12-11-2015

Subspecies Composition, Genetic Variation and Structure of Largemouth Bass in Puerto Rico Reservoirs

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Recommended Citation

Peterson, Nick Ryan, "Subspecies Composition, Genetic Variation and Structure of Largemouth Bass in Puerto Rico Reservoirs" (2015). *Theses and Dissertations*. 4142.
https://scholarsjunction.msstate.edu/td/4142

Subspecies composition, genetic variation and structure of Largemouth Bass in Puerto Rico reservoirs

By

Nick Ryan Peterson

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Wildlife, Fisheries and Aquaculture
in the Department of Wildlife, Fisheries and Aquaculture

Mississippi State, Mississippi

December 2015

Subspecies composition, genetic variation and structure of Largemouth Bass in Puerto Rico reservoirs

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Pages in Study: 114

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Subspecies composition and genetic variation are important to the growth and survival of Largemouth Bass and information on these genetic characteristics is required for proper management of the species. In Puerto Rico, a genetic evaluation is needed to develop management plans for Puerto Rico reservoirs and maintain Florida Largemouth Bass at the Maricao Hatchery. Subspecies composition was characterized among reservoirs and the contemporary Maricao Hatchery broodstock in Puerto Rico, and metrics were compared between subspecies and hybrids among reservoir populations. Genetic variation and structure was determined and compared to Largemouth Bass populations in their native range. Florida subspecies were prevalent in all populations and non-introgressed subspecies seemed to be better adapted to the island than introgressed forms. Genetic diversity was lower in Puerto Rico populations and substantial genetic structure among populations was lacking. These results provide genetic information to guide future management and propagation of Largemouth Bass in Puerto Rico.

DEDICATION

I would like to dedicate this thesis to my family and friends who have supported my journey chasing a career in aquatic science. I am ever indebted to my father, Jim Peterson, for supporting my venture from the family farm to pursue my passion for all things fish and fishing. The countless great experiences and successes in my life would not have been possible without his love and support over the years..

ACKNOWLEDGEMENTS

I am forever indebted to the Texas Parks and Wildlife Department and personnel including Dijar Lutz-Carrillo, Loraine Fries, Megan Elliott, and Greg Southard from the A.E. Wood Laboratory, San Marcos, Texas, for their willingness to collaborate with this research and providing laboratory support. Dijar, in particular, provided countless hours of critical guidance and manuscript reviews throughout the research project, and this study would not have been possible without his help. Special thanks to my advisor, Dr. Wes Neal, for providing the special opportunity to study abroad, and for “having my back.” Thanks to Mississippi State University employees and graduate students Milton Munoz-Hicapié, Cynthia Fox, Karina Olivieri, Samuel Garcia, Clint Lloyd, Rebecca Krogman, and Cliff Hutt, and Puerto Rico Department of Natural and Environmental Resources employees Craig Lilyestrom, Darien Lopez, Gaspar Pons, Marinelly Valentín-Sivico, and María de Lourdes Olmeda for assistance with field collections, statistical analyses, and data support. Thanks to Dr. Peter Allen, Dr. Harold Schramm and Dr. Justin VanDeHey for their critical manuscript reviews. I must also thank the many private fishing club members who allowed private ramp access at many of the reservoirs used in the study. I would also like to thank the Puerto Rico Department of Natural and Environmental Resources, as well as, U.S. Fish and Wildlife Sport Fish Restoration for funding this project (SFR Project F-53R).

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CHAPTER I

LARGEMOUTH BASS IN PUERTO RICO

Research Goals and Objectives

This thesis consists of an introductory chapter, two objective chapters, and a synthesis chapter that provides management recommendations. My main purpose was to provide a contemporary baseline of subspecies composition and genetic variation to assess if subspecies composition and/or limited genetic diversity could be a root of uncharacteristic life history traits (e.g., slow growth and high mortality) observed among Largemouth Bass populations in Puerto Rico. This information is needed to guide future production of Largemouth Bass at the Maricao Hatchery and management of this species islandwide. In this chapter, I describe the history of Largemouth Bass introductions and current population characteristics in Puerto Rico, and I provide an introduction to genetic concepts and techniques important to subsequent chapters of this thesis. Chapter 2 uses six novel diagnostic microsatellite genetic markers to refine estimates of population subspecies composition among Puerto Rico reservoirs and evaluates population metrics (length-frequency distributions, proportional size distribution [PSD], mean total length) between Florida Largemouth Bass, northern Largemouth Bass, and/or intergrade Largemouth Bass to help decipher which of these genetic forms appears most suited for the tropical environment of Puerto Rico. I also evaluated subspecies composition of the 2011 Maricao Hatchery broodstock to determine if the Puerto Rico Department of

Natural and Environmental Resources was meeting management goals to maintain pure Florida Largemouth Bass at the Maricao Hatchery. Chapter 3 quantifies genetic variation among Largemouth Bass populations in Puerto Rico and compares this to populations in their native range (Florida and Texas), and determines if genetic structure is apparent among reservoir populations. The final chapter synthesizes the results of this study and proposes management recommendations and future research needs for Largemouth Bass in Puerto Rico.

History of Largemouth Bass in Puerto Rico

Largemouth Bass *Micropterus salmoides* were first introduced into Comerío Reservoir in Puerto Rico from the mainland U.S. in 1915 and 1916, consisting of 600 Largemouth Bass of unpublished gender and size each year (Iñdigo 1949); however, establishment was generally considered unsuccessful. In 1946, a shipment of 1,067 juvenile and 88 adult fish were transported from Georgia and Mississippi to Maricao Fish Hatchery and successfully introduced into reservoirs throughout the island (Erdman 1984). From 1946 to 1951, additional introductions of Largemouth Bass consisted of offspring of the 1946 importation (Erdman 1984; Neal et al. 2004). Since subspecies were not yet differentiated during this period, most introductions and stockings prior to 1957 were documented ambiguously as *Micropterus salmoides* (Table 1). The only documented importation of Florida Largemouth Bass to Puerto Rico occurred in 1957 from Welaka National Fish Hatchery in Welaka, Florida, which consisted of only 30 individuals of unpublished gender; no additional imports of Florida Largemouth Bass had been documented since 1957 at the time of this thesis research (Neal et al. 2004). The hypothesized lakes of origin for the broodstock raised in Waleka Hatchery in 1957 were the St. Johns River and Lake George (Welaka hatchery personnel, pers. comm.). Records indicate that, following the introduction of Largemouth Bass to Puerto Rico, large numbers of offspring were produced at the Maricao Hatchery and distributed to the island's reservoirs (Table 1). They quickly became the most popular freshwater sport fish in Puerto Rico (Soler 1951; Neal and Lopez-Clayton 2001).

In 1973, the authority to manage and introduce freshwater sport fisheries in Puerto Rico was transferred from the Department of Agriculture to the new Department of Natural and Environmental Resources (PRDNER; Schulte 1974; Rivera-González 1979). Subsequently, there have been increasing efforts towards freshwater fish management and research, with the majority of research focused on Largemouth Bass. Largemouth Bass research has covered a wide spectrum of subjects including gonadal development and spawning season (Gran 1995; Waters 1999; Ozen and Noble 2000; Ozen 2002), foraging dynamics and movement (Alicea et al. 1999; Ashe et al. 2000; Neal et al. 2005), and age, growth and mortality (Neal et al. 1997; Neal and Noble 2002; Neal et al. 2002).

It's currently unknown which of the early introductions have sustained the contemporary populations in Puerto Rico and thus what subspecies, or combination of subspecies, exist on the island. Introductions of Largemouth Bass from Georgia and Mississippi were likely hybrids of both subspecies in the Largemouth Bass lineage (northern, *M. s. salmoides*, and Florida Largemouth Bass, *M. s. floridanus*), as Philipp et al. (1983) showed that all sampled populations in these states were introgressed. Introductions from the Welaka Hatchery, on the other hand, likely represented non-introgressed Florida Largemouth Bass, although recent collections indicate low levels of introgression in these populations (Barthel et al. 2010). Neal et al. (1999) used three allozyme loci to evaluate subspecies-specific genetic composition in ten reservoirs and found that two were non-introgressed and all but one displayed a majority of Florida Largemouth Bass alleles; however, the sample sizes for several populations were less than ten individuals (Table 2).

Growth of Largemouth Bass in Puerto Rico has been shown to be atypical of many populations in temperate climates (Carr 1942). Growth of juvenile Largemouth Bass is rapid, and they usually reach maturity by age 1 (Neal et al. 2002). However, growth slows considerably after maturation and is nearly negligible by age 2 (Neal et al. 2002). Neal and Noble (2006) suggested that multiple spawning events per spawning season and an extended reproductive period resulted in reduced growth rates of Largemouth Bass. Subsequently, Neal and Noble (2008) stocked triploid bass into reservoirs in Puerto Rico to elucidate the effects of reproduction on somatic growth; however, triploid bass did not show a growth advantage over juveniles through age-1. Neal (2014) extended this study and reported no growth advantage through age-2, and no difference in von Bertalanffy growth equations over the life of both ploidy groups. Additionally, abnormally high mortality rates of adult Largemouth Bass in Puerto Rico were inconsistent with annual fishing mortality estimates and suggested natural mortality was disproportionately high in these populations (Neal and Lopez-Clayton 2001). Fishable stocks in most Puerto Rico reservoirs consisted of only three distinct age-classes, with few large, older fish present (Churchill et al. 1995; Neal 2003).

The introduction of a species outside of its native range may be beneficial, negligible, adverse, or a combination of these for the species itself and the rest of the ecological community (Philipp et al. 1981; Courtenay and Stauffer 1984). Adverse effects can be direct, such as competition and predation, or indirect, such as the introduction and transmission of disease or parasites (Plumb et al. 1996) and genetic alteration (Buckmeier et al. 2003; Edmands 2007). Subspecific variability such as in differential growth, survival, and reproductive success among subspecies of Largemouth Bass is well known (Maceina et al. 1988; Maceina and Murphy 1992; Neal and Noble 2002; Philipp et al. 2002). However, few studies have qualified adverse genetic effects (i.e., loss of genetic variation and inbreeding depression) between subspecies of Largemouth Bass and their intergrades after introduction in temperate (Gilliland and Whitaker 1989) and tropical environments.

Limited research on somatic growth and mortality of Largemouth Bass in Puerto Rico has considered subspecies-specific growth rates and genetics. Neal et al. (1999) evaluated the subspecies composition of Largemouth Bass in Puerto Rico reservoirs using allozymes and discovered that only two of ten primary reservoirs contained a pure Florida subspecies population (Cerrillos and Guajataca), while all but one (La Plata) showed a preponderance of Florida subspecific alleles. However, significance of these results was limited due to small sample sizes (less than 10 individuals analyzed) for genetic analysis and lower resolution of allozymes (Table 2). Neal and Noble (2002) found that Florida Largemouth Bass displayed greater longevity (i.e., 76% of total recaptures at age 2 and 100% at ages 3 and 4) than introgressed (Florida x northern hybrid) Largemouth Bass when stocked concurrently; therefore, only Florida subspecies were recommended for future stocking in Puerto Rico (Neal and Noble 2002).

While most of the reservoirs are spatially isolated from each other and unconnected by lotic systems, it's unclear if the effects of natural selection and genetic drift have been enough to allow genetic structure to develop in the face of the homogenizing effects of multiple hatchery stockings. While initial population sizes would have been small, minimizing the efficiency of selection, genetic drift could have produced strong stochastic effects among populations. Furthermore, although small populations do increase identity-by-descent (IBD) probabilities at each locus and, thus, increase homozygosity in general and allow for the purging of deleterious recessives in natural populations, the fact that more than one hybridizing lineage may be represented in each population probably offsets these effects. However, only a few migrants (or stocked fish) per generation are likely needed to counteract the divergence due to genetic drift (Hedrick 2011).

There are only a handful of documented introductions of Largemouth Bass (of either subspecies or their hybrids) to Puerto Rico. In all, fewer than 150 adults were successfully imported to the island. Thus, all subsequent stocking events are likely the result of production from a small number of individuals. Such a founder event may result in increased rates of inbreeding, the loss of genetic variation, and the stochastic fixation of deleterious alleles reducing adaptive potential in the population (Allendorf and Luikart 2007).

In addition to observations of the loss of allelic diversity and reduced heterozygosity, which may be the product of other causes, a severe reduction in population size would leave specific genetic signatures in the population. Allele numbers and frequency are the result of an equilibrium between mutations that accumulate in a population and the processes leading to the loss or fixation of alleles due to genetic drift (Hedrick 2011). However, when a population is the product of a founder event that includes a severe reduction in population size, allelic diversity is lost at a faster rate than heterozygosity. Thus, there should be an excess of heterozygosity observed relative to that expected from the observed number of alleles (Cornuet and Luikart 1996). In addition, the number of alleles (k) is expected to be reduced at a faster rate than the range of allele sizes (r). Thus, the ratio of k/r is expected to be smaller in recently-reduced populations than in populations at equilibrium (Garza and Williamson 2001). Additionally, low frequency alleles are expected to be lost at greater rates than moderate frequency alleles, ultimately leading to a distortion in the occurrence of low frequency alleles relative to moderate frequency alleles in a population (Luikart et al. 1998). Each of these genetic signatures should be present in the contemporary populations unless the documented demographic history of Largemouth Bass in Puerto Rico has not captured the true history of these populations.

The adaptive significance between increased genetic diversity and fitness, adaptation, and persistence of populations is widely recognized (Wright 1982; Koehn and Hilbish 1987; Nelson and Soulé 1987), as are negative effects (e.g., reduced fitness) of introducing a species outside of its native range (Philipp et al. 2002). Largemouth Bass are non-native to freshwater ecosystems in Puerto Rico; therefore, genetic processes (e.g., natural selection) could be amplifying the loss of genetic diversity in these populations. However, genetic diversity of Largemouth Bass in Puerto Rico freshwater reservoirs is currently unknown. Stocking records suggest that all populations of Florida Largemouth Bass in Puerto Rico were founded from only 30 individuals in 1957. This small number of founders may have severely limited the amount of genetic variation transferred to the island and in each subsequent generation levels of genetic drift in such a small population would have resulted in limited levels of contemporary genetic diversity. Depending on hatchery practices, the effects of this small founder population and subsequent genetic drift could have been compounded by inbreeding. While inbreeding would not have affected allele frequencies alone, there would have been a surplus of homozygosity among loci allowing for deleterious recessives to be expressed. This provides a template for observations of slow growth and short life spans in Largemouth Bass in Puerto Rico. Even in the absence of inbreeding, if levels of genetic variation were reduced to low enough levels over time, there would be no segregating alleles left in the population, possibly resulting in the same phenomena. Therefore, the lack of information on the current genetic population structure, records of low numbers of founding individuals from unspecific origins, and reductions of growth and longevity of Largemouth Bass in Puerto Rico warrants the application of genetic techniques to resolve such issues.

Largemouth Bass: Subspecies Determination, Ecology, and Life History

Black basses *Micropterus* spp. are some of the most economically and ecologically important fishes in the world (Heidinger 1975). In a recent survey by the U.S. Department of the Interior, black basses were the most popular fish taxon among the 25.0 million U.S. anglers who fished freshwaters other than the Great Lakes (USFWS 2006). Due to their popularity, black bass fisheries provide many economic benefits. For example, over the duration of one year, 74% of anglers fishing for trophy Largemouth Bass at Lake Fork, Texas, were nonlocal state residents and contributed to about 92% of the total expenditures in the Lake Fork area. At the state level, nonresident angler expenditures created an additional 163 jobs in Texas, with the total economic output for this fishery being $9,585,057 (Chen et al. 2003).

Largemouth Bass are an apex predator known to have substantial impacts on aquatic ecosystems (Swingle and Smith 1950; Swingle 1956; Takamura 2007) and have been categorized into two subspecies (Bailey and Hubbs 1949; Philipp et al. 1983), the northern Largemouth Bass *M. salmoides salmoides* (NLMB) and Florida Largemouth Bass *M. salmoides floridanus* (FLMB). The NLMB is native to central and eastern United States as well as northeastern Mexico and southeastern Canada, while the native range of the FLMB is restricted to peninsular Florida (MacCrimmon and Robbins 1975; Philipp et al. 1983).

Largemouth Bass have become one of the most widely disseminated freshwater aquatic species in the world (e.g., Robbins and MacCrimmon 1974; Azuma and Motomura 1999; Gratwicke and Marshall 2001). Both subspecies of Largemouth Bass coexist in introgressed populations throughout a hybrid zone that includes most of the southeastern United States, a number of the mid-Atlantic-coast states, several southwestern states, and California (Philipp et al. 1983; Barthel et al. 2010). Most of these introgressed populations are the result of stocking activities; however, some are likely the result of natural hybridization that occurred when the two subspecies came into secondary contact (Philipp et al. 1983). Currently, non-introgressed populations of northern Largemouth Bass are primarily restricted to the Midwestern and northeastern United States and southern parts of Canada, while non-introgressed populations of Florida Largemouth Bass are only found in peninsular Florida (Philipp et al. 1983; Barthel et al. 2010).

Because of their natural latitudinal distributions, each subspecies has displayed distinct physiological and morphological characteristics (Hart 1952; Thrasher 1974; Parker et al. 1985; Philipp et al. 1985a; Williamson and Carmichael 1986; Carmichael et al. 1988). Many differences have been observed in spawning periodicity (Isely et al. 1987; Rodgers et al. 2006), temperature tolerances (Coutant 1975; Cichra et al. 1980; Guest 1982, 1985; Philipp et al. 1985b; Fields et al. 1987; Koppelman et al. 1988), survival (Wright and Wigtil 1982; Philipp and Whitt 1991; Neal and Noble 2002), growth and catchability (Clugston 1964; Addison and Spencer 1971; Inman et al. 1976; Zolczynski and Davies 1976; Kleinsasser et al. 1990).

The greater growth potential of FLMB over NLMB in warm climates (Chew 1975; Rudd 1985) has contributed to the widespread stocking of FLMB outside of their native range (Stenenson 1973; Heidinger 1976; Philipp et al. 1983; Forshage and Fries 1995) and increased use in aquaculture (Williamson 1986). Supplemental stocking and introductions have successfully introduced FLMB into pre-existing populations of the northern Largemouth Bass in California (Bottroff 1967; Smith 1971; von Geldern and Mitchell 1975; Bottroff and Lembeck 1978; Moyle and Holzhauser 1978; Pelzman 1980), Texas (Inman et al. 1976; Maceina et al. 1988), and Oklahoma (Reiger and Summerfelt 1978), as well as many other locations around the world (Corral 1936; Toots 1972; Heidinger 1976; Godinho and Ferreira 1993; Takamura 2007).

Before the development of molecular genetics techniques, only morphological and meristic characters were available to describe variation among, and origins of, fish populations. Speculations were made based on the relationship between the morphological phenotype (an organism's appearance) and the organism's genotype (the precise genetic information encoded by its deoxyribonucleic acid [DNA]). However, this relationship is not easily discerned because of influences from factors such as the environment, anthropogenic influences, nutrition, and evolutionary history among populations.

The two subspecies of Largemouth Bass were first distinguished using meristic and morphometric techniques (Bailey and Hubbs 1949); however, early attempts to define subspecies yielded erratic results (Clugston 1964; Addison and Spencer 1971; Johnson 1975; Inman et al. 1976; Zolczynski and Davies 1976). By means of allozyme analysis, Philipp et al. (1983) distinguished genetic differences between subspecies of Largemouth Bass. Since then, additional markers have been developed including more allozyme loci (Carmichael et al. 1986; Williamson et al. 1986), restriction fragment length polymorphism of mitochondrial DNA (mtDNA; Nedbal and Philipp 1994; Williams et al. 1998) and microsatellites (Lutz-Carrillo et al. 2008). Such markers have been applied to determine genetic variation (Dutton et al. 2005; Barthel et al. 2010), taxonomic (subspecies) status, and distributions (Dunham et al. 1992; Alvardo Bremer et al. 1998; Johnson and Fulton 2004; Lutz-Carrillo et al. 2006; Barthel et al. 2010) in populations of Largemouth Bass throughout the world.

There have been contrasting views about whether northern Largemouth Bass and Florida Largemouth Bass should be considered separate species. Based on meristic characters, allozymes, and mtDNA, Kassler et al. (2002) recommended the division of northern Largemouth Bass and Florida Largemouth Bass into separate species (*M. salmoides* and *M. floridanus*). This argument was mainly supported by Philipp et al. (2002), who expressed how the two subspecies alone do not mate fully assortively where introgression is induced by anthropogenic dispersal of F_1 individuals produced in hatcheries, which could eventually lead to outbreeding depression. Additional mtDNA analysis by Near et al. (2003) further supported the determination as separate species. Despite the genetic evidence provided by Kassler et al. (2002) and Near et al. (2003), provisions to recognize the Florida Largemouth Bass as a separate species were not globally accepted among fisheries professionals. Currently, the American Fisheries Society's Committee on Names of Fishes recognizes the Florida Largemouth Bass as a subspecies of the Largemouth Bass (Nelson et al. 2004).

Population Genetics: Processes and Applications

Using common garden experiments, Gregor Mendel (1866) was one of the first to provide genetic support for the inheritance of phenotypic expression of traits from parents to offspring. Soon after, Charles Darwin (1896) was the first to consider the importance of genetics in the persistence of natural populations, expressing concern for a loss of vigor among wildlife species with small, isolated populations. Darwin (1896) developed a theory of evolution by means of natural selection that required the presence of genetic variation, often expressed as phenotypic variation, among individuals (Hartl and Clark 2007). Darwin's research initiated the study of evolution and genetic variation of wild populations and provided the framework for the field of population genetics (Ryman and Utter 1987; Hallerman 2003c).

Over the last 30 years, the field of population genetics has broadly extended into the study of natural resources conservation and management. Biologists are often concerned with genetic variation among individuals within and among populations (Hallerman 2003c). These data can provide insights into the demographic structure and evolutionary history of populations and provide support for management of wild species (Hartl and Clark 2007). In fisheries, population genetics techniques are commonly applied for identification of distinct genetic stocks in mixed-stock fisheries, determination of genetically appropriate hatchery programs for stocking efforts, genetic marking for fish identification, and forensic investigation for wildlife and fisheries law enforcement (Ryman and Utter 1987; Hallerman 2003c).

To understand the influence of genetic processes (i.e., natural selection, genetic drift, mutation, migration) in natural populations, one must be able to describe and quantify genetic variation within and among populations (Hedrick 2011). Genetic variation within populations is known as polymorphism (Hartl and Clark 2007) and can be described as the presence of more than one allele at any locus (Allendorf and Luikart 2007). A relationship exists between genetic variation and many aspects of fitness such as growth, disease resistance, adaptive capability, fecundity and overall survival (Hallerman 2003b). Fitness describes the ability to survive to reproductive age and leave viable offspring (Hallerman 2003b) and is the mechanism that determines the genetic variation in subsequent cohorts. Furthermore, genetic variation is essential for any evolutionary change in a population (Allendorf and Luikart 2007).

Genetic variation in populations is the collective result of the gain of genetic variation through processes of mutation and migration and the loss of genetic variation through natural selection and genetic drift (Hallerman 2003c). These processes may be interrelated, where one process occurs because of, or intensifies the effects of, another process. The principal regulating component in the production of novel genetic variation within a population is mutation, an error in the transmission of genetic information from parent to progeny (Hallerman and Epifanio 2003). Mutations accumulate much more rapidly (per unit time) in species with short generation times than species with longer generation times (Drake et al. 1998). In most species, mutation rates are generally rare on a per locus or per nucleotide level, but are very common from a genomic perspective where each individual may possess hundreds of new mutations among the billions of base pairs in the genome (Lynch et al. 1999). Most mutations are silent and do not have an obvious effect on the phenotype of an individual because they occur in the plethora of non-coding regions of the genome where mutation is not selected against (Allendorf and Luikart 2007). Mutations that have phenotypic effects are often deleterious (Hallerman and Epifanio 2003).

Conversely, the principal component regulating loss of genetic variation is random genetic drift (Hallerman 2003d). Genetic drift can be described as the change in allele frequencies due to random sampling and consequent sampling error (Hallerman 2003d). Genetic drift is a stochastic process in which the actual outcome cannot be predicted because it is affected by random elements (chance). Genetic drift causes changes in the genetic composition of populations resulting in changes in allele frequencies and the loss of genetic variation (Allendorf and Luikart 2007). In small populations, the effect of genetic drift predominates and results in some alleles becoming more common or even fixed at a given locus, while others become less common or completely lost, over time (Allendorf and Luikart 2007). The separation of a small group of individuals from a larger group can cause founder effects, where separated individuals would be unlikely to have all heritable variations, with different proportions, as the original group (Gulick 1905; Hallerman 2003d). Ultimately, founder effects and genetic drift limit genetic diversity and small populations increase the probability of inbreeding.

Hardy-Weinberg Equilibrium and Linkage Disequilibrium

Hardy-Weinberg equilibrium (HWE) is based on the assumption that allele and genotype frequencies remain constant throughout successive generations, which provides a fundamental approach to track allele and genotype frequencies between generations. Testing for departures from HWE can reveal insights into the mating system, social behavior, and genetic structure of populations (Hardy 1908; Weinberg 1908). The Hardy-Weinberg equilibrium model has six assumptions: (1) large and constant population size between generations, (2) random mating (i.e., panmictic population), (3) diploid organisms, (4) non-overlapping generations, (5) sexual reproduction, and (6) negligible effects of mutation, migration, and selection (Hallerman 2003a). When any of the assumptions of the HWE equation are violated, allele frequencies are subject to change. Genotype frequencies that deviate from HWE signal that some environmental or evolutionary process (i.e., mutation, migration, genetic drift, selection) may be acting on the population (Hallerman 2003a; Hedrick 2011). Linkage disequilibrium is the statistical association between alleles at different loci and occurs when loci do not segregate independently, an essential assumption in almost all measures of population differentiation and inbreeding (Raymond and Rousset 1995; Hedrick 2011).

Inbreeding and F-statistics

Detrimental effects of inbreeding, or inbreeding depression, can be found in virtually all outcrossing species, and the more intense the inbreeding, typically the more harmful the effects (Allendorf and Luikart 2007). Inbreeding depression results from increased homozygosity (Crow 1948). Most negative effects have been related to a greater expression in the number of deleterious recessive alleles in inbred individuals due to unmasking of recessive deleterious alleles (Lande 1994; Lynch et al. 1995), increased homozygosity, and reduced variability (Falk and Holsinger 1991; Brock and White 1992; Pray et al. 1994; Virijenhoek 1994).

The level at which inbreeding depression becomes evident is not clearly defined; it varies among species and depends on the particular alleles in a pedigree (Hallerman 2003a). Predicting the relative risk of inbreeding in nature is extremely complicated due to variation among taxa, characters being measured, mating history, environmental conditions, and the potential for a plethora of other effects to be occurring simultaneously (Edmands 2007). However, in many wild species, negative effects of inbreeding on reproductive and meristic traits directly or indirectly related to fitness have been discovered (Crnokrak and Roff 1999).

Inbreeding depression in fish can be expressed as an increased percentage of fish with deformities, abnormalities in gonadal development, decreased fecundity, decreased growth rate, and ultimately decreased survival (Golmelsky 2011). The effects of inbreeding on productive traits are well known in several fish species used for aquaculture. For example, after two generations of full sibling mating in Rainbow Trout *Oncorhynchus mykiss*, the percentage of deformed fry doubled, fry survival decreased by 30%, and mean weight after 364 days of rearing decreased by 33.5% (Kincaid 1976). Moreover, two successive generations of full sibling mating in Channel Catfish *Ictalurus punctatus* resulted in a 19% decrease in body weight (Bondari and Dunham 1987).

The effects of inbreeding can be minimized by managing the population to maximize the effective population size, which equalizes reproductive success among individuals, or potentially alleviated through proper breeding and stocking schemes (Hallerman 2003a; Allendorf and Luikart 2007). It is recommended that the broodstock utilized should be from different lines, strains, or populations and the effective population size for the hatchery stock should be known (Golmelsky 2011).

It is important to delineate whether patterns of genetic variation (i.e., heterozygosity) in a population were caused by the effects of inbreeding, the mating among related individuals, or by random genetic drift, such as a limited number of individuals are breeding rather than just breeding among relatives (Hallerman 2003d; Allendorf and Luikart 2007). In small populations, the probability of inbreeding increases, increasing homozygosity above the level expected from Hardy-Weinberg equilibrium (Hallerman 2003a).

Wright (1922, 1969) developed a conceptual and mathematical framework to describe the distribution of genetic variation among populations within a species using metrics of genetic differentiation called hierarchical F-statistics: F_{IS} and F_{ST}. The inbreeding coefficient F_{IS} is a measure of departure from Hardy-Weinberg proportions among individuals within local demes or subpopulations, and represents the percent increase in homozygosity above that which would have occurred had breeding been at random (Wright 1978; Hallerman 2003a; Allendorf and Luikart 2007). F_{IS} is calculated by comparing the observed heterozygosity averaged over all subpopulations (H_O) with the expected heterozygosity averaged over all subpopulations (H_S):

$$F_{IS} = 1 - \frac{H_O}{H_S} \qquad \text{(Eq. 1.1)}$$

Inbreeding within local subpopulations will result in positive F_{IS} values, constituting a deficit of heterozygotes, whereas small effective population size can result in negative F_{IS} values, indicative of an excess of heterozygotes (Allendorf and Luikart 2007).

The inbreeding coefficient F_{ST}, commonly called the fixation index, is used to measure how much genetic divergence had occurred since the establishment of subpopulations (Wright 1978). Most often, fish within an isolated population are more closely related (inbred) relative to one another than to those from other isolated populations (Kapuscinski and Miller 2007). F_{ST} is calculated by comparing the expected heterozygosity averaged over all subpopulations (H_S) with the expected Hardy-Weinberg heterozygosity (H_T) if the entire base population were panmictic:

$$F_{ST} = 1 - \frac{H_S}{H_T} \qquad \text{(Eq. 1.2)}$$

F_{ST} ranges from zero, when all subpopulations have equal allele frequencies, to one, when all subpopulations are fixed for different alleles.

These F-statistics can be used to describe the effects of population subdivision on the genetic structure of populations (Allendorf and Luikart 2007). Population subdivision produces inbreeding-like effects even when there is random mating within groups due to the fact that the probability of autozygosity increases as population size decreases; therefore, it is important to determine the appropriate inbreeding coefficient measures for determination of genetic population structure.

Inbreeding coefficients measure the probability that two alleles at a specified locus are identical by descent (IBD), derived from one particular allele possessed by a common ancestor (Hedrick 2011). Assuming F is constant in each generation, the inbreeding coefficient after t generations is calculated by:

$$F_t = 1 - (1 - F_x)^{\mathrm{t}} \quad \text{(Eq. 1.3)}$$

where F_x is the inbreeding coefficient for one generation. The amount of inbreeding that occurs in each generation is considered the rate of inbreeding. The mean rate of inbreeding (ΔF) depends on the effective population size (N_e):

$$\Delta \mathrm{F} = 1/(2N_e) \quad \text{(Eq. 1.4)}$$

(Kapuscinski and Miller 2007). In populations with successive generations of inbreeding, F is increased but the rate of increase is reduced (Golmelsky 2011).

Genetic Variation: Measures and Assessment

Genetic variation in populations is measured through the use of molecular markers. Genetic markers are locations in the genome, called loci, which can be identified and compared across individuals to measure variation (Hallerman 2003c). When more than one allele is found at a locus, the individual is considered to be heterozygous, or population polymorphic, at that particular locus. The levels of polymorphism vary among molecular markers and consequently can be used to answer a variety of genetic questions (Beebee and Rowe 2005). The best marker to examine genetic variation in populations depends on cost, sample size, and the variation at each genetic marker (Sunnucks 2000; Theodorakis and Wirgin 2002; Schlötterer 2004).

The development of protein electrophoresis contributed to the expansion of the many new forms of genetic markers used today and made the screening of genetic variation in natural populations technically viable, allowing a large number of loci to be studied with relative ease, speed, and cost efficiency (Powell 1994). Protein electrophoresis places DNA on one end of a gel and institutes an electric charge, separating protein molecules of different charge (Hartl and Clark 2007). Molecules of different size and charge move at different rates through the gel, and the positions of the molecules at the completion of the trial can be used to detect specific genotypes and polymorphisms among individuals (Hartyl and Clark 2007). The genetic basis for variation of protein loci can often be inferred directly from electrophoretic patterns. Data sets from different laboratories can be combined when the same loci are examined at identical allelic destinations. However, this technique can examine only genes that code for water-soluble enzymes and cannot detect genetic changes that do not affect the amino acid sequence of a protein subunit. Moreover, electrophoresis is easily biased by improper sample storage and preservation, requires sacrifice of individuals, and shows low levels of heterozygosity, which limits estimates of genetic variation (Allendorf and Luikart 2007).

Also developed to determine the amount of nucleotide sequence variation present in populations is the polymerase chain reaction (PCR; Hartl and Clark 2007). During the PCR, short primers (i.e., small pieces of single-stranded DNA commonly shorter than 20 nucleotides in length) are used to initiate the replication of DNA. During each round of replication, DNA is heated to separate the strands and then cooled to allow new primers to anneal. Subsequently, *Taq* polymerase extends a synthesized strand of DNA from the

3’ end of each primer to add nucleotides across the target sequence, producing exponentially more replicates of a specific target sequence (Hartl and Clark 2007). The development of the PCR has made the use of molecular markers less complicated and more cost effective (Conner and Hartl 2004). Further, the PCR-amplification technique allows small amounts of tissue to be collected by nonlethal sampling methods or isolated from preserved or partially degraded material (Billington 2003).

Microsatellites are currently one of the most widely used nuclear markers in population genetics (Schlötterer 1998; Allendorf and Luikart 2007). Microsatellites are caused by replication or recombination errors (Eisen 1999) and consist of tandem repeats of a short sequence motif of two to six nucleotides (e.g., ACACACACAC, which can be expressed by AC^5) randomly distributed throughout the nuclear genome (Tautz 1989; Hedrick 2011). The number of tandem repeats in microsatellites can vary at different loci and in different individuals. Microsatellites have many advantages over other genetic markers (e.g., allozymes) because they exhibit more polymorphism in natural populations, with more alleles, and heterozygosities often greater than 0.5 (Estoup et al. 1998). In addition, microsatellites are considered neutral markers, not selected for or against by natural selection and provide higher sensitivity to changes in populations (Hedrick 2011).

The resolution of microsatellites requires minimally invasive, nonlethal sampling of small amounts of tissue (e.g., fin clips) from which all potential loci may be resolved. Furthermore, tissues may be stored under an array of conditions making field sampling efficient, long-term storage viable, and analysis of degraded and archived samples possible, which allows assessment of genetic variation over relatively long time frames

(Nielsen et al. 1999). Microsatellites can be restrictive in long-term studies that require addition of data from multiple laboratories because of the difficulties in calibrating size-based alleles, which often differ by only a few nucleotides (Amos et al. 1996; Broquet and Peti 2004); however, recent publications have successfully alleviated such issues (e.g., Stott et al. 2010). Microsatellites are commonly used for assessment of inbreeding, parentage, and population structure and gene flow in fisheries research (Neff et al. 2000; Allendorf and Lukart 2007).

Current literature suggests that microsatellites are the ideal marker for assessing genetic variation and hybridization among sub-species of Largemouth Bass. For example, Barthel et al. (2010) found that microsatellite genotypes alone resolved the genetic structure and introgression among regional groups of Largemouth Bass in Florida, over allozymes and mtDNA restriction fragment length polymorphisms. Moreover, Lutz-Carrillo et al. (2006) suggested that microsatellites offer the best alternative for estimating the proportions of contributions to the genomes of putative Largemouth Bass hybrids (intergrade northern Largemouth Bass x Florida Largemouth Bass) and the origins of individuals (Primmer et al. 2000; Randi et al. 2001).

Genetic variation can be quantified in a standardized way by measures of heterozygosity, which can be expressed as observed (H_o) and expected (H_e) heterozygosity (Lewontin and Hubby 1966; Hedrick 2011). Observed heterozygosity is the observed proportion of heterozygotes, or the proportion of individuals in a population (or sample) that possess two different alleles at a given locus. Observed heterozygosity at each locus is calculated by dividing the number of heterozygous individuals by the total number of individuals assessed at each locus (Allendorf and Luikart 2007).

Expected heterozygosity is the expected proportion of heterozygotes if the population is mating at random, and provides a standard to compare the relative amount of variation in different populations (Allendorf and Luikart 2007). Expected heterozygosity values (H_e) are calculated from sample allele frequencies according to the Hardy-Weinberg principle using the expression:

$$H_e = 1 - \sum_{i=1}^{\substack{Number\ of \\ alleles}} p_i^2 \qquad \text{(Eq. 1.5)}$$

where p_i is the frequency of the i^{th} allele (Frankham et al. 2001). Estimates of heterozygosity can be averaged across all microsatellite loci to evaluate the reliability of single locus estimates and to more accurately represent genome-wide levels of genetic diversity within populations (Hedrick 1999).

Genetic variation can also be measured at the individual or population level using measures of allelic diversity (A_d), effective number of alleles per locus (A_e), and allele and genotype frequencies (Hedrick 2011). Allele frequencies from genetic data are commonly used for comparative and predictive purposes across populations and across loci (Hartl and Clark 2007). Allele frequencies (p_i) are calculated as follows (Hedrick 1999):

$$p_i = \frac{2N_{ii} + \sum_{i=j} N_{ij}}{2N} \qquad \text{(Eq. 1.6)}$$

where N is the total number of individuals sampled, N_{ii} is the number of observed homozygous individuals, and N_{ij} is the number of observed heterozygous individuals for a specific allele (i). The effective number of alleles per locus can be calculated for separate populations to account for uneven allele frequency distributions and to improve comparisons made between samples with diverse distributions of alleles (Hartl and Clark 2007). The effective number of alleles (A_e) is the inverse of the expected homozygosity (Hedrick 2011):

$$A_e = \frac{1}{1-H_e} \tag{Eq. 1.7}$$

The mean number of alleles for each population can be calculated by summing all alleles present and dividing the sum by the total number of loci analyzed using the following formula (Hedrick 1999):

$$\bar{a}_p = \frac{\sum a_1+a_2+\cdots+a_n}{L} \tag{Eq. 1.8}$$

where $\bar{a}_p$is the mean number of alleles for population *p*, *a* is one allele, and *L* is the total number of loci surveyed.

In this chapter, I outlined basic genetic processes and applications that were used to provide a contemporary baseline of genetic subspecies composition, genetic variation and structure among introduced Largemouth Bass populations in Puerto Rico. These can also be applied to common questions about the role of genetics on management of other Largemouth Bass populations such as "What subspecies is best for my pond or lake? What role does genetics have on the size of fish in my pond or lake? What does genetics have to do with it?" Like most answers in fisheries, the universal answer to these questions are, "It depends" because nearly every situation is different. The process of local adaption, where a gene pool of a population evolves to be most productive under the environmental conditions experienced by the population, is actively shaping the gene pool of all Largemouth Bass populations throughout the world. Even though the answer is not always clear, the genetic principles and metrics outlined and used in this research provide an alternative tool for fisheries managers to respond to difficult genetic questions that cannot be answered otherwise.

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CHAPTER II

SUBSPECIES COMPOSITION OF LARGEMOUTH BASS IN PUERTO RICO

Introduction

Largemouth Bass are represented by two subspecies (Bailey and Hubbs 1949), the northern Largemouth Bass (NLMB) *Micropterus salmoides salmoides* and the Florida Largemouth Bass (FLMB) *M. s. floridanu*s. These subspecies inhabit overlapping ranges with an area of introgression extending west from peninsular Florida to California, and north to Virginia. Non-introgressed populations of the NLMB are primarily restricted to the midwestern and northeastern United States and southern parts of Canada, while non-introgressed populations of the FLMB are only found in peninsular Florida (Philipp et al. 1983; Barthel et al. 2010). Distinct physiological and morphological characteristics have been reported for the two subspecies (Hart 1952; Thrasher 1974; Parker et al. 1985; Philipp et al. 1985a; Williamson and Carmichael 1986; Carmichael et al. 1988). The differences included spawning periodicity (Isely et al. 1987; Rodgers et al. 2006), temperature tolerances (Cichra et al. 1982; Guest 1982, 1985; Philipp et al. 1985b; Fields et al. 1987; Koppelman et al. 1988), survival (Wright and Wigtil 1982; Philipp and Whitt 1991; Neal and Noble 2002), growth, and catchability (Clugston 1964; Addison and Spencer 1971; Inman et al. 1976; Zolczynski and Davies 1976; Wright and Wigtil 1982; Kleinsasser et al. 1990).

Over the last several decades there have been systematic efforts by state and federal agencies to establish the FLMB subspecies outside of its native range due to greater overall size potential observed at southern latitudes (Crawford et al. 2002; Lutz-Carrillo et al. 2006). The FLMB has been successfully introduced into existing populations of NLMB throughout the United States, including California (Bottroff 1967; Smith 1971; von Geldern and Mitchell 1975; Bottroff and Lembeck 1978; Pelzman 1980), Texas (Inman et al. 1976; Maceina et al. 1988), and Oklahoma (Reiger and Summerfelt 1978), as well as many other locations around the world (Corral 1936; Toots 1972; Heidinger 1976; Godinho and Ferreira 1993; Kitagawa et al. 2000; Takamura 2007).

Stocking records indicate that Largemouth Bass *Micropterus salmoides* were introduced to Puerto Rico from the United States as early as 1915 (Johnson 1915; O'Malley 1916; Robbins and MacCrimmon 1974); however, establishment was considered unsuccessful (Table 1). A second introduction was attempted in 1946 when 88 adults and 1,067 juveniles were transported from Georgia and Mississippi to the Maricao Fish Hatchery. Their offspring were subsequently distributed to the island's reservoirs (Erdman 1984; Neal et al. 2004). A third introduction was recorded in 1957 from the Welaka National Fish Hatchery, Florida, which consisted of only 30 adults that are believed to have originated from the St. John's River and Lake George (Welaka Hatchery personnel, pers. comm.). Philipp et al. (1983) found many introgressed Florida x northern Largemouth Bass (ILMB) populations throughout the southeastern United States; therefore, introductions of Largemouth Bass to Puerto Rico from Georgia and Mississippi were likely ILMB. Introductions from the Welaka Hatchery, on the other hand, likely represented non-introgressed FLMB, although recent collections indicate low levels of introgression in these populations (Barthel et al. 2010). Following these introductions, Largemouth Bass became established as the most successful predator and most highly sought-after sport fish in reservoirs and ponds throughout the island (Erdman 1984; Neal et al. 2004).

Studies of Largemouth Bass dynamics in Puerto Rico indicate that, unlike their life history in temperate reservoirs, most do not survive to age-4 and they rarely reach sizes preferred by anglers (Neal et al. 2002). The short life span and relatively small size occur in reservoirs throughout the island despite an apparent abundance of prey and year-round growing season (Alicea et al. 1997; Neal et al.1999). Following rapid growth as juveniles, growth slows at maturation (Gran 1995). Largemouth Bass in Puerto Rico exhibit an extended spawning season of up to 6 months, with multiple spawning events occurring per individual annually. This increased reproductive activity may require an energetic shift from growth to reproduction leading to reduced growth and early mortality (Gran 1995; Neal 2003).

Neal et al. (1999) evaluated the subspecies composition of Largemouth Bass in Puerto Rico reservoirs using three diagnostic allozyme loci and discovered that only two of ten primary reservoirs contained a non-introgressed FLMB population (Cerrillos and Guajataca Reservoirs), while all but one (La Plata Reservoir) showed a preponderance of FLMB subspecific alleles. However, the sample sizes for several populations were less than 10 individuals (Table 2). Neal and Noble (2002) found that FLMB displayed greater longevity (i.e., represented 76% of total recaptures of fish at age-2 and 100% at ages-3 and 4) when compared to concurrently-stocked ILMB in Lucchetti Reservoir. Based on these results the Maricao Hatchery has limited contemporary broodstock collections to Cerrillos Reservoir in an attempt to facilitate the production and propagation of pure FLMB to island reservoirs (Neal and Noble 2002).

In this chapter, I apply an independent marker panel of microsatellite loci with the primary objectives being to 1) refine estimates of population subspecies composition among all major reservoirs and the contemporary Maricao Hatchery broodstock in Puerto Rico, and 2) compare metrics (i.e., length-frequency distributions, proportional size distribution [PSD], mean total length) between FLMB, NLMB, and/or ILMB among reservoir populations to determine whether the patterns observed in Lucchetti Reservoir are conserved across the island**.**

Methods

Study Area

Puerto Rico is the smallest (8,959 km^2) of the Greater Antilles Islands bordering the Caribbean Sea and Atlantic Ocean. The mainland measures 170 km by 60 km and is mostly mountainous, with multiple peaks exceeding 1,000 m. The highest elevation point of Puerto Rico, Cerro de Punta (1,338 m), is located in the main mountainous range called La Cordillera Central (The Central Range).

Most reservoirs in Puerto Rico were constructed in the early and middle 20th century (Soler-López 2001). Puerto Rico has many impoundments that are used for flood control, drinking water supply, storage for irrigation, recreation, and as a source of hydroelectricity (Neal et al. 2004; USGS 2008). Currently, five reservoirs in Puerto Rico have well-developed access and recreational fishing facilities, most including a resident biologist, public ramps, sanitary and picnic amenities, and other facilities. The creation of field stations and associated facilities at these reservoirs (i.e., Dos Bocas, Cerrillos, Lucchetti, La Plata) by the Puerto Rico Department of Natural and Environmental Resources (PRDNER) has improved recreational access and increased reservoir popularity among boating anglers (Churchill et al. 1995).

This study included 12 reservoirs located throughout the tropical island of Puerto Rico. Reservoirs were chosen using three criteria: 1) reservoirs that contained Largemouth Bass, 2) management importance as designated by the PRDNER (Lilyestrom and Neal 2004), and 3) accessibility. Reservoirs exhibited considerable range in surface area (37.2-405.0 ha) and elevation above sea level (46.0-743.1 m)(Table 3).

Most reservoirs on the island receive sporadic supplemental stockings of Largemouth Bass; thus, I also sampled the contemporary Largemouth Bass hatchery broodstock at the Maricao Fish Hatchery (hereafter, the Hatchery) in the Spring of 2011 (N=40). These samples were used to characterize the contemporary genetic contribution from the hatchery source population.

Sample Collections

A minimum of 30 stock-size ((≥ 200 mm total length) Largemouth Bass were collected from each reservoir, except for Guayabal Reservoir (n = 18) and Carite Reservoir (n = 27), and the contemporary Maricao Hatchery broodstock during June and July 2011 in accordance with approved guidelines for the Use of Fishes in Research (available online at http://fisheries.org/docs/wp/Guidelines-for-Use-of-Fishes.pdf) under Mississippi State University Animal Care and Use Committee (IACUC) Protocol 10-074. For standardization purposes, six shoreline sites were established *a priori* in each reservoir with three out of the six sites randomly selected and individually electrofished for 900 seconds using a boom-mounted electrofisher at 240-V pulsed DC. Stock-size Largemouth Bass were collected at each site, measured for total length (mm), and weighed (g). Additional collection sites were used if the predetermined minimum sample size ($n \geq 30$) was not obtained.

For each fish collected, a section (~ 25 mm^2) of the pectoral fin was removed, immediately placed in 2-ml storage vials containing 70% ethanol, and stored at room temperature. Samples were then transported to the A.E. Wood Laboratory (Texas Parks and Wildlife Department), San Marcos, Texas, for sample processing and genetic analyses.

Characterization of Microsatellite Variation

Deoxyribonucleic acid (DNA) was extracted from a portion of each fin clip following a modified version of the Puregene protocol for extraction from fish tissue (Gentra Systems, Inc., Minneapolis, Minnesota). Briefly, 3-5 mm^3 of fin tissue was placed in 300 μL of cell lysis solution (10 mM tris-HCl, 10 mM EDTA [pH 8.0], and 2% sodium dodecyl sulfate) with 5 μL of proteinase K (20 mg/mL) and incubated at 55°C for 3-4 h. After incubation, 120 μL of ammonium acetate (7.5 M) was added and vigorously mixed for 20 s. The solution was then incubated at 0°C for 15 min followed by centrifugation at 13,000 x g for 5 min. The supernatant was then added to 1,000 μL of 100% ethanol, stored at -80°C for 10 minutes, and centrifuged at 13,000 x g for 10 min. The supernatant was then decanted, and the pellet dried at room temperature for 15 minutes. The pellet was resuspended in 200 μL of TLE buffer and left to rehydrate at room temperature for 24 hours. Recovered DNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and adjusted to a concentration of 50 ng/µL with deionized water to ensure consistency in subsequent genotyping.

Two multiplex reactions (MPX8 and MPX9) encompassing six diagnostic microsatellite loci (TPW111, TPW112, TPW169, *Msa*21, *Mdo*6, and *Msa*29; Lutz-Carrillo et al. 2008) were used to analyze subspecies composition. Genotyping was performed using the polymerase chain reaction (PCR) to amplify alleles from each locus in all sampled individuals. The PCR products were combined with stop solution, denatured by heat, and analyzed on an NEN 4200 Global IR2 DNA Sequencer (LICOR) using a 50-350 base pair ladder. Allele sizes were estimated using BioNumerics v. 6.5 (Applied-Maths, Belgium). All genotypes were confirmed visually and quantitative data were transferred to Microsoft Excel 2010.

Statistical Analyses

Diagnostic alleles at six loci were used to assign genotypes (FLMB, NLMB, ILMB) at the individual level. Hybrid index values, defined as the proportion of FLMB alleles detected over all diagnostic loci, were determined for each fish. Mean hybrid index values, the mean proportion of FLMB alleles within each population represent the probability that a randomly selected allele was derived from a FLMB ancestor.

To test the null hypothesis of no differences in length-frequency distributions between FLMB and ILMB, length-frequency distributions were pooled among all populations and compared using a two-way Kolmogrov-Smirnov test and the NPAR1WAY procedure in SAS (SAS Institute, Cary, North Carolina). Length-frequency distributions between FLMB and ILMB were not compared within individual populations because sample sizes were inadequate to meet assumptions with an adequate level of confidence (Miranda 1997). Mean total length was determined for FLMB and ILMB within each population and compared using the GLM procedure in SAS (SAS Institute, Cary, North Carolina). To avoid the effects of gear bias, only stock-size (200 mm; Gabelhouse 1984) or larger Largemouth Bass were used to compare length-frequency distributions and mean total length among populations. Before analyses, total length values were log-transformed to stabilize variance to mean ratios.

A chi-square test and the frequency procedure (FREQ) in SAS (SAS Institute, Cary, North Carolina) were used to test the null hypothesis that the frequency of observations among proportional size distribution (PSD) size groups (proportional size distribution of stock-to-quality [PSD-S-Q], quality-to-preferred [PSD-Q-P], preferred-memorable [PSD-P-M], memorable-to-trophy [PSD-M-T] size; Gabelhouse 1984) was independent of subspecific composition. Independent Wilcoxon rank-sum tests and a nonparametric one-way analysis of variance procedure (NPAR1WAY) in SAS (SAS Institute, Cary, North Carolina) were then used to determine if the frequencies of fish among and within PSD size groups differed between FLMB and ILMB. Significance was established at $\alpha = 0.05$ for all tests.

Results

All 12 reservoir populations, as well as the Maricao Hatchery broodstock, contained some ILMB alleles, but FLMB alleles were most common (Figure 1). Among reservoir populations, the proportion of FLMB alleles ranged from 0.77 to 0.95 (mean 0.86, SD 0.07), with the smallest and greatest proportions observed in Garzas and Cerrillos Reservoirs, respectively (Table 4; Figure 1). Only 2 of 12 populations contained an individual with <50% FLMB influence (Garzas: N = 3; Toa Vaca: N = 1).

The proportion of FLMB genotypes within reservoirs ranged from 0.03 to 0.64 (mean 0.29, SD 0.22), with the least and greatest proportions observed in Garzas and Dos Bocas Reservoir, respectively (Table 4). Among all largemouth examined, 33% (139/418) were resolved as FLMB genotypes and no pure NLMB genotypes were recovered (Table 4; Figure 2). All populations showed lower levels of FLMB alleles and genotypes than the Mariaco Hatchery broodstock (Table 4).

Florida Largemouth Bass and ILMB exhibited significantly different length-frequency distributions (P=0.026; Figure 3). Cumulative distribution functions for FLMB and ILMB were similar from 200 to 350 mm, and distributions separated at 350 mm and higher. Differences were most apparent at size ranges > 400 mm (Figure 3). Introgressed Largemouth Bass were 16% more frequent than FLMB at lengths < 400 mm, while FLMB were 16% and 8% more frequent than ILMB at lengths > 400 mm and 500 mm, respectively (Table 5).

Mean total length of FLMB was greater than ILMB in 9 out of 12 populations (Figure 4). Collectively, differences (F=2.17; df=11; P=0.016) were observed between mean total length of FLMB (mean=378.6; SD=60.2; SE=17.4; range: 298.0 to 490.7 mm) and ILMB (mean=358.1; SD=47.7; SE=13.8; range: 311.1 to 481.4 mm). Overall, a difference (χ^2=8.28, df=3, P=0.041) was observed between the frequency of FLMB and ILMB among PSD size groups (Figure 5). The majority of the overall χ^2-value was explained within PSD Q-P and PSD M-T size groups. Less than 1% of FLMB were trophy-sized and no trophy (PSD-T) ILMB were collected (Figure 5).

Discussion

Largemouth Bass populations in Puerto Rico are primarily composed of Florida Largemouth Bass alleles and genotypes. The prevalence of FLMB alleles could be the result of the genetic composition of founding populations, the effects of recent hatchery releases, natural selection, or genetic drift. Largemouth Bass were introduced to Puerto Rico from mainland North America from the native range of both NLMB and the FLMB. Offspring of these imports were produced at the Maricao Hatchery and released to many reservoirs throughout the island, often without documentation. Starting in 2000, stocking records improved and most documented stocking events consisted of fish presumed to be pure FLMB, although results from the current study suggest some introgression. Since hatchery practices can greatly alter the genetic composition of wild fish populations (Busack and Currens 1995), it is very likely that the current prevalence of FLMB alleles is a product of stocking pure (or mostly pure) FLMB from the Maricao Hatchery into introgressed populations. However, the genetic composition of previous hatchery broodstock fish is not known, and historical records of individual stocking events from the Maricao Hatchery to island reservoirs are vague or absent (Table 1). Therefore, the influence of past stocking events on subspecific composition is unclear. Furthermore, subspecific alleles and genotypes provided by each importation event and precise populations of origin are not known.

Compared to previous genetic analyses (Neal et al. 1999), we found that 4 out of 8 populations addressed in 1999 increased in the proportion of FLMB influence, whereas the other populations did not appreciably change. Excluding potential effects of hatchery stocking, an increase in the proportion of FLMB influence may suggest that natural selection favored FLMB genotypes in these populations. Natural selection is the differential contribution of genotypes to the next generation that results from improved fitness of those genotypes, or co-adapted gene complexes, under the given environmental conditions. Both environmental conditions and genetic factors (e.g., genetic drift, migration) can influence the efficiency and direction of selection (Allendorf and Luikart 2007). Environmental conditions are fairly homogeneous in Puerto Rico (USGS 2005), with stable, warm temperatures and moderated photoperiods much like those found in the FLMB native range. If natural selection was influenced by genetic factors and favored FLMB alleles and genotypes, one would expect to see this unidirectional pattern of selection (i.e., an increase in FLMB influence) among populations over time. However, since this study was not developed to address the mechanisms (e.g., survival, reproduction, or environmental conditions) for the increase in FLMB influence, I cannot definitively say FLMB are the favored genotype in Puerto Rico based on this research alone. Furthermore, there may be issues with comparing these two studies due to differences in sample size and genetic techniques and markers. Therefore, future studies are warranted where many of these mechanisms could be directly controlled (e.g., application of hatchery trials) and similar genetic techniques and markers can be utilized.

Due to longevity differences among subspecies of Largemouth Bass in Puerto Rico, it was previously suggested that Cerrillos Reservoir, then considered a pure FLMB population (Neal et al. 1999), be designated as the solitary broodstock source for the Maricao Hatchery (Neal and Noble 2002). However, my results indicate that the Cerrillos Reservoir population contains a small proportion of NLMB alleles. Although this difference could be due to introduction of ILMB in Cerrillos Reservoir after the Neal et al. (1999) samples, it is more likely that allozyme analyses, which are not as powerful as microsatellite analyses, missed this low-level introgression (Barthel et al. 2010). Although the subspecies status of Cerrillos Reservoir has changed, it continues to contain the highest mean proportion of FLMB alleles among all reservoir populations, and no pure FLMB populations were identified in other island reservoirs in the present study (Figure 1). Three reservoir populations (Caonillas, Dos Bocas, and Guayabal Reservoir) had similar proportions of FLMB alleles and genotypes as Cerrillos Reservoir (Table 4), and these populations could serve as alternative source populations for broodstock collection. In fact, Caonillas and Dos Bocas Reservoir contained a greater proportion of pure FLMB individuals in their populations than all other reservoirs, including Cerrillos Reservoir (Table 4). Although the management objective of the Maricao Fish Hatchery is to maintain and propagate pure FLMB, many current broodstock are introgressed and regular genetic verification is not currently conducted on hatchery broodstock. If regular genetic testing is implemented, new broodstock collections could target populations in Cerrillos, Caonillas, and Dos Bocas Reservoirs. Pure FLMB could be identified from these collections and retained as broodstock. Other factors, including reservoir access, travel and transport distance, and Largemouth Bass catch rates should be considered in broodstock source selection.

My study found evidence of possible artificial selection occurring due to hatchery practices. The existing Maricao Hatchery broodstock contained a higher mean proportion (98%) of FLMB alleles than all reservoir populations (Table 4) despite being collected from Cerrillos Reservoir. This may be indicative of artificial genetic selection, as hatchery broodstock collection efforts generally target large (>350 mm), mature individuals (Maricao Hatchery personnel, pers. comm.). In this study, FLMB genotypes were observed 11.5% more frequently than ILMB among Largemouth Bass that were greater than 350 mm in Cerrillos Reservoir. Thus, the selection of larger fish during broodstock collection events may have resulted in the higher proportions of FLMB alleles and genotypes found at the Maricao Hatchery as compared to the reservoir population.

A central management goal of the PRDNER is to improve size structure among Largemouth Bass populations. The Maricao Fish Hatchery currently collects broodstock from one island reservoir, but these collection procedures can have negative consequences (see Miller and Kapuscinski 2003). Genetic changes are expected to occur from hatchery procedures (Hindar et al. 1991) based on the relative extent of genetic differentiation (i.e., measures of genetic variation and extent of genetic population structure) among sampled populations (Carvalho 1993), which can have strong selective effects (Doyle and Talbot 1986; Doyle and Talbot 2003). Continuous collections and propagations from one population over time can have inadvertent effects (Keller and Waller 2002), especially on small populations (Leberg 1991), resulting in inbreeding depression (Allendorf and Luikart 2007). To counteract this, future hatchery broodstock collections in Puerto Rico should target only large (> 400 mm) individuals from all populations with high proportions of FLMB alleles and genotypes (i.e., Caonillas, Cerrillos, Dos Bocas, Guayabal). The propagation of offspring from large individuals from several populations could increase growth, longevity, fitness, and augment genetic variation (Leberg 1990; Leberg 1993). Artificial selection for fish with more rapid growth or greater longevity in hatchery operations can increase fitness and survival among wild populations; however, this trend is not always apparent (Gross 1991; Hindar et al. 1991).

Florida Largemouth Bass appear to be achieving larger sizes than ILMB in most reservoirs. For all reservoir data pooled, FLMB displayed a more dispersed size distribution (Figure 3) with more fish attaining larger sizes (i.e., 16% more frequent at lengths > 400 mm) than ILMB in Puerto Rico. Furthermore, mean total length was greater for FLMB than ILMB overall and in 9 out of 12 populations. These results support previous findings that FLMB provide enhanced growth and survival potential in Puerto Rico, as Neal and Noble (2002) found that FLMB displayed greater longevity (i.e., represented 76% of total recaptures of fish at age-2 and 100% at ages-3 and 4) than ILMB, but no differences in mean growth rates. Their study was conducted in only one reservoir (Lucchetti Reservoir), whereas the current study included 12 reservoirs distributed throughout Puerto Rico. However, the current study lacks reservoir-specific growth or mortality estimates that would help distinguish a favored genotype on the island. Nevertheless, it is clear that FLMB offer greater potential of providing larger Largemouth Bass in Puerto Rico.

In conclusion, the observed increase in size potential of FLMB compared to ILMB in Puerto Rico supports the decision to maintain pure FLMB in the Maricao Hatchery. Supplemental stocking of offspring from pure FLMB broodstock could increase FLMB genetic influence, which may improve size structure among the ILMB populations in Puerto Rico. If improvements in size structure are a management goal, I recommend that all Largemouth Bass utilized as broodstock for the Maricao Hatchery be genetically evaluated and verified as pure FLMB before being used as broodstock.

Supplemental importations of pure FLMB from the United States could be used to establish and maintain pure FLMB at the Maricao Fish Hatchery. Their offspring could increase FLMB influence among populations and potentially augment genetic variation to increase fitness in Puerto Rico.

Tables and Figures

Table 2.1 Importation and stocking history of Largemouth Bass in Puerto Rico from 1915 to 2010.

Year	Species	Number	Size	Source	Recipient
1915	***Micropterus salmoides***	**600**	**n/a**	**Unknown**	**Maricao Hatchery**
1916	***Micropterus salmoides***	**600**	**n/a**	**Unknown**	**Maricao Hatchery**
1946	***Micropterus salmoides***	**1,067**	**n/a**	**Georgia and Mississippi**	**Maricao Hatchery**
	Micropterus salmoides	**88**	**adults**	**Georgia and Mississippi**	**Maricao Hatchery**
	Micropterus salmoides	**10,538**	**19-76**	**Maricao Hatchery**	**Island's reservoirs; La Plata River**
1947	***Micropterus salmoides***	**22,977**	**n/a**	**Maricao Hatchery**	**Island's reservoirs**
1948	***Micropterus salmoides***	**8,866**	**n/a**	**Maricao Hatchery**	**Island's reservoirs**
1949	***Micropterus salmoides***	**298**	**n/a**	**Maricao Hatchery**	**Island's reservoirs**
1950	***Micropterus salmoides***	**7,455**	**n/a**	**Maricao Hatchery**	**Island's reservoirs**
1951	***Micropterus salmoides***	**100**	**n/a**	**Maricao Hatchery**	**Island's reservoirs**
1957	**Florida Largemouth Bass (*M. s. floridanus*)**	**30**	**n/a**	**Waleka Hatchery, Florida**	**Maricao Hatchery**
1994	***Micropterus salmoides***	**3,966**	**38.1**	**Maricao Hatchery**	**Loiza Reservoir**
1995	***Micropterus salmoides***	**64,653**	**25-63.5**	**Maricao Hatchery**	**Carite, Carraizo, Cidra, Dos Bocas, Guayabal, Guayo, Patillas, Ponce I, Ponce II, Ponce III, Prieto, Toa Vaca reservoirs; Tortuguero Lagoon**
1996	***Micropterus salmoides***	**49,467**	**38-76**	**Maricao Hatchery**	**Carite, Dos Bocas, La Plata, Lucchetti, Toa Vaca reservoirs**
	M. s. floridanus	**1,412**	**n/a**	**Maricao Hatchery**	**Lucchetti Reservoir**
1997	***Micropterus salmoides***	**38,026**	**38-76**	**Maricao Hatchery**	**Carite, Carraizo, Cidra, Dos Bocas, Garzas, Las Curias, Lucchetti, Toa Vaca reservoirs; Tortuguero Lagoon; private pond**
	M. s. floridanus	**48,520**	**25-76**	**Maricao Hatchery**	**Cerrillos, Guajataca, and Lucchetti reservoirs**
1998	***Micropterus salmoides***	**41,114**	**25-76**	**Maricao Hatchery**	**Dos Bocas, Guayabal, La Plata, Las Curias, Patillas, Toa Vaca reservoirs; private ponds**
2000	***M. s. floridanus***	**193,024**	**38-69**	**Maricao Hatchery**	**Carraizo, Cidra, Dos Bocas, Garzas, Guajataca, Guayabal, Guayo, La Plata, Matrullas, Patillas, Toa Vaca, reservoirs; Tortuguero Lagoon**
2001	***M. s. floridanus***	**133,421**	**25-171**	**Maricao Hatchery**	**Carraizo, Cidra, Dos Bocas, Guajataca, Guayabal, La Plata, Las Curias, Lucchetti, Toa Vaca, reservoirs; private ponds**
2002	***M. s. floridanus***	**84,419**	**44.4-63.5**	**Maricao Hatchery**	**Caonillas, Cidra, Dos Bocas, Garzas, Guajataca, Guayo, La Plata, Loco reservoirs; private ponds**
2003	***M. s. floridanus***	**70,474**	**37-62**	**Maricao Hatchery**	**Cidra, Dos Bocas, Guajataca, La Plata, Toa Vaca, reservoirs; private ponds**
2004	***M. s. floridanus***	**62,514**	**37-62**	**Maricao Hatchery**	**Caonillas, Carite, Dos Bocas, Guajataca, La Plata, Lucchetti reservoirs; private ponds**
2005	***M. s. floridanus***	**41,675**	**37-62**	**Maricao Hatchery**	**Cidra, Dos Bocas, Guajataca, Lucchetti reservoirs; private ponds**
2006	***M. s. floridanus***	**57,767**	**37-62**	**Maricao Hatchery**	**Caonillas, Carite, Cidra, Dos Bocas, La Plata, Patillas reservoirs; private ponds**
2007	***M. s. floridanus***	**64,297**	**37-62**	**Maricao Hatchery**	**Cidra, Caonillas, Carraizo, Curias, Guajataca, Guayabal reservoirs; private ponds**
2008	***M. s. floridanus***	**44,360**	**37-62**	**Maricao Hatchery**	**Caonillas, Carraizo, Dos Bocas, Guayabal, Lucchetti reservoirs; private ponds**
2009	***M. s. floridanus***	**47,380**	**37-62**	**Maricao Hatchery**	**Caonillas, Guajataca, La Plata reservoirs; private ponds**
2010	***M. s. floridanus***	**35,308**	**37-62**	**Maricao Hatchery**	**Garzas, Guajataca, La Plata reservoirs**

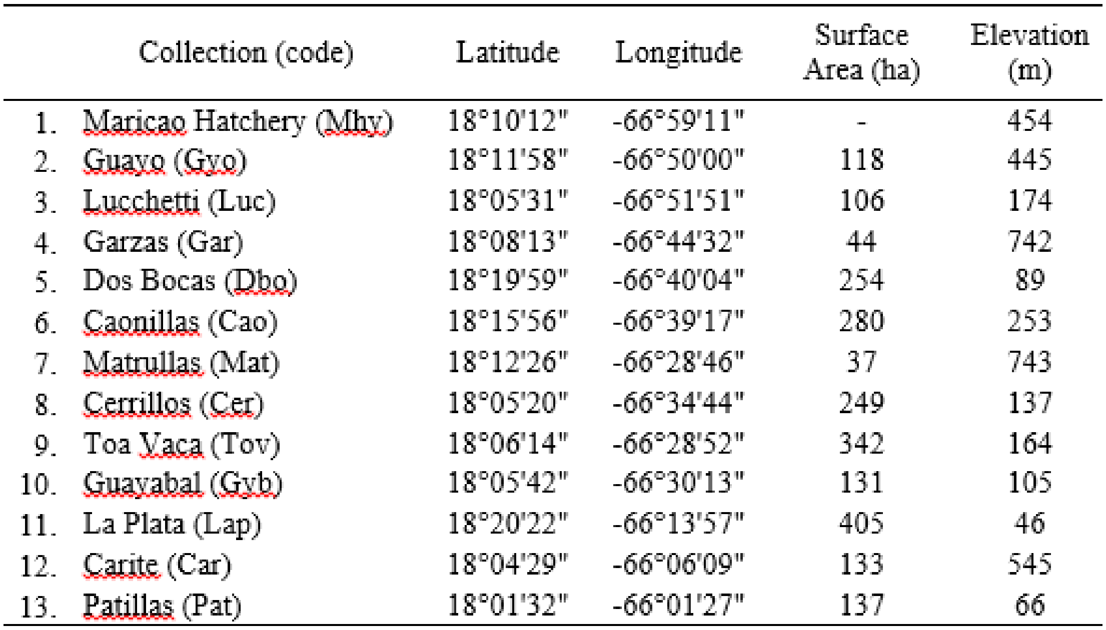
Micropterus salmoides represents suspected introgressed Largemouth Bass genotypes, and *M. s. floridanus* represents the Florida Largemouth Bass genotype, although some introgression cannot be ruled out. Numbers of fish, size range (total length; mm), and source/recipient population data are given where available (n/a is not available; derived from Erdman 1984 and Neal et al. 2004).

Table 2.2 Allele frequencies of three diagnostic allozyme loci for Largemouth Bass from ten reservoirs in Puerto Rico.

		*AAT-B**			*IDHP-B**			*MDH-B**		
Reservoir	N	**1*	**3*	**4*	**1*	**3*	**4*	**1*	**2*	$FLMB_A$
Caonillas	33	0.167	0.652	0.182	0.212	0.788	0.000	0.182	0.818	0.81
Carite	7	0.000	0.571	0.429	0.214	0.786	0.000	0.000	1.000	0.89
Cerrillos	25	0.000	0.760	0.240	0.000	1.000	0.000	0.000	1.000	1.00
Cidra	9	0.611	0.278	0.111	0.222	0.778	0.000	0.333	0.667	0.58
Dos Boca	7	0.000	0.857	0.143	0.286	0.714	0.000	0.286	0.714	0.86
Guajataca	6	0.000	0.583	0.417	0.000	1.000	0.000	0.000	1.000	1.00
Guayabal	30	0.267	0.233	0.500	0.177	0.883	0.000	0.317	0.683	0.78
Guayo	120	0.091	0.341	0.577	0.025	0.975	0.000	0.525	0.475	0.94
La Plata	7	0.571	0.286	0.143	0.071	0.857	0.071	0.500	0.500	0.64
Toa Vaca	6	0.167	0.167	0.666	0.000	1.000	0.000	0.167	0.833	0.92

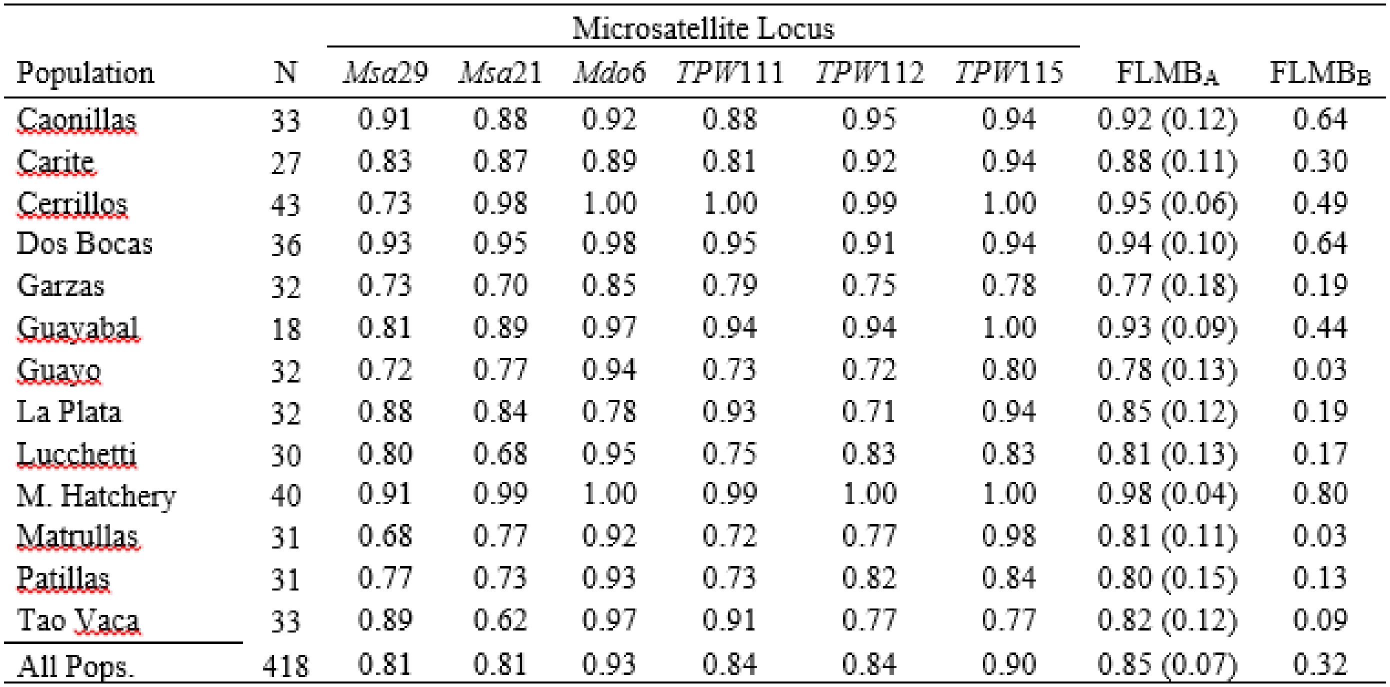
The proportion of Florida Largemouth Bass alleles ($FLMB_A$) among loci are also provided (modified from Neal and Noble 2002). Locus *MDH-B** was not included in the proportion of Florida Largemouth Bass alleles because it was only used as a secondary degree of intergradation by Neal and Noble (2002).

Table 2.3 Locations, surface area (ha), and elevation (m) above sea level of the Maricao Fish Hatchery and 12 reservoir populations from which Largemouth Bass were sampled in this study.



Collection (code)	Latitude	Longitude	Surface Area (ha)	Elevation (m)
1. Maricao Hatchery (Mhy)	18°10'12"	-66°59'11"	-	454
2. Guayo (Gyo)	18°11'58"	-66°50'00"	118	445
3. Lucchetti (Luc)	18°05'31"	-66°51'51"	106	174
4. Garzas (Gar)	18°08'13"	-66°44'32"	44	742
5. Dos Bocas (Dbo)	18°19'59"	-66°40'04"	254	89
6. Caonillas (Cao)	18°15'56"	-66°39'17"	280	253
7. Matrullas (Mat)	18°12'26"	-66°28'46"	37	743
8. Cerrillos (Cer)	18°05'20"	-66°34'44"	249	137
9. Toa Vaca (Tov)	18°06'14"	-66°28'52"	342	164
10. Guayabal (Gyb)	18°05'42"	-66°30'13"	131	105
11. La Plata (Lap)	18°20'22"	-66°13'57"	405	46
12. Carite (Car)	18°04'29"	-66°06'09"	133	545
13. Patillas (Pat)	18°01'32"	-66°01'27"	137	66

Table 2.4 Proportion of Florida Largemouth Bass alleles ($FLMB_A$; ± *S.D.*) and genotypes ($FLMB_B$) and the average proportion of Florida Largemouth Bass alleles and genotypes observed among all populations (All Pops.) at 6 diagnostic microsatellite loci for 12 Puerto Rico reservoirs and the 2011 Maricao Fish Hatchery captive broodstock.



		Microsatellite Locus							
Population	N	*Msa*29	*Msa*21	*Mdo*6	*TPW*111	*TPW*112	*TPW*115	$FLMB_A$	$FLMB_B$
Caonillas	33	0.91	0.88	0.92	0.88	0.95	0.94	0.92 (0.12)	0.64
Carite	27	0.83	0.87	0.89	0.81	0.92	0.94	0.88 (0.11)	0.30
Cerrillos	43	0.73	0.98	1.00	1.00	0.99	1.00	0.95 (0.06)	0.49
Dos Bocas	36	0.93	0.95	0.98	0.95	0.91	0.94	0.94 (0.10)	0.64
Garzas	32	0.73	0.70	0.85	0.79	0.75	0.78	0.77 (0.18)	0.19
Guayabal	18	0.81	0.89	0.97	0.94	0.94	1.00	0.93 (0.09)	0.44
Guayo	32	0.72	0.77	0.94	0.73	0.72	0.80	0.78 (0.13)	0.03
La Plata	32	0.88	0.84	0.78	0.93	0.71	0.94	0.85 (0.12)	0.19
Lucchetti	30	0.80	0.68	0.95	0.75	0.83	0.83	0.81 (0.13)	0.17
M. Hatchery	40	0.91	0.99	1.00	0.99	1.00	1.00	0.98 (0.04)	0.80
Matrullas	31	0.68	0.77	0.92	0.72	0.77	0.98	0.81 (0.11)	0.03
Patillas	31	0.77	0.73	0.93	0.73	0.82	0.84	0.80 (0.15)	0.13
Tao Vaca	33	0.89	0.62	0.97	0.91	0.77	0.77	0.82 (0.12)	0.09
All Pops.	418	0.81	0.81	0.93	0.84	0.84	0.90	0.85 (0.07)	0.32

Table 2.5 Overall percent frequency by length group for all Largemouth Bass, pure Florida Largemouth Bass (FLMB), and intergrade (Florida x northern) Largemouth Bass (ILMB) collected from 12 Largemouth Bass populations in Puerto Rico.

	Percent Frequency			
Size Group	All (365)	FLMB (101)	ILMB (264)	Difference (FLMB-ILMB)
PSD S-Q	17.81	16.83	18.18	-1.35
PSD Q-P	48.77	42.57	51.14	-8.56
PSD P-M	25.21	25.74	25.00	0.74
PSD M-T	7.95	13.86	5.68	8.18
PSD-T	0.00	0.99	0.00	0.99
> 300 mm	82.19	83.17	81.82	1.35
> 400 mm	24.93	36.63	20.45	16.18
> 500 mm	8.49	14.85	6.06	8.79
> 600 mm	0.27	0.99	0.00	0.99

Frequencies of each proportional size distribution length increment and cumulative frequencies by length class are presented.

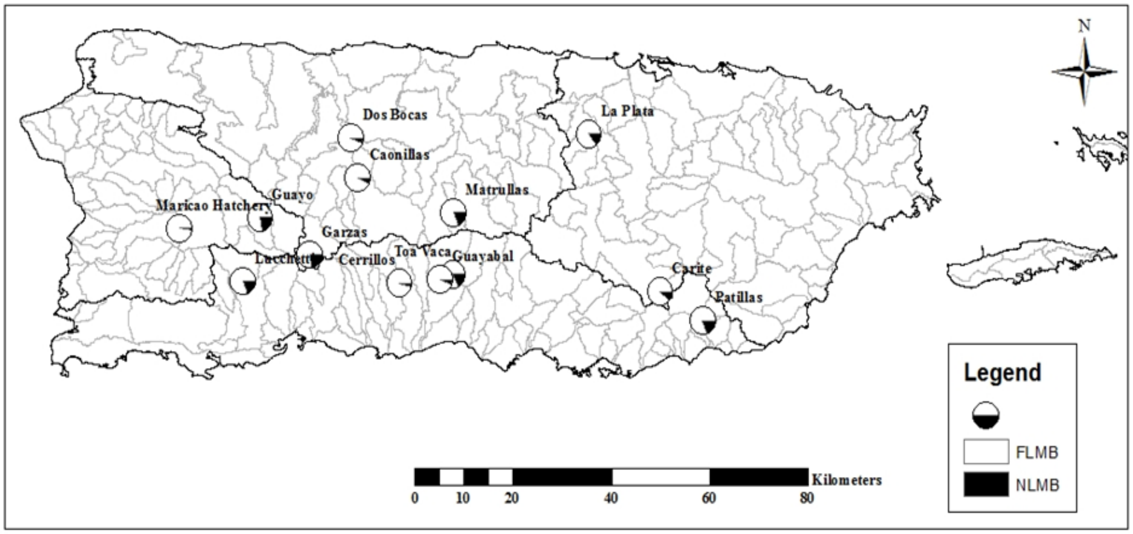


Figure 2.1 Locations of 12 reservoirs and 1 hatchery population in Puerto Rico sampled for this study.

Pie charts depict the frequency of northern Largemouth Bass and Florida Largemouth Bass alleles at six diagnostic microsatellite loci (Msa29, Msa21, Mdo6, TPW111, TPW112, TPW115) within populations. The black lines delineate the major hydrologic units designated by the U.S. Geological Survey, and the gray lines indicate watershed boundaries.

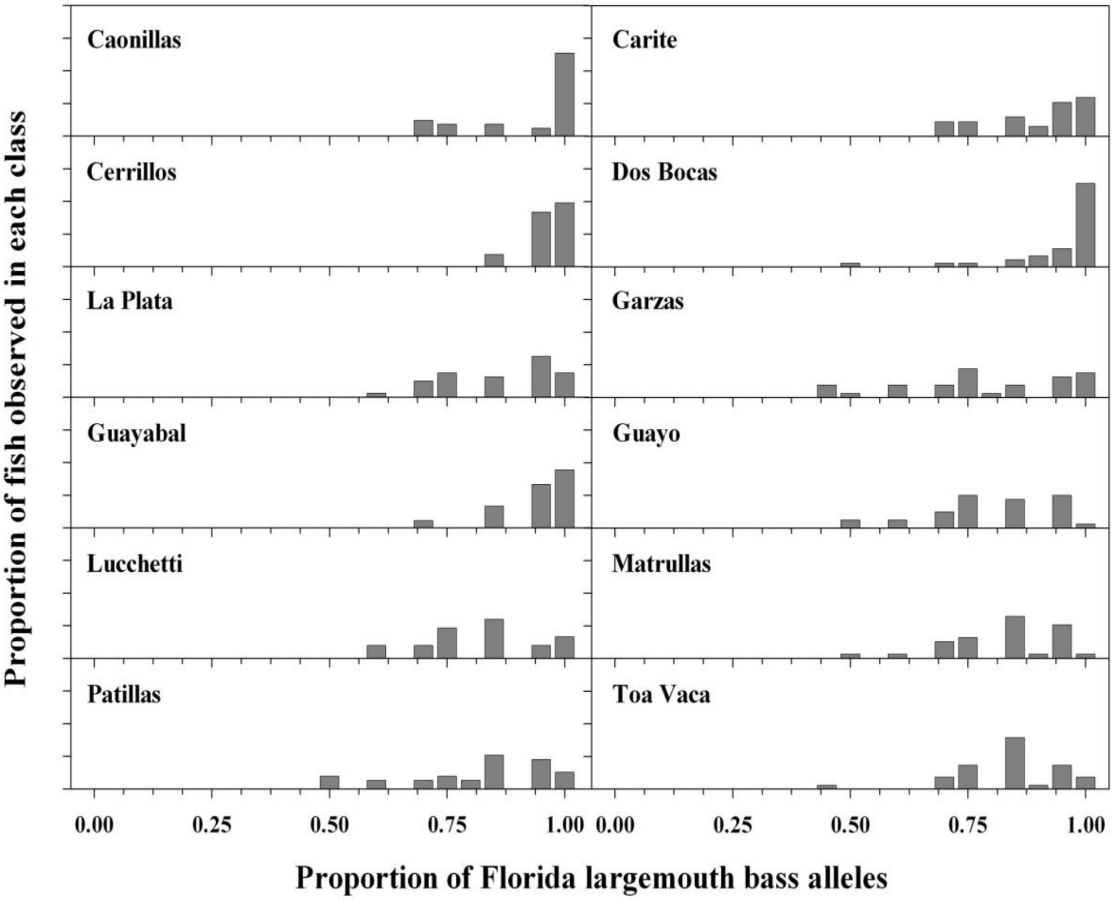


Figure 2.2 Hybrid index frequency histogram for Florida Largemouth Bass x northern Largemouth Bass intergrade populations in several Puerto Rico reservoirs.

Estimates of introgression are based on six diagnostic microsatellite loci (Msa29, Msa21, Mdo6, TPW111, TPW112, TPW115; Lutz-Carrillo et al. 2008). The scale ranges from 0.0 (individuals with all northern Largemouth Bass alleles) to 1.0 (individuals with all Florida Largemouth Bass alleles) via one-allele increments.

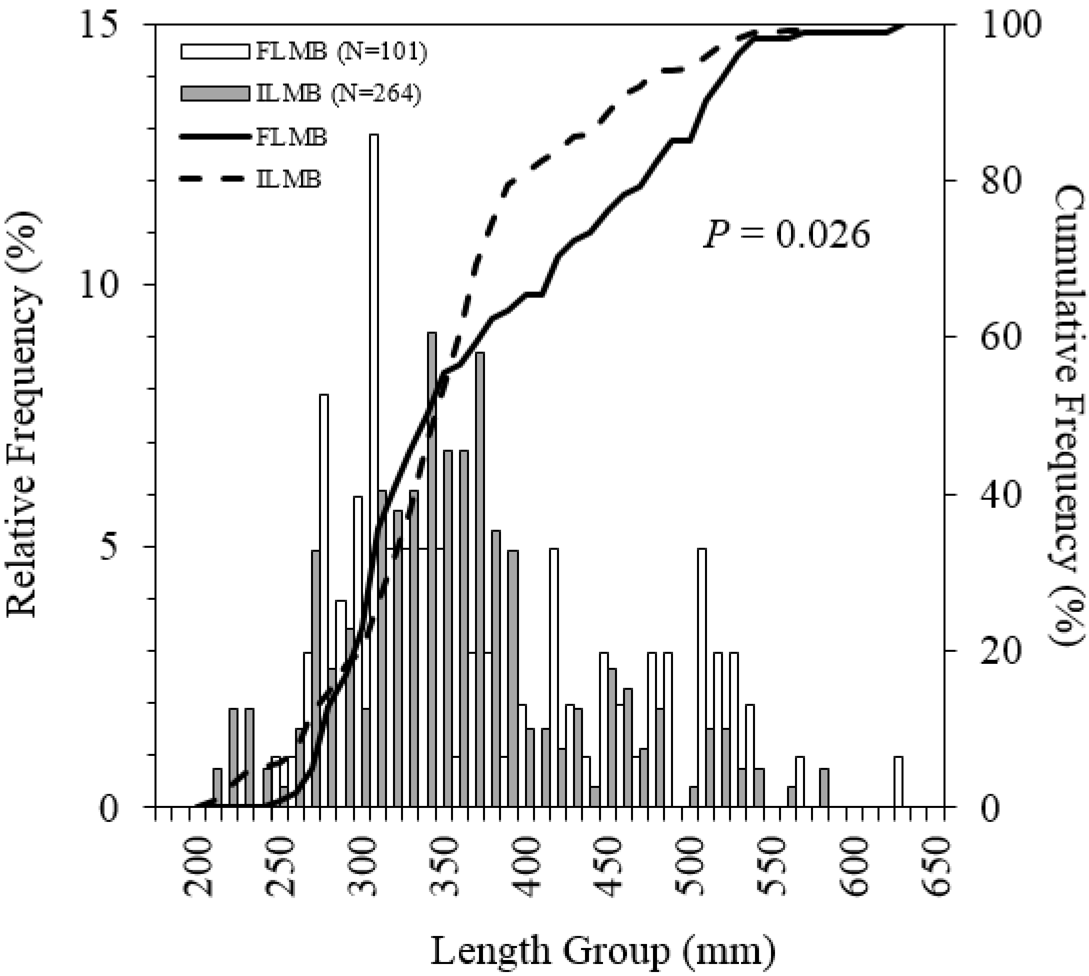


Figure 2.3 Relative length-frequency histogram (%; bars) and cumulative frequency (%; lines) for pure Florida Largemouth Bass (FLMB) and introgressed (Florida x northern) Largemouth Bass (ILMB) from 12 reservoirs in Puerto Rico.

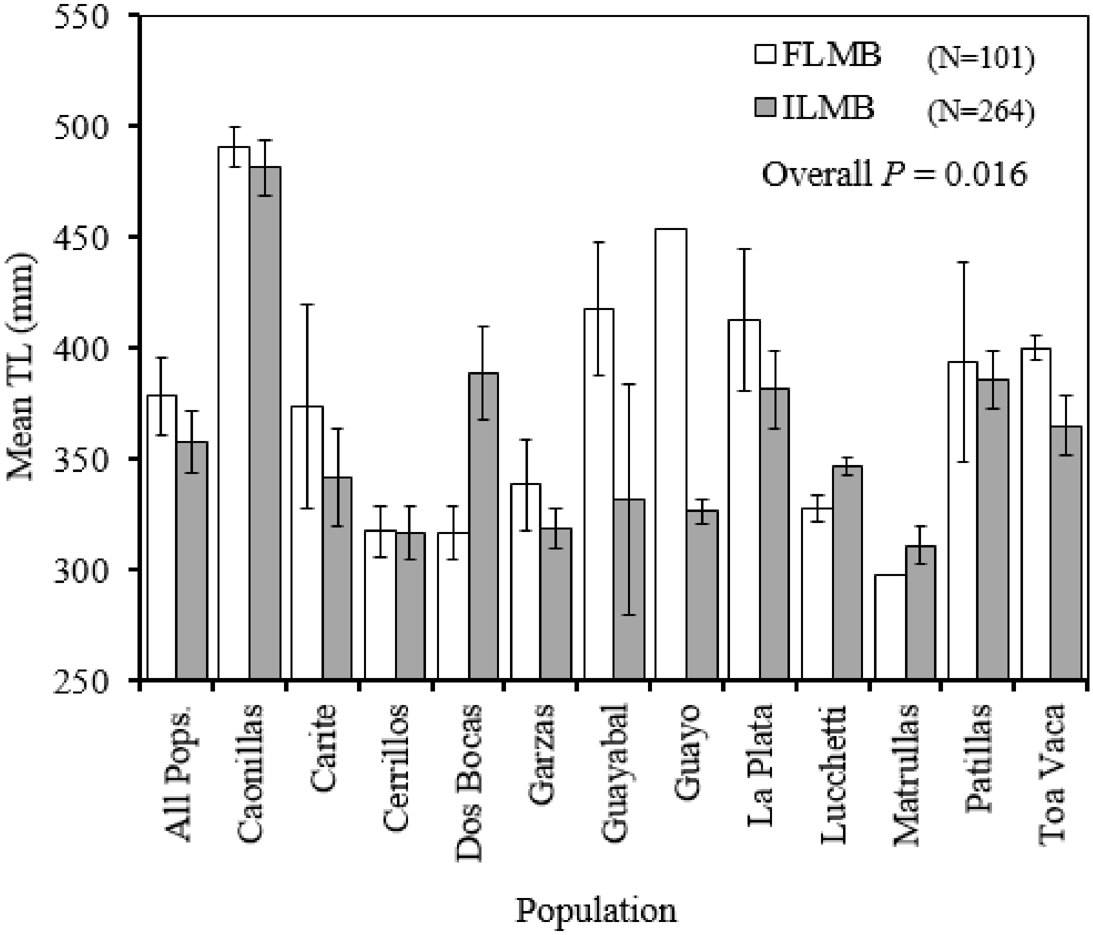


Figure 2.4 Mean total length (mm) of Florida Largemouth Bass (FLMB) and introgressed (Florida x northern) Largemouth Bass (ILMB) from 12 populations in Puerto Rico.

The Overall *P*-value indicates a test of mean total length of FLMB vs. ILMB pooled among all populations. Error bars represent standard error.

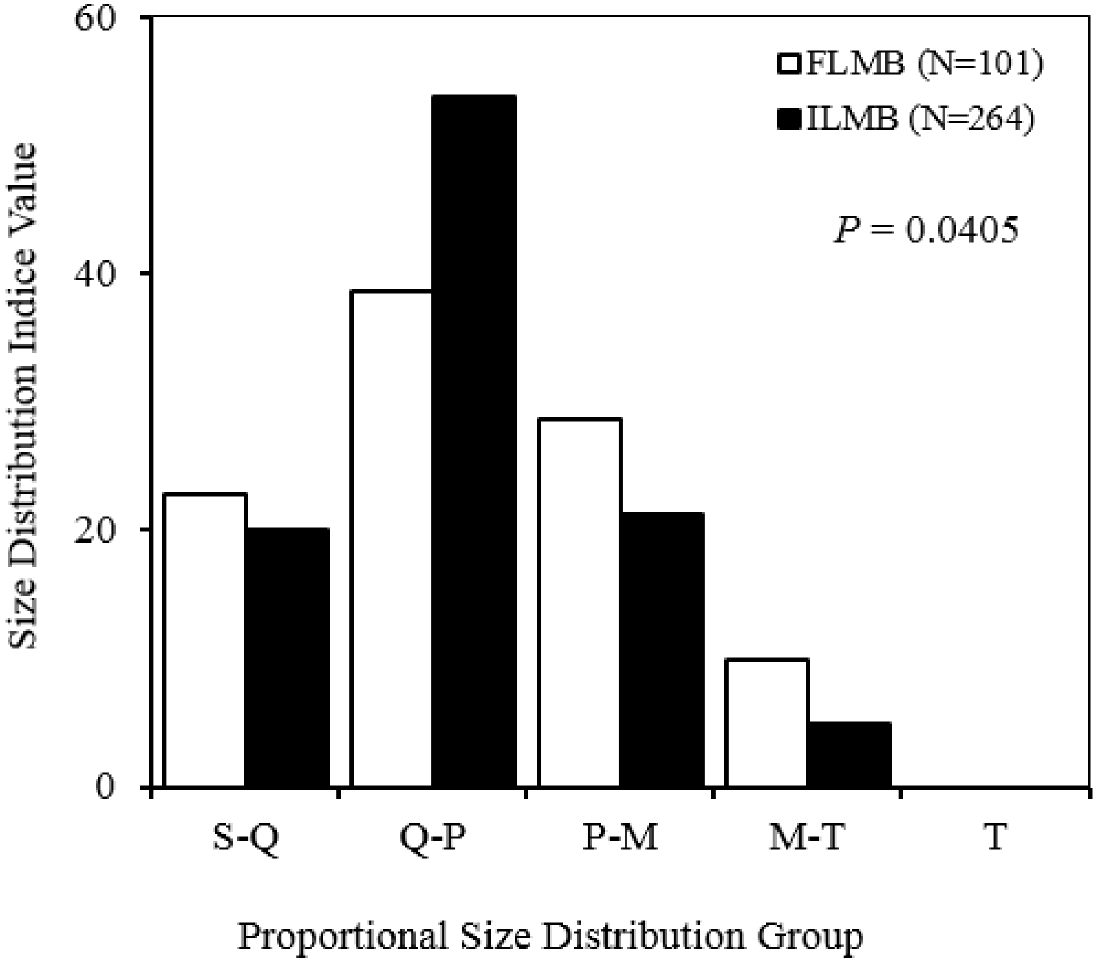


Figure 2.5 Proportional size distribution (PSD) value by incremental size categories for pure Florida Largemouth Bass (FLMB) and introgressed (Florida x northern) Largemouth Bass (ILMB) collected from 12 reservoirs in Puerto Rico.

PSD categories represent fish 200-299 mm (PSD-S-Q), 300-379 mm (PSD-Q-P), 380-510 mm (PSD-P-M), 510-630 mm (PSD-M-T), and 630 mm or greater (PSD-T) determined from proposed length categories for Largemouth Bass (Gabelhouse 1984).

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CHAPTER III

GENETIC VARIATION AND STRUCTURE OF LARGEMOUTH BASS IN PUERTO RICO

Introduction

Intentional introductory and supplemental stockings of Largemouth Bass *Micropterus salmoides* in Puerto Rico reservoirs have been occurring for nearly a century. Neal et al. (2004) defined four phases of fish stocking activities in Puerto Rico as: pre-hatchery, cold-water, warm-water, and modern phases. The pre-hatchery phase was characterized by species introductions from around the world, with many species establishing populations on the island. In 1937, the Maricao Fish Hatchery (hereafter, the Hatchery) was constructed for production of cold-water species, ushering in the short-lived cold-water phase, during which Rainbow Trout *Oncorhynchus mykiss* and Brown Trout *Salmo trutta* were reared for high-altitude stream stocking. The Hatchery shifted emphasis to warm-water species in 1946, and the early warm-water phase (1942-1973) consisted of numerous species introductions and supplementations with little attention paid to determination of need or stocking success. There is a 21-year gap in the stocking record following the early warm-water phase due to poor record keeping and limited management-oriented activities. The Hatchery was not active during this period and official stocking activities were limited; most stocking events consisted of unauthorized angler movements and undocumented transfers.

In 1994, the directive of the Hatchery was reestablished and the modern era of fish propagation and stocking began. The modern Hatchery has the capacity to produce fingerling Largemouth Bass any month of the year, but peak production is generally during summer months. Modern stocking allocations are based on perceived need and reservoir priority. Need is determined via young-of-year sampling results and recommendation of field biologists. Reservoir priority is set by the *Puerto Rico Reservoir Fisheries Management Manual*, where reservoirs are ranked based on size, fishing effort, access, and fisheries-related factors (Lilyestrom and Neal 2004).

The modern era has begun to incorporate genetic concerns into stocking decisions. Initial transfers of Largemouth Bass to Puerto Rico apparently consisted of fish with alleles of both the northern *M. s. salmoides* and Florida *M. s. floridanus* (hereafter, Florida bass) subspecies (Erdman 1984). A more recent transfer in 1957 from Florida introduced the pure Florida subspecies (Neal et al. 2004). Neal and Noble (2002) reported greater longevity of the pure Florida subspecies, and recommended that only this genetic group be cultured and released. Two freshwater systems, Guajataca and Cerrillos Reservoir, were reported to contain only pure Florida Largemouth Bass, and all Hatchery broodstock were collected from these two reservoirs (Neal et al. 1999). More recently, broodstock collections are conducted exclusively at Cerrillos Reservoir.

Geographical, ecological, behavioral, and anthropogenic (e.g., human introductions) factors may influence genetic differentiation among populations and can lead to population structure. Population subdivision often results from genetic or geographic isolation, or reduced genetic connectedness, and can reduce gene flow, the movement between groups that results in genetic exchange. When genetic connectedness is high, gene flow will tend to homogenize the genetic variation over the groups. If gene flow is low, genetic processes (e.g., genetic drift) can lead to genetic differentiation among groups. Even when genetic connectedness is low, a small number of migrants can counteract the effect of genetic drift (Hedrick 2011).

Hybrid populations that receive migrants from two or more other populations can undergo rapid genetic change and show genetic differences (Hedrick 2011). Genetic differentiation may result from natural selection favoring different genotypes in different populations, from random process of transmission of alleles during meiosis, or from chance differences in allele frequencies among the initial founders of the populations (Hartl and Clark 2007). Genetic variation is provided by mutation and migration and is eroded by genetic drift and natural selection. The interactions between these genetic processes greatly influence the fitness of Largemouth Bass in different environments (Philipp et al. 1985; Philipp and Whitt 1991; Philipp 1992).

Atypical life history characteristics have provided a challenge for Largemouth Bass management in Puerto Rico. Compared to Largemouth Bass in temperate reservoirs, most fish in tropical reservoirs do not survive to age-4 and rarely reach large sizes (Neal and Noble 2002). This short life span and small size occurs in reservoirs throughout the island despite an apparent abundance of prey and year-round growing season (Alicea et al. 1997; Neal 2003). Management authorities have questioned if similar poor growth and survival in Largemouth Bass among Puerto Rico reservoirs is restricted by a small gene pool that resulted from limited initial introductions, and/or if management strategies (e.g., stocking frequency and rate) have maintained similar genetic diversity among populations.

There were only a handful of documented introductions of Largemouth Bass to Puerto Rico. Records indicate that 118 adults and 1,067 fish of undocumented size (assumed to be juveniles) were successfully imported to the island. Subsequent stocking events (from the surviving brood fish) are likely the result of production from only a few individuals. Such a founder event may have resulted in the loss of genetic variation, increased probabilities of inbreeding, and the stochastic fixation of deleterious alleles reducing adaptive potential in the population (Allendorf and Luikart 2007). If genetic variation is significantly lower in Puerto Rico populations compared to populations in their native range, it would be likely that a small gene pool was provided from initial introductions to the island. In this case, future research would be justified to determine if genetics influence growth and survival among reservoirs, and if introductions from their native range may be warranted to introduce new genetic material.

Although a limited number of individuals were introduced to the island, more than one hybridizing lineage is represented in Puerto Rico Largemouth Bass populations (Erdman 1984), which could influence genetic variation and fitness. Genetic variation typically increases with mating of two or more parental populations over an extended period of time as long as random mating occurs between lineages (Hedrick 2011). Puerto Rico reservoirs have been sporadically stocked at different rates over the last decade. Stocking is known to disrupt the relative stability of genetic diversity within a population over time and homogenize genetic characteristics among populations (Allendorf and Luikart 2007). Although Largemouth Bass stocking rates have differed among Puerto Rico reservoirs, only a few migrants (or stocked fish) per generation could homogenize genetic differences within and among populations (Hedrick 2011). Similar genetic variation among populations in Puerto Rico would indicate that initial limited introductions to the island and/or subsequent management strategies (stocking) have homogenized genetic variation among populations.

The genetic makeup of Largemouth Bass in Puerto Rico could also be heavily influenced by natural selection because different reservoirs provide much different habitats and experience vastly different environments (e.g., productivity, elevation, precipitation patterns, and geology) depending on location. If genetic structure was apparent among reservoirs with similar characteristics (e.g., elevation), regardless of stocking influences, genetic differences resolved may be due to natural selection rather than genetic drift (small population size). In this Chapter, my objectives were to 1) quantify genetic variation in Largemouth Bass populations in Puerto Rico using microsatellite genetic markers and compare to populations in their native range, and 2) determine if genetic structure is apparent among reservoir populations in Puerto Rico. I hypothesized that the initial introductions provided limited genetic variation compared to Largemouth Bass populations in their native range, and that reservoirs with lower assigned management priority, and presumably lower stocking frequency, will exhibit greater genetic differentiation from each other than populations in reservoirs assigned higher management priority.

Methods

Study Area

This research was conducted on the tropical island of Puerto Rico (Figure 1). Reservoirs (n = 12) were chosen based on three criteria: 1) presence of Largemouth Bass, 2) management importance as determined by the Puerto Rico Department of Natural and

Environmental Resources, and 3) accessibility. Chosen reservoirs were distributed across the island and encompassed a range of sizes (37.2 – 405.0 ha) and elevations (46.0 – 743.1 m) (Table 1; Figure 1).

Puerto Rico collections were compared to three intergrade Florida Bass x northern Largemouth Bass populations and six Florida Bass populations in Florida, and one intergrade Florida Bass x northern Largemouth Bass population and one northern Largemouth Bass population in Texas (Table 1). Florida populations were chosen to incorporate introgressed and non-introgressed Florida Bass populations from four significant genetic structure groupings defined by Barthel et al. (2010), and based on proximity to the hypothesized populations of origin of initial introductions of Largemouth Bass to Puerto Rico (Erdman 1984). Texas populations were chosen to include an introgressed population outside of Florida, and a northern Largemouth Bass population.

Most reservoirs on the island receive sporadic supplemental stockings of Largemouth Bass, thus I also sampled the contemporary Largemouth Bass broodstock at the Hatchery in the Spring of 2011. These samples (N = 40) were used to characterize the contemporary genetic contribution from the Hatchery source population.

Sample Collections

For standardization purposes, six shoreline sites were established *a priori* in each reservoir. Three out of the six sites were then randomly selected in each reservoir and individually electrofished for 900 seconds using a boom-mounted electrofisher at 240-V pulsed DC. All Largemouth Bass were measured (total length; nearest mm) and weighed (nearest g). Additional collection sites were sampled if the necessary minimum sample size (N = 30) was not obtained.

All samples were obtained during June and July 2011. For each fish collected, a section (~25 mm^2) of the pectoral fin was removed, placed in a 2-ml storage vial, preserved in 70% ethanol, and stored at room temperature. Tissues were collected in accordance with American Fisheries Society (AFS) approved guidelines for the Use of Fishes in Research (available online at http://fisheries.org/docs/policy_useoffishes.pdf) under Mississippi State University Animal Care and Use Committee (IACUC) Protocol 10-074. Samples were then transported to the Texas Parks and Wildlife Departments' A.E. Wood Laboratory in San Marcos, Texas, for processing and genetic analyses. Processing took place during the summer and fall of 2011 under the supervision of Loraine Fries and Dijar Lutz-Carrillo.

Characterization of Microsatellite Variation

Deoxyribonucleic acid (DNA) was isolated from each tissue sample using the following procedure. Fin tissue (3-5 mm^3) was placed in 300 µL of cell lysis solution (10 mM tris-HCl, 10 mM EDTA [pH 8.0], and 2% sodium dodecyl sulfate), along with 5 µL of proteinase K (20 mg/mL), and incubated at 55°C for at least 3.0 hours. After incubation, 120 µL of ammonium acetate (7.5 M) was added and mixed. The solution was then incubated at 0°C for 15 min followed by centrifugation at 13,000 x g for 5 min. The supernatant was then mixed with 1,000 µL of 100% ethanol, stored at -80°C for 10 minutes, and centrifuged at 13,000 x g for 10 min. After decanting the supernatant the pellet was washed in 600 uL 70% ethanol and re-centrifuged at 13,000 x g for 5 min. The supernatant was again decanted and the pellet was dried at room temperature for 20 min before being rehydrated in 200 μL of TLE buffer and left overnight at room temperature. Recovered DNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and adjusted to a concentration of 50 ng/μl using water.

Each sample was genotyped using eleven polymorphic microsatellite loci within three multiplex reactions previously developed for Largemouth Bass, including MPX8, MPX11 and MPX16 (Lutz-Carrillo et al. 2008; Table 2 and 3). Primers identical in sequence to the tails on the site-specific primers were labeled with IRDye700 or IRDye800 tags (IDT, Coralville, Iowa, USA). Segments of DNA were amplified using an Eppendorf MasterCycler® Pro S thermalcycler (Eppendorf, Hamburg, Germany) and polymerase chain reaction (PCR). Thermocycler temperature profiles for all loci and multiplexes used included 10x PCR Buffer without $MgCl_2$ (all buffer concentrations were 1X; Thermo Fisher Scientific Inc., Waltham, MA), dNTP mix, 50 mM magnesium chloride solution ($MgCl_2$), and 5 U/ul of Taq DNA polymerase. The PCR products were combined with stop solution [2.5 mM EDTA, 7.5 mM Bromophenol Blue, in formamide] and denatured by heat before separation on a NEN 4200 Global IR2 DNA Sequencer (LICOR). Alleles were sized against standards [LICOR 50-350 ladder] within BioNumerics v. 6.5 (Applied-Maths, Belgium). All genotypes were confirmed visually and recorded.

Hardy-Weinberg Equilibrium and Linkage Disequilibruim

Largemouth Bass were initially grouped according to collection sites for statistical analyses (Table 1). All collections were tested for deviations from Hardy-Weinberg equilibrium (HWE) and evidence of linkage disequilibrium (LD; i.e. non-random association of alleles among loci) using the software GenAlEx version 6.5 (10,000 Markov-chain permutations; Peakall and Smouse 2006). Hardy-Weinberg tests employed 10^5 recorded Markov-Chain-Monte-Carlo (MCMC) chains, following a burn-in of 10^3 chains. Linkage tests employed 10^5 randomizations. Population (sample) conformance to HWE expectations were tested using a chi-square test. Significant population x locus comparisons that did not conform to HWE were summed for rare genotypes (expected and corresponding observed values that were less than 1.0; Hedrick 2011) to create a single value, and then re-evaluated for the observed to expected values using a chi-square test (see Kapuscinski et al. 2013). Test results were then reevaluated using sequential Bonferroni corrections for multiple comparisons (Rice 1989) for a global $\alpha = 0.05$.

Genetic Diversity

Allele frequencies and measures of genetic diversity including the mean number of alleles per locus (N_A), number of private alleles (N_P), observed heterozygosity (H_O), expected heterozygosity (H_E), and F_{IS} (a measure of departure from HWE and inbreeding within populations) were determined for each locus within populations using GenAlEx v. 6.5 (Peakall and Smouse 2006). Allelic richness (A_R) was corrected for differences in sample size among different locations and calculated based on a minimum of 10 genes using the program HP-Rare v1.0 (Kalinowski 2004).

Genetic Structure

I used three methods to delineate the genetic structure of Largemouth Bass in Puerto Rico. These methods included: 1) Model-based Bayesian clustering

(STRUCTURE), 2) pairwise estimates of F_{ST} among populations, and 3) estimates of Cavalli-Sforza and Edwards (1967) genetic distance.

I first used the program STRUCTURE version 2.3.3 (Pritchard et al. 2000) to estimate the extent of population genetic structure among sampling sites. Estimates of nuclear genetic structure were performed with the Bayesian model implemented in STRUCTURE version 2.3.3 (Pritchard et al. 2000; Falush et al. 2003). This model allows an admixed ancestry and attempts to partition multilocus genotypes into an optimal number of groups whose composition minimizes Hardy-Weinberg and linkage disequilibrium. Simultaneously, genotypes are assigned to each resolved group with an associated membership coefficient (q). Runs were performed with multilocus microsatellite genotypes (11 loci) from all 12 populations. An admixture model with independent allele frequencies and non-informative priors was used as suggested by Hubisz et al. (2009) to minimize the chances of overestimating the number of groups. The ad hoc statistic ΔK was used in conjunction with the distribution of likelihood and α values (i.e., admixture among resolved groups) to evaluate each solution as suggested by Evanno et al. (2005). After preliminary experimental runs were conducted, 10 independent replicates of the number of genetic clusters (K) from 1 to 10 were performed with 10^4 burn-in steps and 10^5 Markov-chain Monte Carlo iterations. Genotypes composing each resolved cluster at the optimal K were then re-evaluated under independent STRUCTURE runs as suggested for data sets containing genotypes from contact zones (Evanno et al. 2005). Values of *q* were averaged over all 10 runs for individual fish and then over populations to allow the assignment of populations to groups similar to Barthel et al. (2010).

An initial test of population differentiation was performed to test a global hypothesis of panmixia for all sampled Largemouth Bass populations. This hypothesis was tested using the Exact test of population differentiation employed in ARLEQUIN v3.5 (Excoffier et al. 2005). Significance was determined through a Markov-chain method (10,000 dememorization steps) for a nominal α of 0.05. These tests were used to describe how much of the total observed genetic variation could be explained by partitioning the variation among alternative sources of differences, with a null hypothesis assuming no allelic frequency differences among samples.

Indications of genetic structure were then explored as genetic distances between populations using a consensus neighbor-joining (NJ) and Cavalli-Sforza and Edwards (1967) genetic distances in PHYLIP version 3.6 (Felsenstein 1993). A majority rule consensus dendogram was constructed using CONSENSE package in the PHYLIP version 3.6 (Felsenstein 1993). Confidence in the topology was estimated by bootstrapping over 5,000 pseudoreplicates in PHYLIP. The dendogram was then visualized with FigTree v1.3.1 (Rambaut 2006). Resolved relations/groups of populations were used to determine hypothetical population groups for subsequent analysis.

I hypothesized that reservoirs with higher management priority, and presumably greater stocking frequency, would be genetically similar. The complete stocking history for most Largemouth Bass populations in Puerto Rico is ambiguous or lacking. Therefore, I used the contemporary management priority rankings outlined by Lilyestrom and Neal (2004) as a surrogate for the genetic impact of stocking in each population. Lilyestrom and Neal (2004) ranked each reservoir based on six criteria (e.g., size, fishing pressure, multiple top-level predators) and recommended that sampling priority be given to those reservoirs with a ranking of 5 or greater; reservoirs with lower priority should receive sampling and management efforts according to time and resource constraints. I assigned each reservoir into a low-priority group (priority rankings ≤ 5) and a high-priority group (priority ranking > 5) to test our null hypothesis that management priority did not influence genetic structure. Groupings were tested for significance using an analysis of molecular variance (AMOVA) in ARLEQUIN v3.5 (Excoffier et al. 2005). Significance was computed by non-parametric permutation of the data set with 99,999 permutations (Lowe et al. 2004).

Results

A total of 419 Largemouth Bass samples were collected and genotyped from Puerto Rico. From this total, 379 were collected from reservoirs and 40 were collected from the 2011 Hatchery broodstock. Two-hundred twelve Largemouth Bass genotypes from Florida (N=157) and Texas (N=55) were used to compare genetic variation between Puerto Rico and populations in their native range (Table 1).

Genetic Diversity

All 11 microsatellite loci were polymorphic in Puerto Rico populations except for Cerrillos Reservoir and the Hatchery, which were monomorphic at the locus *Mdo*6 (Table 4). Initially, 22 population x locus comparisons (132 total tests) were shown out of HWE (excluding the 2 monomorphic loci); only one (Caonillas Reservoir; *locus: Msa*21; acronyms provided in Table 1) did not conform to HWE after applying Hedrick adjustments (Table 4). All loci were polymorphic in Florida and Texas populations except for Lake Panasoffkee, which was monomorphic at locus *Mdo6* (Table 4). Three loci from three Florida populations were initially out of HWE (GEOR: loci=*Lar7* and *Tpw96*; PANA: *Msa29* and *Msa21*; STFE: *Tpw157* and *Tpw55*), but all loci conformed to HWE after applying Hedrick adjustments (Table 4). Fifty-two locus x locus populations comparisons were in linkage disequilibrium, but only 3 were significant after applying Bonferroni corrections. Therefore, these loci were assumed not to be physically linked and all eleven loci were used in subsequent analyses.

The total number of alleles observed at individual loci (N_A) in Puerto Rico populations ranged from 1 [CERR] to 16 [LUCC], which was lower than Florida (range=1 [PANA] to 25 [STJO]), and Texas (range = 2 [KICK] to 26 [FORK]; Table 4). The average number of alleles per locus among Puerto Rico reservoirs (A_n = 6.159, A_nSD = 3.081, range = 5.091 to 7.182) was lower than Florida (A_n = 9.894, A_nSD = 6.566, range = 7.818 to 11.091) and Texas (A_n = 9.909, A_nSD = 6.316, range = 7.091 to 12.727; Table 5). The average observed heterozygosity (0.616, SD = 0.248, range = 0.540 to 0.701) and the average expected heterozygosity (0.628, SD = 0.241, range = 0.531 to 0.689) among Puerto Rico reservoirs was slightly lower than Florida (H_O = 0.660, H_OSD = 0.258, range = 0.591 to 0.722; H_E = 0.683, H_ESD = 0.258, range = 0.629 to 0.725) and Texas (H_O = 0.698, H_OSD = 0.211, range = 0.606 to 0.79; H_E = 0.724, H_ESD = 0.194, range = 0.638 to 0.810; Table 5). The average allelic richness (A_R) among reservoirs was 3.856 (range = 3.230 to 4.440), which was less than Florida (4.780, range = 4.230 to 5.080) and Texas (5.000, range = 4.200 to 5.800; Table 5). Inbreeding coefficients (F_{IS}) ranged from -0.034 [MATR] to 0.109 [CAON] in Puerto Rico reservoirs, from 0.003

[STJO] to 0.117 [STFE] in Florida, and from 0.041 [FORK] to 0.071 [KICK] in Texas (Table 5).

The Hatchery broodstock had lower levels of genetic variation than Puerto Rico reservoir populations. The average number of alleles per locus (A_n) was 5.636 (A_nSD =2.908), and N_A observed at individual loci ranged from 1 to 11 alleles per locus. Observed heterozygosity was 0.571 (H_OSD = 0.336), expected heterozygosity was 0.541 (H_ESD = 0.314), and F_{IS} was -0.041. Allelic richness (3.380) was lower than all reservoirs except Cerrillos Reservoir (A_R = 3.230; Table 5).

Fewer private alleles (alleles found in one group but not another) were found among Largemouth Bass populations in Puerto Rico than Florida and Texas (Table 5 and 6; Figure 2). Based on a combined dataset that included all fish genotyped from Puerto Rico, Florida and Texas, only 4 loci had private alleles among Largemouth Bass in Puerto Rico (TAOV: locus = *Tpw60*, allele = 537, freq = 0.019; PATI: locus = *Tpw96*, allele = 591, freq = 0.017; GARZ: locus = *Tpw70*, allele = 344, freq = 0.016; MARH: locus = *Tpw96*, allele = 656, freq = 0.013); all private alleles were in separate individuals. Private alleles were resolved in 72 alleles from 61 individuals in Florida, and 31 alleles from 28 individuals in Texas. Where private alleles were observed within a reservoir population (excluding the Hatchery), the percent of individuals that contained private alleles was much lower in Puerto Rico (average = 3.1%, range = 3.0% [TAOV] to 3.2% [PATI]), than Florida (average = 34.7%, range = 23.3% [GEOR and STJO] to 53.3% [HOMO]), and Texas (average = 50.8%, range = 40.0% [FORK] to 61.5% [KICK]). The private alleles found among Puerto Rico populations came from reservoirs at high elevations (e.g., CARI, GARZ and MATR), with less stocking influences (e.g., MATR), and at greater distances from the Hatchery (e.g., CARI). The percent of Hatchery fish that contained private alleles was 2.5% (N = 1; Table 5; Figure 3).

Genetic Structure

Genetic structure was apparent among Largemouth Bass populations in Puerto Rico. Likelihood values increased asymptotically from a K-value of 1 (mean = -13,052.2; SD = 0.070) to a K-value of 10 (mean = -11,334.7; SD = 38.005). Initially, the model provided insight toward two ($K = 2$), three ($K = 3$), and six ($K = 6$) genetically distinct groups; the profile of ΔK showed the strongest peak at a K-value of 2 followed by a slight indication at a K-value of 3 and 6. Values of alpha (admixture) showed an asymptotic trend, steadily decreasing from a K-value of 1 to a K-value of 10 (Table 7; Figure 2). The overall average membership coefficient among groups at K=2 were relatively similar (Group 1 Mean q = 0.486: Group 2 Mean q = 0.514). Likewise, the average membership coefficients among populations within groups was relatively low, whereas the highest membership coefficients in Group 1 and Group 2 were shown in Cerrillos (q = 0.6966) and Garzas (0.6885) Reservoir, respectively (Table 8; Figure 3). The STRUCTURE results suggest that genetic structure was most apparent at K=2. Reservoir genetic structure was apparently due to the subspecies composition of individuals in each population and was not equivalent to population structure. The overall average membership coefficient among groups at K = 3 indicates that there may be some genetic and population structure with Matrullas Reservoir being different from other populations. The separation at K=6 is unclear.

The global test of genetic differentiation (global F_{ST}) among reservoirs was not significant (overall F_{ST}=0.082, SE=0.007, P=0.7576). However, pairwise F_{ST} between reservoirs showed significant differences in 33% (22/66) of all comparisons after Bonferroni corrections (corrected *P*=0.0004). Sixty-six percent of reservoirs (N=8) showed significant allelic differences from the Maricao Hatchery (Table 9). Population structure was not apparent among reservoirs based on the unrooted Neighbor-Joining (NJ) tree and subsequent analysis of molecular variance (AMOVA). No obvious groupings were found based on the unrooted NJ tree (Figure 4). An AMOVA of the hypothesis of low and high priority management groups was not significant (Among groups: *P* = 0.2005; 0.90% of total variance).

Discussion

Largemouth Bass populations in Puerto Rico displayed lower levels of genetic diversity than Florida and Texas populations. Microsatellite diversity levels exhibited slightly lower variation than native Florida Bass populations in Florida (Barthel et al. 2010) and introgressed Florida Bass x northern Largemouth Bass populations in Texas (Lutz-Carrillo et al. 2008), but higher than reported for cultured populations in China (Bai et al. 2008). Although genetic diversity was lower than other native and introduced Largemouth Bass populations, my dataset met specific criteria (H_O and H_E~0.60) for appropriate genetic diversity described in other studies of fishes (Douglas et al. 1999; Turgeon et al. 1999; Lu et al. 2001; Douglas and Brunner 2002; Hammen 2009; Barthel et al. 2010). However, the adaptive significance between increased genetic diversity and fitness, adaptation, and persistence of populations is widely recognized (Wright 1982; Koehn and Hilbish 1987; Nelson and Soulé 1987), as are negative effects (i.e., reduced fitness) of introducing a species outside of its native range (e.g., Philipp et al. 2002; Springbett et al. 2003). Environmental effects can be more influential on fitness than genetics and genetic variation usually has to be extremely low before fitness is reduced (Hedrick 2011). This study was developed to define the contemporary genetic diversity and possible structure among populations in Puerto Rico, so it is unknown at this time whether genetic diversity is low enough to reduce fitness and adaptive potential of these populations.

Genetic structure was apparent among Largemouth Bass populations in Puerto Rico. Genetic structure mostly corresponded with the presence of two lineages (northern Largemouth Bass and Florida Bass), but local environmental conditions (elevation) and recent stockings were also likely contributors. Most significant genetic differences were found between reservoirs with higher proportions of Florida Bass influence (CERR and DOSB), and populations with lower proportions of Florida Bass influence that were stocked less frequently and at higher elevations (GARZ and MATR). The presence of genetic differences between populations indicated that some populations had started to develop a localized gene pool despite sporadic stocking over the last century, which indicated that observed genetic differences were likely due to natural selection rather than genetic drift (small population size). Many of the genetically similar Largemouth Bass populations in Puerto Rico have been stocked more frequently, which could have homogenized the local gene pools. At this time, it is unknown how localized vs. homogenized gene pools will influence population dynamics and fitness of Largemouth Bass among Puerto Rico reservoirs.

Although minor genetic structure was found, substantial population structure was not apparent among Largemouth Bass populations in Puerto Rico. Population structure has been resolved in the native range of northern Largemouth Bass (Philipp et al. 2002), Florida Largemouth Bass, and interspecific hybrids (Barthel et al. 2010), including areas near the hypothesized populations of origin for Largemouth Bass introduced to Puerto Rico (St. John's River and Lake George, Florida; Erdman 1984). Lack of genetic structure has been reported for many other fish species (Hoarau et al. 2002; Florin and Hoglund 2007; An et al. 2013), but very few reports exist for non-native and/or introduced Largemouth Bass populations, particularly in tropical environments. The lack of population structure and the low and homogeneous genetic diversity in Puerto Rico supports the hypothesis that management strategies (stocking) and/or the presence of two hybridizing lineages have homogenized genetic variation on the island. Other potential reasons for the lack of population structure are contemporary hatchery production, stocking protocols, the relatively short time-frame since original introduction, a limited gene pool on the island compared to the native range, and/or the reproductive biology of Largemouth Bass in Puerto Rico.

The 2011 Hatchery broodstock showed lower genetic diversity than all other populations, which could be an indication of artificial selection during broodstock collection. Current hatchery protocols consist of sporadic broodstock collections, where approximately 40 adult Largemouth Bass from Cerrillos Reservoir are captured and transported to the hatchery and spawned over multiple years. Artificial selection during broodstock collections, hatchery culture and propagation can highly influence genetics of the recipient populations and result in a genetic bottleneck (Allendorf and Luikart 2007). Fish stocking has been shown to disrupt the relative stability of genetic diversity within a population over time (termed genetic integrity) and to homogenize genetic characteristics among populations (Hindar et al. 1991; Allendorf and Luikart 2007; Franckowiak et al.

2009). Franckowiak et al. (2009) found that a series of supplemental Walleye *Sander vitreus* stockings in Escanaba Lake, Wisconsin, led to the complete disruption of the genetic integrity of the population and resulted in a complete replacement of the genetic characteristics of the original population with that of stocked fish. Similar to populations in Puerto Rico, Escanaba Lake is a fish population founded through a series of stockings.

Although management priority rankings could not explain genetic differences among populations, it was still apparent that hatchery and management strategies, particularly stocking, have influenced genetic diversity in Puerto Rico. Populations that received more frequent stocking tended to have more similar genetic diversity metrics and were more closely grouped in the NJ tree and STRUCTURE. Populations that receive less stocking showed indications of development of a localized gene pool. The influence of stocking on genetic variation in Puerto Rico is not discernable due to the lack of detailed historic stocking records. However, the relatively low and homogeneous genetic diversity and lack of population structure among Puerto Rico populations is characteristic for stocked populations (Allendorf and Luikart 2007).

This study assumed that migrations between populations was null or minimal because most reservoirs were created with high elevation dams likely limiting fish migrations (Cooney and Kwak 2013). Furthermore, most reservoirs are in separate drainage basins. This assumption should be evaluated in future studies. While it is highly unlikely that interbasin movement of Largemouth Bass is occurring naturally, there is potential for angler translocation of fish between reservoirs, which would influence the results of this study. However, this mode of gene flow was not observed and is assumed to be minimal.

The lack of population structure could be a product of the relatively short time-frame from the introduction of Largemouth Bass to Puerto Rico. Long term isolation and genetic drift will ultimately lead to genetic differentiation (structure) between populations (Kamonrat 1996; Lafontaine and Dodson 1997; Björklund et al. 2007; Elmer et al. 2008; Guy et al. 2008). If historic records are correct, Puerto Rico populations have been established for up to 100 years. This is a relatively short era in the genetic timeline and likely not enough time to allow significant population structure to develop among populations. However, significant genetic population structure could develop among isolated Puerto Rico populations in the absence of stocking over time.

A limited gene pool with low genetic diversity may also explain the lack of population structure in Puerto Rico. Low genetic diversity compared to most native populations supports the hypothesis that a small gene pool was likely provided from initial introductions to the island. Largemouth Bass populations in Puerto Rico are currently composed of largely Florida Largemouth Bass alleles and genotypes, which supports the hypothesis of selection toward Florida Largemouth Bass genotypes in Puerto Rico. It is likely that limited broodstock collections from one reservoir with high Florida Largemouth Bass influence (Cerrillos Reservoir) and improved production and propagation of Largemouth Bass at the Hatchery over the last few decades further reduced the number of genes among reservoirs and limited the genetic tools required for populations to diverge over the last century.

Reduced genetic variation and lack of population structure could also be an artifact of reproductive biology and strategies of Largemouth Bass in Puerto Rico. Reproductive ability of translocated and hatchery produced fish populations are intimately tied to the reproductive capabilities of these fish in the wild (Fleming and Petersson 2001). Reproductive strategies (e.g., embryonic development, spawning frequency and timing, reproductive behavior and success) vary among native and introduced Largemouth Bass populations (Reiger and Summerfelt 1978; Philipp et al. 1985; Rudd 1985; Rodgers et al. 2006; Shaw et al. 2014). Puerto Rico populations have been developed from importing stocks of Largemouth Bass from the native range of northern Largemouth Bass and Florida Bass, including the recently characterized introgression zone (Barthel et al. 2010). However, these genetically different individuals may not experience the same reproductive success, which could lead to reduction of the available genes to the most successful genetic combinations. Spawning period in Largemouth Bass is driven by both environmental and genetic factors (Rodgers et al. 2006). Successful spawning and recruitment, leading to transmission of genes from parent to offspring, may depend on a genetic strain’s affinity for the local thermal environment (Philipp et al. 1985). The most successful strain in Puerto Rico would need to reproduce and develop the best at temperatures that greatly exceed its evolutionary optimum for reproduction because temperatures in Puerto Rico reservoirs exceed native spawning temperatures year-round (Gran 1995).

Conversely, what is advantageous for population genetics may not be the best fit for fisheries management. Largemouth Bass management in Puerto Rico has been limited by suboptimal growth, reproduction, and longevity compared to fish in their native range (Gran 1995; Waters 1999; Neal 2003). However, information on how reproductive strategy, reproductive success, and mating scheme influence population dynamics and genetics of Largemouth Bass is limited or lacking for most reservoirs.

Repeated interbreeding between the wild and hatchery populations can result in positive- or negative-assortative mating if the mated pairs in a population are composed of individuals with the same phenotype more often, or less often, than expected by chance (Hedrick 2011). Future production and management of Largemouth Bass in Puerto Rico would benefit from more information on the reproductive strategy, reproductive success, and mating scheme among reservoirs.

Resource managers have expressed concerns about outbreeding depression, a loss of fitness resulting from interbreeding of two distinct taxonomic groups (i.e., Florida Largemouth Bass and northern Largemouth Bass). Outbreeding depression has been recognized as a significant concern for management and conservation of Largemouth Bass (Childers 1975; Kassler et al. 2002; Philipp et al. 2002). Philipp et al. (2002) found a substantial loss in fitness for an Illinois population of Largemouth Bass after it became introgressed with two other non-native stocks of northern Largemouth Bass and Florida Largemouth Bass. Typically the native stock is assumed to have evolved with the native environment and be more likely to possess a suitable genotype/phenotype. Stocking a native population with a divergent group might not have any affect during the first generation because a complete half of the native genome is still intact (i.e., no crossing over among nuclear chromosomes between lineages would have occurred at this point), but subsequent introgression would disrupt nuclear chromosomes between lineages and potentially result in outbreeding depression (Templeton 1986; Hedrick 2011). All Largemouth Bass populations in Puerto Rico were introduced and exist as naturalized Florida Largemouth Bass x northern Largemouth Bass hybrid populations. Since there is no native stock that has evolved in Puerto Rico, the extent of disruption of nuclear chromosomes between lineages is unclear. It is also unclear if interbreeding between Florida Largemouth Bass and northern Largemouth Bass results in a loss in fitness in introduced Largemouth Bass populations in the tropics. It remains unknown whether outbreeding depression is a legitimate management concern in Puerto Rico.

Inbreeding depression has been a concern on the basis that some historic importations of Largemouth Bass to Puerto Rico consisted of a limited number of individuals. Many Puerto Rico populations had lower genetic diversity (H_o, H_E and F_{IS}) than Florida and Texas populations, which would support that Puerto Rico populations have a greater potential for reduced fitness due to inbreeding depression. Conversely, the lack of significant deviations from HWE and LD among loci within reservoirs suggests that inbreeding depression from the small founder size and subsequent genetic drift has not yet manifested. Increasing genetic variation in a population can offset the reductions in fitness due to inbreeding depression (Kincaid 1976; Hedrick and Kalinowski 2000; Hallerman 2003), which may be accomplished by additional imports of Largemouth Bass to the island. However, the magnitude and specific effects of inbreeding depression are highly variable because they depend on the genetic constitution of the species or populations and on how these genotypes interact with the environment (Hedrick and Kalinowski 2000; Hallerman 2003). Additional research is warranted to determine if future introductions would increase genetic variation within Puerto Rico populations and if inbreeding depression is a legitimate management concern.

This study was the first to quantify genetic variation and structure of established, non-native Largemouth Bass populations in the tropics. It provides a new tool and genetic baseline that can be used in future culture and management of Largemouth Bass in Puerto Rico.

Tables and Figures

Table 3.1 Collection locations, GPS coordinates, surface area (ha), and elevation (m above sea level) for 12 reservoirs and 1 hatchery in Puerto Rico, 6 Florida populations and 2 Texas populations.

Map ID	Region	Collection (code)	Latitude	Longitude	Surface Area (ha)	Elevation (m)	Sub. Comp.
1.	Puerto Rico	Caonillas Reservoir (CAON)	18°15'56"	-66°39'17"	280	253	I
2.		Carite Reservoir (CARI)	18°04'29"	-66°06'09"	133	545	I
3.		Cerrillos Reservoir (CERR)	18°05'20"	-66°34'44"	249	137	I
4.		Dos Bocas Reservoir (DOSB)	18°19'59"	-66°40'04"	254	89	I
5.		Garzas Reservoir (GARZ)	18°08'13"	-66°44'32"	44	742	I
6.		Guayabal Reservoir (GYBL)	18°05'42"	-66°30'13"	131	105	I
7.		Guayo Reservoir (GUYO)	18°11'58"	-66°50'00"	118	445	I
8.		La Plata Reservoir (LAPL)	18°20'22"	-66°13'57"	405	46	I
9.		Lucchetti Reservoir (LUCC)	18°05'31"	-66°51'51"	106	174	I
10.		Maricao Hatchery (MARH)	18°10'12"	-66°59'11"	-	454	I
11.		Matrullas Reservoir (MATR)	18°12'26"	-66°28'46"	37	743	I
12.		Toa Vaca Reservoir (TAOV)	18°06'14"	-66°28'52"	342	164	I
13.		Patillas Reservoir (PATI)	18°01'32"	-66°01'27"	137	66	I
14.	Florida	Lake George (GEOR)	29°16'27"	-81°35'01"	140	4	I
15.		Homosassa River (HOMO)	28°46'13"	-82°41'49"	-	0	F
16.		St. John's River (STJO)	29°51'40"	-81°35'29"	-	5	I
17.		Lake Panasoffkee (PANA)	28°48'15"	-82°07'18"	1,805	12	F
18.		Lake Okeechobee (OKEE)	26°56'02"	-80°49'52"	182,108	5	F
19.		Lake Santa Fe (STFE)	29°44'26"	-82°04'42"	2,370	45	I
20.	Texas	Lake Fork (FORK)	32°49'32"	-95°34'22"	11,206	123	I
21.		Lake Kickapoo (KICK)	33°38'36"	-98°49'20"	2,440	330	N

Under Subspecies Composition (Sub. Comp.), I represents an introgressed population and F represents a pure Florida subspecies population.

Table 3.2 Polymerase chain reaction (PCR) recipes, fluorescent labels, and thermocycler temperature profiles for all loci and multiplexes used in this study.

Locus	Multiplex	dNTPs	$MgCl_2$	Primer Forward (Conc.)	Primer Reverse (Conc.)	Label
Lar7		0.20mM	3 mM	0.10uM	0.20uM	M13(-29)
MiSaTPW060		0.20mM	1.5 mM	0.05uM	0.20uM	CAG
MiSaTPW070		0.20mM	2 mM	0.05uM	0.20uM	CAG
MiSaTPW096		0.20mM	1.5 mM	0.05uM	0.20uM	CAG
Mdo6	MPX8	0.20mM	1.9 mM	0.04uM	0.20uM	M13(-29)
Msal21				0.04uM	0.10uM	M13(-29)
Msa29				0.04uM	0.20uM	M13(-29)
MiSaTPW076	MPX11	0.20mM	1.5 mM	0.20uM	0.04uM	CAG
MiSaTPW157				0.15uM	0.04uM	CAG
MiSaTPW055	MPX16	0.20mM	1.5 mM	0.30uM	0.04uM	CAG
MiSaTPW062				0.08uM	0.04uM	CAG

MPX8	94°C for 1 min 30 s. 1 series of 31 cycles each at 94°C for 30 s, then 55°C annealing for 30 s. 72°C for 30 s then a final elongation of 72°C for 10 min.
MPX11	94°C for 1 min 30 s. 1 series of 32 cycles each at 94°C for 30 s, then 60°C annealing for 30 s. 72°C for 45 s then a final elongation of 72°C for 10 min.
MPX16	94°C for 1 min 30 s. 1 series of 33 cycles each at 94°C for 30 s, then 55°C annealing for 30 s. 72°C for 45 s then a final elongation of 72°C for 10 min.
Lar7	94°C for 1 min 30 s. 1 series of 31 cycles each at 94°C for 30 s, then 50°C annealing for 20 s. 72°C for 45 s then a final elongation of 72°C for 10 min.
MiSaTPW060	94°C for 1 min 30 s. 1 series of 29 cycles each at 94°C for 30 s, then 60°C annealing for 30 s. 72°C for 45 s then a final elongation of 72°C for 10 min.
MiSaTPW070	94°C for 1 min 30 s. 1 series of 31 cycles each at 94°C for 30 s, then 55°C annealing for 30 s. 72°C for 45 s then a final elongation of 72°C for 10 min.
MiSaTPW096	94°C for 1 min 30 s. 1 series of 31 cycles each at 94°C for 30 s, then 60°C annealing for 30 s. 72°C for 45 s then a final elongation of 72°C for 10 min.

All buffer concentrations were 1x PCR Buffer without $MgCl_2$ (Thermo Fisher Scientific Inc., Waltham, MA), dNTP mix, 50 mM magnesium chloride solution ($MgCl_2$), and 5 U/ul of Taq DNA polymerase.

Table 3.3 Locus, primer sequence (5’-3’), allele size (base pairs), number of alleles, references and Genbank accession number for all loci used in this study.

Locus		Primer Sequence (5'-3')	Allele Size (bp)	Number of Alleles	Reference	GenBank Accession no.
Lar7	F:	GTGCTAATAAAGGCTACTGT	121 - 237	19	DeWoody et al. 2000	NA
	R:	TGTTCCCTTAATTGTTTTGA				
MiSaTPW060	F:	TATAGTTTGGTCCAGCAGGTGGCGT	294–561	20	Lutz-Carrillo et al. 2008	EF590079
	R:	(CAG)TGTGGAATGACATTTAGCCGAGGCC				
MiSaTPW070	F:	ACTTCGCAAAGGTATAAC	171–494	24	Lutz-Carrillo et al. 2008	EF590084
	R:	(CAG)CCTCATGCAGAAGAT				
MiSaTPW096	F:	CTTCTAAATGTGTGTAGGGTTGC	345–581	19	Lutz-Carrillo et al. 2008	EF590088
	R:	(CAG)AGCTTAGCATAAAGACTGGGAAC				
Mdo6	F:	TGAAATGTACGCCAGAGCA	160 - 176	3	Malloy et al. 2000	AF294494
	R:	TGTGTGGGTGTTTATGTGGG				
Msa21	F:	CACTGTAAATGGCACCTGT	214 - 224	3	DeWoody et al. 2000	NA
	R:	GTTGTCAAGTCGTAGTCCGC				
Msa29	F:	CACGACGTTGTAAAACGACGCGTGTGGTTAGGATGTAGA	266 - 292	9	Designed from the listed genbank accession	DQ211538
	R:	GTTCTGAATGGAGCAATCC				
MiSaTPW076	F:	ACACAGTGTCAGTTCTGCA	250–325	12	Lutz-Carrillo et al. 2008	EF590085
	R:	(CAG)GTGAATACCTCAGCA				
MiSaTPW157	F:	GACCTCAATGCGGATACTGTGACC	169–202	15	Lutz-Carrillo et al. 2008	EF590106
	R:	(CAG)AGGCACTCATCTGAATTGTCCATGT				
MiSaTPW055	F:	ATCATGGTCTAAAAACTATT	132–148	5	Lutz-Carrillo et al. 2008	EF590077
	R:	(CAG)TTTTGAATGGAATTAC				
MiSaTPW062	F:	ATGCTTTCTGCTGAAGTGC	190–222	6	Lutz-Carrillo et al. 2008	EF590080
	R:	(CAG)TCCCTTTTGGAAGTAGAAGT				
M13(-29)	F:	CACGACGTTGTAAAACGAC				
CAG	R:	CAGTCGGGCGTCATCA				

A three primer PCR approach was used for all reactions using M13(-29) and CAG primers. One primer from each primer pair was tailed at the 5' end with the CAG locus sequence. Lar7, Msa21 and Mdo6 were also tagged with M13(-29) on the forward sequence.

Table 3.4 Number of alleles (N_A), observed heterozygosity (H_O), and adherence to Hardy-Weinberg Equilibrium (P_{HWE}) per locus for 13 Largemouth Bass populations in Puerto Rico, the contemporary Maricao Hatchery broodstock (2011; MARH), 6 Florida populations and 2 Texas populations.

Locus		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Lar7	N_A	7	8	6	6	8	6	7	8	4	6	8	8	4	11	5	10	10	14	9	9	3
	H_O	0.783	0.833	0.595	0.733	0.903	0.692	0.688	0.677	0.667	0.650	0.839	0.862	0.724	0.727	0.500	0.833	0.714	0.967	0.786	0.767	0.231
	P_{HWE}	0.074	0.390	0.644	0.512	0.391	0.996	0.740	0.976	0.968	0.694	0.786	0.844	0.908	0.392	0.479	0.781	0.635	0.877	0.917	0.827	0.207
Msa29	N_A	3	2	2	3	4	2	4	4	4	2	2	4	4	3	2	3	3	2	2	8	5
	H_O	0.182	0.259	0.442	0.147	0.313	0.278	0.500	0.250	0.367	0.179	0.452	0.212	0.419	0.552	0.111	0.647	0.308	0.267	0.192	0.900	0.409
	P_{HWE}	0.954	0.729	0.402	0.975	0.496	0.631	0.749	0.095	0.821	0.538	0.853	0.998	0.929	0.755	0.860	0.274	0.113	0.399	0.588	0.676	0.075
Msa21	N_A	4	5	2	3	3	3	4	3	4	3	3	3	3	6	5	4	4	4	5	5	2
	H_O	0.152	0.577	0.047	0.091	0.750	0.167	0.516	0.344	0.467	0.051	0.452	0.485	0.452	0.567	0.600	0.483	0.480	0.567	0.370	0.633	0.333
	P_{HWE}	**0.031**	0.117	0.876	0.875	0.198	0.320	0.718	0.895	0.546	0.999	0.451	0.976	0.905	0.999	0.953	0.976	0.225	0.907	0.815	0.965	0.344
Mdo6	N_A	3	3	1	2	3	2	2	2	3	1	2	2	3	3	2	2	1	3	4	3	2
	H_O	0.152	0.222	0.000	0.031	0.226	0.056	0.125	0.375	0.200	0.000	0.167	0.061	0.167	0.200	0.033	0.100	0.000	0.200	0.207	0.600	0.440
	P_{HWE}	0.974	0.936	Mono	0.928	0.489	0.940	0.706	0.583	0.946	Mono	0.619	0.858	0.970	0.946	0.926	0.773	Mono	0.946	0.135	0.959	0.473
MiSaTPW060	N_A	11	10	9	9	10	12	14	10	16	11	12	13	13	21	13	23	24	20	20	26	15
	H_O	0.840	0.792	0.700	0.571	0.871	0.733	0.867	0.607	0.900	0.903	0.900	0.821	0.808	0.867	0.833	0.929	0.923	0.933	0.931	0.897	0.846
	P_{HWE}	0.982	0.479	0.936	0.242	0.621	0.819	0.642	0.807	0.415	0.233	0.492	0.612	0.690	0.987	0.553	0.793	0.620	0.966	0.164	0.055	0.465
MiSaTPW070	N_A	4	7	4	8	11	6	8	3	6	6	8	5	8	23	17	19	19	17	16	21	11
	H_O	0.700	0.840	0.677	0.867	0.839	0.800	0.933	0.400	0.600	0.875	0.710	0.655	0.759	0.933	0.862	0.862	0.840	0.833	0.786	0.867	0.808
	P_{HWE}	0.063	0.986	0.564	0.183	0.650	0.686	0.649	0.172	0.197	0.791	0.999	0.623	0.646	0.895	0.833	0.305	0.306	0.430	0.831	0.278	0.391
MiSaTPW096	N_A	10	6	9	6	12	8	11	15	8	8	9	15	11	18	11	25	13	24	13	19	10
	H_O	0.636	0.480	0.683	0.552	0.833	0.714	0.844	0.846	0.567	0.650	0.867	0.962	0.793	0.846	0.783	0.962	0.818	0.867	0.706	0.897	0.846
	P_{HWE}	0.773	0.433	0.851	0.616	0.995	0.734	0.935	0.650	0.941	0.632	0.788	0.512	0.900	0.563	0.982	0.969	0.397	0.919	0.365	0.704	0.258
MiSaTPW076	N_A	8	5	6	6	6	5	8	7	9	7	4	8	8	10	10	10	8	7	13	11	6
	H_O	0.679	0.583	0.762	0.839	0.767	0.923	0.906	0.839	0.833	0.725	0.742	0.920	0.828	0.867	0.741	0.862	0.840	0.800	0.875	0.862	0.500
	P_{HWE}	0.971	0.847	0.743	0.644	0.601	0.442	0.497	0.284	0.151	0.981	0.742	0.184	0.166	0.120	0.993	0.936	0.997	0.992	0.736	0.756	0.311
MiSaTPW157	N_A	7	6	5	6	6	5	6	6	6	8	3	5	5	13	9	10	9	15	11	12	5
	H_O	0.483	0.917	0.762	0.636	0.656	0.769	0.781	0.516	0.733	0.875	0.552	0.692	0.871	0.767	0.741	0.857	0.846	0.867	0.786	0.586	0.560
	P_{HWE}	0.216	0.602	0.130	0.541	0.882	0.457	0.996	0.068	0.832	0.461	0.558	0.396	0.334	0.564	0.741	0.753	0.404	0.434	0.458	0.159	0.943
MiSaTPW062	N_A	7	7	6	5	7	6	9	5	8	5	6	7	6	7	6	8	5	7	6	15	10
	H_O	0.750	0.826	0.634	0.778	0.750	0.692	0.848	0.704	0.786	0.789	0.613	0.857	0.696	0.810	0.565	0.792	0.550	0.600	0.563	0.966	0.846
	P_{HWE}	0.236	0.114	0.313	0.261	0.796	0.922	0.321	0.626	0.992	0.748	0.997	0.797	0.704	0.416	0.055	0.112	0.131	0.100	0.068	0.950	0.059
MiSaTPW055	N_A	7	6	6	6	5	5	6	5	5	5	4	6	4	7	6	7	6	6	4	11	9
	H_O	0.692	0.800	0.643	0.839	0.571	0.615	0.706	0.828	0.733	0.579	0.742	0.731	0.458	0.619	0.727	0.615	0.913	0.714	0.318	0.724	0.846
	P_{HWE}	0.641	0.799	0.851	0.196	0.625	0.183	0.074	0.501	0.897	0.838	0.601	0.489	0.384	0.943	0.731	0.577	0.481	0.837	0.246	0.279	0.754

Bold P_{HWE} values indicate statistically significant deviation from Hardy-Weinberg expectations. Monomorphic loci are noted by Mono. Population names corresponding to numbers on map are found in Table 3.1.

Table 3.5 Genetic diversity parameters observed among 11 microsatellite loci are shown for Puerto Rico, Florida and Texas Largemouth Bass populations.

Region	Population	N	N_a	N_a SD	H_o	H_o SD	H_E	H_E SD	F_{IS}	A_R	P_A
Puerto Rico	Caonillas	26	6.455	2.697	0.550	0.265	0.601	0.279	0.109	3.910	0.000
	Carite	25	5.909	2.212	0.648	0.243	0.619	0.222	-0.026	3.890	0.000
	Cerrillos	40	5.091	2.663	0.540	0.270	0.541	0.281	0.014	3.230	0.000
	Dos Bocas	31	5.455	2.115	0.553	0.317	0.531	0.292	-0.025	3.340	0.000
	Garzas	31	6.818	3.125	0.680	0.225	0.689	0.189	0.029	4.250	0.091
	Guayabal	15	5.455	2.841	0.585	0.284	0.578	0.265	0.024	3.770	0.000
	Guayo	32	7.182	3.401	0.701	0.241	0.680	0.246	-0.016	4.440	0.000
	La Plata	28	6.182	3.763	0.581	0.261	0.626	0.223	0.102	3.840	0.000
	Lucchetti	30	6.636	3.668	0.623	0.211	0.632	0.220	0.031	3.950	0.000
	Matrullas	31	5.545	3.297	0.639	0.221	0.609	0.218	-0.034	3.510	0.000
	Patillas	28	6.273	3.349	0.634	0.225	0.639	0.226	0.026	3.980	0.091
	Tao Vaca	28	6.909	4.011	0.660	0.293	0.644	0.281	-0.006	4.160	0.091
	Maricao Hatchery	38	5.636	2.908	0.571	0.336	0.541	0.314	-0.041	3.380	0.091
Florida	Homosassa	25	7.818	4.665	0.591	0.280	0.629	0.302	0.081	4.230	0.545
	St. Johns River	26	11.000	7.912	0.722	0.252	0.710	0.262	0.003	5.080	0.455
	George	27	11.091	6.949	0.705	0.211	0.725	0.227	0.047	5.050	0.727
	Santa Fe	25	9.364	5.732	0.593	0.275	0.655	0.261	0.117	4.650	0.545
	Panasoffkee	23	9.273	7.016	0.657	0.294	0.687	0.267	0.066	4.720	0.545
	Okeechobee	29	10.818	7.494	0.692	0.260	0.691	0.271	0.016	4.950	0.818
Texas	Fork	29	12.727	6.973	0.791	0.136	0.810	0.121	0.041	5.800	0.818
	Kickapoo	25	7.091	4.206	0.606	0.238	0.638	0.219	0.071	4.200	0.273
Puerto Rico	All Reservoirs	343	6.159	3.081	0.616	0.248	0.616	0.241		3.856	0.023
Florida	All	157	9.894	6.566	0.660	0.258	0.683	0.258		4.780	0.606
Texas	All	55	9.909	6.316	0.698	0.211	0.724	0.194		5.000	0.545

including sample size (N), average number of alleles per locus (N_a) with standard deviation (N_aSD), observed heterozygosity (H_O) with standard deviation (H_OSD), expected heterozygosity (H_E) with standard deviation (H_ESD), inbreeding coefficient (F_{IS}), allelic richness (A_R), and the proportion of private alleles (P_A)

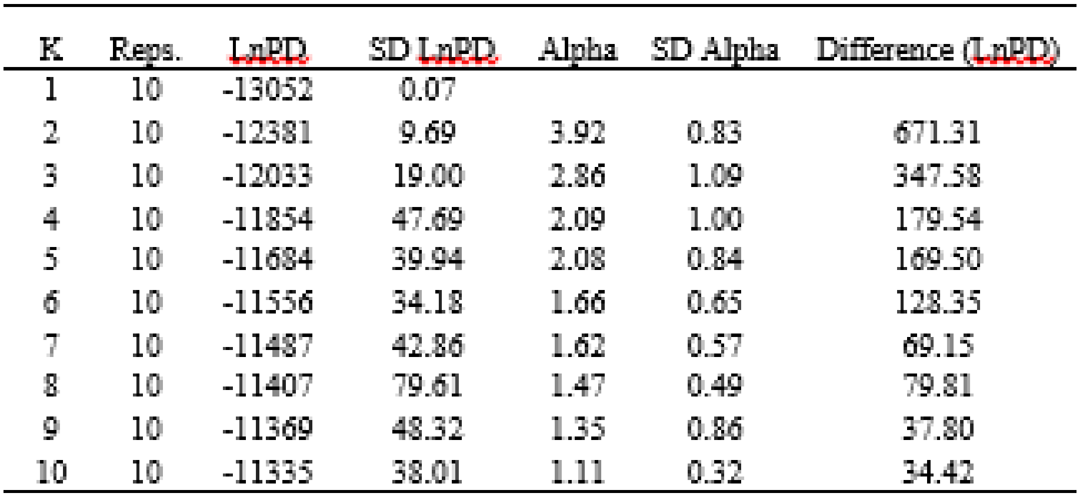
Table 3.6 Summary of private alleles observed at 11 microsatellite loci within and among population groupings (Group) of Largemouth Bass from Puerto Rico, Florida and Texas.

Group	Pops.	Ind.	% Pops.	% Ind.	PA
PR Reservoirs	12	379	92	13	24
PR All	13	419	92	12	25
Florida	6	175	100	55	75
Texas	2	56	100	96	92
Florida and Texas	8	231	100	44	101
All Pops	21	650	57	14	77

Data are shown as the number of populations (Pops.), number of individuals (Ind.), percent of populations with private alleles (% Pops.), the percent of individuals with private alleles (% Ind.), and the total number of private alleles observed (PA) within groups.

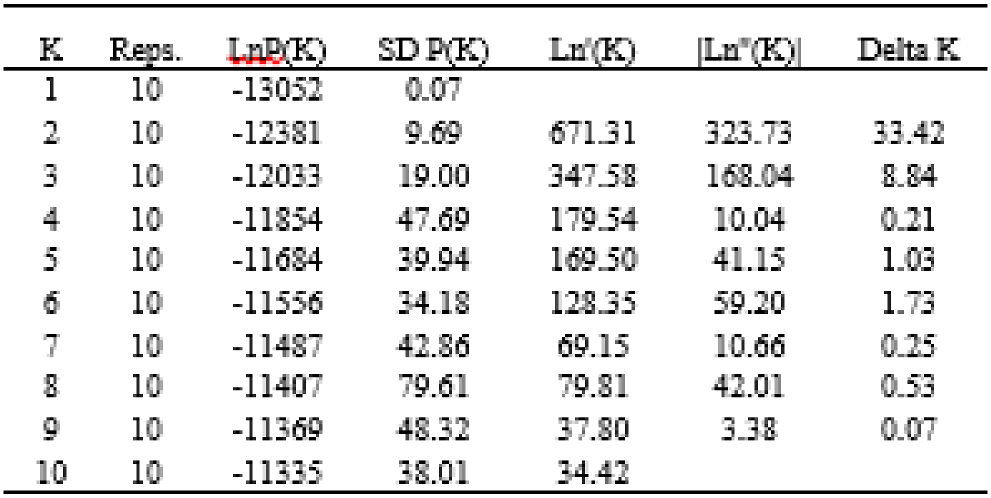
Table 3.7 Evanno et al. (2005) table output from STRUCTURE 2.3.3 (Pritchard et al. 2000) and STRUCTURE HARVESTER (Earl and von Holdt 2011).

a)



K	Reps.	LnPD	SD LnPD	Alpha	SD Alpha	Difference (LnPD)
1	10	-13052	0.07			
2	10	-12381	9.69	3.92	0.83	671.31
3	10	-12033	19.00	2.86	1.09	347.58
4	10	-11854	47.69	2.09	1.00	179.54
5	10	-11684	39.94	2.08	0.84	169.50
6	10	-11556	34.18	1.66	0.65	128.35
7	10	-11487	42.86	1.62	0.57	69.15
8	10	-11407	79.61	1.47	0.49	79.81
9	10	-11369	48.32	1.35	0.86	37.80
10	10	-11335	38.01	1.11	0.32	34.42

b)



K	Reps.	LnP(K)	SD P(K)	Ln'(K)	|Ln"(K)|	Delta K
1	10	-13052	0.07			
2	10	-12381	9.69	671.31	323.73	33.42
3	10	-12033	19.00	347.58	168.04	8.84
4	10	-11854	47.69	179.54	10.04	0.21
5	10	-11684	39.94	169.50	41.15	1.03
6	10	-11556	34.18	128.35	59.20	1.73
7	10	-11487	42.86	69.15	10.66	0.25
8	10	-11407	79.61	79.81	42.01	0.53
9	10	-11369	48.32	37.80	3.38	0.07
10	10	-11335	38.01	34.42		

In table a), when Alpha is close to zero, most individuals are essentially from one population or another, while alpha >1 means that most individuals are admixed (Falush et al. 2003). In table b), the most likely number of groups is indicated by the highest difference in LnP(K) and Delta K values (Evanno et al. 2005).

Table 3.8 Mean membership coefficients for individuals in each population to each cluster (i to vi) assuming K = II, K = III and K = VI.

	Inferred Clusters at Specified *K*										
	K = II		*K* = III			*K* = VI					
Population	i	ii	i	ii	iii	i	ii	iii	iv	v	vi
Carite	**0.508**	0.492	0.344	0.303	**0.352**	**0.282**	0.189	0.116	0.173	0.043	0.196
Caonillas	0.418	**0.582**	**0.345**	0.329	0.327	**0.243**	0.162	0.167	0.121	0.154	0.153
Cerrillos	0.303	**0.697**	0.399	0.201	**0.400**	**0.287**	0.187	0.185	0.011	0.212	0.118
Dos Bocas	0.353	**0.647**	**0.380**	0.245	0.375	0.187	0.090	0.136	0.091	**0.280**	0.215
Garzas	**0.689**	0.311	0.400	0.110	**0.490**	0.200	**0.292**	0.014	0.099	0.291	0.105
Guayabal	0.390	**0.610**	0.400	0.182	**0.418**	**0.274**	0.206	0.177	0.055	0.166	0.123
Guayo	**0.641**	0.359	0.405	0.122	**0.474**	0.118	0.130	**0.288**	0.169	0.202	0.092
La Plata	0.487	**0.513**	0.394	0.160	**0.446**	**0.285**	0.193	0.132	0.140	0.080	0.169
Lucchetti	**0.583**	0.417	0.400	0.143	**0.456**	0.117	0.158	**0.258**	0.175	0.167	0.125
Maricao Hatchery	0.314	**0.686**	0.398	0.203	**0.399**	**0.280**	0.195	0.188	0.017	0.193	0.127
Matrullas	**0.512**	0.488	0.210	**0.678**	0.112	0.108	0.100	0.191	**0.291**	0.107	0.204
Patillas	**0.510**	0.490	0.383	0.200	**0.417**	0.129	0.146	**0.236**	0.160	0.179	0.150
Toa Vaca	**0.609**	0.391	0.371	0.215	**0.414**	0.199	0.181	0.195	**0.214**	0.060	0.151
All Puerto Rico	0.486	0.514	0.371	0.238	0.391	0.208	0.171	0.176	0.132	0.164	0.148

See text and Figure 3 for additional details. The greatest coefficients for each population are indicated by bold cells.

Table 3.9 Pairwise population F_{ST} analyses for 12 reservoir populations and the Maricao Hatchery.

	La Plata	Caonillas	Toa Vaca	Guayabal	Carite	Guayo	Lucchetti
La Plata	--	0.0559	0.0004	0.0243	0.0013	0.0080	0.0080
Caonillas	0.0365	--	0.0026	0.2042	0.0034	0.0024	0.0076
Tao Vaca	0.0427	0.0387	--	0.0033	0.0008	0.0102	0.2304
Guayabal	0.0402	0.0207	0.0415	--	0.0020	0.0643	0.0587
Carite	0.0431	0.0251	0.0412	0.0427	--	0.0016	0.0063
Guayo	0.0439	0.0430	0.0311	0.0484	0.0458	--	0.3573
Lucchetti	0.0442	0.0420	0.0211	0.0460	0.0536	0.0284	--
Cerrillos	0.0538	0.0295	0.0652	0.0182	0.0539	0.0623	0.0654
Dos Bocas	0.0539	0.0201	0.0559	0.0293	0.0471	0.0617	0.0577
Patillas	0.0328	0.0285	0.0267	0.0320	0.0407	0.0327	0.0215
Matrullas	0.0638	0.0418	0.0533	0.0555	0.0537	0.0598	0.0628
Garzas	0.0514	0.0594	0.0441	0.0676	0.0521	0.0315	0.0420
Maricao Hatchery	0.0451	0.0204	0.0538	0.0194	0.0505	0.0604	0.0589

F_{ST} values are shown in the lower triangle matrix and corresponding *P*-values are shown in the upper triangle matrix. Tests were performed in ARLEQUIN v3.5 (Excoffier et al. 2005). Significant values after Bonferroni corrections (adjusted *P*=0.0003; Rice 1989) are shown in bold.

Table 3.9 (Continued)

	Cerrillos	Dos Bocas	Patillas	Matrullas	Garzas	Mariaco Hatchery
La Plata	**0.0000**	0.0103	0.0312	**0.0001**	**0.0000**	**0.0000**
Caonillas	**0.0001**	0.8712	0.0319	**0.0003**	**0.0000**	0.0368
Tao Vaca	**0.0000**	**0.0000**	0.1310	**0.0003**	0.0005	**0.0000**
Guayabal	0.1635	0.2461	0.0397	0.0012	**0.0001**	0.0925
Carite	**0.0000**	**0.0002**	0.0228	0.0048	0.0012	**0.0000**
Guayo	**0.0000**	**0.0000**	0.5014	0.0012	0.0008	**0.0000**
Lucchetti	**0.0000**	**0.0002**	0.4968	0.0011	0.0012	**0.0000**
Cerrillos	--	0.0009	**0.0000**	**0.0000**	**0.0000**	0.0056
Dos Bocas	0.0273	--	0.0012	**0.0000**	**0.0000**	0.3107
Patillas	0.0448	0.0412	--	0.0320	0.0005	**0.0000**
Matrullas	0.0763	0.0643	0.0468	--	**0.0000**	**0.0000**
Garzas	0.0877	0.0861	0.0465	0.0701	--	**0.0000**
Maricao Hatchery	0.0120	0.0142	0.0382	0.0695	0.0858	--

F_{ST} values are shown in the lower triangle matrix and corresponding *P*-values are shown in the upper triangle matrix. Tests were performed in ARLEQUIN v3.5 (Excoffier et al. 2005). Significant values after Bonferroni corrections (adjusted *P*=0.0003; Rice 1989) are shown in bold.

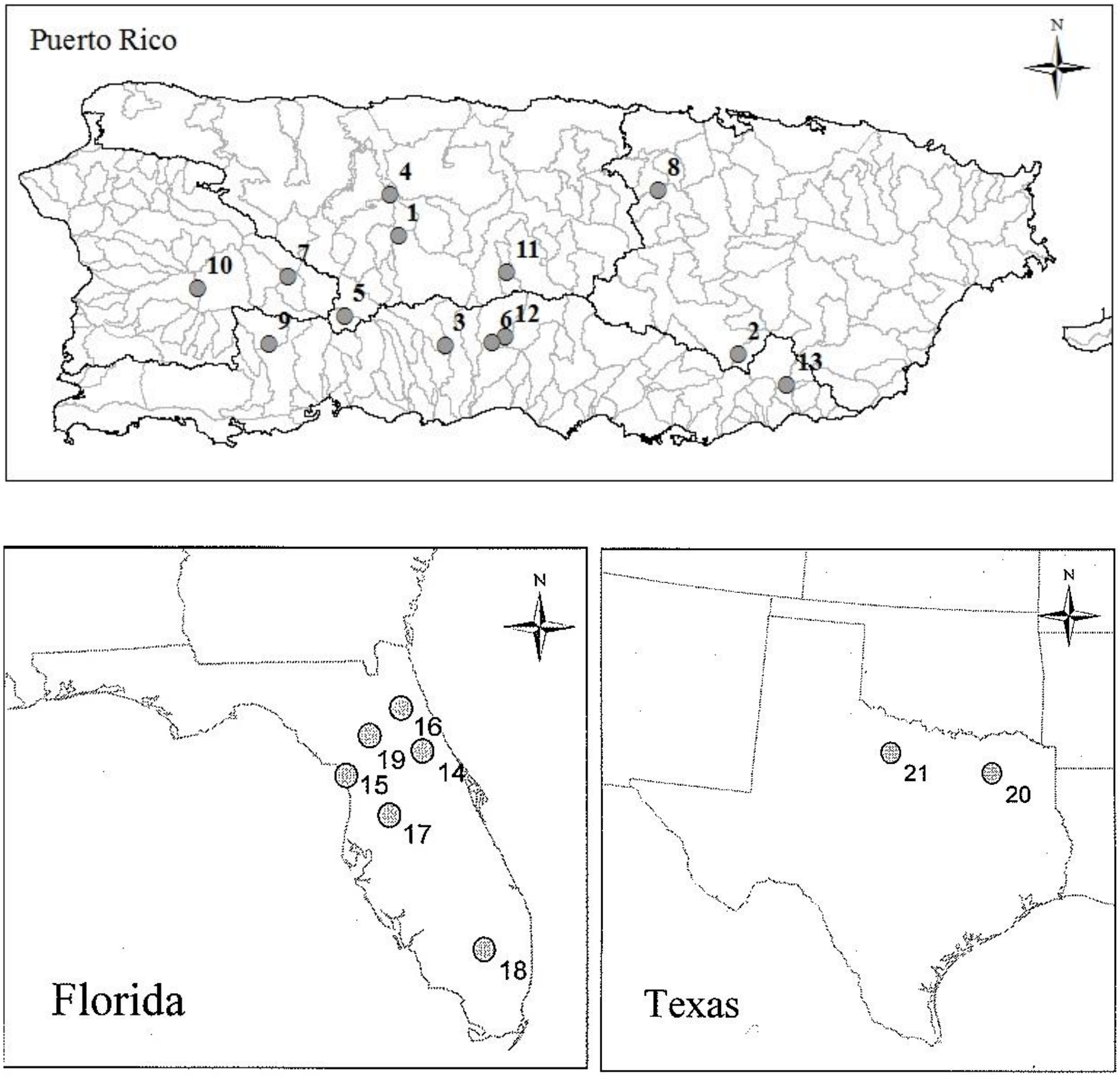


Figure 3.1 Locations of Largemouth Bass populations in Puerto Rico, Texas and Florida used in this study.

Population names corresponding to numbers on map are found in Table 3.1.

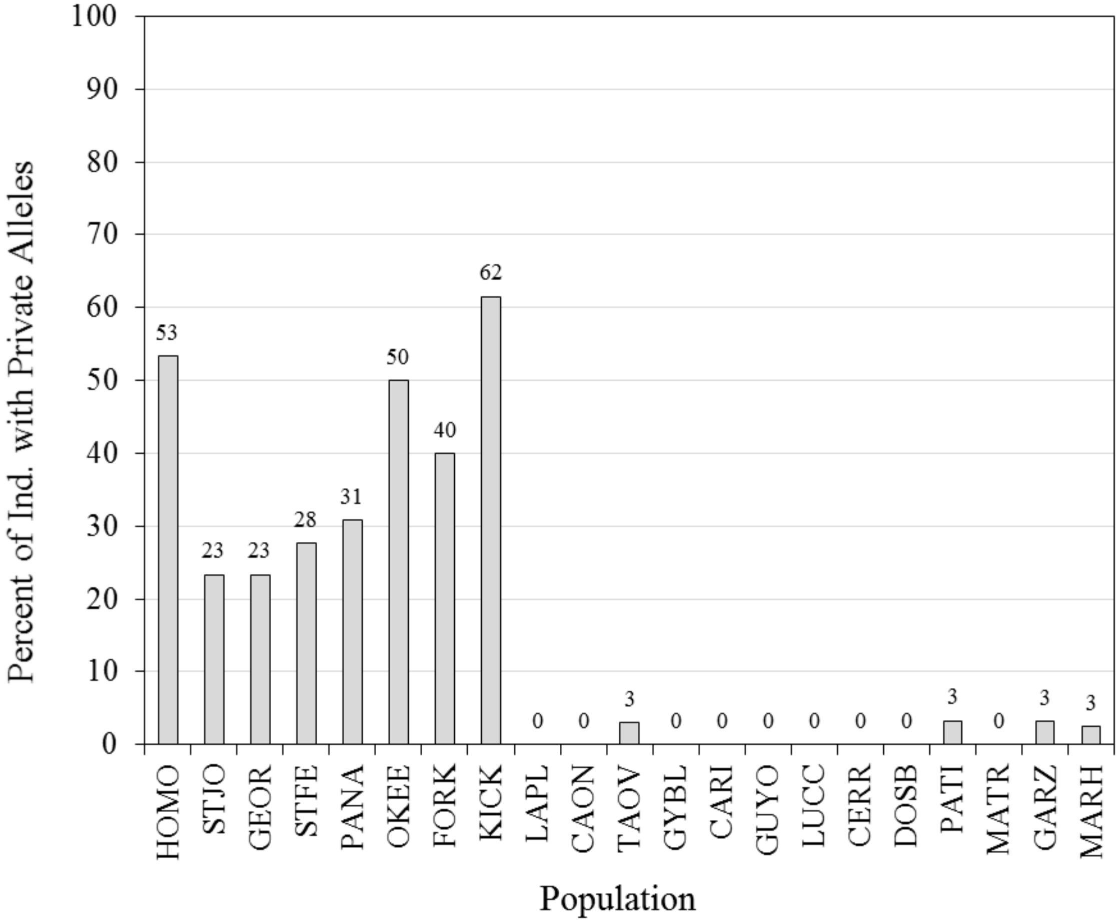


Figure 3.2 The percent of Largemouth Bass in each population with private alleles determined using 11 microsatellite loci and a combined dataset from Puerto Rico, Texas, and Florida.

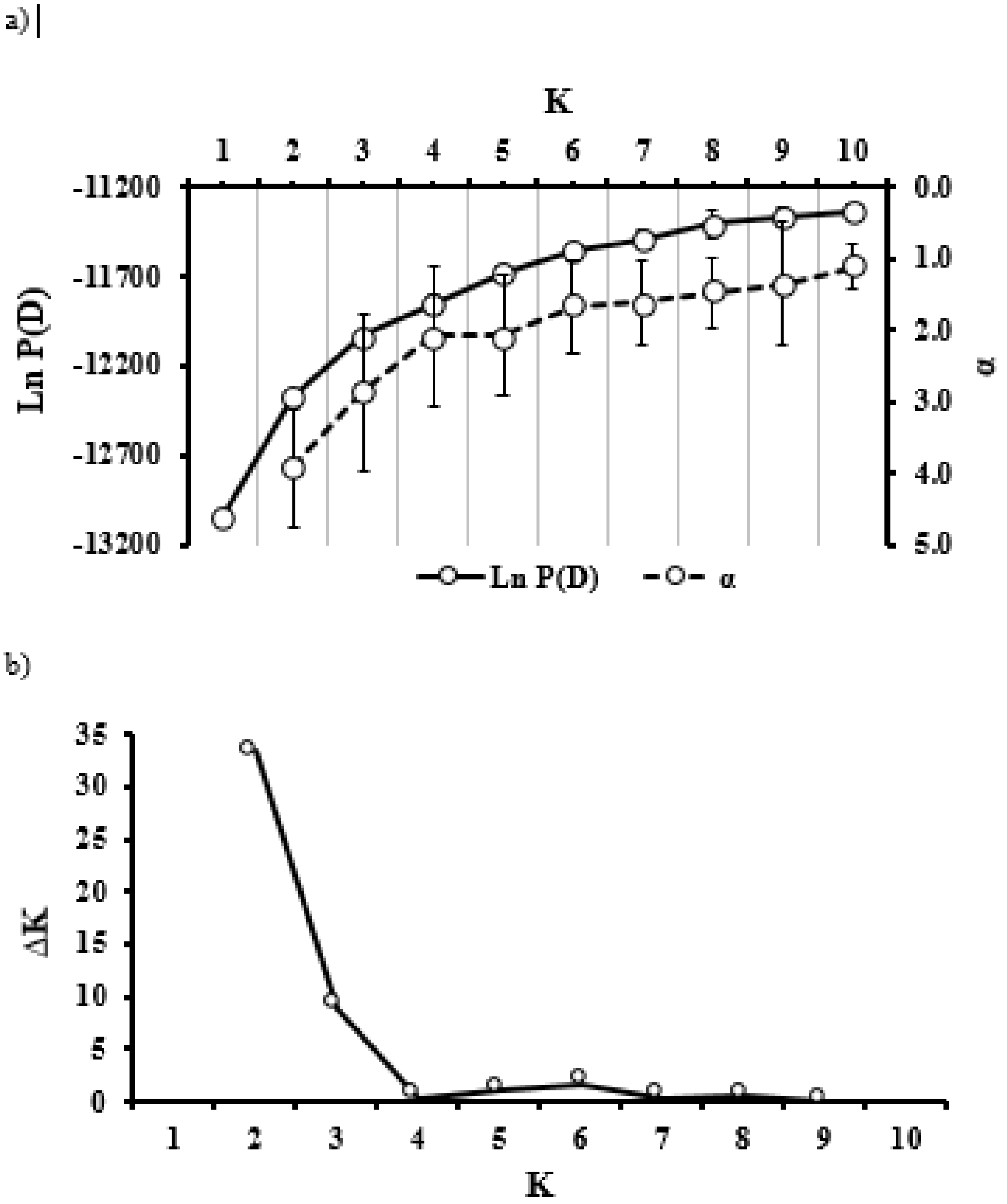


Figure 3.3 Likelihood (LnP[D]; mean ± SD), admixture coefficient (α; mean ± SD), and number of clusters (K) for STRUCTURE runs using all Florida Bass and intergrade Largemouth Bass genotypes sampled in Puerto Rico Reservoirs.

In a) Ln P(D) is the probability of the data under each clustering solution (K). In b) The K-values plotted in relation to ΔK are shown for all Florida Bass and intergrade Largemouth Bass genotypes. All genotypes were composed of 11 microsatellite loci and all runs were performed for K-values of 1-10 with 10 independent replicates.

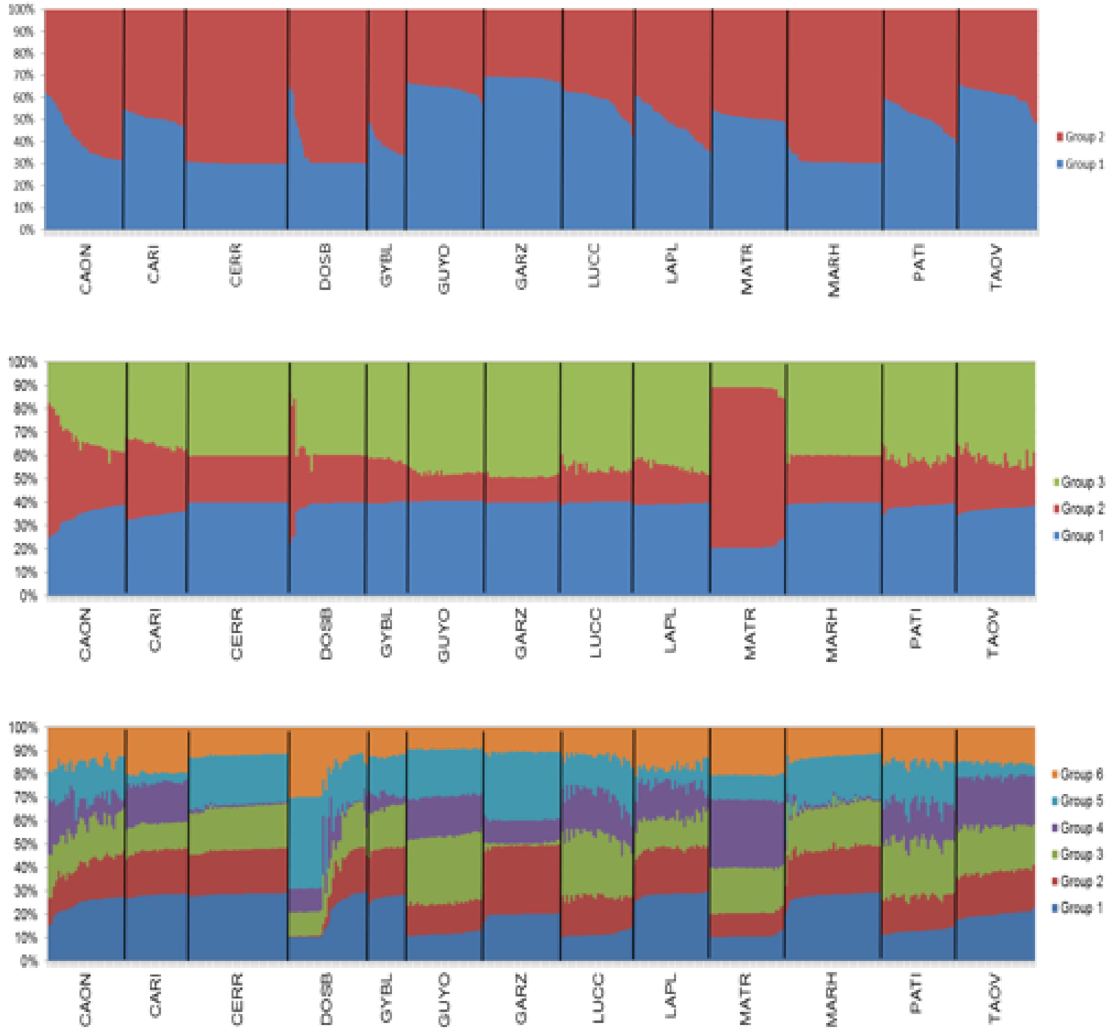


Figure 3.4 Estimated population composition from Bayesian STRUCTURE analysis for *K*=2 (top), *K*=3 (middle) and *K*=6 (bottom) population groups.

Each graph represents the highest probability run among 10 separate runs at that *K*. Each individual fish is represented by a thin vertical line, which is partitioned into colored segments that represent the individual's estimated membership fractions. Black thin lines separate each population. Ten STRUCTURE runs at each K produced nearly identical individual membership coefficients, and this figure is based on the highest probability run at each K. Typically, groups that originally emerge at lower K are the most differentiated. K=2 had the greatest mean likelihood and posterior probability values, and thus represents the closest estimate to the true number of population groups according to STRUCTURE.

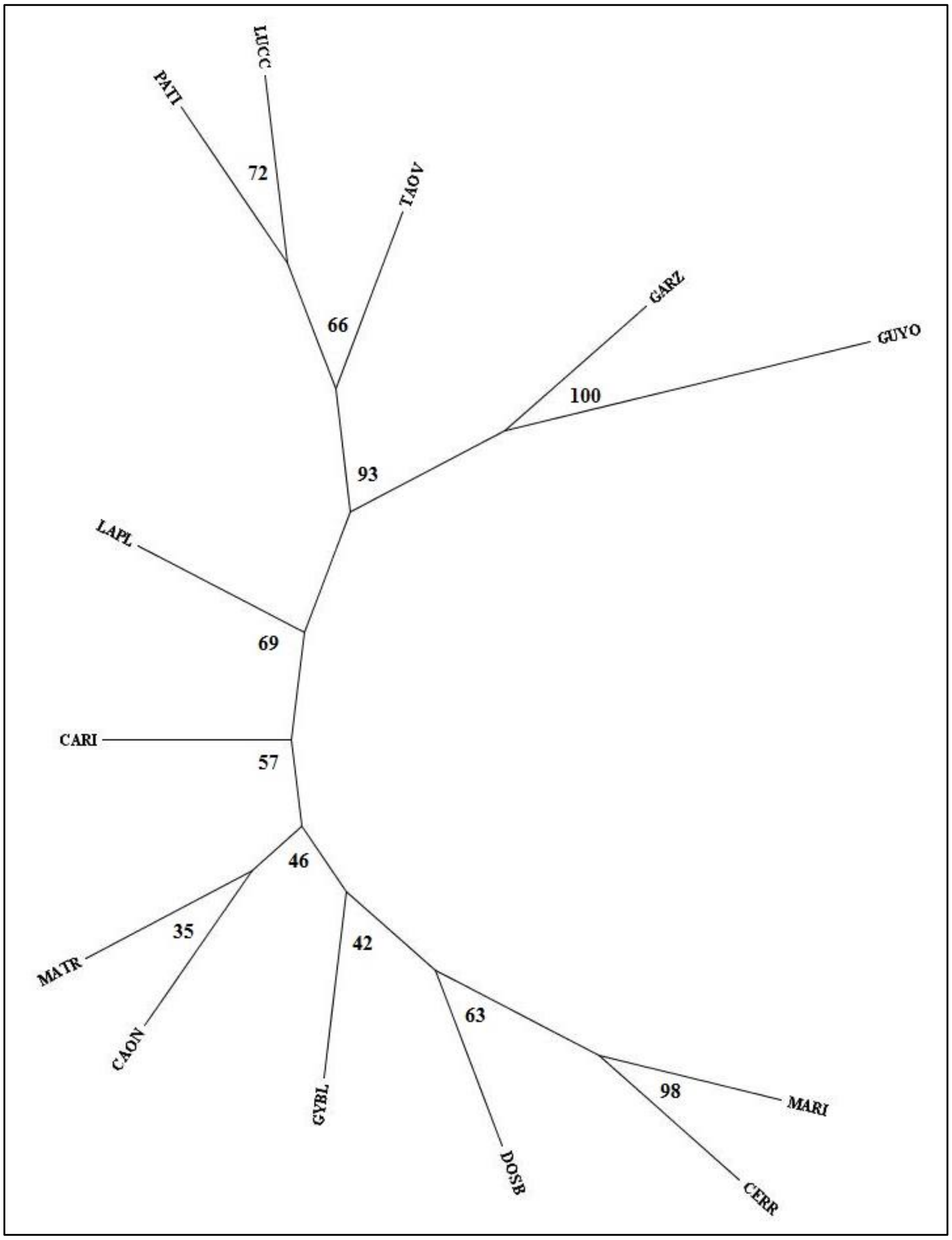


Figure 3.5 Neighbor-joining tree (Cavalli-Sforza and Edwards 1967) constructed in PHYLIP showing relationships among populations of intergrade Florida Largemouth Bass x northern Largemouth Bass populations in Puerto Rico.

The values shown at each node represent the percent of times out of 5,000 simulations that the groups separated into each branch.

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CHAPTER IV

SUMMARY

Management Implications and Future Research

Given the cultural, ecological and economic importance of Largemouth Bass to Puerto Rico, results of this thesis and other recent studies (e.g., Neal and Noble 2002; Neal et al. 2004) indicate the need for a strategic management plan for Largemouth Bass in Puerto Rico. Detailed genetic sampling and assessment protocols are necessary if the PRDNER intends to develop and maintain pure Florida Bass broodstock at the Maricao Hatchery and incorporate genetic concerns into future management decisions. The plan should also consider guidelines for determining census population size (N_c), generally determined via mark-recapture population estimates, and effective (genetic) population size (N_e). Effective population size genetically estimates the number of adult Largemouth Bass that successfully provide offspring that contribute to the next generation. Together, N_c and N_e will better depict reproductive biology, survival, and fitness among populations. Additionally, these factors can help identify threats to the genetic integrity of each population through increased genetic drift and susceptibility to inbreeding and outbreeding depression.

The goal of sampling for genetic analyses is to obtain a single genetically representative sample for the entire reservoir. Staff must sample in multiple areas to account for any localized variation in genetic structure. Samples taken from only a portion of a reservoir can be biased, and thus not be representative of the entire population. Random spring electrofishing surveys should be used as the primary means for obtaining samples. A sample size of at least 30 fish per reservoir should ensure that the percent of Florida Bass allele estimate will be within 5% of the true value 80% of the time, and within 10% of the true value 95% of the time (Dumont and Schlechte, Texas Parks and Wildlife Department, unpublished data). Insignificant size- and age-related bias has been found when estimating genetic composition in introgressed Largemouth Bass populations (Dumont and Lutz-Carrillo 2011). Therefore, the selection of specific age-classes or size-classes to be sampled should be based on the efficiency with which the age-classes or size-classes can be collected.

Microsatellite markers provide more polymorphic loci and genetic resolution than other markers such as allozymes. At a minimum, the same microsatellite loci used in this study should be used in future studies to allow applicable comparisons of genetic diversity and structure over time.

My data suggest that Puerto Rico Largemouth Bass populations contain reduced genetic diversity, which could be limiting the adaptive potential of Largemouth Bass on the island. Therefore, I recommend that additional importations of Florida Largemouth Bass are warranted to improve genetic diversity and fulfill the Maricao Hatchery objective to culture and stock only pure Florida Bass. Detailed information on the source population and the number of fish successfully imported should be recorded in detail for all future fish introductions. More research will be needed to determine if the limited genetic variation among island Largemouth Bass populations is, in fact, a mechanism behind reduced growth and longevity in Puerto Rico. Tissue samples should be archived from a subsample (N=50) of successfully imported and introduced fish to allow a baseline for future genetics evaluations to elucidate the influence of importations on subspecies composition, population dynamics (e.g., recruitment, growth, mortality), genetic diversity, and fitness. Currently, data are limited for how genetics and fitness of temperate species or stocks react when introduced to non-native, tropical environments, and management of Largemouth Bass in Puerto Rico and other non-native, tropical populations would benefit from such analyses.

Largemouth Bass populations in Puerto Rico are genetically similar due to their shared introduction history, limited time at large, and periodic stocking influences; therefore, they should continue to be managed as a single genetic unit. However, two hybridizing lineages currently exist in Puerto Rico, and genetic integrity within populations over time will continue to undergo dynamic change due to anthropogenic and environmental pressures. At this time, it is unknown whether a locally adapted gene pool would provide adaptive benefits to these fisheries (e.g., improve size structure).

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