould in Moss Landing, central California: evidence from genetic identification of spat. Journal of Experimental Marine Biology and Ecology 328:136-145.

Johnson, S. B., A. Waren, and R. C. Vrijenhoek. 2008. DNA barcoding of *Lepetodrilus* limpets reveals cryptic species. Journal of Shellfish Research 27:43-51.

Jones, G., and S. M. Vanparijs. 1993. Bimodal echolocation in pipistrelle bats: are cryptic species present? Proceedings of the Royal Society of London- B Biological Sciences 251:119-125.

Kamermans, P., H. W. Van der Veer, J. I. J. Witte, and E. J. Adriaans. 1999. Morphological differences in *Macoma balthica* (Bivalvia, Tellinacea) from a Dutch and three southeastern United States estuaries. Journal of Sea Research 41:213-224.

Karatayev, A. Y., D. Boltovskoy, D. K. Padilla, and L. E. Burlakova. 2007. The invasive bivalves *Dreissena polymorpha* and *Limnoperna fortunei*: parallels, contrasts, potential spread and invasion impacts. Journal of Shellfish Research 26:205-213.

Kelly, D. W., H. J. MacIsaac, and D. D. Heath. 2006. Vicariance and dispersal effects on phylogeographic structure and speciation in a widespread estuarine invertebrate. Evolution 60:257-267.

King, J. L., and R. Hanner. 1998. Cryptic species in a "living fossil" lineage: taxonomic and phylogenetic relationships within the genus *Lepidurus* (Crustacea: Notostraca) in North America. Molecular Phylogenetics and Evolution 10:23-36.

Klautau, M., C. A. M. Russo, C. Lazoski, N. Boury-Esnault, J. P. Thorpe, and A. M. Sole-Cava. 1999. Does cosmopolitanism result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. Evolution 53:1414-1422.

Knowlton, N. 1986. Cryptic and sibling species among the decapod crustacea. Journal of Crustacean Biology 6:356-363.

Knowlton, N. 1993. Sibling species in the sea. Annual Review of Ecology and Systematics 24:189-216.

Knowlton, N. 2000. Molecular genetic analyses of species boundaries in the sea. Hydrobiologia 420:73-90.

Knowlton, N., and J. B. C. Jackson. 1994. New taxonomy and niche partitioning on coral reefs: jack of all trades or master of some? Trends in Ecology and Evolution 9:7-9.

Kyle, C. J., and E. G. Boulding. 1998. Molecular genetic evidence for parallel evolution in a marine gastropod, *Littorina subrotundata*. Proceedings of the Royal Society of London- B Biological Sciences 265:303-308.

Larsen, J. R., M. E. Frischer, L. J. Rasmussen, and B. W. Hansen. 2005. Single-step nested multiplex PCR to differentiate between various bivalve larvae. Marine Biology 146:1119-1129.

Lavoie, D. M., L. D. Smith, and G. M. Ruiz. 1999. The potential for intracoastal transfer of non-indigenous species in the ballast water of ships. Estuarine, Coastal and Shelf Science 48:551-564.

Lee, C. E. 2000. Global phylogeography of a cryptic copepod species complex and reproductive isolation between genetically proximate "populations". Evolution 54:2014-2027.

Lee, T., and D. Ó Foighil. 2004. Hidden Floridian biodiversity: mitochondrial and nuclear gene trees reveal four cryptic species within the scorched mussel, *Brachidontes exustus*, species complex. Molecular Ecology 13:3527-3542.

Lee, T., and D. Ó Foighil. 2005. Placing the Floridian marine genetic disjunction into a regional evolutionary context using the scorched mussel, *Brachidontes exustus*, species complex. Evolution 59:2139-2158.

Leite, T. S., M. Haimovici, W. Molina, and K. Warnke. 2008. Morphological and genetic description of *Octopus insularis*, a new cryptic species in the *Octopus vulgaris* complex (Cephalopoda: Octopodidae) from the tropical southwestern Atlantic. Journal of Molluscan Studies 74:63-74.

Linnaeus, C. v. 1758. Caroli Linnaei...Systema naturae per regna tria naturae: secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Impensis Direct. Laurentii Salvii, Holmiae.

Liu, H. P., R. Hershler, and K. Clift. 2003. Mitochondrial DNA sequences reveal extensive cryptic diversity within a western American springsnail. Molecular Ecology 12:2771-2782.

Lopez-Piñión, M. J., A. Insua, and J. Méndez. 2002. Identification of four scallop species using PCR and restriction analysis of the ribosomal DNA internal transcribed spacer region. Marine Biotechnology 4:495-502.

Marshall, H. D., K. A. Johnstone, and S. M. Carr. 2007. Species-specific oligonucleotides and multiplex PCR for forensic discrimination of two species of scallops, *Placopecten magellanicus* and *Chlamys islandica*. Forensic Science International 167:1-7.

Matabos, M., E. Thiebaut, D. Le Guen, F. Sadosky, D. Jollivet, and F. Bonhomme. 2008. Geographic clines and stepping-stone patterns detected along the East Pacific Rise in the vetigastropod *Lepetodrilus elevatus* reflect species crypticism. Marine Biology 153:545-563.

Mathews, L. M. 2006. Cryptic biodiversity and phylogeographical patterns in a snapping shrimp species complex. Molecular Ecology 15:4049-4063.

May, R. M. 1988. How many species are there on earth? Science 241:1441-1449.

Mayden, R. L. 1997. A hierarchy of species concepts: the denouement in the saga of the species concept. Pp. 381-424 *in* M. F. Claridge, H. A. Dawah, and M. R. Wilson, eds. Species: the Units of Biodiversity. Chapman and Hall, London.

Mayer, F., and O. von Helversen. 2001. Cryptic diversity in European bats. Proceedings of the Royal Society of London- B Biological Sciences 268:1825-1832.

Mayr, E. 1942. Species concepts and definitions. Pp. 1-22 *in* E. Mayr, ed. The Species Problem. American Association for the Advancement of Science, Washington.

Mayr, E. 1963. Animal Species and Evolution. Harvard University Press, Cambridge, Massachusetts.

Meehan, B. W. 1985. Genetic comparison of *Macoma balthica* (Bivalvia: Telinidae) from the eastern and western north-Atlantic ocean. Marine Ecology Progress Series 22:69-76.

Miglietta, M. P., and H. A. Lessios. 2009. A silent invasion. Biological Invasions 11:825-834.

Mikkelsen, P. M., and R. Bieler. 2007. Seashells of Southern Florida: Living Marine Mollusks of the Florida Keys and Adjacent Regions: Bivalves. Princeton University Press, Princeton.

Mizi, A., E. Zouros, N. Moschonas, and G. C. Rodakis. 2005. The complete maternal and paternal mitochondrial genomes of the Mediterranean mussel *Mytilus galloprovincialis*: implications for the doubly uniparental inheritance mode of mtDNA. Molecular Biology and Evolution 22:952-967.

Moore, W. S. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evolution 49:718-726.

Morard, R., F. Quillévéré, G. Escarguel, Y. Ujiie, T. de Garidel-Thoron, and C. de Vargas. 2009. Morphological recognition of cryptic species in the planktonic foraminifer *Orbulina universa*. Marine Micropaleontology 71:148-165.

Morton, B. 1988. The population dynamics and reproductive cycle of *Brachidontes variabilis* (Bivavlia: Mytilidae) in a Hong Kong mangrove. Malacological Review 21:109-117.

Morton, B. 1991. The biology of *Brachidontes erosus* (Bivalvia: Mytilidae) in Princess Royal Harbour, Albany, Western Australia. Pp. 693-712 *in* F. E. Wells, W. D. I., H. Kirkman, and R. Lethbridge, eds. The Marine Flora and Fauna of Albany, Western Australia. Western Australia Museum, Perth.

Nakano, T., and H. Spencer. 2007. Simultaneous polyphenism and cryptic species in an intertidal limpet from New Zealand. Molecular Phylogenetics and Evolution 45:470-479.

Nalesso, R. C., L. F. L. Duatre, and E. G. Mendes. 1992. Phenotypic plasticity in *Brachidontes darwinianus* (Bivalvia: Mytilidae). Revista Brasileira de Biologia 52:245-249.

Nichols, S. A., and P. A. G. Barnes. 2005. A molecular phylogeny and historical biogeography of the marine sponge genus *Placospongia* (Phylum Porifera) indicate low dispersal capabilities and widespread crypsis. Journal of Experimental Marine Biology and Ecology 323:1-15.

Omland, K. E., C. L. Tarr, W. I. Boarman, J. M. Marzluff, and R. C. Fleischer. 2000. Cryptic genetic variation and paraphyly in ravens. Proceedings of the Royal Society of London- B Biological Sciences 267:2475-2482.

Packer, L., J. Gibbs, C. Sheffild, and R. Hanner. 2009. DNA barcoding and the mediocrity of morphology. Molecular Ecology Resources 1:42-50.

Palumbi, S. R. 1994. Genetic divergence, reproductive isolation, and marine speciation. Annual Review of Ecology and Systematics 25:547-572.

Patil, J. G., R. M. Gunasekera, B. E. Deagle, and N. J. Bax. 2005. Specific detection of Pacific oyster (*Crassostrea gigas)* larvae in plankton samples using nested polymerase chain reaction. Marine Biotechnology 7:11-29.

Penton, E. H., P. D. N. Hebert, and T. J. Crease. 2004. Mitochondrial DNA variation in North American populations of *Daphnia obtusa*: continentalism or cryptic endemism. Molecular Ecology 13:97-107.

Pfenninger, M., and K. Schwenk. 2007. Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. B M C Evolutionary Biology 7:121.

Pie, M. R., R. O. Ribeiro, W. A. Boeger, A. Ostrensky, R. M. Falleiros, and L. Angelo. 2006. A simple PCR-RFLP method for the discrimination of native and introduced oyster species (*Crassostrea brasiliana, C. rhizophorae* and *C. gigas*; Bivalvia: Ostreidae) cultured in southern Brazil. Aquaculture Research 37:1598-1600.

Power, A. J., R. L. Walker, K. Payne, and D. Hurley. 2004. First occurrence of the nonindigenous green mussel, *Perna viridis* (Linnaeus, 1758) in coastal Georgia, United States. Journal of Shellfish Research 23:741-744.

Quattro, J. M., M. R. Chase, M. A. Rex, T. W. Greig, and R. J. Etter. 2001. Extreme mitochondrial DNA divergence within populations of the deep-sea gastropod *Frigidoalvania brychia*. Marine Biology 139:1107-1113.

Reeb, C. A., and J. C. Avise. 1990. A genetic discontinuity in a continuously distributed species: mitochondrial DNA in the American oyster, *Crassostrea virginica*. Genetics 124:397-406.

Reimer, O., and M. Tedengren. 1996. Phenotypical improvement of morphological defenses in the mussel *Mytilus edulis* induced by the predator *Asterias rubens*. Oikos 75:383-390.

Rice, S. A., S. A. Karl, and K. A. Rice. 2008. The *Polydora cornuta* complex (Annelida: Polychaeta) contains populations that are reproductively isolated and genetically distinct. Invertebrate Biology 127:45-64.

Rios, E. C. 1985. Seashells of Brazil. Funadção Universidade do Rio Grande, Museu Oceanografico, Rio Grande.

Ruiz, G. M., T. K. Rawlings, F. C. Dobbs, L. A. Drake, T. Mullady, A. Huq, and R. R. Colwell. 2000. Global spread of microorganisms by ships. Nature 408:49-50.

Sáez, A. G., and E. Lozano. 2005. Body doubles. Nature 433:111-111.

Sáez, A. G., I. Probert, M. Geisen, P. Quinn, J. R. Young, and K. K. Medlin. 2003. Pseudo-cryptic speciation in coccolithophores. Proceedings of the National Academy of Sciences 100:7163-7168.

Schama, R., A. M. Sole-Cava, and J. P. Thorpe. 2005. Genetic divergence between east and west Atlantic populations of *Actinia* spp. sea anemones (Cnidaria: Actiniidae). Marine Biology 146:435-443.

Schlick-Steiner, B. C., B. Seifert, C. Stauffer, E. Christian, R. H. Crozier, and F. M. Steiner. 2007. Without morphology, cryptic species stay in taxonomic crypsis following discovery. Trends in Ecology and Evolution 22:391-392.

Seed, R. 1968. Factors influencing shell shape in the mussel *Mytilus edulis*. Journal of the Marine Biological Association of the United Kingdom 48:561-584.

Seed, R. 1980a. A note on the relationship between shell shape and life habits in *Geukensia demissa* and *Brachidontes exustus* (Mollusca: Bivalvia). Journal of Molluscan Studies 46:293-299.

Seed, R. 1980b. Shell growth and form in the Bivalvia Pp. 23-67 *in* D. C. Rhoads, and R. A. Lutz, eds. Skeletal Growth of Aquatic Organisms. Plenum Press, New York.

Seed, R. 1992. Systematics, evolution and distribution of mussels belonging to the genus *Mytilus*: an overview. American Malacological Bulletin 9:123-138.

Siddall, S. E. 1980. A clarification of the genus *Perna* (Mytilidae). Bulletin of Marine Science 30:858-870.

Simison, W. B., and D. R. Lindberg. 1999. Morphological and molecular resolution of a putative cryptic species complex: a case study of *Notoacmea fascicularis* (Menke, 1851) (Gastropoda: Patellogastropoda). Journal of Molluscan Studies 65:99-109.

Solé-Cava, A. M., and J. P. Thorpe. 1992. Genetic divergence between color morphs in populations of the common intertidal sea anemones *Actinia equina* and *A. prasina* (Anthozoa: Actiniaria) in the Isle of Man. Marine Biology 112:243-252.

Soot-Ryen, T. 1969. Superfamily Mytilacea Rafinesque, 1815. Pp. N271-N281 *in* R. C. Moore, ed. Treatise on Invertebrate Paleontology. The Geological Society of America and University of Kansas Press, Kansas.

Strelkov, P., R. Nikula, and R. Väinölä. 2007. *Macoma balthica* in the White and Barents Seas: properties of a widespread marine hybird swarm (Mollusca: Bivalvia). Molecular Ecology 16:4110-4127.

Sturmbauer, C. 1999. Mitochondrial DNA reveals cryptic oligochaete species differing in cadmium resistance. Molecular Biology and Evolution 16:967-974.

Sweeney, M. L., and R. L. Walker. 1998. The gametogenic cycle of *Brachidontes exustus* (Bivalvia: Mytilidae) at Wassaw Island, Georgia. American Malacological Bulletin 14:149-156.

Tanaka, M. O., and C. Alveres de Magalhaes. 1999. Morphometric species recognition in *Brachidontes darwinianus* and *Brachidontes solisianus* (Bivalvia: Mytilidae). The Veliger 43:267-274.

Tarjuelo, I., D. Posada, K. Crandall, and M. Pascual. 2001. Cryptic species of *Clavelina* (Ascidiacea) in two different habitats: harbours and rocky littoral zones in the northwestern Mediterranean. Marine Biology 139:455-462.

Taylor, J. 1971. Reef associated molluscan assemblages in the western Indian Ocean. Symposium of the Zoological Society of London 28:501-534.

Taylor, J., and E. Glover. 2005. Cryptic diversity of chemosymbiotic bivalves: a systematic revision of worldwide *Anodontia* (Mollusca: Bivalvia: Lucinidae). Systematics and Biodiversity 3:281.

Terranova, M. S., S. Lo Brutto, M. Arculeo, and J. B. Mitton. 2007. A mitochondrial phylogeography of *Brachidontes variabilis* (Bivalvia: Mytilidae) reveals three cryptic species. Journal of Zoological Systematics and Evolutionary Research 45:289-298.

Teske, P. R., H. Winker, C. D. McQuaid, and N. P. Barker. 2009. A tropical/subtropical biogeographic disjunction in southeastern Africa separates two Evolutionarily Significant Units of an estuarine prawn. Marine Biology 156:1265-1275.

Tilman, D. 1977. Resource competition between plankton algae: an experimental and theoretical approach. Ecology 58:338-348.

Väinölä, R. 2003. Repeated trans-Arctic invasions in littoral bivalves: molecular zoogeography of the *Macoma balthica* complex. Marine Biology 143:935-946.

Veliz, D., F. M. Winkler, and C. Guisado. 2003. Developmental and genetic evidence for the existence of three morphologically cryptic species of *Crepidula* in northern Chile. Marine Biology 143:131-142.

Verling, E., G. M. Ruiz, D. L. Smith, B. Galil, A. W. Miller, and K. R. Murphy. 2005. Supply-side invasion ecology: characterizing propagule pressure in coastal ecosystems. Proceedings of the Royal Society of London- B Biological Sciences 272:1249-1256.

Warwick, R. M., and J. Robinson. 2000. Sibling species in the marine pollution indicator genus *Pontonema leidy* (Nematoda: Oncholaimidae), with a description of *P. mediterranea* sp. nov. Journal of Natural History 34:641-662.

Wellborn, G., and R. Cothran. 2004. Phenotypic similarity and differentiation among sympatric cryptic species in a freshwater amphipod species complex. Freshwater Biology 49:1-13.

Wellborn, G., and R. Cothran. 2007. Niche diversity in crustacean cryptic species: complementarity in spatial distribution and predation risk. Oecologia 154:175-183.

Westheide, W., and H. Schmidt. 2003. Cosmopolitan versus cryptic meiofaunal polychaete species: an approach to a molecular taxonomy. Helgoland Marine Research 57:1-6.

White, L. R., B. A. McPheron, and R. J. J. Stauffer. 1994. Identification of freshwater mussel glochidia on host fishes using restriction fragment length polymorphisms. Molecular Ecology 3:183-195.

Wilson, E. O. 1988. The current state of biological diversity. Pp. 3-18 *in* E. O. Wilson, ed. Biodiversity. National Academy Press, Washington.

Wilson, N. G., M. Schrodl, and K. M. Halanych. 2009. Ocean barriers and glaciation: evidence for explosive radiation of mitochondrial lineages in the Antarctic sea slug *Doris kerguelenensis* (Mollusca, Nudibranchia). Molecular Ecology 18:965-984.

Winker, K. 2005. Sibling species were first recognized by William Derham (1718). Auk 122:706-707.

Won, Y., C. R. Young, R. A. Lutz, and R. C. Vrijenhoek. 2003. Dispersal barriers and isolation among deep-sea mussel populations (Mytilidae: Bathymodiolus) from eastern Pacific hydrothermal vents. Molecular Ecology 12:169-184.

Wood, A. R., S. Apte, E. S. MacAvoy, and J. P. A. Gardner. 2007. A molecular phylogeny of the marine mussel genus *Perna* (Bivalvia: Mytilidae) based on nuclear (ITS1&2) and mitochondrial (COI) DNA sequences. Molecular Phylogenetics and Evolution 44:685-698.

Xu, Z., X. M. Guo, P. M. Gaffney, and J. C. Pierce. 2001. Chromosomal location of the major ribosomal RNA genes in *Crassostrea virginica* and *Crassostrea gigas*. The Veliger 44:79-83.

Yeatman, J., and J. A. H. Benzie. 1994. Genetic structure and distribution of *Photololigo* spp. in Australia. Marine Biology 118:79-87.

Yu, E. T., M. Juinio-Menez, and V. D. Monje. 2000. Sequence variation in the ribosomal DNA internal transcribed spacer of *Tridacna crocea.* Marine Biotechnology 2:511-516.

Zhang, D.-Y., K. Lin, and I. Hanski. 2004. Coexistence of cryptic species. Ecology Letters 7:165-169.

Zouros, E., A. O. Ball, C. Saavedra, and K. R. Freeman. 1994. An unusual type of mitochondrial DNA inheritance in the blue mussel *Mytilus*. Proceedings of the National Academy of Sciences 91:7463-7467.

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2006 R. A. Lutz, A. G. Collins, E. R. Ansis, A. J. Reed, K. F. Bennett, K. M Halanych, and R. C. Vrijenhoek. Stauromedusan populations inhabiting deep-sea hydrothermal vents along the southern East Pacific Rise. *Cahiers de Biologie Marine.* 47(4): 409-413