REPRODUCTIVE ISOLATION AND GENETIC DIVERGENCE IN A YOUNG "SPECIES FLOCK" OF PUPFISHES (*CYPRINODON SP.*) FROM SAN SALVADOR ISLAND, BAHAMAS

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Abstract

The study of the process of speciation is instrumental to understanding the species diversity observed today. Diverging populations are intriguing, because speciation has not reached an endpoint, yet the process that may eventually lead to distinct species can be studied. Systems that contain many putative species and/or parallel divergences, such as many species flocks and species pairs, are extraordinary examples of divergence and therefore are critical to the understanding of the speciation process. A "miniature" species flock of pupfish (Cyprinodon variegatus) discovered in lakes on San Salvador Island, Bahamas has evolved in less than 6 000 years, and is, therefore, important to the study of the pace of evolutionary processes. The San Salvador Island pupfish flock is composed of a normal form, which resembles coastal *C. variegatus*, and bulldog and bozo morphs, which diverge ecologically and morphologically from the normal morph.

In Chapter 1, I sequenced the mtDNA control region and used haplotype frequency analyses to assess the level of differentiation between sympatric normals and bulldogs sampled from Osprey Lake and Little Lake on San Salvador Island. The bozo morph was too rare to include in the study. I also included samples of normals that occur in lakes without bulldog and bozo morphs to assess any differences between lakes on the island. All haplotype frequency comparisons for sympatric normals and bulldogs were highly significant, which suggests these morphs are distinct populations in sympatry and, therefore, have characteristics of biological species. Further, an estimation of Time for Speciation supports geological data that suggest this fauna is very young (6 000 years). The San Salvador Island pupfish species flock is, therefore, the youngest known species flock and presents an important model system for the study of how morphological and ecological divergence can promote speciation in *Cyprinodon*.

In Chapter 2, I first compared the San Salvador Island pupfishes to other Bahamian *C. variegatus* populations to assess the level of inter- and intra-island pupfish

population differentiation in the Bahamas. The mtDNA control region was sequenced for bulldogs and normals from San Salvador Island and normals sampled from New Providence and Exuma Islands. San Salvador Island bulldogs were found to be distinct from all normal populations sampled, and comparisons of shared haplotypes suggest they originated on San Salvador Island rather than any of the other islands sampled. This was intriguing, because a "bulldog-like" morph has recently been observed in a lake on New Providence Island, which suggests parallel divergences may be occurring throughout the Bahamas. I also sequenced the mtDNA cytochrome *b* gene to assess the phylogeography of *C. variegatus*. Populations were sampled from the Bahamas and the east coast of North America, and the results suggest the Bahamas were only recently colonized by the Southern coastal lineage of *C. variegatus*. A distinct Northern lineage of *C. variegatus*, which may warrant species designation, was also supported by the cytochrome *b* data. Overall, the results supported a San Salvador Island origin for the Little Lake and Osprey Lake bulldog morphs, and also suggest the Bahamian *C. variegatus* populations are very young.

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Background

A brief introduction to *Cyprinodon variegatus*

The genus *Cyprinodon* comprises fish species collectively referred to as "pupfishes". These fish species are distributed only in the New World and rarely occur sympatrically with each other (Liu 1969). Introgression can occur when *Cyprinodon* species do come in contact with each other. For instance, the sheepshead minnow *C. variegatus* was introduced into the Pecos River, TX between 1980 and 1984 (Echelle & Connor 1989), and within five years it had almost completely introgressed with native Pecos pupfish (*C. pecosensis*) populations throughout a 300 km portion of the river (Wilde and Echelle 1997). Many populations in the river now consist of pupfish with the *C. variegatus* morphology, which suggests certain *Cyprinodon* species are very efficient colonizers and can outcompete other members of the genus. Further, reproductive isolating mechanisms between *Cyprinodon* species may not be very complex, and apparently can break down when certain species come in contact with each other. In general, pupfishes also are considered to be good colonizers because of their wide temperature and salinity tolerances. For instance, *C. variegatus* can withstand temperatures ranging from 5^0C to 42^0C (Bennett and Beitinger 1997) and can survive in salinities ranging from hypersaline to almost freshwater (Martin 1968). This genus is, therefore, of general interest because many species have been able to colonize and thrive in diverse and even harsh environments.

The "sheepshead minnow" (*Cyprinodon variegatus* in the broadest sense) is the most widespread species in the genus. As presently defined, this pupfish species ranges coastwise from Cape Cod, MA to the Gulf of Mexico (reaching to the mouth of the Rio Tuxpan, Mexico), throughout the Bahamas, most of the Greater Antilles, and Yucatan/Belize. Other members of the genus have much smaller ranges as compared to *C. variegatus*; the most extreme example being *C. diabolis*, which only occurs in a very small habitat in the Death Valley drainage system of southern California and Nevada (Liu 1969). The distribution of *C. variegatus* populations has been described as "patchy", and geographically isolated populations are known. Several of these are morphologically divergent, which has resulted in the description of subspecies and even distinct species;

many of which are poorly known or equivocally classified. For example, *C. v. hubbsi* was thought to be endemic to 8 lakes in the St. Johns River drainage of central Florida, and had slight morphometric and meristic divergence from *C. variegatus* (Guillory and Johnson 1986). However, allozyme studies showed no divergence between *C. v. hubbsi* and *C. variegatus*, and resulted in *C. v. hubbsi* being considered synonymous with *C. variegatus (*Duggins *et al.* 1983). Slight morphological divergence from *C. variegatus* also occurs in *C. v. artifrons*, which ranges along the Yucatan coast to Belize (and possibly to Nicaragua) and presumably has been isolated from the main portion of the *C. variegatus* distribution for a considerable amount of time (Humphries and Miller 1981). The most obvious morphological divergence between *C. v. artifrons* and *C. v. variegatus* is the presence of a black spot on male dorsal fins in the latter (it is present on mature females in most populations of *C. variegatus*). Some authors have considered *C. artifrons* a distinct species (Humphries and Miller 1981; Heath *et al*. 1993). Because very few direct genetic comparisons of *C. v. variegatus* and *C. v. artifrons* have been made, the phylogenetic relationship of these two "species" is still unknown. Overall, many of the relationships of populations throughout the range of *C. variegatus* are uncertain and the phylogeographic structure of the species is largely unresolved.

Although some subspecies and populations of *C. variegatus* appear to have slight morphological divergence, a significant level of divergence recently was discovered in populations of *C. variegatus* from Little Lake and Osprey Lake on San Salvador Island, Bahamas. Dr. Grant Gilmore (Harbor Branch Oceanographic Institute, Fort Pierce, Florida) discovered 4 presumably divergent pupfish morphs occurring in these 2 lakes. The first morph, termed "normal", was indistinguishable from coastal *C. variegatus*, while the other 3 morphs ("bulldog", "gums", "wide mouth") were apparently morphologically divergent. Recently, Cami Holtmeier (Cornell University, Ithaca, New York; now at DuPaul University, Chicago, Illinois) collected samples from Little Lake and Osprey Lake and only recognized the "normal", "bulldog", and "gums" morphs. She refers to the "gums" morph as "bozo", and her terminology will be used here. Morphometric studies showed significant divergence among these three morphs (Holtmeier 2000; Holtmeier 2001). The normal morph is the most common and is found in all of the lakes on San Salvador Island as well as throughout the Bahamas. It has a

terminal mouth and is presumably a benthic omnivore; characteristics typical of mainland *C. variegatus*. The bulldog morph has a longer jaw that is almost vertically oriented. Stable isotope and dietary analyses suggest it is piscivorous (Holtmeier 2000). Bozo morphs are the rarest and have not been collected enough for thorough study, but preliminary studies suggest their dentition is highly modified and that they eat gastropods in addition to the general pupfish diet of algae and detritus. Furthermore, these divergent morphologies are recognizably maintained through the F_2 laboratory generation and intermorph crossings result in intermediate phenotypes (Holtmeier 2001). These observations suggest that these morphs have a genetic basis and do not simply reflect phenotypic plasticity.

The San Salvador Island morphs are of particular interest because this degree of morphological divergence and apparent ecological specialization is not seen in coastal populations of *C. variegatus*. Secondly, sea level has been high enough to sustain lakes on this karst island for only the past 3 000 - 6 000 years, which suggests the pupfish divergence has occurred very recently and could be developed into a model system of a rapid evolutionary process. This time frame has been estimated from studies of carbonate (Carew and Mylroie 1997), ostracod (Teeter 1995), and pollen deposition (Pachecho and Foradas 1986) from core samples of the beds of San Salvador lakes. The San Salvador morphs, therefore, are interesting because they could be the youngest species flock known. Genetic studies must be conducted to assess the level of gene flow between the San Salvador morphs in order to explore this divergence as well as to aid in classifying these unique populations of the *C. variegatus* complex.

The San Salvador morphs are also of interest because of their similarity to components of a presumptive "species flock" of pupfishes from Laguna Chichancanab on the Yucatan Peninsula, Mexico. A species flock is a group of monophyletic species that occur in a circumscribed area, such as a lake (Greenwood 1984). The Chichancanab flock is comprised of 5 endemic species, which presumably evolved from coastal *C.* (*v.*) *artifrons* in only 8 000 years (Humphries and Miller 1981; Strecker *et al.* 1996). *C. beltrani* is the most common species of this flock, and is ecologically and morphologically quite difficult to distinguish from *C. artifrons*. The remaining species are divergent from *C. artifrons*/C. *beltrani* in a similar way that bulldogs and bozos are

divergent from the normal morph from San Salvador. For instance, *C. simus* has a distinct upturned jaw, which is similar to the jaw morphology found in the San Salvador bulldogs (Stevenson 1992). However, *C. simus* apparently feeds on plankton and is not a piscivore, unlike the bulldog morph. On the other hand, *C. maya* was found to be piscivorous and also has this upturned jaw morphology. Interestingly, mtDNA control region sequence comparisons revealed that *C. maya* was the only component of the Laguna Chichancanab flock that was reproductively isolated (Strecker *et al.* 1996). The evolution of reproductive isolation, therefore, may not be complete in this species flock. This degree of incomplete isolation is likely due to the youth of this system. The pupfish morphs on San Salvador Island may compose a "miniature" species flock that has strong similarities to the Laguna Chichancanab flock. Molecular analyses must be conducted to assess the level of gene flow between the San Salvador morphs to fully characterize their value as a model system. *Cyprinodon* species may be prone to diverge into trophic morphs, and eventually into species, when they invade particular environments. It is, therefore, important that divergences, such as that in the San Salvador system, be studied in order to understand such evolutionary processes.

Introduction to species flocks and species pairs

Species flocks, such as the 5 endemic pupfish species of Laguna Chichancanab, are important to the study of evolutionary process because many flocks are composed of morphologically and ecologically distinct forms that are endemic to a circumscribed area and are often still in the process of diverging from one another. The most noted species flocks are the African cichlid flocks of Lakes Malawi, Victoria, and Tanganyika. Each of these flocks is composed of hundreds of endemic species that have considerable trophic specialization and morphological differences from one another and that, in some cases, have diverged in a short period of time (reviewed in Kornfield and Smith 2000). For instance, the time of divergence between 2 major cichlid lineages in Lake Malawi has been estimated to be 1 million years (Moran *et al*. 1994), but the divergence among taxa within these lineages apparently has occurred very recently (Moran and Kornfield 1993). The Lake Tanganyika flock is considered to be much older

(9-12 my) than the Victoria and Malawi flocks (Martens *et al.* 1994). It has been difficult for many to accept the surprising amount of diversity that has evolved in a relatively short period of time in the Malawi and Victoria flocks. However, consideration of the Laguna Chichancanab pupfish flock and other young flocks makes it hard to ignore the presumably rapid pace of certain evolutionary processes. The range of trophic specialization in the African cichlids includes algae grazing, piscivory, planktivory, and ambush predation, but there is evidence that food-switching occurs when prey abundance fluctuates (McKaye and Marsh 1983). Species flocks are, therefore, important to understanding the speed of divergence and the correlation between trophic specialization and the maintenance of species diversity over time, as well as why divergence begins in certain species and not others.

The degree of morphological and ecological divergence and even reproductive isolation that has occurred in a relatively short period of time in many species flocks has shifted thinking toward speciation as a process rather than as an endpoint. Patchy distributions and phenotypic similarities among populations in these systems have made classification difficult. Furthermore, there is some inter- and intra-flock parallelism in the trophic specialization and associated behavior in species from the three major African flocks, which is problematic when attempting to assess phylogenetic relationships using classical ichthyological characteristics (Kornfield and Smith 2000). However, this ambiguity makes study of species flocks and similar systems important to understanding of the speciation process. It has, therefore, been important that morphological, genetic, and mating studies be used together to better understand the relationships of populations/species that are often still in the process of diverging from one another. These flocks have been developed into model systems to test speciation hypotheses, such as allopatric vs. sympatric speciation, as well as to test whether sexual selection has been an important factor mediating divergence (reviewed in Kornfield and Smith 2000). These evolutionary mechanisms have been proposed for a long time, but the amazing divergence in the cichlid flocks provides fertile ground for testing and further developing these hypotheses. However, the three major cichlid flocks have very high species diversities and are consequently difficult to study. Smaller species flocks, such as the

putative miniature flock on San Salvador Island, therefore are easier to study in order to test hypotheses that may eventually explain the diversity found in larger flocks.

Species pairs, which are morphologically and ecologically divergent sympatric morphs that are classified under the same latin binomial, are also important to the understanding of the speciation process due to their similarities to species flocks (Taylor 1999). The most studied species pairs are the morphologically divergent and trophically specialized "benthic" and "limnetic" pairs of threespine stickleback (*Gasterosteus aculeatus*) morphs that occur in lakes in the Strait of Georgia region of southwestern Bristish Columbia (McPhail 1984, 1992, 1994). Interestingly, the degree of morphological and trophic divergence between these sympatric pairs varies among the lakes, and mtDNA haplotype frequency analyses revealed that the degree of genetic differentiation between sympatric morphs also varies among lakes (Taylor and McPhail 1999). These mtDNA data also suggested that the sympatric morphs have multiple origins throughout their range, and that each lake presumably contains a monophyletic species pair. The variation among the species pairs of the threespine stickleback species complex has been used to test evolutionary hypotheses and even to assess the period of time it takes for reproductive isolation to evolve, which presumably has occurred in 10 000 - 15 000 years in this case (reviewed in Taylor 1999). Species pairs also have been described for several salmonid species, coregonid species, and other fish species, which suggests this type of divergence has been historically important to the diversity seen today (reviewed in Taylor 1999). It is, therefore, important that species flocks and species pairs be collectively used to develop and test evolutionary hypotheses.

Their relative youth makes the divergent pupfish morphs on San Salvador Island of particular interest. The pace of evolutionary processes classically has been considered to be very slow, but this "miniature" flock could provide strong support for divergence occurring rapidly in some cases. It is, therefore, important that genetic analyses be conducted in this system to assess the level of genetic differentiation between sympatric morphs as well as to determine whether there is support that these morphs have multiple origins on San Salvador Island.

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Chapter 1: Molecular evidence for rapid evolution of reproductive isolation between sympatric ecomorphs of pupfish (*Cyprinodon variegatus*) on San Salvador Island, Bahamas

Abstract

Three "ecomorphs" of pupfish (*Cyprinodon*) occur in several lakes of Holocene vintage on San Salvador Island, Bahamas. The "normal" morph is morphologically and ecologically similar to a widespread coastal species (*Cyprinodon variegatus*), while the "bulldog" and "bozo" morphs have divergent jaw morphologies and presumably also ecological specialization. Haplotype frequency analysis of mitochondrial DNA control region sequences was used to assess genetic isolation of sympatric normal and bulldog morphs from Osprey Lake and Little Lake and to test a multiple origin hypothesis for the bulldog morph. The bozo morph was too rare to include in the analysis. Data analysis suggests that there is restricted gene flow between sympatric morphs, and that reproductive isolation between bulldogs and normals has evolved in less than 6 000 years. Intra-bulldog morph comparisons supported a single origin hypothesis for the bulldog morph on the island. High haplotype diversity (54 haplotypes from 190 normal and bulldog pupfish morphs sampled from 6 lakes) was found in this system, so there is no indication that bottlenecks were historically important in the evolution of these trophic morphs. Thus, sympatric bulldogs and normals are morphologically, ecologically, and genetically distinct entities with characteristics of biological species and have evolved in populations with large effective population sizes. Analysis of mtDNA haplotype frequencies suggested that sympatric bulldogs and normals are sorting into distinct lineages, which is a level of mtDNA differentiation that has not been reported in all other young species flocks. The San Salvador Island pupfish "species flock" is unique because mtDNA haplotype frequency comparisons provided evidence of genetic distinctiveness and considerable lineage sorting among bulldogs and normals, which was not expected in such a young species flock.

Introduction

Lineage sorting and reproductive isolation are often incomplete in diverging populations. Assessing their status as either species or simply divergent populations is difficult and sometimes controversial. For example, "species flocks", i.e., assemblages of monophyletic species that occur in a circumscribed area (Greenwood 1984), often include lineages that are at different stages of divergence. In addition, many lakes in North America contain "species pairs", i.e., populations of fishes that are at least partially reproductively isolated from each other in sympatry, but are still similar enough to be classified under a common Latin binomial (Taylor 1999). These sympatric species pairs and many components of species flocks often have considerable behavioral, morphological, and/or ecological differentiation despite their recent divergence from a common ancestor. While classifying these entities can be difficult, they are, by virtue of their ambiguity, valuable model systems for studying the process of speciation.

Much of the species flock literature discusses fish species flocks; however, there are examples of species flocks that range from birds to fish to even gastropods (reviewed in Echelle and Kornfield 1984), which suggests that species flocks historically have been important to contemporary biodiversity. The most noted species flocks are the African cichlids of Lake Malawi, Lake Victoria, and Lake Tanganyika, which each are comprised of hundreds of endemic species of fish that are morphologically and ecologically distinct. The cichlid flocks of Lake Malawi and Lake Victoria are considered to be much younger than the Tanganyikan flock, yet there is considerable convergence of morphology and correlated ecological niches between members of these flocks. The absence of synapomorphies between species and convergent morphologies makes it difficult to classify these flocks solely on morphology (Stiassny 1991). Therefore, molecular markers that are not directly related to morphology have been instrumental in helping resolve the relationships between populations and species in these complex assemblages (Kornfield and Smith 2000). For instance, mtDNA control region sequences were used to show that morphologically convergent species from Lake Tanganyika and Lake Malawi were more closely related to sympatric species than to each other. Furthermore, the mtDNA sequences supported a more recent origin of Lake Malawi cichlids than their

Tanganyikan counterparts (Kocher *et al*. 1993). Mitochondrial markers also have shown that most of the Tanganyikan genera are polyphyletic (Sturmbauer *et al*. 1994) and that multiple modes of speciation have occurred in the African cichlid flocks (reviewed in Kornfield and Smith 2000). Thus, the recent origin, species richness, and incomplete lineage sorting of the African cichlid flocks have presented great difficulty in resolving the phylogenetic relationships of these species, as well as the evolutionary processes which have produced them. Smaller species flocks and species pairs can be developed into model systems for characterizing the process of speciation which may be appropriate for explaining portions of more complex systems such as the African cichlids.

Species pairs of threespine sticklebacks (*Gasterosteus aculeatus*) from North America provide examples of powerful model systems. "Benthic" and "limnetic" species of sticklebacks found in many lakes throughout the Strait of Georgia are morphologically, behaviorally, ecologically, and genetically distinct from one another (McPhail 1992, 1994). However, the level of genetic distinction between sympatric species pairs is not the same in each lake, which suggests these species pairs are at different stages of a parallel speciation process (Taylor and McPhail 1999). Further, recent mtDNA studies have shown that the paired species of sticklebacks have evolved multiple times throughout their distribution, and that each species pair may have arisen from a separate evolutionary process (Taylor and McPhail 1999). Laboratory studies have shown that limnetic-benthic hybrids have intermediate morphologies and reduced foraging efficiencies, which suggest that divergent selection may be promoting speciation in these sticklebacks (Bentzen and McPhail 1984; Schluter 1993). Model systems such as the threespine stickleback species pairs are therefore very powerful, because morphological, ecological, behavioral, molecular, and reproductive studies can be conducted and the results compared in order to better understand not only how speciation proceeds, but also why divergence even begins.

The discovery of 3 ecomorphs of pupfish (*Cyprinodon*) in lakes on San Salvador Island, Bahamas sparked interest in developing a model system for the study of rapid divergence. The sheepshead minnow (*Cyprinodon variegatus*) is a coastal fish which ranges along the east coast of the United States from Cape Cod to the Gulf of Mexico and throughout the Bahamas. Pupfish can tolerate broad temperature and salinity ranges,

which makes them extremely efficient colonizers of Bahamian lakes and ponds (Martin 1968; Bennett and Beitinger 1997). *Cyprinodon* species generally do not occur sympatrically with one another, which is why the discovery by Dr. Grant Gilmore (Harbor Branch Oceanographic Institute, Fort Pierce, Florida) of several morphologically and possibly ecologically divergent forms of *Cyprinodon variegatus* in Little Lake, San Salvador Island was surprising (Figure 1.1). The "normal" morph was so named because it resembles typical *Cyprinodon variegatus* in morphology, while the "bulldog" and "bozo" morphs were named due to their divergence from the "normal" morphology (see Holtmeier 2000, 2001). The normal morph is found in all of the lakes on San Salvador Island as well as the coast, while the bulldog and bozo morphs are found only sympatrically with normals in Osprey Lake and Little Lake on the island. Osprey Lake and Little Lake are geographically isolated from one another (Figure 1.2), which suggests the bulldog and bozo morphs may have multiple origins on the island. The three morphs have significantly different jaw morphologies as well as divergent diets (Holtmeier 2000). Gut content analysis exhibited that normal morphs eat algae and detritus, while bulldogs prey upon small fish and consume fish scales in addition to eating algae and detritus. Bozo morphs were found to consume snails, algae, and detritus. This apparent ecological specialization has been attributed to the divergent jaw morphologies of these morphs. Further, the morphologies are maintained through two generations in captivity, and intermediate morphologies result from hybridization (Holtmeier 2000). The current data and the absence of intermediate morphologies in samples from the island suggest that there is a genetic component to these morphologies and that these morphs may represent distinct sympatric gene pools (Holtmeier 2001). Sea level was not high enough to provide hydrostatic support to sustain the lakes on San Salvador Island until about 5 000 ybp or perhaps even later (J. Mylroie, Dept. of Geosciences, Mississippi State University, Mississippi, pers. comm.), which suggests these pupfish morphs have evolved in one of the shortest recorded time spans. Other geological studies, including data on carbonate deposition (Carew and Mylroie 1997), pollen grain deposition (Pacheco and Foradas 1986), and ostracod deposition (Teeter 1995) in lakes on San Salvador further support the recent origin of these lakes (4 000 - 6 000 ybp). Therefore, the San Salvador

pupfish morphs may be the youngest example of rapid morphological and ecological divergence in sympatry.

It is evident that the rapid morphological and ecological divergence of the San Salvador pupfishes parallels that of the African cichlids and threespine sticklebacks, which makes the San Salvador system important to the study of speciation. Furthermore, the putative San Salvador flock is strikingly similar to a species flock of five endemic pupfish species that has been described from Laguna Chichancanab on the Yucatan Peninsula, Mexico. The lake has been estimated to be only 8 000 years old (Covich and Stuiver 1974), and contains a species flock that has presumably evolved from the common pupfish (*Cyprinodon artifrons*) that is found along the coast of the Yucatan Peninsula. *Cyprinodon beltrani* is the most common species in the lake and occupies the general pupfish niche of eating algae and detritus, while the other 4 species have divergent jaw morphologies and some degree of trophic specialization (Humphries and Miller 1981; Humphries 1984). Mitochondrial control region sequences have shown that the 5 endemic species are monophyletic in the lake, but only one of the species, *Cyprinodon maya*, was found to be reproductively isolated from the other endemic species. Haplotype diversity was low among the 5 species from the lake, which suggests a very recent origin of these species and coincides with geological data in suggesting that this is one of the youngest species flocks known. Common haplotypes were found in all of the endemic species except *C. maya*, which suggests incomplete lineage sorting or hybridization between the other 4 endemics (Strecker *et al.* 1996). The Laguna Chichancanab pupfish flock is a very young flock that appears to have species that are still diverging from one another in sympatry. The San Salvador Island flock strongly resembles the Chichancanab flock in that it is suspected to be very young and that the flock is comprised of a "normal" form that resembles a coastal species and several divergent forms that have morphological characteristics that are convergent with the Chichancanab flock. For instance, there is noticeable convergence of the robust jaw morphology between *Cyprinodon labiosus* from the Laguna Chichancanab flock and the bulldog morph found on San Salvador Island (Stevenson 1992). The distinct upturned jaw of the bulldog morph also resembles the nearly vertical jaw orientation of *Cyprinodon simus* from Laguna Chichancanab. The study of the putative species flock of

pupfishes on San Salvador Island, therefore is, not only important to the study of speciation, but also to the understanding of adaptation in the genus *Cyprinodon* and its apparent ability to rapidly diverge into several species in lake environments.

Investigation of mitochondrial DNA variability was used to further develop the pupfishes of San Salvador Island, Bahamas as a model system for the study of the process of speciation. The mitochondrial DNA control region ("d-loop") has exhibited genetic structure at the species and population level for *Cyprinodon* species including the Laguna Chichancanab pupfish flock (Strecker *et al*. 1996) and the Death Valley pupfishes (Duvernell and Turner 1998). Therefore, the control region was expected to be useful in assessing gene flow between diverging populations/morphs on San Salvador Island. Furthermore, the mtDNA control region is a neutral marker that is considered hypervariable (Brown 1985), which suggests that it may be one of the few markers that has accumulated "phylogenetic signal" in such a short period of time (4 000 - 6 000 years). The mtDNA control region was sequenced to estimate the amount of gene flow among the bulldog and normal pupfish morphs on the island. Samples of bozo morphs were not large enough to include in the study. My first objective was to determine the amount of genetic differentiation between allopatric normal pupfish populations that has accumulated over the 4 000 - 6 000 years that they may have been isolated from each other. The second objective was to apply the Biological Species Concept (BSC) to this system by determining the level of genetic isolation between sympatric bulldog and normal morphs from Osprey Lake and Little Lake. The San Salvador Island system is so young that determining phylogenetic relationships may be impossible, so instead, haplotype frequencies were assessed to determine if there is evidence of reproductive isolation between sympatric morphs and thus evidence that they are acting as biological species in sympatry. My final objective was to test a multiple origin hypothesis for the bulldog morphology on San Salvador island by determining whether or not the sympatric bulldog and normal populations were monophyletic in their respective lakes.

Materials and Methods

Sampling

A total of 190 *Cyprinodon variegatus* individuals were collected using minnow traps from 6 lakes on San Salvador Island (Figure 1.2). Bulldogs and normals were sampled from Little Lake and Osprey Lake, while only normals were found in the other 4 lakes. Specimens were collected in January 1998 (C. Holtmeier[1] & M. Barton[2]; [1]Dept. of Biological Sciences, DePaul University, Chicago; [2]Dept. of Biology, Centre College, Kentucky) and June 1999 (Bunt, Barton, Holtmeier). Each specimen was fixed in 100% ethanol and then labeled with a tag inserted into the operculum.

DNA extraction and PCR amplification

DNA was extracted from tail muscle tissue by phenol:chloroform extraction (Kocher *et al*., 1989) or the Puregene™ DNA Isolation Kit protocol (Gentra Systems, Minneapolis, MN). Proteinase K was used during extractions to promote cell lysis and protein digestion. Extracted DNA was resuspended in water and stored at -20^{0}C.

A portion of the mitochondrial control region ("d-loop") that extends from the threonine tRNA gene to the central conserved region was amplified using primers E (5'-CCTGAAGTAGGAACCAGATG-3') and K (5'-AGCTCAGCGCCAGAGCGCCGGTCTTGTAAA-3'). Primers E and K were designed specifically for amplification in *C. variegatus* by Michael Fisher (Department of Biology, Virginia Tech, Blacksburg, VA) using DNA fragments amplified with other fish mtDNA control region primers (Lee *et al*. 1995). MasterAmp™ PCR Premix F and MasterAmp™ DNA *Taq* Polymerase (Epicentre, Madison, WI) were used to optimize PCR amplification of this region. Standard 3-Step PCR amplification was performed in a PTC-100 Thermal Cycler (MJ Research, Inc.), with an initial denaturation step of 94^{0}C for 1 min., and 30 cycles of 94^{0}C for 20 secs., 50^{0}C for 30 secs., and 72^{0}C for 45 secs., and then a final elongation step of 72^{0}C for 5 mins. PCR products were electrophoresed through a 1.0% agarose gel in TBE buffer. DNA bands that were approximately 550 bp long were excised from the gels and cleaned using QIAquick™ Spin Columns and the

QIAquick™ Gel Extraction Kit protocol (Qiagen Inc., Valencia, CA). Portions of the cleaned PCR products were electrophoresed through 1.0% TBE agarose gels to ensure proper cleaning before sequencing.

DNA sequencing

Cleaned PCR products were sequenced on an ABI Prism 377 sequencer or an ABI Prism 310 genetic analyzer at the Virginia Tech Core Sequencing Facility and Virginia Tech Biology Department. Sequencing reaction mixtures contained 3 μl of ABI Prism Big Dye™ Terminator Cycle Sequencing Mix, 0.8 μl of primer E (2 pmol/μl), and cleaned PCR template. Reactions were performed in a PTC-100 Thermal Cycler (MJ Research, Inc.) with 25 cycles of a denaturing step of 96^0C for 30 sec., an annealing step of 50^0C for 15 sec., and an extension step of 60^0C for 4 min. Sequencing Reactions were then cleaned by an ethanol precipitation (PE Applied Biosystems, Foster City, CA) or DyeEx™ Spin Columns (Qiagen Inc., Valencia, CA) using the DyeEx™ Spin Kit protocol.

DNA sequence analysis

DNA sequences were visually edited and aligned using the EditSeq, SeqMan and MegAlign programs in the Lasergene software package (DNASTAR Inc., Madison, WI). Electropherograms also were visually edited using the EditView program (PE Applied Biosystems, Foster City, CA). Redundant sequences and invariant sites were filtered out of the sequence alignment using MacClade (Maddison and Maddison 1992). Unique DNA sequences described from the San Salvador Island pupfish were designated as haplotypes. The number of individuals possessing particular haplotypes from each normal and bulldog population was recorded.

Minimum spanning network

A Minimum Spanning Network (MSN) derived from pairwise differences of haplotypes (Excoffier *et al*. 1992; Excoffier and Smouse 1994; Bandelt *et al*. 1995) was constructed using Arlequin 2.0 output. The network was overlayed on a map of San Salvador Island to observe geographic and infer historic haplotype distributions. A haplotype frequency-based network was constructed from the original network, where the size of haplotypes in the network correlate to the frequency of those haplotypes on the island.

Inter- and intra-population differentiation analysis

Haplotype frequencies were used to assess inter- and intra-morph population genetic differentiation and diversity in Arlequin 2.0 (Schneider *et al*. 2000). Analysis of Molecular Variance (AMOVA), which compares haplotype frequencies and nucleotide differences among haplotypes between populations in a modified ANOVA, was done to assess inter- and intra-population variance from haplotype data (Excoffier *et al*. 1992). Samples were grouped based on morphology in order to include variance attributed to morphology in the AMOVA model. Variance among populations within morphs as well as the variance within populations also were assessed. Haplotype diversity ($\hat{h}$), which is the probability that 2 randomly chosen haplotypes are different, was calculated for each sample (Nei 1987). Nucleotide diversity (π), which is the probability that 2 randomly chosen homologous nucleotides are different, also was calculated for all populations (Tajima 1983; Nei 1987). Populations were compared further using Tamura and Nei's (1993) distance method that was designed specifically for the hypervariable mitochondrial control region. This model was used to compute pairwise F_{ST} values and "M" values from haplotype data. F_{ST} values are estimates of genetic isolation that are calculated by permutating haplotypes between populations. Values range from 0 to 1, where a value of 0 suggests the 2 samples are 1 panmictic population and a value of 1 suggests the 2 samples are 2 distinct populations. The significance of a given F_{ST} estimate is computed as the proportion of permutations leading to an equal or larger F_{ST}

estimate for a given comparison (Reynolds *et al*. 1983; Slatkin 1995). The number of migrants per generation (M) between populations was estimated using F_{ST} values (Slatkin 1991), and was used to assess genetic isolation of sympatric and allopatric pupfish populations. Arlequin 2.0 computed a standard error for each *p*-value calculated for F_{ST}s and M values in order to account for the loss of statistical power from multiple comparisons. An exact test of population differentiation, which tests a random distribution of haplotypes among samples using a modified Fisher's Exact test, was done using the suggested 100 000 steps in the Markov Chain with 6 000 dememorization steps (Raymond and Rousset 1995). Markov Chain methods were developed to give an unbiased probability by exploring all possible contingency tables, while keeping the number of haplotypes and sample sizes constant. The resulting *p*-value for each comparison is the probability of observing another contingency table that has an equal or lower probability than the actual data (Raymond and Rousset 1995). The total number of steps for each exact test was subdivided into batches in order to estimate a standard error of the *p*-value (Guo and Thompson 1992).

Estimation of Time for Speciation (TFS)

Intra- and Inter-morph sequence divergences were calculated from haplotype data using MEGA version 2.0 (Kumar *et al*. 2001). The maximum intramorph and minimum intermorph sequence divergences were used as upper and lower bounds, respectively (McCune and Lovejoy 1998), to estimate the amount of divergence that has occurred between bulldogs and normals. McCune and Lovejoy (1998) used this estimate for examples of allopatric speciation, but did not apply it to sympatric speciation because of the lack of intra-species divergence. Due to the level of mtDNA control region intramorph variation, the San Salvador Island pupfishes presented an opportunity to use the Time for Speciation (TFS) estimate for sympatric speciation. TFS was then calculated using the newly estimated sequence divergence between morphs, and the mtDNA control region divergence rate (3.6 ± 0.46 % per million years) calibrated in Snook by Donaldson and Wilson (1999).

Results

MtDNA variation among populations on San Salvador Island

A 357 bp portion of the mitochondrial control region was sequenced from 190 *Cyprinodon variegatus* individuals collected from 6 lakes on San Salvador Island (Figure 1.2). Invariant sites were filtered out of the analysis, which revealed 49 variable sites and defined 54 haplotypes from the island (Table 1.1; Appendix A). Sympatric bulldogs and normals from Little Lake and Osprey Lake were treated as separate populations in order to test population differentiation using haplotype data. Several haplotypes were found in all of the 8 pupfish populations sampled from San Salvador Island, while most of the populations contained several rare haplotypes (Table 1.2). Little Lake and Osprey Lake Bulldogs shared 7 out of the 54 haplotypes, which was the highest number of shared haplotypes between populations. Little Lake and Osprey Lake normals shared 4 haplotypes, while Little Lake bulldogs and Osprey Lake normals also shared 4 haplotypes. Crescent Pond normals did not share any haplotypes with any of the other pupfish populations sampled. Haplotypes 1, 7 and 27 had the greatest frequency among the populations, while the majority of the other haplotypes were very rare on the island (Table 1.3).

Haplotype diversity (Nei 1987) and nucleotide diversity (Tajima 1983; Nei 1987) were calculated to assess haplotypic composition among populations. Total haplotype diversity, which is the probability that 2 randomly chosen individuals have different haplotypes, was 0.8927±0.0150, while population values ranged from 0.9644±0.0224 in Little Lake bulldogs to 0.2092±0.1163 in Blue Hole normals (Table 1.4). Little Lake and Osprey Lake bulldogs and normals had very high haplotype diversity, while the other normal morph populations generally had much lower haplotype diversity (Table 1.4). Total nucleotide diversity was 0.006654±0.004028, and those for populations ranged from 0.009435±0.005652 in Osprey Lake bulldogs to 0.000586±0.000835 in Blue Hole normals (Table 1.4).

A Minimum Spanning Network (MSN) was constructed from the 54 haplotypes using pairwise differences in Arlequin 2.0 (Figure 1.3). Most haplotypes are separated by a single nucleotide difference, while a few haplotypes differ by 2 or more nucleotides.

Alternate connections were included in the network, because the 54 haplotypes are so closely related that the actual position of several haplotypes in the network could not be determined. Most of the haplotypes are derived from haplotypes 1 and 7 (Figure 1.3), which are also two of the most frequent haplotypes on the island (Table 1.3).

The frequencies of the 54 haplotypes on San Salvador Island were used to construct a Minimum Spanning Network where the size of a circle representing a haplotype correlates to its frequency on the island (Figure 1.4). The frequency of haplotype 1 on the island was so great that the size of the circle representing haplotype 1 had to be decreased in order to show the less frequent haplotypes. Haplotypes 1, 27, and 40 represent the most frequent haplotypes (Figure 1.4). However, haplotypes 1 and 27 are found in most of the populations sampled, while haplotype 40 is only found in the Crescent Pond sample (Table 1.2). The majority of the haplotypes on the network were rare and appear to branch off of the larger, more frequent haplotypes on the network (Figure 1.4). Several haplotypes such as haplotypes 2, 5, and 40 appear to be fairly frequent on the island (Figure 1.4), but these frequencies are attributed to their observation several times in only 1 or 2 populations sampled (Table 1.2). The haplotype frequency-based Minimum Spanning Network shows there are several haplotypes found in most populations on the island, and the majority of the other haplotypes are population-specific haplotypes.

The minimum spanning network (Figure 1.3) was overlayed on a map of San Salvador Island, which is a conventional use of haplotype networks to infer geographic differences as well as sympatric subdivision of populations (Figure 1.5). Haplotypes found in a population were shown in black on its network. Population haplotype arrays then were compared. Crescent Pond is the only population that does not contain haplotype 1. Osprey Lake and Little Lake bulldogs and normals are the only populations sampled that have haplotype 7, while Little Lake bulldogs and Osprey Lake, Blue Hole, and Reckley Pond normals have haplotype 27. Furthermore, Little Lake and Osprey Lake bulldogs have a cluster of haplotypes that is derived from haplotype 7. This haplotype cluster is not found in any of the normal populations sampled. Crescent Pond also has a cluster of haplotypes that are not found in any of the other populations sampled. Furthermore, each population sampled, except Blue Hole normals, appears to

have its own unique, star-like, array of haplotypes, which is derived from 1 or 2 common haplotypes found in all of the populations. These results suggest that the normal populations from San Salvador Island represent distinct populations, and further suggest that normals and bulldogs also represent distinct populations.

The Analysis of Molecular Variance (AMOVA) revealed that inter- and intra-morph population variation contributed considerably to genetic subdivision of the San Salvador *Cyprinodon variegatus* populations (Table 1.5). Differences between normals and bulldogs contributed to 18.7% of the molecular variance, while intra-morph variation accounted for 18.2%. The pupfish populations on San Salvador, therefore, appear to be relatively distinct from one another. The majority of the molecular variance (63.1%) was attributed to variance within populations on the island, which correlates to the relatively high level of haplotype diversity in the populations sampled (Table 1.4).

Analysis of Population Pairwise F_{ST} estimates suggested that Osprey Lake and Little Lake bulldogs are the only samples compared that do not represent 2 distinct populations (Table 1.6). F_{ST} values ranged from 0.86240 in the comparison of Blue Hole normals with Crescent Pond normals to -0.02608 (effectively zero) in the comparison of Osprey Lake bulldogs with Little Lake bulldogs. Comparisons of normals generally resulted in highly significant F_{ST} values, which suggests these lakes contain "normal" populations that have been isolated from each other long enough to accumulate significant genetic distinctiveness. Crescent Pond normals share no haplotypes with any other sample and consequently comparisons involving Crescent Pond normals had the highest F_{ST} values. The comparison between Little Lake and Osprey Lake normals resulted in a fairly low F_{ST} value (0.03205), but there was still a significant difference between the 2 samples. Several other comparisons also resulted in low, yet significant F_{ST} values, which may be attributed to the high frequency of common haplotypes throughout all the samples. Osprey Lake and Little Lake bulldogs were both found to be significantly different from all normal populations on the island. Comparisons of Osprey Lake normals to Osprey Lake and Little Lake bulldogs resulted in the lowest F_{ST} values out of all the comparisons between morphs, which suggests Osprey Lake normals have more genetic overlap with the two bulldog samples than any of the other normal populations on the island.

Analysis of the number of migrants exchanged between 2 populations per generation (M) supported the findings from F_{ST} estimates (Table 1.6). Values for M ranged from infinity in the comparison between Osprey and Little Lake bulldogs to 0.07978 from the comparison between Blue Hole and Crescent Pond normals. Several comparisons exhibited values for M that were greater than 1, which suggests some populations have recent or historic gene flow between them or at least share a recent ancestry on the island.

An Exact Test of Population Non-differentiation, which tests a null hypothesis that haplotypes are randomly distributed among populations, suggested that all normal pupfish populations on the island, as well as sympatric morphs, are distinct populations (Table 1.7). Non-differentiation Exact *p*-values ranged from 0.76968 in the comparison between bulldogs from Osprey Lake and Little Lake to 0.00000 in the majority of comparisons.

A bulldogs vs. normals minimum spanning network was constructed to assess the level of lineage sorting between these two putative species (Figure 1.6). All previous analyses exhibited significant differences between normals and bulldogs, but the bulldog populations were not found to be significantly different. Certain analyses suggested that the normals from Osprey Lake and Little Lake also were not distinct populations. Therefore, pooled samples of bulldogs and normals from Little Lake and Osprey Lake were used to construct the MSN in order to assess the potential bulldog and normal lineages. Normals and bulldogs share only 4 haplotypes and both putative lineages seem to have unique haplotype arrays (Figure 1.6). Furthermore, the majority of "normal" haplotypes are derived from haplotype 1, while "bulldog" haplotypes are derived mainly from haplotype 7. Lineage sorting between sympatric morphs therefore appears to be well underway in this system, which suggests these morphs have been reproductively isolated for some time.

The estimate of time for speciation (TFS) supported a rapid evolutionary process hypothesis for the San Salvador Island pupfish "species flock". Intermorph sequence divergence between bulldogs and normals from Osprey Lake and Little Lake ranged from 0.011±0.001 and 0.012±0.001. Intramorph divergence ranged from 0.000±0.000 to 0.026±0.008 (Figure 1.7). TFS could not be directly calculated using McCune and

Lovejoy's (1998) method, because intramorph sequence divergence was greater than intermorph divergence. Therefore, the intermorph sequence divergence (0.012 ± 0.003) and the maximum intramorph sequence divergence (0.026± 0.008) were used as lower and upper bounds, respectively, to calculate TFS using the mtDNA control region divergence rate (3.6 ± 0.46 % per million years) calibrated by Donaldson and Wilson (1999). TFS was thus estimated to be 7 200 - 3 300 years.

Discussion

Genetic distinctiveness of "normal" populations on San Salvador Island

Haplotype frequency analysis of mitochondrial control region sequences revealed that each lake contained a genetically distinct normal morph pupfish population. All populations were found to be genetically isolated from each other by F_{ST} estimates (Table 1.6). Each population was characterized by its own unique haplotype pattern on the minimum spanning network constructed for the entire island (Figure 1.5). Haplotypes 1 and 27 were found at high frequencies in all of the populations except Crescent Pond, and all other haplotypes are derived from one of these 2 common haplotypes. Older haplotypes tend to occur in the interior of minimum spanning networks and are characterized by high frequencies in populations (Crandall and Templeton 1993). Therefore, haplotypes 1 and 27 are likely ancestral haplotypes present in the founding *Cyprinodon variegatus* population(s) of San Salvador Island. During the ensuing estimated 6 000 year period of isolation, each population presumably has acquired a unique array of haplotypes derived by mutation from haplotypes 1 and 27 and modified by subsequent genetic drift.

Haplotype diversity, which is the probability that two randomly chosen individuals in a sample have different haplotypes, varied among the normal morph populations. Effective population size is positively correlated with haplotype diversity, which suggests effective population size also differs among these pupfish populations. The total haplotype diversity for pupfish populations on San Salvador was very high (0.8927±0.0150), which suggests there is a large population of pupfish on the island. High haplotype diversity (0.79±0.033) of the mtDNA control region has also been reported in coastal *Cyprinodon variegatus* from Florida (Duvernell and Turner 1998). Therefore, *Cyprinodon variegatus* may form populations with large effective sizes in lake as well as coastal environments. Furthermore, Little Lake and Osprey Lake normals, which are the only populations in the study that occur sympatrically with bulldog morphs, exhibited 2 of the highest haplotype diversities on the island (0.7065±0.0995 and 0.9016±0.0376 respectively), and likely have very high effective population sizes. All of the other normal populations, except Reckley Pond, have considerably lower haplotype

diversities, and thus likely represent pupfish populations with small effective population sizes. Therefore, effective population size, suggested by haplotype diversity, varies considerably among the normal populations on San Salvador and may be positively correlated with the occurrence of the bulldog morph.

The genetic distinctiveness of normal morph populations on San Salvador Island is surprising because of the close proximity of many of these lakes and ponds. For instance, Osprey Lake, Crescent Pond, and Reckley Pond are clustered closely together on the island (Figure 1.2), yet they contain very distinct pupfish populations. Thus, many normal morph populations on San Salvador have been geographically isolated long enough to accumulate significant differences in mtDNA control region haplotype frequencies, which suggests there are very few, if any, subsurface connections between these lakes, or if there are, that pupfish do not use them to migrate among lakes.

Reproductive isolation of normal and bulldog morphs

The mitochondrial control region provided evidence of reproductive isolation between sympatric bulldogs and normals from Osprey Lake and Little Lake. AMOVA exhibited a significant amount of variation in haplotype frequencies (18.7%; p = 0.00000±0.00000) was due to differentiation between normals and bulldogs (Table 1.5). Pairwise F_{ST}s from inter-morph comparisons were highly significant, and were similar to the average F_{ST} estimated from intra-normal morph comparisons. Sympatric bulldog and normal morph populations, therefore, represent distinct gene pools, which are also distinct from all of the other normal morph populations on the island. Further, statistical significance is maintained after Bonferroni correction (Rice 1989) of p-value s for all bulldog vs. normal comparisons. The Bonferroni correction may be overly conservative as compared to the standard errors reported for the p-values of the above analyses, but the inference that bulldogs and normals remain distinct entities even after this correction suggests there is considerable reproductive isolation between the two morphs.

The Biological Species Concept (BSC) distinguishes species primarily on their in

ability to interbreed (Mayr 1963). Species pairs and species flocks present difficult systems for application of species concepts, such as the Phylogenetic Species Concept (PSC) (Cracraft 1983), because the morphological, ecological, behavioral, and genetic divergence of their putative species is often incomplete (Taylor 1999). The BSC can be applied to these systems because it requires evidence of reproductive isolation and does not require complete lineage sorting to delineate species. The San Salvador Island pupfishes present an interesting test of the BSC, because there is evidence that sympatric bulldog and normal morphs are distinct gene pools and are, therefore, reproductively isolated from each other. Lineage sorting is incomplete in this system, because several haplotypes are shared between sympatric bulldog and normal morph populations, as well as all of the pupfish populations on the island (Figure 1.5). However, it also can not be discounted that shared haplotypes may be due to similar states of the control region in various populations, as opposed to those haplotypes being identical by decent. Bulldogs and normals from Osprey Lake and Little Lake have accumulated morph-specific mtDNA control region haplotype arrays (Figure 1.6), which suggests lineage sorting is well underway in this system. This miniature species flock is so young that ancestral haplotypes are bound to be present in both lineages, which is apparent from the several shared haplotypes between bulldogs and normals. Founder events likely explain the high frequency of these shared haplotypes (haplotypes 1 and 27) in the bulldog and normal morph populations on the island. Thus, bulldog and normal morphs share a common ancestry, but they have been reproductively isolated from each other in sympatry long enough to accumulate significantly different haplotype frequencies.

Cyprinodon variegatus has been found to readily hybridize with other *Cyprinodon* species (Wilde and Echelle 1997), which suggests that for lineage sorting to progress, that there is significant behavioral, temporal, and/or spatial reproductive isolation of the bulldog and normal *Cyprinodon variegatus* morphs on San Salvador Island. These pupfishes could have evolved allopatrically or sympatrically, but it is evident that reproductive isolation is maintaining these two lineages in sympatry. The finding that the mitochondrial control region exhibited genetic divergence between bulldog and normal morphs further supports the findings of morphological and ecological divergence

of these morphs (Holtmeier 2000), and suggests that these morphs are behaving as biological species.

Single origin of bulldog morphs on San Salvador Island

Sympatric bulldog and normal morphs from Osprey Lake and Little Lake are apparently reproductively isolated, but there is no evidence of separate origins of the bulldog morph in these two lakes. The comparison of bulldog morphs from Osprey Lake and Little Lake yielded the only non-significant pairwise F_{ST} value of all population comparisons (Table 1.6), which suggests these populations have a very recent ancestry or that there is direct gene flow between them. Furthermore, bulldogs from Osprey Lake and Little Lake share more haplotypes than any other pupfish population pairs on the island. An Exact Test of Population Non-Differentiation exhibited no significant difference between Osprey Lake and Little Lake bulldog morphs, and also showed no significant difference between the normal morphs from both of these lakes (Table 1.7). However, bulldogs from both lakes still were significantly different from both normal populations from Osprey Lake and Little Lake, which suggests there has been gene flow between similar morphs from each lake but not between divergent morphs in either lake. Therefore, bulldogs and normals from Osprey Lake and Little Lake behave as biological species, but there seems to be only a single origin of the bulldog ecomorph on San Salvador Island.

Osprey Lake and Little Lake are on opposite sides of San Salvador Island (Figure 1.2), so there has been no direct migration of morphs between these two lakes. Osprey Lake is much smaller than Little Lake, yet the large haplotype diversities found in the bulldog and normal morph populations of both lakes suggests these lakes contain very large populations of bulldog and normal morphs. The Great Lake System runs almost the entire length of San Salvador Island and is composed of very large bodies of water, such as Great Lake (Figure 1.2). Daily tidal fluctuations cause Great Lake to flood over a beach barrier into Osprey Lake (Godfrey *et al*. 1994), which is a perfect opportunity for pupfish to migrate into Osprey Lake from the Great Lake System. Little Lake is directly connected to Great Lake by a man-made channel, which is also a potential migration

route for opportunistic pupfish (Teeter 1983). Therefore, the presence of similar morphs in Osprey Lake and Little Lake is likely due to the indirect connection of these two lakes through the Great Lake System. Bulldog and normal morphs from Osprey Lake and Little Lake share haplotypes, but they also have accumulated their own unique haplotypes in their respective lakes. Therefore, there may be resident pupfish populations in each lake that accumulate "resident" haplotypes, but the connection of these two lakes to Great Lake causes migration of "cosmopolitan" haplotypes into both lakes.

Ecological and behavioral divergence between normal and bulldog morphs may explain the different level of genetic differentiation between similar morphs from Osprey Lake and Little Lake. For instance, bulldogs are piscivorous and may be roving predators that migrate long distances. *Cyprinodon variegatus* characteristically is a philopatric species, but bulldog morphs may be less dependent on substrate and, therefore, may move more freely within the Great Lake System. Therefore, bulldogs may be more likely to spread their haplotypes throughout the Great Lake System because of divergence from the normal *Cyprinodon variegatus* life history. Unfortunately, Great Lake has not been sampled for bulldogs, but the mtDNA control region data suggest that pupfish populations from Osprey Lake and Little Lake have had significant gene flow that can be explained by a Great Lake dispersal hypothesis. Human introduction cannot be discounted, but the geographical isolation of these two lakes makes it an unlikely possibility. Further, Clear Pond and Reckley Pond are approximately the same size as Osprey Lake, but both ponds have smaller haplotype diversities and theoretically smaller pupfish populations than Osprey Lake. The unexpectedly high haplotype diversity found in Osprey Lake may be due to a much larger pupfish population in this lake as compared to Reckley Pond and Clear Pond, but it may also be affected by migration of pupfish into Osprey Lake from the Great Lake System.

The bulldog morph may have evolved in the Great Lake System on San Salvador Island and has now subsequently dispersed to other lakes. Bulldogs are partly piscivorous and are likely dependent on the presence of normal morphs. Bulldog/normal hybrids have not been collected in the wild, which suggests divergent selection and coadaptation may have promoted reproductive isolation of these morphs. The evolution of a piscivorous morph may be dependent on the presence and abundance of prey, which

suggests that the bulldog morph evolved in sympatry with one or both of the only fish species on San Salvador Island, *Cyprinodon variegatus* and *Gambusia puncticulata hubbsi* (Fink 1971). The bulldog morph is much less common than the normal morph in Osprey Lake and Little Lake, which may be due to the population density of its prey, not only in the current distribution of bulldogs, but also in their lake of origin on the island. Bulldogs have been found historically in Osprey Lake and Little Lake, which are connected to the Great Lake System. This Great Lake System probably supports a very large pupfish population, which presumably could also support the evolution of a piscivorous morph. Therefore, I hypothesize that the bulldog morph evolved in sympatry with the large normal morph populations of the Great Lake System, and subsequently found its way into Osprey Lake and Little Lake.

The monophyly of bulldogs and normals in the Great Lake System (evidence from Osprey Lake and Little Lake) cannot be definitely supported, but it is suggested based on the higher percentage of shared haplotypes between these sympatric morphs as compared to the lakes that have only normal morphs. Haplotype 7 was found in normals and bulldogs from both Osprey Lake and Little Lake, but it was not found in any of the other populations sampled (Table 1.2). The lowest F_{ST} values for normal vs. bulldog comparisons were those involving normal morphs from Osprey Lake. Therefore, bulldogs and normals in Osprey Lake and the Great Lake System appear to be more closely related to each other than any other populations on the island, which suggests bulldogs did arise in this interior system of lakes. However, larger sampling efforts could reveal that haplotype 7 as well as other haplotypes are shared among all of the pupfish populations on the island at low frequencies. Further, bulldogs could have evolved allopatrically from normals and subsequently had gene flow with normals in the Great Lake System, which could explain the presence of shared haplotypes. Increased sampling and screening of nuclear markers would have to be done to test the Great Lake dispersal hypothesis for the sympatric divergence of pupfish in the interior lake system on San Salvador Island. The mitochondrial control region was highly informative in showing significant genetic differences between normals and bulldogs, but one can only speculate about the origin of bulldogs and the evolutionary process that has produced this divergent morphology.

Bulldogs have recently been found in lakes other than Osprey Lake and Little Lake (C. Holtmeier, Dept. of Biological Sciences, DePaul University, Chicago, pers. comm.), but their occurrence is most likely due to flood events or introductions and not to multiple origins of the bulldog morph. Reproductive isolation between bulldogs and normals presumably developed in sympatry, but this reproductive isolation may break down when bulldogs migrate into lakes containing normal morphs that have never been in contact with bulldogs. Therefore, these recent migrations of bulldogs into new lakes and ponds present systems to test and study the maintenance of reproductive isolation between bulldog and normal morphs outside of the original environment.

Rapid divergence of San Salvador Island pupfishes

The divergence of pupfish on San Salvador Island into two reproductively isolated ecomorphs has likely occurred in less than 6 000 years, which makes this system the most rapid example known of this evolutionary process. The estimation of time for speciation (TFS) suggests the amount of mtDNA control region sequence divergence between normals and bulldogs from Osprey Lake and Little Lake has occurred within the last 7 200 - 3 300 years. Estimating TFS using McCune and Lovejoy's (1998) method for allopatric speciation proved problematic due to the greater degree of intramorph divergence compared to intermorph divergence (Figure 1.7). McCune and Lovejoy (1998) had similar difficulties with estimating TFS for species flocks and other putative sympatric speciation events due to the youth of those systems. They also suggested that sister species with presumed sympatric origin have lower sequence divergence than sister species with an allopatric origin, which may suggest sympatric speciation in fishes could be driven by faster evolutionary processes. The mtDNA control region data from the San Salvador Island bulldogs and normals supports such a theory, because the amount of control region haplotype diversity found in this system was surprisingly large, yet the TFS estimate agrees with geological data in suggesting this species flock is very young. Therefore, the San Salvador Island pupfish species flock is a keystone system to the understanding of the pace of evolutionary processes.

In comparison, the five endemic presumptive pupfish species of the Laguna Chichancanab species flock evolved in less than 8 000 years (Covich and Stuiver 1974), although mitochondrial control region data indicated that only one of them was reproductively isolated from the other endemics (Strecker *et al*. 1996). Bulldog and normal morphs on San Salvador Island also exhibited greater haplotype diversity than any of the endemic species of the Chichancanab flock, which is surprising since the San Salvador pupfishes are putatively younger than the Chichancanab flock. High haplotype diversity also has been exhibited in *C. artifrons*, which is the proposed ancestor of the Laguna Chichancanab pupfish flock from the Yucatan Peninsula (Strecker *et al*. 1996). Therefore, effective population size of pupfishes may be very large in the San Salvador system and much smaller in the Chichancanab flock. However, the difference in haplotype diversity between these two flocks also may be due to smaller sample sizes in the study of the Chichancanab flock. The mitochondrial control region exhibited significant differences in haplotype frequencies between bulldog and normal morphs on San Salvador Island, but it was much less variable in the Chichancanab pupfish flock. There could be considerable hybridization between the 4 pupfish species of Laguna Chichancanab that were not found to be reproductively isolated from each other, which suggests the evolution of reproductive isolation may still be in progress in that system. In contrast, mitochondrial control region data supported that bulldog and normal morphs on San Salvador Island are reproductively isolated from each other and suggests that the San Salvador Island system is important for the study of rapid evolution of reproductive isolation between ecomorphs. There is additional parallelism between these two young flocks, evidenced by noticeable morphological and ecological convergence between the San Salvador bulldog morph and *Cyprinodon simus* from Laguna Chichancanab. The genus *Cyprinodon*, therefore, may tend to diverge into several morphs when given the opportunity, which makes the San Salvador and Laguna Chichancanab pupfishes invaluable model systems for study of rapid ecological, morphological, and genetic divergence.

The species pairs of threespine stickleback (*Gasterosteus aculeatus*) in many lakes throughout southwestern British Columbia are similar to the San Salvador pupfishes in that analysis of mtDNA has revealed relationships of recently evolved

sympatric species pairs. The benthic and limnetic forms of threespine sticklebacks have evolved multiple times in approximately 12 000 years (McPhail and Lindsey 1986) and exhibit levels of mtDNA haplotype diversity similar to those found in the younger San Salvador system (Taylor and McPhail 1999). However, mtDNA data supported a multiple origin hypothesis for the numerous occurrences of the threespine stickleback species pairs, but did not support for the bulldog pupfish morph on San Salvador Island. The threespine stickleback species pairs exhibit morphological, ecological, and genetic divergence that has occurred many times in their range, but AMOVA results exhibited very little genetic variation due to differences between phenotypes, whereas genetic differences between bulldog and normal morphs from San Salvador contributed significantly to the variation in the data. The multiple origins of threespine stickleback species pairs causes difficulty when trying to establish phylogenetic relationships or correlations among the benthic and limnetic forms, but it also presents a powerful system for studying how different evolutionary processes can produce convergent species pairs. The bulldog morph has apparently evolved only once on San Salvador Island. However, a bulldog-like morph has recently been collected from Lake Cunningham on New Providence Island (M Barton, Dept. of Biology, Centre College, Kentucky, per. comm.), which suggests that a parallel divergence in *C. variegatus* may be occurring throughout the Bahamian islands.

Mitochondrial DNA has been informative in the Chichancanab species flock of pupfishes, the threespine stickleback species pairs, and the San Salvador Island *C. variegatus* system. Even though reproductive isolation already may have evolved in these systems, the process of divergence is still occurring in all of them. It is difficult even for a hypervariable molecular marker like the mtDNA control region to resolve phylogenetic relationships between putative species of such recent origin. Sample size may be the important variable in determining how informative mtDNA markers can be in these young systems. For example, the mitochondrial control region and the cytochrome *b* gene were not phylogenetically informative in the *Alcolapia* species flock of cichlids from Lakes Natron and Magadi (Seegers *et al*. 1999). The 5 putative species in this flock have evolved in less than 9 000 years and are morphologically distinct, but still share several mtDNA haplotypes, which created difficulty in constructing a phylogenetic tree.

Haplotypes did not correlate with morphotypes. However, 18 haplotypes were described from 61 specimens in this cichlid flock, which suggests there is a large amount of haplotype diversity in this system. In comparison, 54 haplotypes were described from 190 pupfish specimens from the San Salvador flock, which is very similar to the haplotype diversity found in the *Alcolapia* flock.

Lineage sorting is often incomplete in young species flocks like the *Alcolapia* flock, which makes it difficult to apply species concepts that require lineage sorting. Young flocks and species pairs inevitably will have shared ancestral mtDNA haplotypes, which suggests that haplotype frequency analysis is likely to be one of the few successful ways to examine inter-species differentiation from mtDNA data. However, haplotype frequency analysis is dependent on a large sample size at each locality. The San Salvador system is an important flock, because mtDNA haplotype frequency analysis exhibited considerable differentiation of sympatric morphs and suggested these morphs are biological species. The sample size of each morph at each locality was large enough to allow haplotype frequencies to be analyzed in the San Salvador system, but sample sizes have been too small in the other young flocks to attempt this analysis. Lineage sorting is not complete in any of these young flocks, but larger sample sizes may very well show that these flocks have considerable genetic differentiation among their putative species. These analyses could potentially provide evidence that speciation in the biological sense has already occurred in several of these young systems, yet the species involved are still diverging/lineage sorting from one another and, therefore, cannot be delineated as species by many other species concepts, such as the Phylogenetic Species Concept (Cracraft 1983). Thus, these systems are extremely valuable for the study of the speciation process and how reproductive isolation, morphological divergence, and ecological divergence can begin in certain populations and also how these isolating mechanisms can be maintained as the populations diverge. The value of mtDNA markers may be underestimated in these systems due to the historically small sample sizes.

Ramifications

Haplotype frequency analysis of the mtDNA control region revealed significant differences among bulldog and normal morph populations on San Salvador Island. A

large number of mtDNA control region haplotypes were described from the island, which was unexpected due to the very young age (6 000 years) of these pupfish populations and from comparisons of mtDNA data from other *Cyprinodon* species. However, the mtDNA control region did exhibit significant differences between sympatric bulldog and normal morph populations, which suggests that reproductive isolation, in addition to the morphological and ecological divergence, has evolved rapidly in this system. The overall monophyly of sympatric morphs was not supported by the mtDNA data, but it is consistent with the number of shared haplotypes between bulldog and normal morphs from Osprey Lake and Little Lake. The mtDNA control region, therefore, was informative in this system, but there was a large number of shared haplotypes between populations that could be due to incomplete lineage sorting or hybridization. The high haplotype diversity on the island also made it difficult to construct a minimum spanning network for the island, because there were several homoplasious connections among haplotypes on the network. Therefore, there is evidence that bulldogs and normals are acting as biological species in sympatry, but other molecular markers need to be applied to this system in order to determine further the phlyogenetic relationships between these putative species. Hypervariable nuclear markers need to be applied in order to assess gene flow between populations in this extremely young system. Such future genetic studies should include exhaustive sampling of San Salvador and other Bahamian islands as well as in conjunction with further morphological, ecological, behavioral, and life-history studies.

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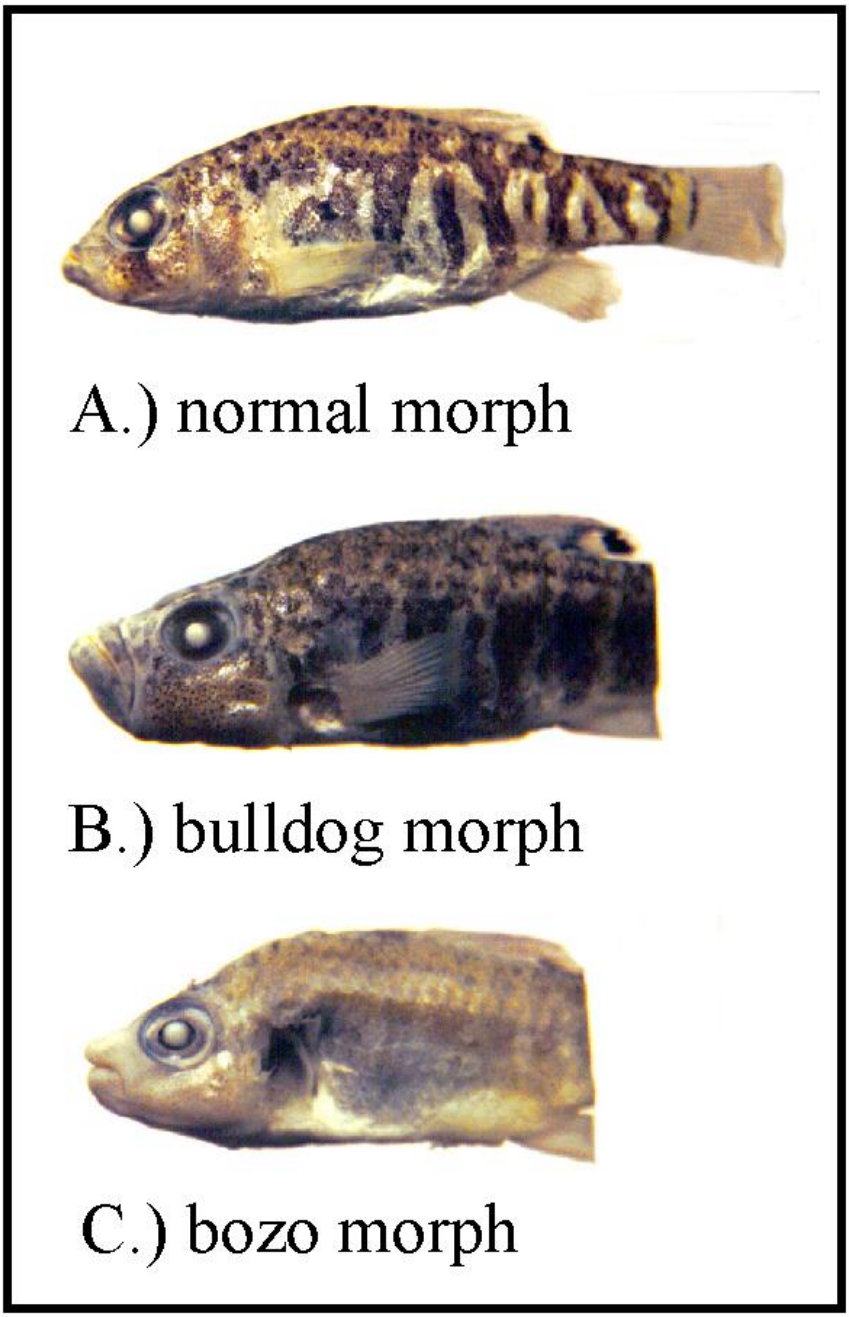

Figure 1.1: Three trophic morphs of *Cyprinodon variegatus* described from Little Lake and Osprey Lake on San Salvador Island, Bahamas.

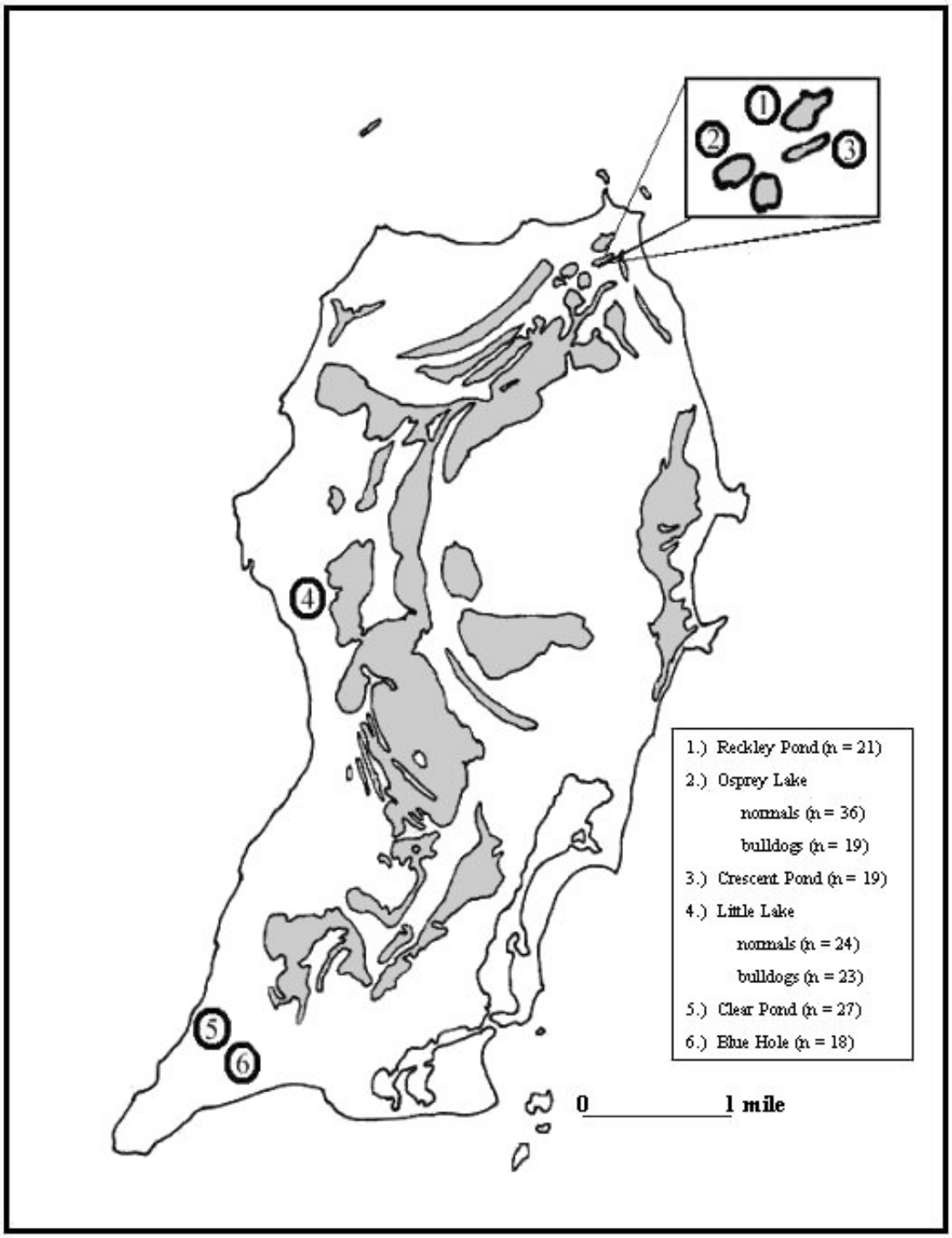

Figure 1.2: Locations of C. variegatus sampled from San Salvador Island, Bahamas. Sample sizes are denoted in parentheses.

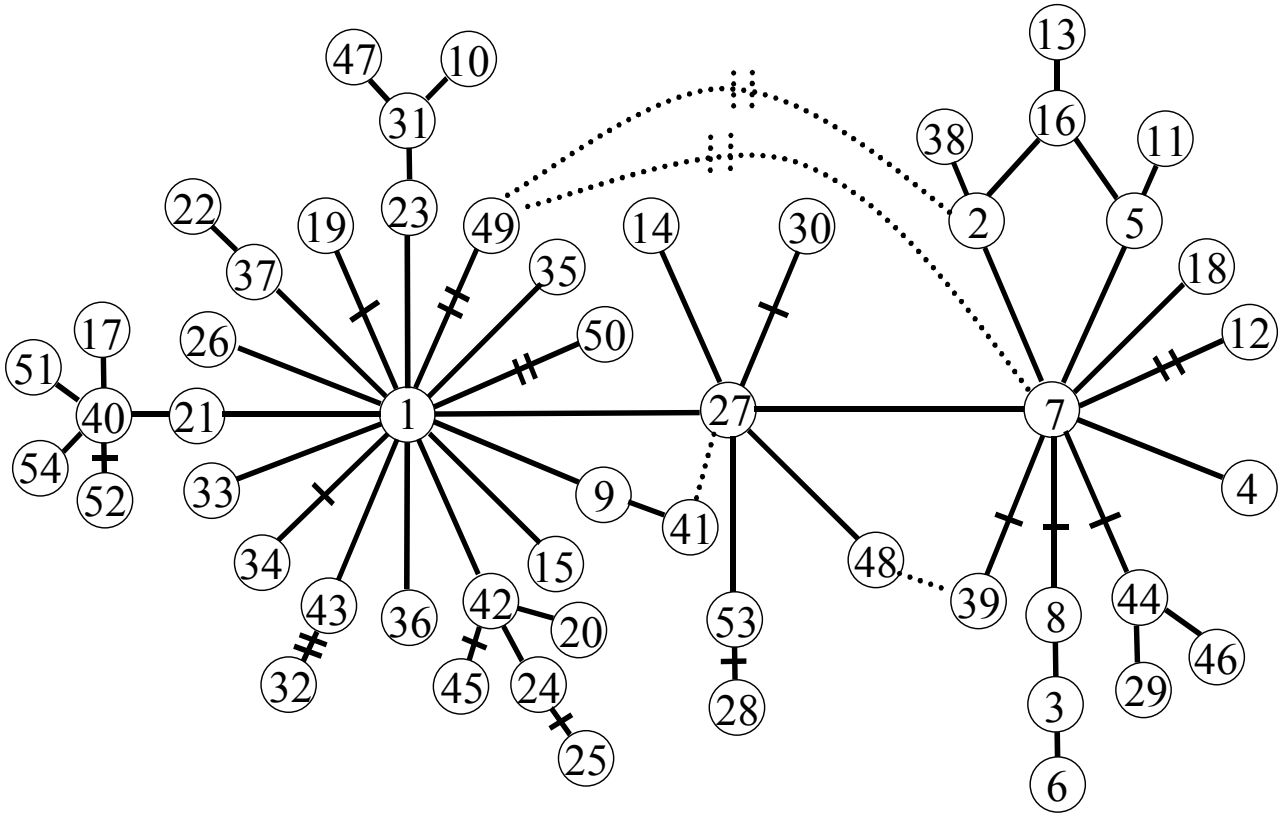

Figure 1.3: Minimum spanning network (MSN) of the 54 haplotypes described from normal and bulldog pupfish populations from San Salvador Island, Bahamas. Connections between haplotypes represent single nucleotide changes, while additional nucleotide changes are denoted by hash marks on the connections. Alternative connections between haplotypes are denoted as "* * * * ".

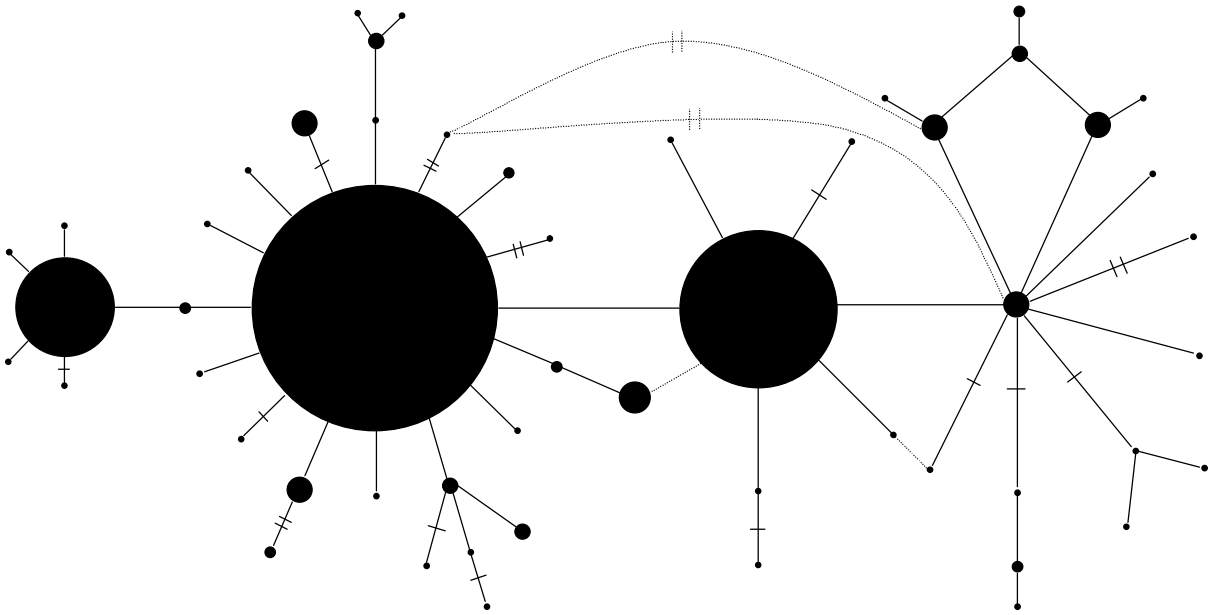

Figure 1.4: Minimum spanning network of mtDNA control region haplotypes described from C. variegatus populations. The size of each haplotype is proportional to its frequency on the island. Connections between haplotypes represent a single nucleotide change, while additional nucleotide changes are denoted as hash marks on the connections. Due to the high frequency of haplotype 1 on San Salvador, the size of haplotype 1 was made smaller than its actual frequency on the island in order to show this network.

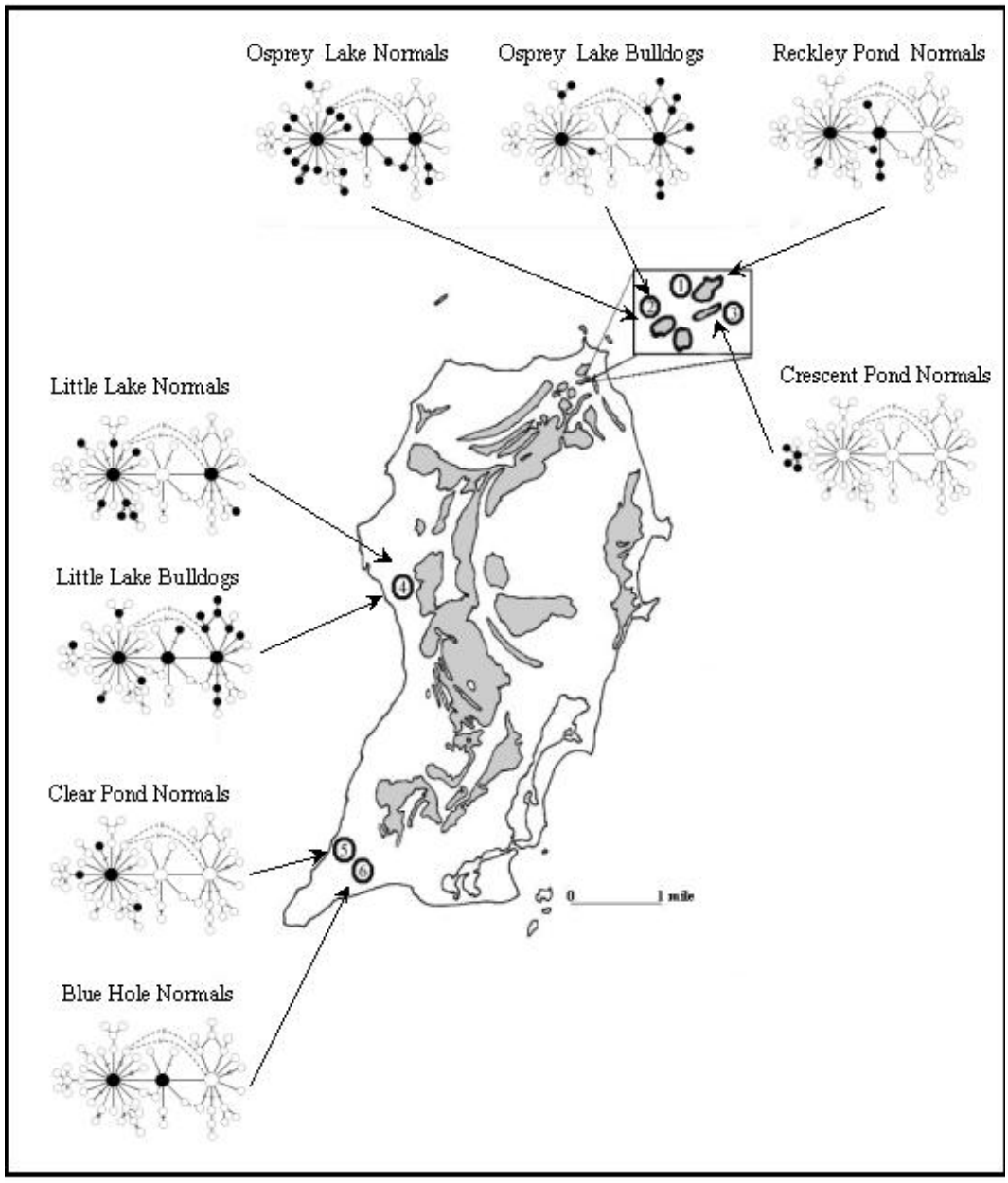

Figure 1.5: Haplotype diversity and distribution in pupfish populations on San Salvador Island are represented on the minimum spanning network for the entire island. Haplotypes described from a population are colored black, while haplotypes not found in the population are left white.

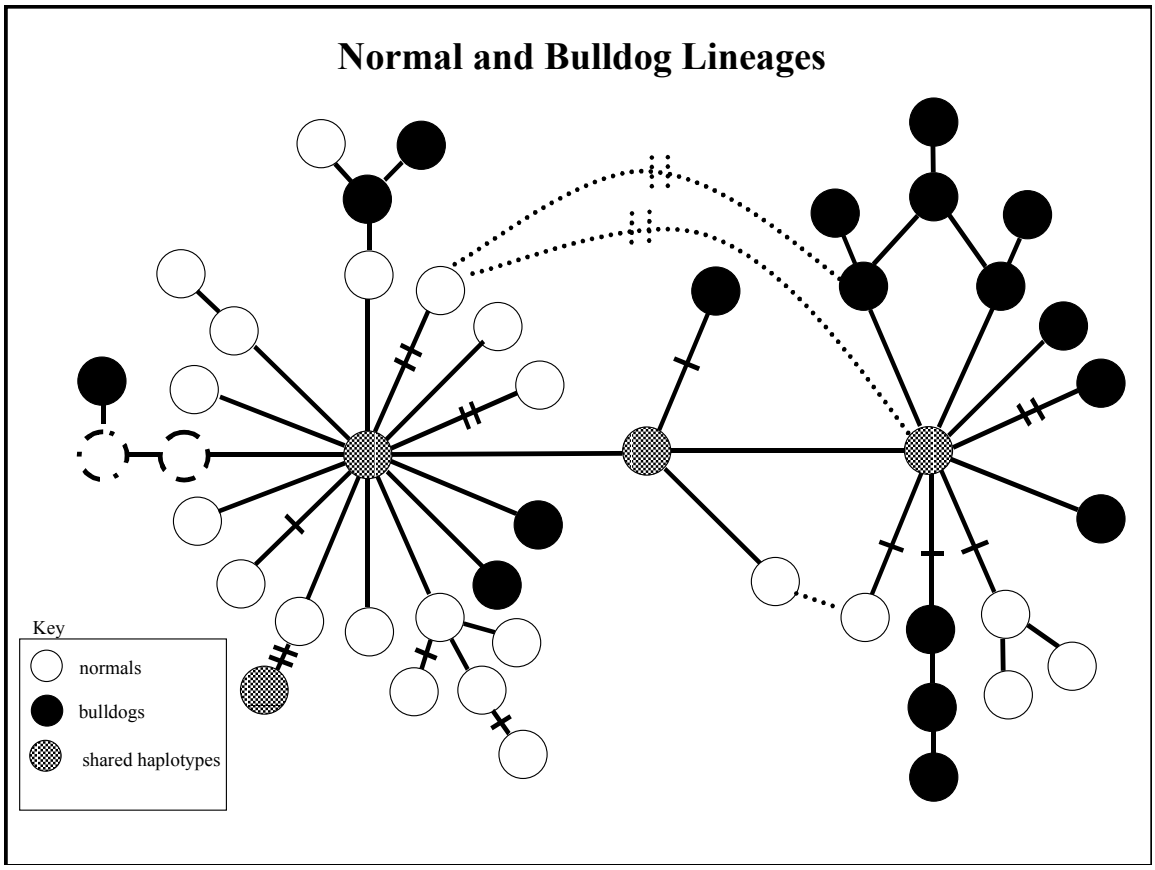

Figure1.6: Minimum Spanning Network comparison of pooled samples of bulldogs and normals from Osprey Lake and Little Lake. Haplotypes described from other lakes were removed from the MSN, while intermediate haplotypes found outside of the 2 lakes have dashed borders.

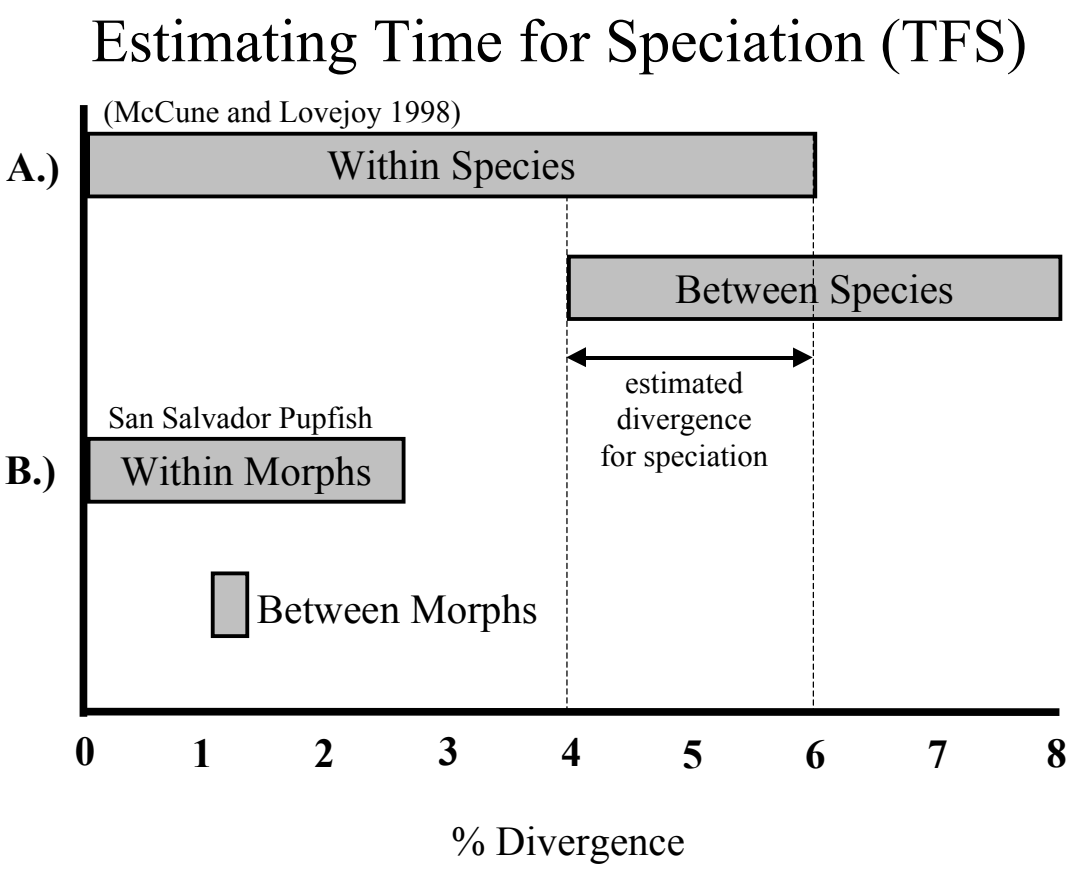

Figure 1.7: Estimation of Time for Speciation (TFS) using interspecific and intraspecific sequence divergence. A.) McCune and Lovejoy (1998) suggested TFS can be calculated using the maximum intraspecific divergence and the minimum interspecific divergence as upper and lower bounds, respectively. B.) Estimating TFS for sympatric normals and bulldogs from San Salvador was problematic because intramorph divergence had a much wider range than intermorph divergence, which suggested these morphs diverged very rapidly.

Table 1.1: Haplotypes described from normal and bulldog populations. Invariant sites were filtered out the analysis from the amplified 357 bp portion of the mitochondrial control region.

Haplotype	Polymorphic Sites
1	A C G G A A G C A T A A A A A T T A G T A G A C T T T G A A T A G T T T T A A C G C T G G A A C A
2	. . . . . A G G . . . . .
3	. . . . . A . G . . . . . C . . T . . . . G . . .
4	. . . . . G . . . . . A . G . . . . .
5	. . A . . . . . A . G . . . . .
6	. . . . . A . . . . . A . G . . . . . C . . T . . . . G . . .
7	. . . . . A . G . . . . .
8	. . . . . A . G . . . . . T . . . . G . . .
9	. . . . . G . . . . .
10	. T . . . . . G . . . . . A . . . . .
11	. . A . . . . . A . G . . . . . T . . . . .
12	. . . . . A . . . . . T . . . A . G . . . . . G . . .
13	. . A . . . . . A G G . . . . . C . . . . .
14	. . . . . T . . . A . . . . .
15	. . . . . C . . . . .
16	. . A . . . . . A G G . . . . .
17	. . . G . . . . . G . . . . .
18	. . . . . C . . . . . A . G . . . . .
19	. . . . . G . . . . . C . . . . .
20	. . . . . A . . . . . T . . . . .
21	. . . . . C . . . . .
22	. . . . . A . . . . . A . . . . .
23	. T . . . . .
24	. . . . . T . . . . A . . . .
25	. . . A . . . . . A . . . .
26	. . . . . C . . . . .
27	. . . . . A . . . . .
28	. . . . . A G . . . A . . . . .
29	. . . . . T . . . . . A . G . . . . . T . T . . . . .
30	G . . . . . C A . . . . .
31	. T . . . . . A . . . . .
32	. . . . . C . . G . . . . . G . . . . . G
33	. . . . . A .
34	. . . . . A . . . . . C . . . . .
35	. . . . . A . . . . .
36	. . . . . C . . . . .
37	. . . . . A . . . . .
38	. . . . . C . . A G G . . . . .
39	. . . . . G . . . . . A . G . G . . . . .
40	. . . . . G . . . . .
41	. . . . . G . . . . . A . . . . .
42	. . . . . T . . . . .
43	. . . . . G . . . . .
44	. . . . . T . . . . . A . G . . . . . T . . . . .
45	. . . . . C . . . . . G T . . . . .
46	. . . . . T . . . . . A . G . . . . . T . . . . G . .
47	. T . . . . . A . G . . . . .
48	. . . . . G . . . . . A . . . . .
49	. . . . . G . . . . . T G . . . . .
50	. . . . . A . . . . . C . . G . . .
51	. . . . . G . . . . . C . . . . .
52	. . . . . G . . G . . . . . A . . . . .
53	. . . . . C . . . . . A . . . . .
54	. . . . . G . . . . . A . . . . .

Table 1.2: Distribution of mtDNA control region haplotypes described from *C. variegatus* populations from lakes on San Salvador Island, Bahamas.

Haplotype	Little Lake Normals n = 24	Little Lake Bulldogs n = 23	Osprey Lake Normals n = 36	Osprey Lake Bulldogs n = 19	Clear Pond Normals n = 27	Blue Hole Normals n = 18	Crescent Pond Normals n = 22	Reckley Pond Normals n = 21
1	13	2	10	3	18	2	-	1
2	-	2	-	3	-	-	-	-
3	-	1	-	1	-	-	-	-
4	-	-	-	1	-	-	-	-
5	-	3	-	2	-	-	-	-
6	-	-	-	1	-	-	-	-
7	1	1	2	1	-	-	-	-
8	-	1	-	-	-	-	-	-
9	-	-	-	2	-	-	-	-
10	-	-	-	1	-	-	-	-
11	-	-	-	1	-	-	-	-
12	-	-	-	1	-	-	-	-
13	-	1	-	1	-	-	-	-
14	-	-	-	-	-	-	-	1
15	-	1	-	-	-	-	-	-
16	-	3	-	-	-	-	-	-
17	-	1	-	-	-	-	-	-
18	-	1	-	-	-	-	-	-
19	-	-	-	-	5	-	-	-
20	-	-	1	-	2	-	-	-
21	-	-	-	-	2	-	-	-
22	1	-	-	-	-	-	-	-
23	1	-	-	-	-	-	-	-
24	1	-	-	-	-	-	-	-
25	-	-	1	-	-	-	-	-
26	-	-	1	-	-	-	-	-
27	-	1	6	-	-	16	-	9
28	-	-	-	-	-	-	-	1
29	-	-	1	-	-	-	-	-
30	-	1	-	-	-	-	-	-
31	-	2	-	1	-	-	-	-
32	-	1	1	-	-	-	-	-
33	-	-	1	-	-	-	-	-
34	-	-	1	-	-	-	-	-
35	1	-	1	-	-	-	-	-
36	-	-	1	-	-	-	-	-
37	-	-	1	-	-	-	-	-
38	-	1	-	-	-	-	-	-
39	-	-	1	-	-	-	-	-
40	-	-	-	-	-	-	19	-
41	-	-	-	-	-	-	-	6
42	3	-	-	-	-	-	-	-
43	1	-	2	-	-	-	-	2
44	-	-	1	-	-	-	-	-
45	1	-	-	-	-	-	-	-
46	1	-	-	-	-	-	-	-
47	-	-	1	-	-	-	-	-
48	-	-	1	-	-	-	-	-
49	-	-	1	-	-	-	-	-
50	-	-	1	-	-	-	-	-
51	-	-	-	-	-	-	1	-
52	-	-	-	-	-	-	1	-
53	-	-	-	-	-	-	-	1
54	-	-	-	-	-	-	1	-

Table 1.3: Population haplotype frequencies for 54 mtDNA control region haplotypes described from C. variegatus sampled from San Salvador Island, Bahamas.

Haplotype	Little Lake Normals n = 24	Little Lake Bulldogs n = 23	Osprey Lake Normals n = 36	Osprey Lake Bulldogs n = 19	Clear Pond Normals n = 27	Blue Hole Normals n = 18	Crescent Pond Normals n = 22	Reckley Pond Normals n = 21
1	0.5417	0.0870	0.2778	0.1579	0.6667	0.1111	-	0.0476
2	-	0.0870	-	0.1579	-	-	-	-
3	-	0.0435	-	0.0526	-	-	-	-
4	-	-	-	0.0526	-	-	-	-
5	-	0.1304	-	0.1053	-	-	-	-
6	-	-	-	0.0526	-	-	-	-
7	0.0417	0.0435	0.0556	0.0526	-	-	-	-
8	-	0.0435	-	-	-	-	-	-
9	-	-	-	0.1053	-	-	-	-
10	-	-	-	0.0526	-	-	-	-
11	-	-	-	0.0526	-	-	-	-
12	-	-	-	0.0526	-	-	-	-
13	-	0.0435	-	0.0526	-	-	-	-
14	-	-	-	-	-	-	-	0.0476
15	-	0.0435	-	-	-	-	-	-
16	-	0.1304	-	-	-	-	-	-
17	-	0.0435	-	-	-	-	-	-
18	-	0.0435	-	-	-	-	-	-
19	-	-	-	-	0.1852	-	-	-
20	-	-	0.0278	-	0.0741	-	-	-
21	-	-	-	-	0.0741	-	-	-
22	0.0417	-	-	-	-	-	-	-
23	0.0417	-	-	-	-	-	-	-
24	0.0417	-	-	-	-	-	-	-
25	-	-	0.0278	-	-	-	-	-
26	-	-	0.0278	-	-	-	-	-
27	-	0.0435	0.1667	-	-	0.8889	-	0.4286
28	-	-	-	-	-	-	-	0.0476
29	-	-	0.0278	-	-	-	-	-
30	-	0.0435	-	-	-	-	-	-
31	-	0.0870	-	0.0526	-	-	-	-
32	-	0.0435	0.0278	-	-	-	-	-
33	-	-	0.0278	-	-	-	-	-
34	-	-	0.0278	-	-	-	-	-
35	0.0417	-	0.0278	-	-	-	-	-
36	-	-	0.0278	-	-	-	-	-
37	-	-	0.0278	-	-	-	-	-
38	-	0.0435	-	-	-	-	-	-
39	-	-	0.0278	-	-	-	-	-
40	-	-	-	-	-	-	0.8636	-
41	-	-	-	-	-	-	-	0.2857
42	0.1250	-	-	-	-	-	-	-
43	0.0417	-	0.0556	-	-	-	-	0.0952
44	-	-	0.0278	-	-	-	-	-
45	0.0417	-	-	-	-	-	-	-
46	0.0417	-	-	-	-	-	-	-
47	-	-	0.0278	-	-	-	-	-
48	-	-	0.0278	-	-	-	-	-
49	-	-	0.0278	-	-	-	-	-
50	-	-	0.0278	-	-	-	-	-
51	-	-	-	-	-	-	0.0455	-
52	-	-	-	-	-	-	0.0455	-
53	-	-	-	-	-	-	-	0.0476
54	-	-	-	-	-	-	0.0455	-

Table 1.4: Haplotype diversity (Nei 1987) and nucleotide diversity (Tajima 1983; Nei 1987) indices for *C. variegatus* morph population samples from San Salvador Island, Bahamas.

Population	**Total (N)**	**haplotype diversity *h* (±SE)**	**nucleotide diversity π(±SE)**
Little Lake Normals	24	0.7065(0.0995)	0.004425(0.003040)
Little Lake Bulldogs	23	0.9644(0.0224)	0.009400(0.005578)
Osprey Lake Normals	36	0.9016(0.0376)	0.006878(0.004234)
Osprey Lake Bulldogs	19	0.9532(0.0305)	0.009435(0.005652)
Clear Pond Normals	27	0.5299(0.9890)	0.002953(0.002250)
Blue Hole Normals	18	0.2092(0.1163)	0.000586(0.000835)
Crescent Pond Normals	22	0.2597(0.1202)	0.001019(0.001138)
Reckley Pond Normals	21	0.7524(0.0716)	0.003481(0.002561)
Total	190	0.8927(0.0150)	0.006654(0.004028)

Table 1.5: Analysis of Molecular Variance (AMOVA) of mitochondrial control region haplotypes to assess inter- and intra-morph variance as well as intra-population variance for San Salvador Island pupfish morphs and populations.

Source of Variation	d. f.	Sum of Squares	Variance Components	Percentage of Variation
Among Morphs	1	26.399	0.29191 Va	18.72
Among Populations Within Morphs	6	46.42	0.28374 Vb	18.2
Within Populations	182	179.035	0.98371 Vc	63.08
Total	189	251.854	1.55936	

*Va (P = 0.03617±0.000694)
*Vb (P = 0.00000±0.000000)
*Vc (P = 0.00000±0.000000)

Table 1.6: Estimated number of migrants per generation (M) and F_{ST}s estimated from mtDNA control region haplotype frequency data to assess San Salvador Island pupfish morph and population differentiation. M-values are above the diagonal, while population pairwise F_{ST}s estimated using Tamura and Nei's (1993) distance mode are below the diagonal. Non-significant F_{ST}s are in bold with an asterisk.

	Little Lake Normals	Little Lake Bulldogs	Osprey Normals	Osprey Bulldogs	Clear Pond Normals	Blue Hole Normals	Crescent Normals	Reckley Normals
Little Lake Normals		1.31597	15.09945	1.38231	9.19334	0.79298	0.48648	1.02813
Little Lake Bulldogs	0.27533		2.89003	inf	0.96084	2.28958	0.44650	2.00882
Osprey Lake Normals	0.03205	0.14749		3.10663	5.47691	3.95631	0.72794	3.37171
Osprey Lake Bulldogs	0.26563	**-0.02608 ***	0.13863		0.94275	2.00914	0.41292	2.09005
Clear Pond Normals	0.05158	0.34227	0.08366	0.34656		0.50633	0.37128	0.74507
Blue Hole Normals	0.38670	0.17924	0.11220	0.19927	0.49686		0.07978	7.20805
Crescent Normals	0.50685	0.52826	0.40719	0.54769	0.57387	0.86240		0.20763
Reckley Normals	0.32720	0.19930	0.12914	0.19305	0.40158	0.06487	0.70658	

Table 1.7: Exact tests of population differentiation p-values from mtDNA control region haplotype data. Standard error of the p-value is in parentheses. Non-significant p-values ($\alpha = 0.05$) are in bold type.

	Little Lake Normals	Little Lake Bulldogs	Osprey Normals	Osprey Bulldogs	Clear Pond Normals	Blue Hole Normals	Crescent Normals
Little Lake (B)	0.00008(0.0001)						
Osprey Lake (N)	**0.08268(0.0093)**	0.00131(0.0009)					
Osprey Lake (B)	0.00034(0.0002)	**0.76968(0.0047)**	0.00609(0.0020)				
Clear Pond	0.00194(0.0007)	0.00000(0.0000)	0.00070(0.0002)	0.00000(0.0000)			
Blue Hole	0.00000(0.0000)	0.00000(0.0000)	0.01855(0.0032)	0.00000(0.0000)	0.00000(0.0000)		
Crescent Pond	0.00000(0.0000)	0.00000(0.0000)	0.00000(0.0000)	0.00000(0.0000)	0.00000(0.0000)	0.00000(0.0000)	
Reckley Pond	0.00000(0.0000)	0.00003(0.0000)	0.00448(0.0018)	0.00000(0.0000)	0.00000(0.0000)	0.00487(0.0005)	0.00000(0.0000)

Chapter 2: Phylogeography of pupfish (*Cyprinodon*) from islands in the Bahamas: Evidence from mitochondrial DNA sequences

Abstract

As presently recognized, the sheepshead minnow (*Cyprinodon variegatus* in the broadest sense) ranges coastwide from Cape Cod, MA to the Gulf of Mexico (reaching to the mouth of the Rio Tuxpan, Mexico), throughout the Bahamas, most of the Greater Antilles (Bonaire), and Yucatan Peninsula/Belize. The Bahamian populations are of particular interest because of morphological divergence and ecological specialization that occur in certain lakes on several islands. Three sympatric trophic morphs have been described from San Salvador Island, Bahamas, and have presumably evolved in less than 4 000-6 000 years. The "normal" morph is distributed throughout the Bahamas and is morphologically and ecologically indistinguishable from mainland *Cyprinodon variegatus*. The "bulldog" and "bozo" morphs have divergent jaw morphologies and presumably consume scales and snails, respectively, in addition to the general pupfish diet of algae and detritus. Analysis of mitochondrial control region sequence data (Bunt 2001) strongly suggests that sympatric normal and bulldog morphs are reproductively isolated. The recent discovery of "bulldog-like" individuals on New Providence Island suggests that parallel divergences may be occurring on several Bahamian islands and that this system has utility as a model for a rapid evolutionary process. In the present study, the mitochondrial control region was sequenced to assess the phylogeography of Bahamian pupfish populations, while cytochrome *b* sequences were used to determine the relationship of these populations to the coastal distribution of *C. variegatus*. The control region was sequenced for normals and bulldogs sampled from San Salvador Island, and for normals collected from Exuma and New Providence Islands. Bozos and New Providence "bulldogs" were too rare to include in the study. The cyt *b* gene was sequenced for a subset of the Bahamian populations as well as from northern and southern portions of the coastal distribution of *C. variegatus*. Samples of *C. (v.) artifrons* (Belize) and *C. dearborni* (Bonaire) also were included for broader comparisons. Control region haplotypes were generally separated by only single substitutions and supports a recent origin of these Bahamian populations. Analysis of

Molecular Variance (AMOVA) revealed significant variance due to inter- and intra-island differentiation, and suggested that these populations have diverged during a short period of isolation. All haplotype frequency comparisons involving bulldogs vs. normals were highly significant, and a minimum spanning network of control region haplotypes supported a San Salvador Island origin for the bulldogs sampled. The haplotype network also revealed distinct haplotype arrays for San Salvador and New Providence Islands, which could be used in the future to test a multiple origin hypothesis for the bulldogs found on these islands. Cytochrome *b* haplotype data supported earlier findings of distinct Northern and Southern coastal lineages of *C. variegatus* and suggested the Bahamas were very recently founded by members of the southern pupfish lineage. Overall, the level of inter-island and inter-morph differentiation among Bahamian pupfish populations was surprising given the relative youth of this fauna. These data, therefore, suggest that the study of Bahamian pupfish may provide an important contribution toward understanding the pace of divergence and speciation in this species flock.

Introduction

Trophic polymorphism occurs in populations of many species of fish and may be an important process leading to speciation. For instance, lake whitefish (*Coregonus clupeaformis*) occurs as sympatric "normal" and "dwarf" morphs in many lakes throughout its distribution (Fenderson 1964; Chouinard *et al*. 1996). In addition to the considerable morphological divergence, these morphs specialize as benthic and pelagic feeders, respectively, and the presence of the dwarf morph often is negatively related to the presence of other pelagic species (reviewed in Lindsey 1981). Interestingly, the morphological divergence and ecological specialization, as well as the degree of genetic differentiation between sympatric morphs, varies among lakes (Lu and Bernatchez 1999). Certain sympatric normal and dwarf morphs also have been found to have the same mitochondrial DNA lineages, but many lakes contain normal and dwarf morphs with different mitochondrial lineages (Pigeon *et al*. 1997). It, therefore, has been suggested that the origins of many sympatric normal and dwarf morphs are due to parallel processes rather than to a single origin. Systems such as the lake whitefishes are intriguing because the numerous parallel or convergent polymorphisms/divergences can be collectively used as a model system to test evolutionary hypotheses. For instance, ecological divergence was found to be positively correlated with the degree of genetic divergence between sympatric normal and dwarf lake whitefish, which supported an ecological speciation hypothesis for the origin of these divergent morphs (Lu and Bernatchez 1999). Such a hypothesis could not be tested if there were not parallel divergences occurring in this system, which is why it is important that these systems be thoroughly studied.

Another phenomenon that occurs in many of these systems, which include species flocks and species pairs, is that trophic polymorphism is generally negatively related to species diversity (Robinson and Schluter 2000). This suggests that there may be fundamental environmental differences, such as the presence or absence of competing species, between the general range of a species and the areas in which increased trophic polymorphism and/or divergence occurs. Character release, which is the expansion of niche in the absence of competition and/or predation, has been proposed as an explanation for the morphological and ecological divergence that occurs in species that

diverge in many lakes throughout the world (Van Valen 1965; reviewed in Robinson and Schluter 2000). The limited species diversity of a newly-founded lake putatively releases the pressure of natural selection on divergent morphologies and allows the founding species to expand its niche. Diversity of habitat and the presence/absence of other species presumably promote or inhibit this process accordingly, and may explain why species flocks and species pairs occur in certain environments and not in others. Thus, the importance of other species and the diversity of habitat in lakes that now contain divergent morphs cannot be assessed without the use of systems that have parallel divergences.

A putative parallel divergence to the San Salvador Island bulldog and normal pupfish morphs (*Cyprinodon variegatus*) recently was found in Lake Cunningham on New Providence Island, Bahamas (Figure 2.1; M. Barton, Dept. of Biology, Centre College, Kentucky, *per. comm*.). The bulldogs and normals of San Salvador Island are morphologically and ecologically distinct (Holtmeier 2000; Holtmeier 2001), and mitochondrial control region sequences suggest that reproductive isolation has evolved rapidly and has been maintained between these sympatric morphs (Bunt 2001). Trophic polymorphism was likely important in the beginnings of this divergence, but it is apparent that these sympatric morphs now are separate gene pools and, therefore, are further along the speciation process than first thought. The discovery of the "bulldog-like" individuals in Lake Cunningham attracted further interest in this system, because it suggested that *Cyprinodon variegatus* may tend to diverge when provided with the opportunity in Bahamian lakes, and that similar morphs on different islands may be at different stages of a parallel divergence. The apparent absence of bulldog morphs throughout most of the range of *C. variegatus*, as well as in many Bahamian lakes, suggests that there is something unique about the lakes that contain these divergent morphs. Hypotheses, such as character release due to the lack of competition, potentially could be tested in this system using comparisons of populations in lakes with sympatric morphs to those in lakes with only the normal morph. Further, the "miniature" species flock of San Salvador Island pupfish is only 4 000-6 000 years old, which may make this system important to understanding the pace of such evolutionary processes.

The only other known pupfish species flock includes the 5 endemic species from Laguna Chichancanab on the Yucatan Peninsula. These forms apparently were derived from *Cyprinodon (v.) artifrons*, which ranges along the Yucatan coast to Belize (Humphries and Miller 1981; Strecker *et al*. 1996). This flock is only 8 000 years old and parallels the San Salvador Island system in that there is a "normal" form (*C. beltrani* - in this case it is considered a distinct species, though the evidence for this is sparse) that resembles a coastal species. The other 4 endemic species are morphologically and ecologically divergent from *C. beltrani*, which is similar to the differences between normals and bulldogs from San Salvador Island. Mitochondrial DNA sequence data also provided evidence that at least one species (*C. maya*) was reproductively isolated from the other 4 Chichancanab species (Strecker *et al*. 1996), and supports the San Salvador Island data (Bunt 2001) in suggesting that reproductive isolation can develop rather quickly between morphs in these fishes. Interestingly, the morphological divergence and ecological specialization of the Laguna Chichancanab flock has not been found in any other pupfish population on the Yucatan peninsula. This suggests there may be distinct environmental differences between Laguna Chichancanab and the Bahamas that may explain the single divergence in one system and the apparent parallel divergences occurring in the other. The study of the San Salvador Island system is, therefore, important not only to the understanding of rapid evolutionary processes, but also to the understanding of how divergence begins and proceeds in *Cyprinodon*. However, a thorough molecular examination, in concordance with life-history, behavioral, ecological, and morphological studies, of Bahamian pupfish populations is important before the total value of this model system can be realized.

The objective of this study was to further develop the Bahamian pupfish model system by using mtDNA control region and cytochrome *b* gene sequences to determine the level of differentiation among Bahamian pupfish populations and to assess the phylogeography of *Cyprinodon variegatus*. Mitochondrial genes/regions are useful in intraspecific comparisons, because they have a higher rate of substitution than the average nuclear marker (reviewed in Avise 1994). Further, mtDNA is haploid and maternally inherited, which makes it 4 times as sensitive as nuclear markers to characterize differentiation among diverging populations. The control region is a non-

coding portion of the mitochondrial genome that is considered to be the most hypervariable mitochondrial marker. It has been used to assess inter- and intra-population gene flow in the African cichlids (Parker and Kornfield 1997) as well as in many other species flocks and species pairs. The cytochrome *b* gene, on the other hand, is a functional mitochondrial gene that has a much slower rate of substitution. Furthermore, the cyt *b* gene in fishes apparently changes more slowly than in most other vertebrates (Kocher *et al.* 1989). This slower rate of substitution makes cyt *b* useful in assessing deeper phylogenetic relationships in fish species as opposed to faster evolving mitochondrial markers, such as the control region, which are generally used to assess relationships at the population level.

This study was designed to incorporate mtDNA control region and cytochrome *b* data to better understand not only the Bahamian pupfishes, but also the *Cyprindon variegatus* complex as a whole. The main objectives were to: (1) Assess the level of intra- and inter-island differentiation of Bahamian normal and bulldog pupfish populations by analyzing mtDNA control region haplotype frequencies, (2) Determine the level of support for the hypothesis that the bulldog morph of San Salvador originated on that island, (3) Use cytochrome *b* data to test the hypothesis that the Bahamian pupfishes were recently founded by members of the mainland southern *C. variegatus* lineage, and (4) Infer the overall phylogeography of the *Cyprinodon variegatus* complex using the control region and cyt *b* data.

Materials and Methods

Sample collection and DNA extraction

Pupfish were collected from San Salvador Island, New Providence Island, and Exuma Island in the Bahamas (Figure 2.2). Coastal *Cyprinodon variegatus* was sampled from various northern and southern localities (Figure 2.3), while *C. (v.) artifrons* and *C. dearborni* were collected from Twin Cays, Belize and Bonaire (Greater Antilles), respectively. Sample sizes, localities, and collection information are listed in Figure 2.3. Specimens were tagged and then fixed and stored in 95% ethanol. DNA was extracted from tail muscle tissue as described in Chapter 1.

Control region

Primers E (5'-CCTGAAGTAGGAACCAGATG-3') and K (5'-AGCTCAGCGCCAGAGCGCCGGTCTTGTAAA-3'), which were designed for *C. variegatus* from other fish mtDNA control region primers (Lee *et al*. 1995), were used to amplify and sequence the mtDNA control region using the same protocol described in Chapter 1. Sequences were visually edited and then aligned using EditSeq, SeqMan and MegAlign programs in the Lasergene software package (DNASTAR Inc., Madison, WI). The 54 San Salvador haplotypes described in Chapter 1 were aligned with sequences from New Providence and Exuma individuals using MacClade (Maddison and Maddison 1992). Redundant sequences were filtered out of the alignment, while unique sequences were designated as new haplotypes. As in the protocol described in Chapter 1, invariant sites then were filtered out of the alignment and Arlequin 2.0 (Schneider *et al*. 2000) was used to construct a minimum spanning network of the haplotypes as well as calculate F_{ST} and Exact Test *p*-values from pairwise comparisons of haplotype frequencies. The minimum spanning network was used to infer Bahamian pupfish phylogeography by assessing the proportion of shared haplotypes among San Salvador Island normals, San Salvador Island bulldogs, and New Providence Island normals. F_{ST}, which is a measure of gene flow that ranges from 0 to 1 and is calculated from haplotype frequency

differences, was estimated for all pairwise comparisons of samples. Exact Tests of Population Differentiation (Raymond and Rousset 1995), which is the probability of a random result being less likely than the observed difference in haplotype frequencies between two samples, were also done to assess the level of differentiation among these pupfish. Arlequin calculated a standard error of the *p*-value for each F_{ST} and Exact Test pairwise comparison to accommodate the loss of statistical power due to multiple comparisons. The resulting standard errors were used to assess statistical significance ($\alpha = 0.05$) for all Pairwise F_{ST} estimates and Exact Tests of Population Differentiation. Samples were also grouped by island in an Analysis of Molecular Variance (AMOVA) (Excoffier *et al*. 1992) to determine the amount of variance in the control region data that was due to inter- and intra-island differentiation.

Cytochrome *b*

The mitochondrial cytochrome *b* gene was amplfied and sequenced with primers L14841 (5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3') and H15149 (5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3') (Kocher *et al*. 1989) using the same protocol described for the control region in Chapter 1. *C. variegatus* individuals were collected along the east coast of the United States as well as San Salvador Island and New Providence Island in the Bahamas. Individuals were grouped as Northern, Southern, and Bahamas in order to assess phylogeographic patterns of cytochrome *b* haplotype distributions. Samples of *C. dearborni and C. artifrons* were included for broader comparisons and for use as outgroups. Sequences were edited and aligned using the genetics software indicated in Chapter 1. Invariant sites were filtered out of the analysis and cytrochrome *b* haplotypes were described and designated with letters in order to prevent confusion with the control region data. A minimum spanning network of cytochrome *b* haplotypes was constructed using pairwise haplotype comparison data from Arlequin 2.0 (Excoffier *et al*. 1992; Excoffier and Smouse 1994; Bandelt *et al*. 1995). The distribution of haplotypes on the network was then used to infer the phylogeography of *Cyprinodon variegatus*.

Results

MtDNA control region

Invariant sites were filtered out of the 357 bp control region sequences that were aligned for 260 individuals sampled from San Salvador Island, Exuma Island, and New Providence Island. A total of 68 haplotypes were described from 56 polymorphic sites found in the control region sequences (Table 2.1; Appendix A). The first 54 haplotypes were previously described from San Salvador Island in Chapter 1, while the other 14 were newly described from New Providence Island (Table 2.2). The Exuma Island population was the only sample in the study that had but a single haplotype, which suggests this may be a very small pupfish population that was recently founded or has had a recent bottleneck. Haplotype 27 was found on all three islands at a relatively high frequency, but haplotype 1, which is the most frequent haplotype on San Salvador Island, was not recovered from New Providence or Exuma Islands. The presence of haplotypes 7, 14, 20, and 27 in pupfish populations from San Salvador and New Providence Islands suggests these pupfish populations shared a relatively recent ancestry. However, there has still been sufficient time for these pupfish populations to accumulate a number of unique haplotypes.

Analysis of Molecular Variance (AMOVA) revealed that a significant amount of the variance in haplotype frequency data was due to differences between populations from San Salvador Island and New Providence Island/Exuma Island (Table 2.3). Differences between islands contributed to 11.5% of the molecular variance ($p \sim 0.03$), which suggests that there is little gene flow between these Bahamian islands and that there has been sufficient time for these island populations to accumulate significant differences in control region haplotype frequencies. As previously found for the San Salvador Island pupfish, there is considerable variance (22.4%) due to differences among pupfish populations on the same island ($p = 0.00000$), while the major source of variation is within populations (65.3%; $p = 0.00000$). This result was expected due to the large population sizes of pupfish in Bahamian lakes. In contrast, mtDNA control region sequence data from isolated Death Valley pupfish (*Cyprinodon*) populations exhibited

high inter-population variance and low intra-population variance (Duvernell and Turner 1998).

Haplotype frequency analyses revealed significant differences between pupfish populations from all 3 Bahamian islands. Only 4 pairwise F_{ST} comparisons yielded results that were not statistically significant (Table 2.4). As previously reported, the lowest F_{ST} value (-0.0259 ~ 0) was estimated from the comparison of bulldogs from Osprey Lake and Little Lake. However, the highest F_{ST} value (0.91914) was estimated for the comparison between Crescent Pond normals and Exuma normals, which is attributed to the low haplotype diversities of these samples and the absence of shared haplotypes between them. On the other hand, Wilson Pond normals (New Providence) were found to be indistinguishable from Blue Hole normals from San Salvador and Exuma normals, because of the unusually low haplotype diversity in these populations and shared haplotypes among them. The degree of genetic differentiation of San Salvador Island pupfish populations is evidenced by the statistical significance of all other comparisons involving these populations. This level of genetic differentiation among populations on the same island, derived from pairwise F_{ST} estimates, is not as marked on New Providence Island. Exact Tests of Population Differentiation yielded results that support the F_{ST} comparisons (Table 2.4). The Exact Test differed from the F_{ST} results only in that the comparison of Osprey Lake and Little Lake normals was insignificant ($p \sim 0.078$), while Exuma and Wilson Pond normals were found to be significantly different from each other ($p = 0.00000$).

A minimum spanning network was constructed using pairwise differences of the 68 control region haplotypes described from the Bahamian samples (Figure 2.4). Alternate haplotype connections were possible because most haplotypes are separated by a single substitution, but it is still evident that all haplotypes apparently are derived from haplotypes 1, 7 or 27. All of the haplotypes described from New Providence Island can be derived from haplotype 27, while normal and bulldog haplotypes from San Salvador Island can be derived from haplotype 1 and 7, respectively (Figure 2.5). The distinctiveness of San Salvador and New Providence pupfish populations is evident when pooled samples of San Salvador Island normals and bulldogs are compared to pooled samples of New Providence Island normals (Figure 2.6). Haplotype 27 apparently has

been important in the differentiation of haplotypes on New Providence Island, but has not been as important in the derivation of haplotypes on San Salvador Island. However, haplotypes 1 and 7 are directly derived from 27. which might suggest common ancestry of pupfish from these 2 islands as well as Exuma Island.

Molecular diversity indices differed among the Bahamian populations. Haplotype diversity, which is the probability that 2 randomly chosen individuals possess different haplotypes, ranged from 0.9644 in Little Lake bulldogs to 0.0000 in Exuma normals (Table 2.5). Total haplotype diversity was high (0.8742) and suggests that the effective sizes of most Bahamian populations are large. The relatively high haplotype diversity of Lake Cunningham and Lake Killarney normals suggests that the populations in these lakes, as well as the normal and bulldog populations of Osprey Lake and Little Lake on San Salvador Island, have very large effective sizes. Since these lakes either contain bulldogs or bulldog-like individuals, there may be a positive correlation with large effective population sizes of normal morphs and the presence of the divergent bulldog morph. Nucleotide diversity varied among the populations and was positively correlated with haplotype diversity.

MtDNA Cytochrome *b*

A 202 bp portion of the mtDNA cytochrome *b* gene was sequenced from 59 individuals collected from key locations along the range of *C. variegatus*. Nine haplotypes were described from *C. variegatus*, while samples of *C. dearborni* and *C. artifrons* had only 1 and 2 haplotypes, respectively (Table 2.6; Appendix B). There were 24 polymorphic sites found in the 202 bp sequenced, and 12 of these polymorphisms were due to the apparent divergence between *C. variegatus* and *C. artifrons*. Haplotype A was found at high frequencies in samples from San Salvador, New Providence, and Southern US, but it was not recovered in *C. variegatus* sampled from Northern states (Table 2.7). All other haplotypes described from Southern US and Bahamian samples are only one substitution from haplotype A and apparently are derived from this common haplotype, while Northern *C. variegatus* have haplotypes which are at least 3

substitutions from haplotype A (Table 2.7). Further, the Northern US sample does not share any haplotypes with other samples of *Cyprinodon variegatus*. Outgroups yielded haplotypes that ranged from barely divergent to highly divergent from the *C. variegatus* haplotypes. Haplotype D, which was described from the *C. dearborni* sample, was separated from haplotype B from the Bahamas by only a single substitution. Interestingly, the *C. dearborni* haplotype is more closely related to the Southern and Bahamian cyt *b* haplotypes than those haplotypes are to the Northern *C. variegatus* lineage. In contrast, the 2 haplotypes described from *C. artifrons* were separated from all other haplotypes by at least 15 substitutions. This suggests *C. artifrons* is distantly related to *C. variegatus* and *C. dearborni*, which apparently are related very closely to each other. Overall, the cyt *b* results support the hypothesis that pupfish only recently have colonized the Bahamas from Florida, and further support previous findings of distinct Northern and Southern *C. variegatus* lineages (Finne 2001). Furthermore, the current classification of the members of the *Cyprinodon* genus may not accurately reflect the phylogenetic relationships of these species. For instance, the Northern *C. variegatus* lineage is apparently not as closely related to the Southern lineage as is *C. dearborni*, and might merit recognition as a distinct species.

Discussion

Differentiation of Bahamian pupfishes

The mitochondrial control region exhibited significant inter- and intra-island differentiation among Bahamian pupfish populations. Total mtDNA control region haplotype diversity was very high ($\hat{h}$ = 0.8742) in this study, and suggests that the Bahamas support a very large assemblage of *Cyprinodon variegatus* (Table 2.5). Despite the apparent size of this assemblage, each lake appears to contain a unique pupfish population, as evidenced from the haplotype frequency analyses (Table 2.4). Almost every comparison yielded significant results, supporting earlier findings of the high level of differentiation of San Salvador Island pupfish populations (Bunt 2001). A few comparisons involving the Wilson Pond and Exuma samples were not found to be significant, but this is attributed to the observation that both of these populations have very low haplotype diversity and are mostly composed of haplotype 27, the most frequent haplotype in the Bahamas. However, the control region data still support the earlier findings that bulldogs from Osprey Lake and Little Lake are not distinguishable and that normals from these lakes also are related closely to each other. Lake Killarney and Lake Cunningham normals from New Providence Island also were found to be indistinguishable from each other, which is interesting because bulldog-like individuals have been found in both of these lakes. There is no direct or indirect physical connection between Lake Killarney and Lake Cunningham, as was found between Little Lake and Osprey Lake on San Salvador Island. New Providence Island has a much higher human population than San Salvador Island, and human introduction via the use of pupfish for bait may be a more likely explanation for the non-differentiation of the Lake Killarney and Lake Cunningham pupfishes. However, temporary connections between these two lakes due to tidal fluctuations cannot be discounted as an explanation for the homogeneity. All other Bahamian comparisons yielded significant results, which suggests there is a high degree of "endemism" in these pupfishes.

Intra- and inter-island differences as well as diversity within populations contributed significantly to the overall variance of the control region data (Table 2.3). The minimum spanning network comparison (Figure 2.5), which suggests each island

was founded by a unique pupfish population, may explain the inter-island variance. Haplotypes described from New Providence Island are directly derived from haplotype 27, while haplotypes described from San Salvador Island normals are generally derived from haplotype 1 and San Salvador Island bulldog haplotypes are derived from haplotype 7. Interestingly, haplotype 1 was described only from San Salvador Island samples and occurs at a relatively high frequency in these populations. On the other hand, haplotype 27 was found in samples from all three islands and at high frequency on New Providence and Exuma but at a moderate frequency on San Salvador (Table 2.2). Ancestral haplotypes have been suggested to occur at the center of haplotype networks and at relatively high frequencies in samples (Crandall and Templeton 1993). Therefore, the founding population of New Providence Island may have been represented by a high frequency of haplotype 27, while San Salvador Island was apparently colonized by a different population. However, haplotypes 1 and 7 are directly derived from 27 and may have been present in the founding populations of both islands, but have been subsequently lost or reduced to low frequencies in the New Providence Island populations because of genetic drift. Haplotype 27 appears to be the most ancestral haplotype in the Bahamas considering it was found on all three islands. All haplotypes described from the Bahamas are directly derived from the 3 core haplotypes and most are only a single mutation from other haplotypes (Figure 2.4).

The mtDNA control region data suggest that each island may have been founded by a unique pupfish population, or at least by a different subset of the population that originally founded the Bahamas. There has subsequently been sufficient time for each founding population to form distinguishable allopatric populations on its respective island. However, the amount of time available for this to occur has been constrained by sea level fluctuations, and in the case of the San Salvador Island pupfish, this time frame was at most 6 000 years (Pacheco and Foradas 1986; Teeter 1995). The recent origin of the Bahamian pupfishes also is evidenced from the multitude of alternate connections between mtDNA control region haplotypes (Figure 2.4). Alternate connections generally arise because haplotypes are so closely related that their actual relationships cannot be determined. A greater number of substitutions would have separated the haplotypes if there had been a long period of time for these populations to diverge from one another.

Further, cytochrome *b* sequence data showed virtually no haplotype diversity in the Bahamas (Table 2.7). Unlike the selectively neutral mtDNA control region, the cytochrome *b* gene has functional constraints, which causes it to accumulate mutations at a much slower rate (Kocher *et al.* 1989; Cantatore *et al.* 1994). There has not been sufficient time for phylogenetic signal to accumulate in the slower-evolving cytochrome *b* gene, but it apparently has accumulated in the control region, which further supports the hypothesis that the Bahamian pupfish assemblage is very young.

The evolution of the bulldog morphology

Samples of the bulldog morph were statistically distinguishable by mtDNA control region data from all Bahamian samples of normals. This inter-morph differentiation is quite surprising considering these populations have existed only for 4 000-6 000 years. The monophyly of San Salvador Island normals and bulldogs in regards to other Bahamian pupfish could not be unequivocally supported from the mtDNA data, but is suggested by these data. This is evidenced from the presence of haplotype 1 at high frequencies in bulldog and normal populations on San Salvador Island, but its apparent absence in samples from other islands (Table 2.5). Exact Tests of Population Non-differentiation of control region haplotype frequencies suggested that of all of the bulldog vs. normal comparisons, bulldog haplotype frequencies most closely resembled the frequencies found in the Osprey Lake normals sample (Table 2.4). This also is illustrated by the observation that the lowest F_{ST} estimate from bulldog vs. normal comparisons involved Osprey Lake normals. Even with this support for a San Salvador Island origin of bulldogs, the Great Lake System of the island, which is thought to be the place of origin of the bulldog morph, needs to be sampled to further test the monophyly of San Salvador Island normals and bulldogs. Samples of the bulldog-like individuals from New Providence Island also need to be obtained to conduct morphological, gut-content, and genetic analyses in order to compare them to the San Salvador Island bulldogs. The hypothesis that the bulldog morph has multiple origins in the Bahamas then could be tested by assessing the level of genetic differentiation of normals and

bulldogs from New Providence and San Salvador Islands. However, a test of this hypothesis could only be accomplished with a larger sample of the New Providence Island "bulldogs".

Whether bulldogs evolved in allopatry or sympatry with normals on San Salvador Island is of importance, but cannot be answered with these mtDNA data. Further, an allopatric vs. sympatric speciation model may be difficult to test if there has been any recent gene flow between sympatric normals and bulldogs, which could significantly dampen any phylogenetic signal that has accumulated and potentially result in false evidence for sympatric speciation. Instead, the intriguing aspect of this system is that the apparent reproductive isolation between normals and bulldogs has evolved very rapidly and has been maintained in this system. The discovery of bulldog-like individuals on New Providence Island (C. Holtmeier[1] & M. Barton[2]; [1]Dept. of Biological Sciences, DePaul University, Chicago; [2]Dept. of Biology, Centre College, Kentucky, *pers. comm*.) provides the potential to test hypotheses that could explain the evolution and maintenance of this reproductive isolation. Haplotype diversity of normals appears to be positively correlated with the presence of the bulldog morph, which suggests bulldogs may be found throughout the Bahamas in large lakes that support large effective populations of normals. I, therefore, hypothesized that parallel divergences are occurring throughout the Bahamas and that several Bahamian lakes may contain sympatric normals and "bulldogs". The level of morphological and ecological differentiation between these sympatric morphs may vary from lake to lake, and this heterogeneity could be used to help determine how morphological divergence, ecological specialization, and reproductive isolation evolve and how they are subsequently maintained.

Hypotheses regarding the evolutionary process that has produced the apparent morphological divergence, ecological specialization, and reproductive isolation of bulldogs could be developed from collecting and comparing life-history, behavioral, gut-content, and molecular data from normals and bulldogs. For instance, perhaps bulldogs are not very efficient piscivores, and thus small pupfish populations cannot support this divergent morph. In large lakes, on the other hand, pupfish populations could become very large so that an inefficient piscivore could survive. Alternatively, it may be that bulldogs are roving predators, which may maximize their feeding strategy. Male pupfish

are very philopatric and readily hold territories to graze as well as to attract females (Kodric-Brown 1977). A roving scale-eater would not necessarily benefit from holding the typical pupfish territory and, therefore, may tend to be less philopatric. However, this would necessitate changes in mating behavior since pupfish mate in territories held by males. Hence, the bulldog feeding strategy would be coupled with a change in reproductive strategy, which could be the reproductive isolating mechanism between normals and bulldogs, due to the requirements and/or costs of being this type of predator. Reproductive isolation, morphological divergence, and ecological specialization, therefore, may be tightly associated in this system.

The Bahamian pupfishes are similar to the Lake Whitefish (*Coregonus clupeaformis*) complex, which is composed of several assemblages of forms that have considerable morphological divergence and ecological specialization as well as temporal and spatial reproductive isolation (Lu and Bernatchez 1999). The Lake Whitefishes are interesting in that many lakes contain these divergent morphs, but the degree of differentiation between morphs varies among the lakes, which could very well be the case in the Bahamian bulldog and normal morphs. This apparent convergence in morphology coupled with the variance in the degree of genetic differentiation allowed a test of an ecological speciation hypothesis in the Lake Whitefishes. In this test, the degree of trophic specialization between sympatric "ecomorphs" from several lakes was plotted against the degree of genetic differentiation of these morphs (Lu and Bernatchez 1999). It was determined that trophic specialization was positively correlated to the degree of genetic differentiation between sympatric morphs, which suggests ecological specialization is important or is at least tightly associated with the evolution of reproductive isolation in this system. This test of the ecological speciation hypothesis could be done in the Bahamian *Cyprinodon variegatus* system by comparing the level of ecological and/or morphological divergence between sympatric bulldogs and normals to the level of genetic differentiation of these morphs. However, this would require greater sampling of San Salvador and New Providence Islands as well as other Bahamian island lakes that may very well contain bulldog-like individuals. These pupfish lineages are also very young, which may pose difficulties in finding a molecular marker that will be able to give significant resolution of the degree of genetic differentiation between these

morphs. The mtDNA control region provided considerable resolution in this regard, but there was "noise" in these data that presented problems in constructing a minimum spanning network as well as inferring phylogeography. The differentiation of these pupfish populations was very clear, but their relationship to each other was not. Therefore, it is important to find other molecular markers that may be able to resolve such questions.

Phylogeography of *Cyprinodon variegatus*

The cytochrome *b* data supported significant genetic structure between Northern and Southern *C. variegatus* lineages as well as a recent origin for the Bahamian pupfishes. All of the haplotypes described from the Northern sample were at least 3 substitutions from any other cyt *b* haplotypes described from *C. variegatus* (Table 2.6; Table 2.7). This evident differentiation between Northern and Southern pupfishes from the East Coast of the United States supports mtDNA control region data (Finne 2001). Further, haplotype A occurred at very high frequencies in the Bahamian and Southern samples, which suggests that these pupfish populations have not been separated as long as the Northern and Southern lineages have (Table 2.7). Distinct Northern and Southern mtDNA lineages also have been found in *Fundulus heteroclitus*, which is an estuarine teleost that occurs sympatrically with coastal *C. variegatus* populations (Gonzales and Powers 1996; Smith *et al*. 1998). As proposed for *F. heteroclitus*, the Northern lineage of pupfish was presumably founded by populations that occurred in glacial refugia during the last North American glaciation event, which was approximately 10 000-20 000 years ago (Smith *et al.* 1998). The Southern lineage, on the other hand, was not hindered by glacial coverage, but rather by the melting of glaciers, which caused sea level fluctuations that both hindered and promoted their dispersal. These fluctuations also affected the Bahamian islands. For instance, sea level was not high enough to provide hydrostatic support to sustain the San Salvador Island lakes until approximately 6 000 years ago (Teeter 1995; J. Mylroie, Dept. of Geosciences, Mississippi State University, Mississippi, pers. comm.). Many other Bahamian islands were influenced in the same way, which is

evidenced from lake sediment deposits that correlate with rising sea levels (reviewed in Carew and Mylroie 1997). Therefore, the cyt *b* data support the hypothesis that the Northern and Southern pupfish lineages have been separated for a long time, but the Bahamian islands were colonized only recently by a founding pupfish population that was clearly from the Southern lineage and presumably from Florida. The slower rate of substitution, as compared to the control region, of cyt *b* provided phylogenetic resolution in *C. variegatus*, because lineages could be assessed without the interference of intra-population variance in the data. On the other hand, control region data allowed an assessment of gene flow at the population level, which provided evidence of inter- and intra-population differentiation in the Bahamas. The use of multiple molecular markers in this manner is important when inferring recent and historical relationships of *C. variegatus* populations.

Understanding the Bahamian portion of the *Cyprinodon variegatus* complex

The Bahamian populations of *Cyprinodon variegatus* represent a unique assemblage in what is apparently a diverse species complex. Cytochrome *b* data suggest the Bahamian pupfish were founded by members of the Southern *C. variegatus* lineage, but it is evident that these Bahamian pupfish have a greater degree of morphological and ecological divergence than their most recent ancestors. This suggests that certain Bahamian lakes are novel environments that promote or allow morphological and ecological divergence to occur in *C. variegatus*. For instance, the occurrence of divergent pupfish morphs may be positively related to the size and area of lakes. The size and/or area of lakes is thought to be positively related to the presence of diverse habitats, and therefore trophic polymorphisms are more likely to develop in large lake environments (Smith and Skulason 1996). Therefore, ecological opportunity provided by the large interior lake system on San Salvador Island has presumably promoted the evolution of the piscivorous bulldog morph and potentially the bozo morph.

Large lake environments are not available to coastal populations of *C. variegatus*, which likely is why coastal pupfish do not diverge into "ecomorphs". The low level or

lack of interspecific competition in Bahamian lakes may also explain this phenomenon, because Bahamian pupfish do not have many endemic predators and/or competitors. Character release is the concept that niche expansion can occur and persist in a population that enters a novel or depauperate environment because of the availability of niches and the presence of diffuse interspecific competition in this new environment (Robinson and Schluter 2000; Robinson *et al*. 2000). The apparent parallelism occurring in certain Bahamian lakes could be used to determine the significance of particular environmental factors, such as lake size and interspecific competition, that may have promoted, inhibited, or simply allowed morphological and ecological divergence to occur in these pupfish populations. These environmental factors already have been tested in the pumpkinseed sunfish (*Lepomis gibbosus*), which occurs almost strictly as a littoral form, but apparently diverges into a pelagic form in the absence or reduction of interspecific competition (Robinson *et al*. 2000). Lake size and area were not found to be positively correlated to the presence of the divergent sunfish morph, but the level of interspecific competition was negatively correlated to morphological divergence in pumpkinseeds. Therefore, it is evident that environmental factors, such as the level of interspecific competition, also may have been important in promoting or inhibiting trophic polymorphism in the Bahamian pupfish. Such factors could be tested and then used to further develop hypotheses that could explain why divergence begins and proceeds in this model system.

Future research

The mitochondrial control region and cytochrome *b* gene were very informative in this system, but nuclear markers must be applied to the Bahamian pupfishes. The control region provided considerable resolution as to the genetic structure of the Bahamian pupfish populations, but the high haplotype diversity resulted in a lot of noise in the data. For example, construction of a minimum spanning network was hampered by the number of alternative connections generated. These alternate connections occurred because many of the haplotypes were very closely related to each other and, therefore,

their true connections could not be determined. This prevented the use of a nested clade analysis (Templeton 1998) of the control region haplotype data to assess and test historical vs. recent events in the phylogeography of the Bahamian pupfishes. However, the control region data provided evidence of reproductive isolation between sympatric bulldogs and normals and also showed significant structuring among populations and between islands. This miniature species flock is so young that hypervariable markers, like the control region, may be the only markers that can be used to assess gene flow in this system. The use of hypervariable nuclear markers, such as microsatellites, should be implemented in this system so that more robust tests of genetic divergence can be done. The mtDNA data provided convincing evidence and helped generate hypotheses that need to be tested using nuclear markers. However, it is critical that further sampling of San Salvador Island, including the Great Lake System, as well as other Bahamian islands is conducted. Comparing bulldogs to bulldog-like morphs from other islands is also a critical part of realizing the full potential of this model system. Samples of the bozo morph also could prove important to understanding whether character release promoted this divergence in Bahamian lakes or if some other mechanism was involved. It is quite evident that one genetic study is not sufficient to understand this system. It often takes a combination of several markers, with different mutation rates, and modes of inheritance, to understand the evolutionary processes that are involved in a system such as the Bahamian pupfishes. Behavioral, life-history, gut content analysis, observational, morphological, and other studies are needed to further the understanding of this system and the processes that have produced these morphs. The accumulation of all of these types of data will be instrumental in the classification, conservation, and understanding of the *Cyprinodon variegatus* complex.

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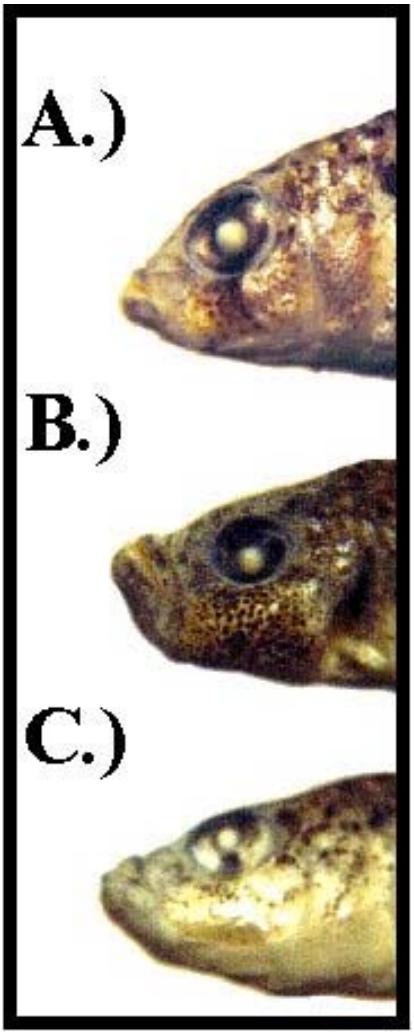


Figure 2.1: Trophic morphs of *Cyprinodon variegatus* found on Bahamian Islands. A.) normal morph (San Salvador) B.) bulldog morph (San Salvador) C.) "bulldog-like" individual (New Providence).

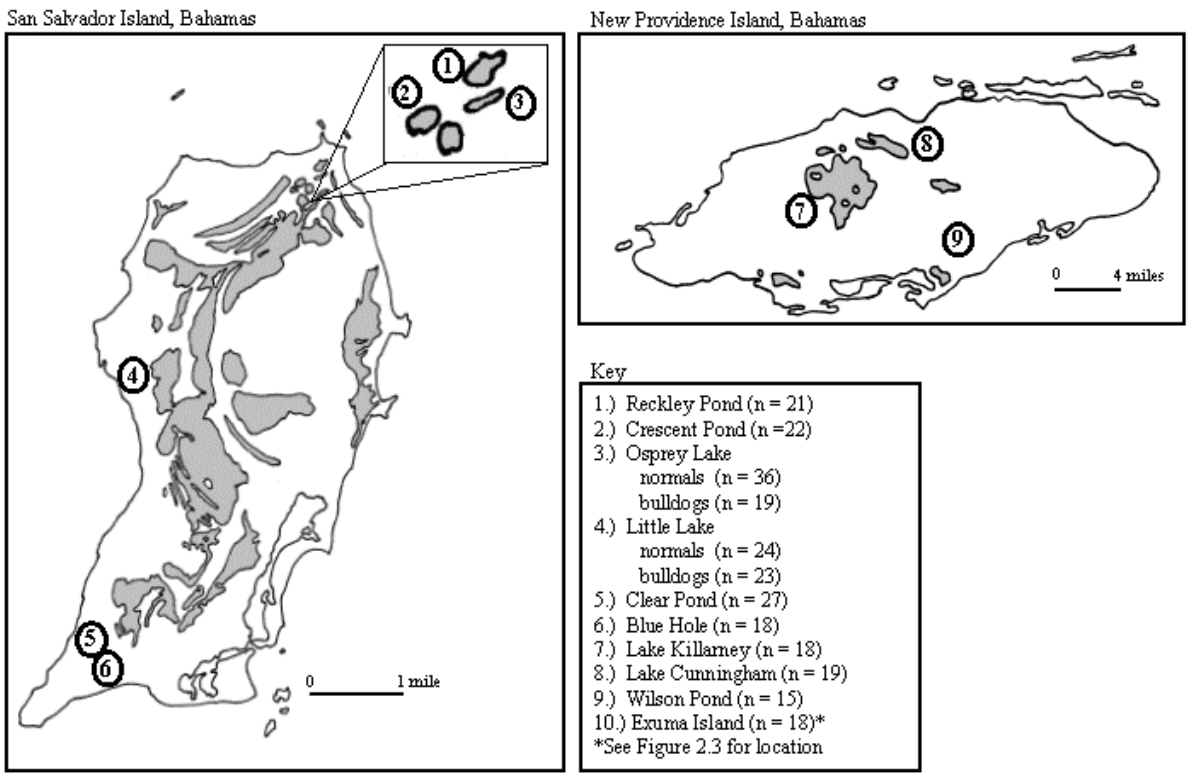
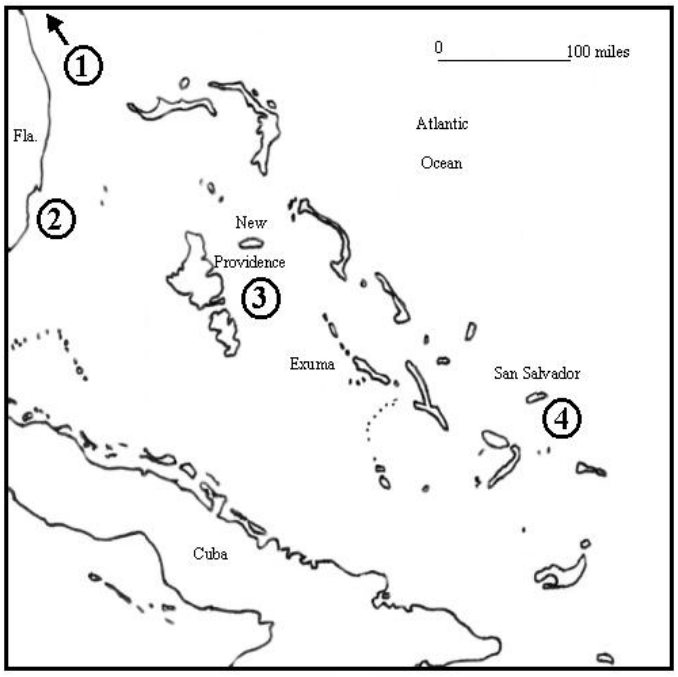


Figure 2.2: Sampling localities for Bahamian pupfish (*C. variegatus*) for mtDNA control region sequence survey.

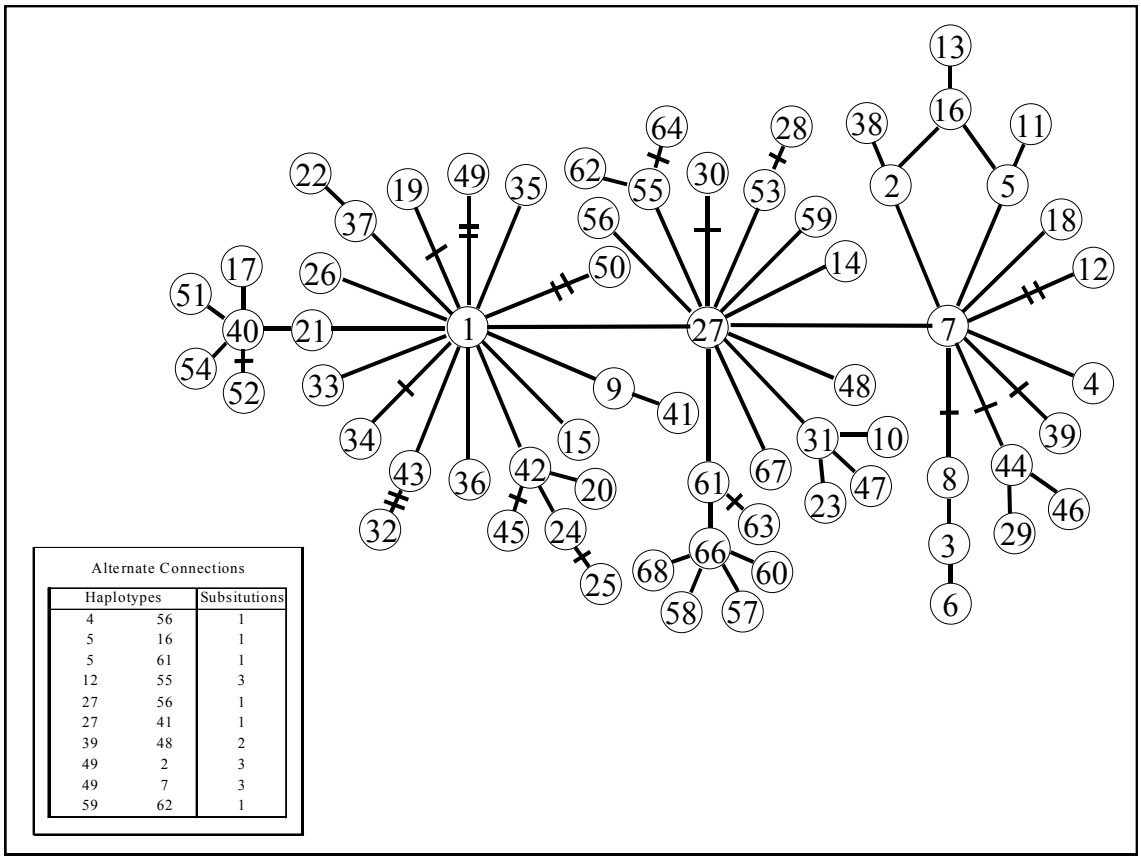
A.)



B.)

Sample	Group	Type	Location	N	Collection
1	Northern US	*Cyprinodon variegatus*	Shelter Island, NY	3	M. Stiassny Nov. 2000
			Hereford Inlet, NJ	1	K.A. Goddard 1992
			Martha's Vineyard, MA	2	B. Stallsmith 1992
2	Southern US	*Cyprinodon variegatus*	Catfish Creek, FL	2	B. Dunson Dec. 1999
			Forest St. Colvert, FL	3	J. Elder 1992
			High Island, TX	2	M. C. Wooten 1992
			Ft. Desota Park, Mullet Key, FL	1	C. Dempsey June 1990
			Sapelo Island, GA	1	J. Elder 1992
			LaFourche Parish, LA	2	B.G. Granier June 1998
3	Bahamas	normals	Lake Cunningham	7	Bunt and Barton June 1999
4	Bahamas	normals	Little Lake	8	Holtmeier & Barton Jan. 1998;
		bulldogs	Little Lake	7	Bunt, Barton, Holtmeier June 1999
		normals	Osprey Lake	9	
		bulldogs	Osprey Lake	11	
5	*Cyprinodon artifrons*		Hummingbird Pond, Twin Cays, Belize	6	B.J. Turner 2000
6	*Cyprinodon dearborni*		Canals of Carlisle Salt Works, Bonaire (Netherlands Antilles)	2	J. Scanlan 1999

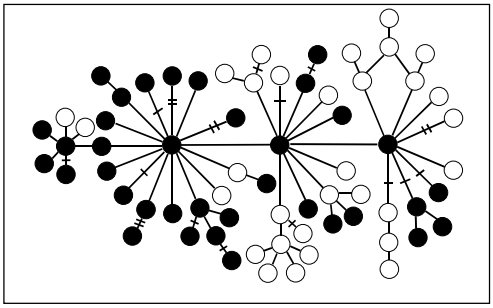
Figure 2.3: A.) Major sampling localities for *Cyprinodon variegatus* collections for mtDNA cytochrome *b* survey. B.) Sampling localities and information for samples of *C. variegatus*, *C. artifrons*, and *C. dearborni*.



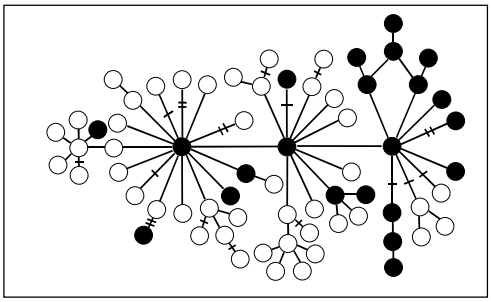
Alternate Connections

Haplotypes		Subsitutions
4	56	1
5	16	1
5	61	1
12	55	3
27	56	1
27	41	1
39	48	2
49	2	3
49	7	3
59	62	1

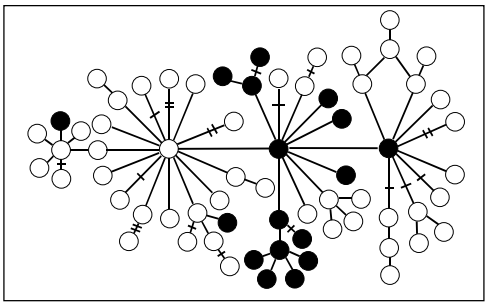
Figure 2.4: Minimum spanning network of mitochondrial control region haplotypes described from Bahamian samples of *C. variegatus*. Connections between haplotypes represent single substitutions, while additional substitutions are denoted as hash marks on the connections. Alternate connections between haplotypes were not included in the network to simplify the diagram. These connections do not significantly alter the conclusions drawn from the analysis of the network.



San Salvador normals



San Salvador bulldogs



New Providence normals

Figure 2.5: Distribution of mtDNA control region haplotypes described from samples of *Cyprinodon variegatus* from the Bahamas. Alternate connections between haplotypes were not included in the networks to simplify the diagrams, but their absence does not significantly alter conclusions drawn from these comparisons.

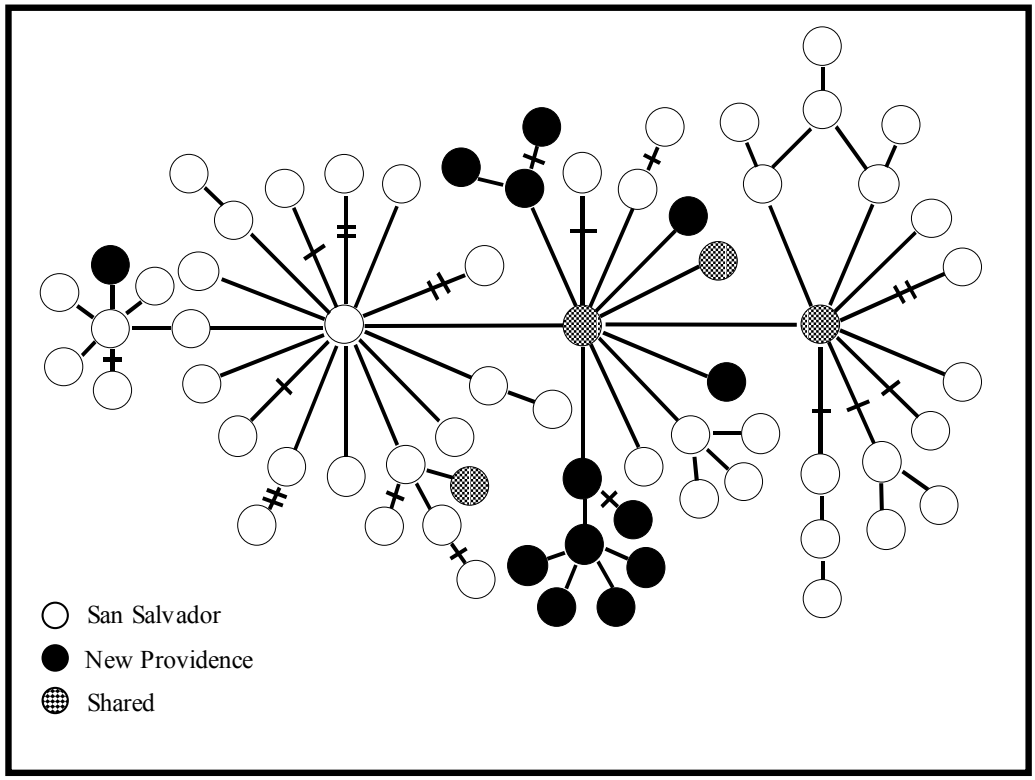


Figure 2.6: Distribution of mtDNA control region haplotypes from pooled samples of San Salvador Island and New Providence Island pupfish, *Cyprinodon variegatus*, populations.

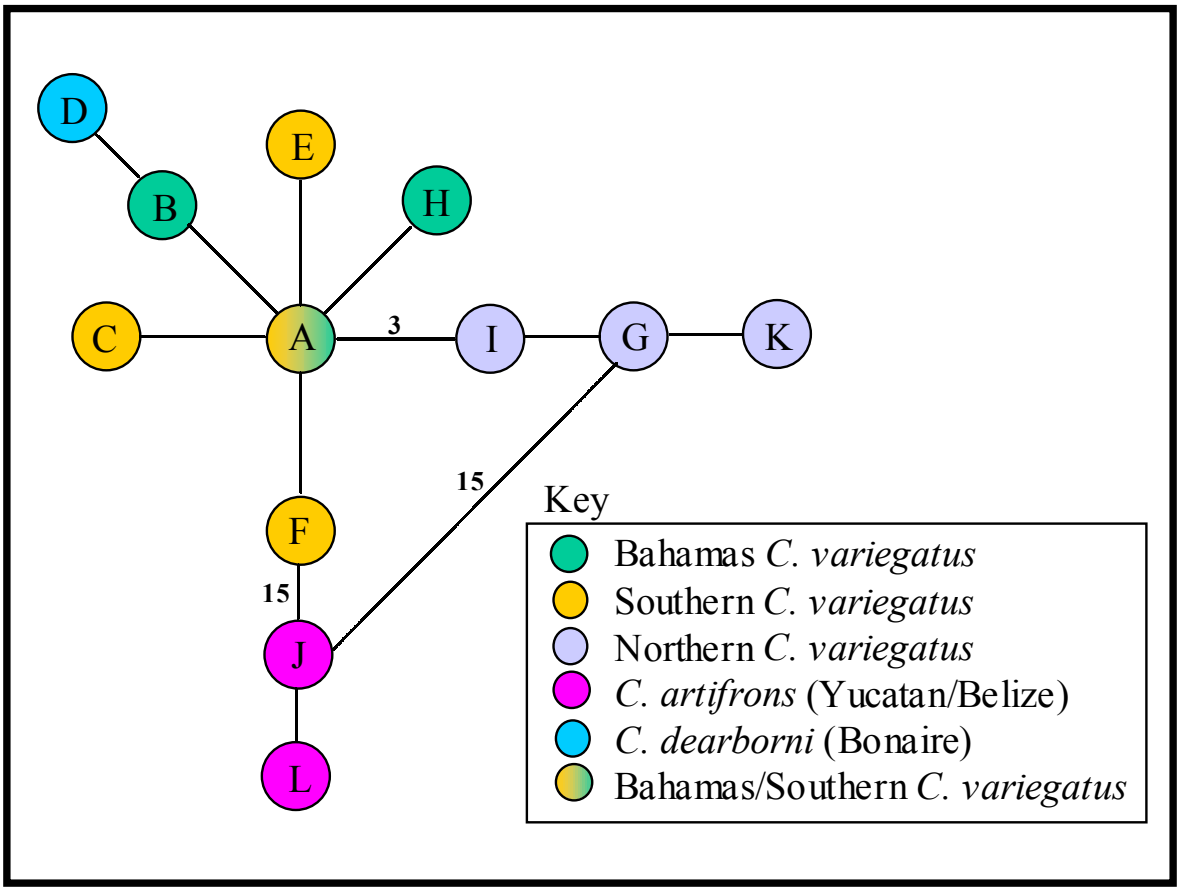


Figure 2.7: Minimum spanning network of cytochrome *b* haplotypes described from samples of *C. variegatus*, *C. artifrons*, and *C. dearborni*. Connections between haplotypes represent a single nucleotide change, while the number of substitutions is used when there is more than a single substitution separating haplotypes.

Table 2.1: Mitochondrial DNA control region haplotypes described for *C. variegatus* sampled from the Bahamas. Invariant sites were filtered out of the alignment of the 357 bp long sequences. Full haplotype sequences are listed in Appendix A.

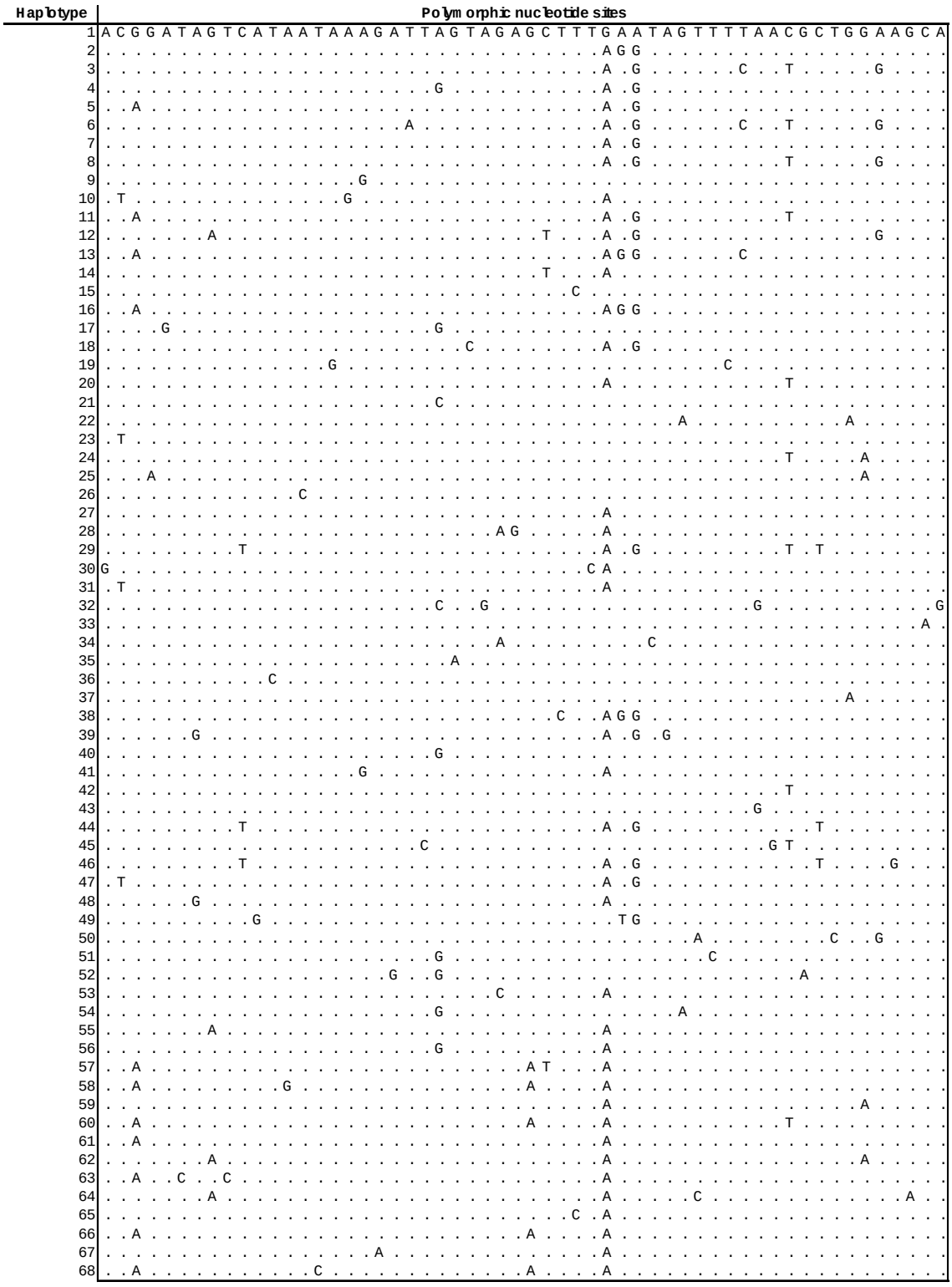


Table 2.2: Distribution of mtDNA control region haplotypes described from *Cyprinodon variegatus* from the Bahamas.

Haplotype	Little Lake Normals n = 24	Little Lake Bulldogs n = 23	Osprey Lake Normals n = 36	Osprey Lake Bulldogs n = 19	Clear Pond Normals n = 27	Blue Hole Hole Normals n = 18	Crescent Pond Normals n = 22	Reckley Pond Normals n = 21	Exuma Island Normals n = 18	Lake Cunningham Normals n = 19	Wilson Pond Normals n = 15	Lake Killarney Normals n = 18
1	13	2	10	3	18	2	-	1	-	-	-	-
2	-	2	-	3	-	-	-	-	-	-	-	-
3	-	1	-	1	-	-	-	-	-	-	-	-
4	-	-	-	1	-	-	-	-	-	-	-	-
5	-	3	-	2	-	-	-	-	-	-	-	-
6	-	-	-	1	-	-	-	-	-	-	-	-
7	1	1	2	1	-	-	-	-	-	1	-	-
8	-	1	-	-	-	-	-	-	-	-	-	-
9	-	-	-	2	-	-	-	-	-	-	-	-
10	-	-	-	1	-	-	-	-	-	-	-	-
11	-	-	-	1	-	-	-	-	-	-	-	-
12	-	-	-	1	-	-	-	-	-	-	-	-
13	-	1	-	1	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	1	-	1	-	3
15	-	1	-	-	-	-	-	-	-	-	-	-
16	-	3	-	-	-	-	-	-	-	-	-	-
17	-	1	-	-	-	-	-	-	-	-	-	-
18	-	1	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	5	-	-	-	-	-	-	-
20	-	-	1	-	2	-	-	-	-	-	1	-
21	-	-	-	-	2	-	-	-	-	-	-	-
22	1	-	-	-	-	-	-	-	-	-	-	-
23	1	-	-	-	-	-	-	-	-	-	-	-
24	1	-	-	-	-	-	-	-	-	-	-	-
25	-	-	1	-	-	-	-	-	-	-	-	-
26	-	-	1	-	-	-	-	-	-	-	-	-
27	-	1	6	-	-	16	-	9	18	4	13	8
28	-	-	-	-	-	-	-	1	-	-	-	-
29	-	-	1	-	-	-	-	-	-	-	-	-
30	-	1	-	-	-	-	-	-	-	-	-	-
31	-	2	-	1	-	-	-	-	-	-	-	-
32	-	1	1	-	-	-	-	-	-	-	-	-
33	-	-	1	-	-	-	-	-	-	-	-	-
34	-	-	1	-	-	-	-	-	-	-	-	-
35	1	-	1	-	-	-	-	-	-	-	-	-
36	-	-	1	-	-	-	-	-	-	-	-	-
37	-	-	1	-	-	-	-	-	-	-	-	-
38	-	1	-	-	-	-	-	-	-	-	-	-
39	-	-	1	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	19	-	-	-	-	-
41	-	-	-	-	-	-	-	6	-	-	-	-
42	3	-	-	-	-	-	-	-	-	-	-	-
43	1	-	2	-	-	-	-	2	-	-	-	-
44	-	-	1	-	-	-	-	-	-	-	-	-
45	1	-	-	-	-	-	-	-	-	-	-	-
46	1	-	-	-	-	-	-	-	-	-	-	-
47	-	-	1	-	-	-	-	-	-	-	-	-
48	-	-	1	-	-	-	-	-	-	-	-	-
49	-	-	1	-	-	-	-	-	-	-	-	-
50	-	-	1	-	-	-	-	-	-	-	-	-
51	-	-	-	-	-	-	1	-	-	-	-	-
52	-	-	-	-	-	-	1	-	-	-	-	-
53	-	-	-	-	-	-	-	1	-	-	-	-
54	-	-	-	-	-	-	1	-	-	-	-	-
55	-	-	-	-	-	-	-	-	-	1	-	1
56	-	-	-	-	-	-	-	-	-	-	1	-
57	-	-	-	-	-	-	-	-	-	1	-	-
58	-	-	-	-	-	-	-	-	-	-	-	1
59	-	-	-	-	-	-	-	-	-	-	-	1
60	-	-	-	-	-	-	-	-	-	2	-	-
61	-	-	-	-	-	-	-	-	-	2	-	3
62	-	-	-	-	-	-	-	-	-	1	-	-
63	-	-	-	-	-	-	-	-	-	1	-	-
64	-	-	-	-	-	-	-	-	-	1	-	-
65	-	-	-	-	-	-	-	-	-	1	-	-
66	-	-	-	-	-	-	-	-	-	1	-	1
67	-	-	-	-	-	-	-	-	-	1	-	-
68	-	-	-	-	-	-	-	-	-	1	-	-

Table 2.3: Analysis of Molecular Variance (AMOVA) of *Cyprinodon variegatus* mtDNA control region haplotype data. Samples were grouped by islands in order to assess inter- and intra-island differentiation.

Source of Variation	df	Sum of Squares	Variance Components	Percentage of Variation
Among Islands	1	22.453	0.15196 Va	11.47
Among Populations Within Islands	10	75.662	0.30820 Vb	23.25
Within Populations	248	214.561	0.86516 Vc	65.28
Total	259	312.675	1.32533	

*Va (p = 0.02737±0.00568)
*Vb (p = 0.00000 ±0.00000)
*Vc (p = 0.00000 ±0.00000)

Table 2.4: Exact Tests of Population Non-differentiation and Pairwise F_{ST} comparisons from mtDNA control region data. A.) Exact Test *p*-values are above the diagonal and F_{ST} values are below the diagonal. B.) Standard errors for Exact Test p-values. Significant *p*-values and F_{ST}s are highlighted in boxes ($\alpha = 0.05$). Populations: 1.) Little Lake normals 2.) Little Lake bulldogs 3.) Osprey Lake normals 4.) Osprey Lake bulldogs 5.) Clear Pond normals 6.) Blue Hole normals 7.) Crescent Pond normals 8.) Reckley Pond normals 9.) Exuma Island normals 10.) Lake Cunningham normals 11.) Wilson Pond normals 12.) Lake Killarney normals

A.)

	1	2	3	4	5	6	7	8	9	10	11	12
1		0.00000	0.07820	0.00000	0.00120	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
2	0.27448		0.00865	0.77235	0.00000	0.00000	0.00000	0.00000	0.00000	0.00375	0.00000	0.00000
3	0.03278	0.14591		0.00605	0.00000	0.01730	0.00000	0.00240	0.00000	0.00380	0.03745	0.00235
4	0.26256	-0.0259	0.13564		0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
5	0.05153	0.34177	0.08450	0.34363		0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
6	0.38821	0.18484	0.11446	0.20294	0.49599		0.00000	0.00690	0.00000	0.00000	0.13915	0.00035
7	0.50600	0.52515	0.40607	0.54292	0.57241	0.86152		0.00000	0.00000	0.00000	0.00000	0.00000
8	0.32684	0.20013	0.12866	0.19257	0.40027	0.06698	0.70434		0.00225	0.00635	0.00815	0.00670
9	0.47296	0.20113	0.17124	0.22503	0.58613	0.05882	0.91014	0.10195		0.00000	0.00000	0.00000
10	0.35526	0.14243	0.21170	0.15783	0.41910	0.13529	0.63374	0.15284	0.13554		0.00190	0.59550
11	0.42254	0.17336	0.15310	0.18899	0.53421	0.03031	0.85456	0.08221	0.01254	0.10819		0.00605
12	0.40300	0.16132	0.19621	0.18300	0.48032	0.11891	0.74313	0.12564	0.12247	-0.01189	0.09648	

B.)

	1	2	3	4	5	6	7	8	9	10	11	12
1		0.0000	0.0274	0.0000	0.0012	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2			0.0052	0.0160	0.0000	0.0000	0.0000	0.0000	0.0000	0.0017	0.0000	0.0000
3				0.0034	0.0000	0.0055	0.0000	0.0008	0.0000	0.0026	0.0128	0.0008
4					0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5						0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
6							0.0000	0.0030	0.0000	0.0000	0.0082	0.0004
7								0.0000	0.0000	0.0000	0.0000	0.0000
8									0.0015	0.0031	0.0032	0.0042
9										0.0000	0.0000	0.0000
10											0.0008	0.0217
11												0.0023
12												

Table 2.5: Haplotype diversity ($\hat{h}$) and nucleotide diversity (π) indices for mtDNA control region data from Bahamian pupfish, *Cyprinodon variegatus*. Standard errors are in parentheses.

Population	**Island**	**Total (N)**	**haplotype diversity (±SE)**	**nucleotide diversity (±SE)**
Little Lake normals	San Salvador	24	0.7065 (0.0995)	0.030914 (0.020785)
Little Lake bulldogs		23	0.9644 (0.0224)	0.067406 (0.039308)
Osprey Lake normals		36	0.9016 (0.0376)	0.048275 (0.029197)
Osprey Lake bulldogs		19	0.9532 (0.0305)	0.066785 (0.039392)
Clear Pond normals		27	0.5299 (0.9890)	0.019759 (0.014848)
Blue Hole normals		18	0.2092 (0.1163)	0.003860 (0.005423)
Crescent Pond normals		22	0.2597 (0.1202)	0.006872 (0.007507)
Reckley Pond normals		21	0.7524 (0.0716)	0.023650 (0.017101)
Lake Cunningham normals	New Providence	19	0.9532 (0.0358)	0.050001 (0.030869)
Wilson Pond normals		15	0.2571 (0.1416)	0.004991 (0.006353)
Lake Killarney normals		18	0.7778 (0.0835)	0.024112 (0.017495)
Exuma normals	Exuma	18	0.0000 (0.0000)	0.000000 (0.000000)
Total		242	0.8742 (0.0150)	0.039335 (0.024134)

Table 2.6: Cytochrome *b* haplotypes described from *Cyprinodon variegatus* and 2 outgroups (*C. dearborni* and *C. artifrons*). Invariant sites were filtered out of the analysis. Entire 202 bp haplotype sequences are listed in Appendix B.

Haplotype	Variant Nulceotide Sites
A	G G A T G A T A T G G G G G A T G T T G C C A A
B	. . . . A . . . .
C	. . . . . G . . . .
D	. . . . A . . . . A . . . .
E	. . . . . G . . . . .
F	. . . . . G . . . . .
G	. . . G . . . . . A . A . . A . . . .
H	. . . . . C . . . . .
I	. . . G . . . . . A . A . . . .
J	C A G C . . . G C . A A A . G . A . G A T . G G
K	. . . G . . G . . . . A . A . . A . . . . . .
L	C A G C . . . G C . A A A . G . A . G A T T G G

Table 2.7: Distribution of cytochrome *b* haplotypes described from samples of *C. variegatus, C. dearborni*, and *C. artifrons*.

			Haplotype											
Sample	**N**	**Location**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
Little Lake normals	8	San Salvador Island, Bahamas	7	1										
Little Lake bulldogs	7	San Salvador Island, Bahamas	7											
Osprey Lake normals	9	San Salvador Island, Bahamas	8							1				
Osprey Lake bulldogs	11	San Salvador Island, Bahamas	10	1										
Lake Cunningham *Cyprinodon variegatus*	7	New Providence Island, Bahamas	7											
Southern US *Cyprinodon variegatus*	11	Florida, Texas, Lousianna	8		1		1	1						
Northern US *Cyprinodon variegatus*	6	New York, New Jersey, Massachusetts							4		1		1	
Cyprinodon dearborni (outgroup)	2	Bonaire				2								
Cyprinodon artifrons (outgroup)	6	Belize										5		1

APPENDIX A

357 bp mtDNA control region haplotypes described from Cyprinodon variegatus populations. GenBank accession numbers in parentheses after haplotype titles.

HAPLOTYPE 1 (AF380435)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 2 (AF380436)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTGTGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 3 (AF380437)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCACTTATGTAGGGTTACATTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCGAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 4 (AF380438)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATGTTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 5 (AF380439)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 6 (AF380440)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATAAATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCACTTATGTAGGGTTACATTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCGAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 7 (AF380441)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCC
GGCAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 8 (AF380442)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACATTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCGAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 9 (AF380443)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTGAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 10 (AF380444)

ACTTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCGATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 11 (AF380445)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACATTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 12 (AF380446)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGATGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGATTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCGAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 13 (AF380447)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTGTGGTTG
ATAACATTATTGTGGAATTTTCACTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 14 (AF380448)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGATTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 15 (AF380449)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTCCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 16 (AF380450)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTGTGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 17 (AF380451)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAGTTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATGTTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 18 (AF380452)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
CAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 19 (AF380453)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGGTAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTCCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 20 (AF380454)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACATTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 21 (AF380455)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATCTTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 22 (AF380456)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTATGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATAGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 23 (AF380457)

ACTTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 24 (AF380458)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACATTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTACTATGAGGCCAAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 25 (AF380459)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGAAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGAGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 26 (AF380460)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GCGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 27 (AF380461)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 28 (AF380462)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAAGGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 29 (AF380463)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCTTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACATTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTATTATGGGGCCAAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 30 (AF380464)

GCCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATCAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 31 (AF380465)

ACTTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 32 (AF380466)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATCTTAGTTCAG
TGGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTGGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGGGTTAG

HAPLOTYPE 33 (AF380467)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATACTAGCTTTGGGAGTTAG

HAPLOTYPE 34 (AF380468)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAAAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ACAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 35 (AF380469)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAATTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 36 (AF380470)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACCGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 37 (AF380471)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATAGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 38 (AF380472)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGCCTTCATAATATGTATTAGTTCTGTGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 39 (AF380473)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGGCTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTGTTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 40 (AF380474)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATGTTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 41 (AF380475)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTGAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 42 (AF380476)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACATTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 43 (AF380477)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTGGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 44 (AF380478)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCTTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTATTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 45 (AF380479)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATACATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTGCATTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 46 (AF380480)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCTTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTATTATGGGGCCAGGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 47 (AF380481)

ACTTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTG
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 48 (AF380482)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGGCTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 49 (AF380483)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTGGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTTTGGTTGA
TAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 50 (AF380484)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAAATTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTACGGGGCCGAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 51 (AF380485)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATGTTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATCTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 52 (AF380486)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATGTATGTTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATATT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 53 (AF380487)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TACAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 54 (AF380488)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATGTTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTATGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 55 (AY034454)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTGGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 56 (AY034455)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATGTTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 57 (AY034456)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAAATTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 58 (AY034457)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGG
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAAACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 59 (AY034458)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGAGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 60 (AY034459)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAAACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACATTACTTAAAATGTTT
AATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGGC
AAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 61 (AY034460)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 62 (AY034461)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGATGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGAGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 63 (AY034462)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATCTGACTTGCAGAGGTGTTGAGCCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 64 (AY034463)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGATGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAACTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAAAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 65 (AY034464)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTCCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 66 (AY034465)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAAACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 67 (AY034466)

ACCTTCAATAACCGTGTACATTAAGGAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGTTATGATAGGGCAATTCAATTTCGTCTTTTTAAAATATATATTAGTTCAG
TAGAGACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

HAPLOTYPE 68 (AY034467)

ACCTTCAATAACCGTGTACATTAAGAAATCAACTGATGGTGGTCTCTTACTAC
ATAGGAATTTGACTTGCAGAGGTGTTGAGTCCTGGTATAACTAGACTGATGA
GAGCTATGATAGGGCAATTCAATTTCGTCTTTTTAAGATATATATTAGTTCAG
TAGAAACTAATGGTTTATGTATGTCTTCATAATATGTATTAGTTCTATGGTTA
ATAACATTATTGTGGAATTTTCATTTATGTAGGGTTACACTACTTAAAATGTT
TAATAAAAATTAATGGGGATAATACATATATGTACTATGGGGCCAAGTCCGG
CAAAGAATAGTTTAATTTAGAATCCTAGCTTTGGGAGTTAG

APPENDIX B

202 bp Cytochrome *b* mtDNA haplotypes described from *C. variegatus*, *C. dearborni*, and *C. artifrons*. GenBank accession numbers are in parentheses after haplotype titles. *Haplotypes J and L have not been submitted to GenBank.

HAPLOTYPE A (AF380426)

GGCCTCGGCCAATATGTAGGTAAATACAGATAAAGAAGAAAGATGCTCCATT
AGCATGTATGTTTCGGATGAGTCAGCCGTAGTTGACGTCACGACAAATGTGA
GCAACGGATGAGAAAGCAGTAGAGATATCTGATGTATAGTGCATTGCCAAAA
ATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE B (AF380427)

GGCCTCGGCCAATATGTAGATAAATACAGATAAAGAAGAAAGATGCTCCATT
AGCATGTATGTTTCGGATGAGTCAGCCGTAGTTGACGTCACGACAAATGTGA
GCAACGGATGAGAAAGCAGTAGAGATATCTGATGTATAGTGCATTGCCAAAA
ATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE C (AF380428)

GGCCTCGGCCAATATGTAGGTAAATGCAGATAAAGAAGAAAGATGCTCCATT
AGCATGTATGTTTCGGATGAGTCAGCCGTAGTTGACGTCACGACAAATGTGA
GCAACGGATGAGAAAGCAGTAGAGATATCTGATGTATAGTGCATTGCCAAAA
ATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE E (AF380429)

GGCCTCGGCCAATATGTAGGTAAATACAGATAAAGAAGAAAGATGCTCCATT
AGCATGTATGTTTCGGATGAGTCAGCCGTAGTTGACGTCACGACAAATGTGA
GCAACGGAGGAGAAAGCAGTAGAGATATCTGATGTATAGTGCATTGCCAAA
AATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE F (AF380430)

GGCCTCGGCCAATATGTAGGTAAATACAGATAAAGAAGAAAGATGCTCCATT
AGCATGTATGTTTCGGATGAGTCAGCCGTAGTTGACGTCACGACAAATGTGA
GCAACGGATGAGAAAGCAGTAGAGATATCGGATGTATAGTGCATTGCCAAA
AATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE G (AF380431)

GGCCTCGGCCAATATGGAGGTAAATACAGATAAAGAAGAAAGATGCTCCATT
AGCATGTATGTTTCGGATGAGTCAGCCGTAATTGACATCACGACAAATGTGA
GCAACGGATGAGAAAGCAGTAGAAATATCTGATGTATAGTGCATTGCCAAAA
ATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE H (AF380432)

GGCCTCGGCCAATATGTAGGTAAATACAGATAAAGAAGAAAGATGCTCCATT
TAGCATGTATGTTTCGGATGAGTCAGCCTAGTTGACGTCACGACAAATGTGA
GCAACGGATGAGAAAGCAGTAGAGACATCTGAGTATAGTGCATTGCCAAAA
ATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE I (AF380433)

GGCCTCGGCCAATATGGAGGTAAATACAGATAAAGAAGAAAGATGCTCCATT
AGCATGTATGTTTCGGATGAGTCAGCCGTAATTGACATCACGACAAATGTGA
GCAACGGATGAGAAAGCAGTAGAGATATCTGAGTATAGTGCATTGCCAAAA
ATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE J

GCCCTCGACCGATATGCAGGTAAATACAGATAAAGAAGAAAGATGCTCCGTT
AGCATGCATGTTTCGGATAAGTCAGCCGTAATTAACGTCACGGCAAATGTGA
GCAACGGATGAGAAAGCAGTAGAAATATCGGATGTATAATGTATTGCCAAAA
ATAGTCCTGTTAGGATTTGGGCAATTAGGCATAGCCCTAGTAGGGA

HAPLOTYPE K (AF380434)

GGCCTCGGCCAATATGGAGGTAAATACAGATAAAGAAGAAAGAGGCTCCAT
TAGCATGTATGTTTCGGATGAGTCAGCCGTAATTGACATCACGACAAATGTG
AGCAACGGATGAGAAAGCAGTAGAAATATCTGATGTATAGTGCATTGCCAAA
AATAGTCCTGTTAAGATTTGGGCAATTAGACATAGCCCTAGTAGGGA

HAPLOTYPE L

GCCCTCGACCGATATGCAGGTAAATACAGATAAAGAAGAAAGATGCTCCGTT
AGCATGCATGTTTCGGATAAGTCAGCCGTAATTAACGTCACGGCAAATGTGA
GCAACGGATGAGAAAGCAGTAGAAATATCGGATGTATAATGTATTGCTAAAA
ATAGTCCTGTTAGGATTTGGGCAATTAGGCATAGCCCTAGTAGGGA

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Graduate Teaching Assistant, Department of Biology, Virginia Polytechnic Institute and State University; Honors Biology Laboratory, General Biology Laboratory, Principles of Biology Laboratory, Majors Biology Laboratory (1998 – 2001)

Chair on Biology Department Seminar Committee as Biology Graduate Student Association (BGSA) representative, Virginia Polytechnic Institute and State University (2000 – 2001).

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Phylogeography of Bahamian Pupfish Populations

Freshwater Mussel Culture and Identification of Algal Species

Manuscripts in Preparation:

Molecular evidence for rapid evolution of reproductive isolation between sympatric ecomorphs of pupfish (*Cyprinodon variegatus*) on San Salvador Island, Bahamas. T. M. Bunt, B. J. Turner, D. Duvernell, C. Holtmeier, M. Barton. for submission to: Proceedings of the National Academy of Science.

A species level phylogeny of *Cyprinodon variegatus* using mitochondrial markers. M. T. Fisher, T. M. Bunt, and B. J. Turner. for submission to: Molecular Ecology.

Principle Research Interests:
Population Genetics
Speciation
Species Flocks
Pace of Evolutionary Processes

Grants and Awards Received:

Graduate Student Research Development Project Grant, Virginia Polytechnic Institute and State University, Graduate Student Assembly (1999)

Grant-in-Aid of Research, Sigma Xi (1999)

Small Grant, Graduate Student Assembly Budget Board, Virginia Polytechnic Institute and State University, Graduate Student Assembly Budget Board (20001)

Presentations and Abstracts:

Bunt TM, D Duvernell, C Holtmeier, M Barton, and BJ Turner. A young species flock of pupfishes from San Salvador Island: Molecular evidence for reproductive isolation between sympatric trophic morphs. Joint Meeting of American Society of Ichthyologists and Herpetologists (ASIH) and American Elasmobranch Society (AES), State College, Pennsylvania, July 2001.

Fisher MT, Finne K, Bunt TM, and BJ Turner. Phylogeographic structure of the Atlantic pupfish (*Cyprinodon variegatus*): Evidence from mitochondrial nucleotide sequences. Joint Meeting of American Society of Ichthyologists and Herpetologists (ASIH) and American Elasmobranch Society (AES), State College, Pennsylvania, July 2001.

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Bunt TM, BJ Turner, D Duvernell, C Holtmeier, and M Barton. Molecular Evidence for reproductive isolation between two sympatric trophic morphs of San Salvador pupfish (*Cyprinodon*). Proceedings of the 8th Symposium on the Natural History of the Bahamas, San Salvador Island, Bahamas, June 1999.

Bunt, TM, BJ Turner, D Duvernell, C Holtmeier , and M Barton. Molecular evidence for reproductive isolation between two sympatric trophic morphs of San Salvador pupfishes (*Cyprinodon*). 31st Annual Meeting of the Desert Fishes Council, Ciudad Victoria, Tamaulipas, México, November 1999.

Bunt, TM, BJ Turner, D Duvernell, C Holtmeier , and M Barton. Molecular Evidence for Reproductive Isolation Between Two Sympatric Trophic Morphs of San Salvador Pupfish (*Cyprinodon*). Eastern Ecological, Population Genetics and Evolution (SEEPAGE) Conference, Mountain Lake Biological Station, Virginia, U.S.A., September 1999.