COMPARATIVE GENETIC ANALYSIS IN INSULAR AND MAINLAND POPULATIONS OF THE FLORIDA COTTONMOUTH, AGKISTRODON PISCIVORUS CONANTI

By

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by

Andrew Warner Roark
To my wife, Alison McCombe Roark, for her endless patience and support.
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Seahorse Key (Levy County, Florida) is an important rookery for wading birds and supports an unusually dense population of Florida cottonmouth snakes (*Agkistrodon piscivorus conanti*) that are dependent on the rookery. The snakes are entirely terrestrial and scavenge on dead or rotting fish that are dropped or regurgitated by the nesting birds. Sensitivity of snakes to seawater and the apparent differentiation of certain character states relative to mainland populations suggest the cottonmouths on Seahorse Key comprise a relatively isolated population. DNA fingerprinting was used to study genetic divergences among the Seahorse Key and two mainland cottonmouth populations. Amplified fragment length polymorphism (AFLP) analyses indicated little genetic variation ($\Phi_{st} = 0.005 - 0.020$) and small genetic distances ($D = 0.003 - 0.012$) among the populations, although genetic distance was least between the two mainland populations. The small differences measured suggest the population at Seahorse Key either
experiences significant exchange with the mainland or has not been isolated for a long evolutionary period. The highly conserved nature of reptilian DNA, rather than continual gene flow, may also account for the overall lack of genetic divergence. Heterozygosity estimates indicate that the Seahorse Key population has the lowest percent heterozygosity of the three populations, potentially supporting the hypothesis that this population has experienced decreased gene flow for an extended period of time. Consequently, quantitative genetic distances and variation among populations, though very small, might still be considered useful data in assessing the degree of isolation in this unusual insular population of snakes.
CHAPTER 1
HABITAT FRAGMENTATION AND THE FLORIDA COTTONMOUTH

The population of Florida cottonmouth snakes (*Agkistrodon piscivorus conanti*) found on Seahorse Key in the Cedar Keys National Wildlife Refuge maintains a high population density, exploits a seemingly novel food source, and appears to be characterized by generally larger body sizes and darker coloration than mainland cottonmouths (Wharton 1966, 1969; H. Lillywhite, unpublished data). Cottonmouths on Seahorse Key are believed to be relatively sedentary, and thus these ecological and morphological differences from cottonmouths on the Florida mainland may indicate that this population is experiencing the effects of long-term geographic isolation.

**Habitat Fragmentation**

Habitat fragmentation, the division of ecosystems into “habitat islands,” is an increasingly prevalent problem in wildlife conservation and management (Lande and Barrowclough 1987, Templeton et al. 1990). Vast deforestation and expanding agricultural land use are destroying natural areas throughout the world, leaving behind fragmented habitats that resemble islands in their isolation, limited area, and distance from each other (Mader 1984).

Habitat fragmentation may interfere with migration and consequently with gene flow, leading to the eventual retardation and degradation of evolutionary processes and to a high probability of eventual extinction within fragmented populations (Erwin 1991, Frankham 1995). The magnitude of the effects of this fragmentation is critically dependent on the degree to which dispersal of individuals between habitat islands is
limited (Templeton et al. 1990). Reduced potential for dispersal may heighten the
probability of inbreeding and genetic drift within isolated populations, especially when
population size is small (Bushar et al. 1998). Isolated populations are more susceptible to
inbreeding depression than are non-fragmented populations, because inbreeding may
cause more frequent expression of recessive deleterious alleles, and genetic drift may fix
these deleterious alleles in the population. Once deleterious alleles are fixed in an
isolated population, no mechanisms for their removal exist (Templeton et al. 1990).

Even without fixation of deleterious alleles, inbreeding and genetic drift ultimately
reduce genetic diversity within a population (Templeton et al. 1990, Bushar et al. 1998).
Just as fixed deleterious mutations are known to accumulate within isolated populations
until these populations can no longer survive (Muller 1964), cumulative loss of genetic
diversity has been linked to decline and extinction in wild populations (Westemeier et al.
1998). Genetic variability has thus been proposed as an indicator of a population’s
vulnerability to both natural and anthropogenic stressors (D’Surney et al. 2001).

As decreasing genetic diversity caused by inbreeding and genetic drift within one
species promotes extinction of local populations, the number of populations of that
species existing at the global level declines, and genetic variants unique to individual
populations are lost. In an “extinction ratchet,” the loss of each habitat island population
that cannot be recolonized is an irreversible step towards the total extinction of the
species. As the extinction ratchet decreases the total number of populations globally, the
rate of genetic diversity loss at the species level accelerates, thus potentially driving the
entire species toward extinction (Templeton et al. 1990).
Unfortunately, it is not feasible to directly study the effect of habitat fragmentation on dispersal and susceptibility to extinction for most species (Templeton et al. 1990, Lawson and King 1996). Indirect investigation of a population’s degree of isolation (i.e., using genetic techniques) has thus become necessary for species management and conservation (Moritz 1994, Avise 1995, Frankham 1995, Bushar et al. 1998). Surveys capable of determining genetic variability within and between potentially isolated populations are now used to effectively identify populations at high risk of extinction (Templeton et al. 1990, Palumbi and Baker 1994, Avise 1994).

Island Biogeography

Populations that have experienced limited genetic exchange for an extended period of time due to geographic isolation are natural laboratories in which to study the long-term ecological, physiological, and behavioral effects of habitat fragmentation. Islands, as examples of fragmented and isolated habitats, are useful systems for investigating patterns and mechanisms in ecology and evolution at both species and population levels (MacArthur and Wilson 1967, Grant 1998). Genetic drift, combined with selection pressures different from those experienced by mainland populations, facilitates trait divergence in isolated populations (Galis and Metz 1998, Bonnet et al. 1999). Furthermore, individual isolated populations have the potential to make each island system distinctive in regard to the suite of traits displayed there.

In many cases, unoccupied ecological niches, combined with the founder effect, have allowed populations on islands to evolve life-history traits vastly divergent from those found in mainland relatives (Miller et al. 2000). Adaptations in island populations are believed to occur more rapidly and with greater directionality due to both a small gene pool and decreased gene flow between populations (Foster 1964). Characteristic
divergences attributable to population isolation frequently include differences in behavior, morphology, color, longevity, and fecundity (Miller et al. 2000). Populations experiencing isolation and decreased geographic area often maintain higher and more stable population densities, higher individual survival rates, and reduced aggressiveness and reproductive output relative to non-fragmented populations (Gliwicz 1980, Adler and Levins 1994, Padmanabhan and Yom-Tov 2000). Variation in mean body mass between isolated and mainland populations is also common (Adler and Levins 1994, McNab 1994, Adler 1996, Anderson and Handley 2002). These differences can be attributed to both the inverse relationship between geographic separation and rate of immigration (Soulé 1966) and the decrease in habitat diversity and density-depressing factors (i.e., predation and interspecific competition) created by decreased geographical area and decreased species richness (Adler and Levins 1994, Wiggins et al. 1998).

In reptiles, examples of characteristic divergences between insular and mainland populations include differences in body size, coloration, scalation, reproductive output, clutch size, size of offspring, and territorial behavior. Variation in mean body size between insular and mainland reptile populations has been found in tiger snakes (*Notechis scutatus*) on islands off the coast of south-eastern Australia (Schwaner and Sarre 1988; Schwaner 1990); in side-blotched lizards (*Uta stansburiana*) on islands in the Gulf of California, Mexico (Soulé 1966); in Chinese pit-vipers (*Gloydius shedaoensis*) on Shedao island in northeast China (Li-xin et al. 2002); and in the eastern cottonmouth snake (*Agkistrodon piscivorus conanti*) at the northern edge of its range near Hopewell, Virginia, USA (Blem 1981, Blem and Blem 1995). Body sizes in reptiles on islands are not dependent on island size, latitude, age, or distance to the mainland, but instead tend to
be bimodal based on prey size and abundance (Li-xin et al. 2002, Boback and Guyer 2003). However, mean population body size comparisons in reptiles are rare, presumably because sampling is problematic and continuous growth in reptiles makes these comparisons difficult (Soulé 1966). Morphological variation in coloration between populations has been reported in cottonmouth snakes (Blem and Blem 1995), variation in scale counts and dorsal scale size has been observed in tiger snakes (Schwaner 1990), and variable scale serration has been reported in side-blotched lizards (Soulé 1966).

Reproductive output, offspring size, and offspring number also vary between insular and mainland reptile populations. Total reproductive output is expected to correlate directly with resource availability, and thus a high degree of variability is expected among insular populations. A strong trade-off between offspring body size and number of offspring produced also exists: larger offspring and reduced clutch sizes are expected when large body size increases offspring viability, whereas small body size and large clutch size are expected when offspring survival is low (Roff 1992). Large body size in island population offspring is believed to be strongly favored when a larger gape size allows the exploitation of a food source that could otherwise not be consumed at an early age (Li-xin et al. 2002).

Large body sizes in adult reptiles may reduce the population’s vulnerability to predation, thereby increasing the population’s survival rate and allowing the population size to be sustained by a smaller number of offspring (Williams 1966, Bull and Shine 1979). Females of large size and better body condition have been shown to produce larger offspring in water snakes (*Nerodia sipedon*, Weatherhead et al. 1999), Chinese pit-vipers (Li-xin et al. 2002), and cottonmouth snakes (Blem 1981). Although not
statistically significant, this trend was further observed in smooth snakes (*Coronella austriaca*, Luiselli et al. 1996) and European adders (*Vipera berus*, Andren and Nilson 1983). However, the opposite trend (large females producing numerous, small offspring) was reported in the North American brown snake (*Storeria dekayi*, King 1993).

The primary behavioral difference between insular and mainland reptile populations appears to be a heightened tolerance to proximity between individuals. If food is the limiting environmental factor for insular population growth, then abundant food availability may create a high population density (Soulé 1966). Soulé (1966) found the population of side-blotched lizards on the island of San Pedro Mártir to maintain a population density two to three times higher than mainland populations and to exhibit decreased levels of territorial behavior. Territorial behavior is still expected to exist at some level, however, as it prevents resource exhaustion by controlling population density (Wynne-Edwards 1962). In terms of species abundance, reptiles and amphibians are the vertebrate taxa most significantly impacted by island isolation, followed by mammals, resident birds, and finally migratory birds (Harris 1984).

*Agkistrodon piscivorus* as a Study Species

The combination of unique characteristics and relatively high probability of population extinction in island systems in general, and in snakes in particular (Dodd 1993), makes locating and researching established insular populations of reptiles and other vertebrates important to conservation. The population of Florida cottonmouth snakes (*Agkistrodon piscivorus conanti*) found on Seahorse Key (Levy County, Florida) is a potentially useful system for the study of insular vertebrate populations experiencing significantly reduced gene flow due to geographic or habitat isolation. This population appears to exhibit behavioral, morphological, and reproductive character divergences
commonly used to identify isolated populations (i.e., darker coloration, larger adult body sizes, greater tolerance of close proximity to others, and increased offspring body size relative to mainland populations) (Wharton 1966), and could be useful to both evolutionary and conservation biologists.

Cottonmouth snakes are New World pit vipers of the monophyletic clade *Agkistrodon*, in the family Viperidae, subfamily Crotalinae (Parkinson 1999). New World pit vipers are currently believed to have moved into North America in a single invasion (Krauss et al. 1996, Parkinson 1999, Parkinson et al. 2000, but see Burger 1971, Gloyd and Conant 1990). Fossil records suggest that divergence between *Agkistrodon* and the North American rattlesnake genera, *Crotalus* and *Sistrurus*, occurred no later than the late Miocene era (10-12 million years ago; Conant 1990). The genus *Agkistrodon* comprises three widely distributed species: the copperhead, *A. contortrix*, the cottonmouth, *A. piscivorus*, and the cantil, *A. bislineatus*. Restriction fragment length polymorphism and mitochondrial DNA sequence analyses suggest that *A. contortrix* is the basal species of the genus and that *A. bislineatus* subsequently arose from *A. piscivorus* (Knight et al. 1992, Parkinson et al. 1997, Parkinson 1999, Parkinson et al. 2000). *Agkistrodon piscivorus* and *A. contortrix*, along with most rattlesnake species, resided in temperate ancestral habitats (Klauber 1972), whereas *A. bislineatus* diverged into tropical ancestral habitats (Parkinson et al. 2000). Fossil records indicate that *A. piscivorus* has been present in Florida since the Pleistocene era (Brattstrom 1953, Auffenberg 1963).

*Agkistrodon piscivorus* has a broad head relative to its neck, a pair of facial pits, a single row of ventral scales under the major part of the tail, and a single anal plate. It
reaches the largest size of any member of the genus *Agkistrodon*, especially in girth (may exceed 1.8 meters in length and 4.6 kg in body mass). It is the only semi-aquatic member of the genus, has the greatest number of scale rows (25) at midbody, and lacks a loreal scale (Gloyd and Conant 1990). Cottonmouths are largely nocturnal during hot weather but bask during daylight hours in the cooler months of the year. The typical cottonmouth diet is generalized and includes carrion, small mammals, birds, snakes, amphibians, turtles, and fish (Burkett 1966, Savitzky 1992). These snakes are opportunistic feeders and take the prey item that is most readily available to them (Ernst 1992). The maximum known lifespan for a cottonmouth is 21 years (Gloyd and Conant 1990).

The Florida cottonmouth (*Agkistrodon piscivorus conanti*) is found from Southern Georgia to the Florida Keys and inhabits many coastal islands (Conant and Collins 1998). This subspecies can be distinguished from other *Agkistrodon piscivorus* subspecies by its 11 to 16 dark dorsal cross-bands, dark cheek stripe, dark vertical stripes at the first supralabials and edges of the rostral and adjacent prenasals, and pair of dark blotches extending from the mental along the first four or five infralabial scales and the outside of the chin shield (Gloyd and Conant 1990). Pattern and color variations are generally slight, and although dorsal body markings may become subdued by a uniform black or dark brown coloration in adults, some indication of facial markings is generally retained (Gloyd and Conant 1990).

**Seahorse Key**

Insular populations of the Florida cottonmouth inhabiting the Cedar Keys of Florida have been studied by Carr (1936) and, more extensively, by Wharton (1966, 1969), who focused his investigations on an unusually dense population of snakes inhabiting Seahorse Key. This island, a crescent-shaped land mass just over 1.6 km in
length (0.67 ha in area), is the largest in the Cedar Keys National Wildlife Refuge (Levy County, FL). It lies approximately 3.5 km offshore, is of sand dune origin, has a maximum elevation of 15.85 meters, and consists of salt marsh, mangrove, and mixed hardwood forest habitats. Seahorse Key is part of a wide continental shelf and is separated from the mainland by water approximately 2 – 3.5 meters in depth. Given that sea levels have continuously risen in Florida since the late Holocene (some 8,000 years before present) at an estimated rate of 4 cm per 100 years over the last 3,000 years and 25 cm per 100 years prior to that (Wanless 1982), the separation of Seahorse Key from the mainland can be roughly estimated at 3,300 – 3,900 years before present.

The island’s peripheral vegetation consists mostly of black mangrove (*Avicennia nitida*) and salt marsh. The inland hammock consists primarily of dwarf palmetto (*Sabal minor*), red bay (*Persea borbonia*), sand live oak (*Quercus geminata*) and Virginia live oak (*Quercus virginiana*). The upland hammock and mangroves are heavily populated by white pelicans (*Pelecanus erythrorhynchos*), brown pelicans (*Pelecanus occidentalis carolinensis*), nesting cormorants (*Phalacrocorax auritus*), osprey (*Pandion haliaetus*), and wading birds like white ibis (*Gaira alba*), snowy egret (*Leucophoyx thula*), American egret (*Casmerodius albus*), as well as several heron species (Wharton 1966, 1969).

Seahorse Key has no permanent fresh water, and resident cottonmouths are thus denied their characteristic semi-aquatic habitat. The primary food source for this population comes from scavenging fish dropped or regurgitated by colonial wading birds that nest in large numbers on the island, usually from March through September or
October. Marine foraging is not considered a possible feeding strategy due to saltwater avoidance by cottonmouths (Wharton 1966).

During the years 1954-56, Wharton captured 545 cottonmouths on this island and estimated its total population at 600 individuals. He noted that snakes occur throughout the island but are concentrated in the area of highest nesting bird abundance, a low peninsula at the west end of the island. Cottonmouth density was estimated at 56.1 individuals per hectare beneath these rookeries and 4.6 individuals per hectare on the main ridge of the island, where rookeries are relatively scarce. Although Seahorse Key snakes may face less pressure from predators relative to their mainland counterparts, food availability varies seasonally with the presence of nesting birds, and an estimated 77% are in danger of starvation during the winter months when rookeries are largely empty (Wharton 1969). This strong dependence on nesting bird populations, seasonal food supply, and high probability of starvation-induced mortality caused Wharton to refer to this population as living at a “critical survival level” (Wharton 1969).

Cottonmouths are also present on the keys adjacent to Seahorse Key, but the density of these snakes appears to correlate with the presence and numbers of nesting birds, which are by far most common on Seahorse Key (H. Lillywhite, unpublished data). These island populations of cottonmouths presumably rafted to the islands sometime in the undetermined past and then became established or have been present since the islands became disconnected from the mainland. Observations of coloration and behavior suggest divergence between island and mainland snake populations (H. Lillywhite, unpublished data). The occurrence of island snakes lacking one or both eyes (Wharton 1969; Roark and Lillywhite, unpublished data) could support this theory and indicate that
Phenotypic divergence could be rooted in the genetic composition of these island populations if this condition is congenital. Such phenotypic variation between populations is frequently used to identify microevolutionary processes including gene flow, natural selection, and genetic drift (Lawson and King 1996).

Preliminary field and laboratory data suggest Seahorse Key cottonmouths are sensitive to salt water and avoid seawater (Wharton 1966, 1969; Roark and Lillywhite, unpublished data), thereby potentially isolating island snakes from their mainland counterparts. The sharing of refugia, which has been observed in the Seahorse Key cottonmouth population, could also decrease population genetic diversity, as it facilitates inbreeding within snake species that copulate upon emergence in the spring (Bushar et al. 1998). The potential for reduced gene flow and increased inbreeding, combined with recognized phenotypic differences relative to mainland populations, suggests that genetic divergence from mainland cottonmouth populations may characterize the Seahorse Key population. However, genetic analyses of the current degree of gene flow and genetic variation within and between this island population and surrounding mainland populations are required to determine the degree of isolation affecting this population and, thus, its susceptibility to eventual extinction.
CHAPTER 2
COMPARATIVE GENETIC ANALYSIS

Geographically isolated populations are interesting to biologists because they have a high potential for extinction as predicted by island biogeography theory (MacArthur and Wilson 1967), may exhibit long-term ecological, physiological, and behavioral effects of habitat fragmentation, and are likely to experience inbreeding depression and reduced intrapopulation genetic diversity (Templeton et al. 1990). The cottonmouth population residing on Seahorse Key, an island in the Cedar Keys National Wildlife Refuge, appears to be a potentially useful system in which to study the characteristics of an isolated population. However, before this system can be studied effectively, a determination of whether this population is truly isolated must be made. Studies attempting to determine phylogenetic relationships and divergences based on morphological and physiological traits are often flawed because these traits are affected by environmental factors and phenotypic plasticity (Avise 1995). Problems building these types of phylogenies are further compounded in snakes because they possess few variable morphological traits relative to other groups, retain primitive character traits, and frequently exhibit evolutionary convergence between taxa (Knight et al. 1992).

Population isolation can, however, be measured with the use of molecular techniques such as amplified fragment length polymorphism (AFLP, Vos et al. 1995) analysis.

The AFLP technique developed by Keygene BV (Wageningen, The Netherlands) is a multilocus marker protocol wherein genomic DNA undergoes fragmentation through double-restriction digestion and subsequent ligation to specific oligonucleotide adapters.
that alter the restriction site to prevent reformation and post-ligation digestion. A subset of fragments is then amplified by polymerase chain reaction (PCR) as selective primers are added. Amplified fragments are separated and identified using polyacrylamide gel electrophoresis. The AFLP technique can generate a number of potential markers per genome ten times greater than can be accomplished using simple sequence repeats (SSR) or random amplified polymorphic DNA (RAPD) techniques (D’Surney et al. 2001). AFLP markers have gained popularity due to their relatively low cost (Giannasi et al. 2001), utility in the absence of prior sequence information (Busch et al. 2000; Negi et al. 2000), high multiplex ratio (Negi et al. 2000), and high degree of reproducibility (Ribeiro et al. 2002). AFLP markers are also ideal for fluorescent dye labeling and gel-based automated analyses (D’Surney et al. 2001). The primary deterrent to using AFLP markers is their dominant nature, which does not allow direct measurement of heterozygosity (Negi et al. 2000, although see Nei 1978 for an indirect measure of heterozygosity).

**Objective**

The objective of this study was to test two hypotheses: 1) the Seahorse Key cottonmouth population is more genetically distinct from select mainland populations than these mainland populations are from each other, and 2) gene flow into the Seahorse Key population is greatly reduced relative to that between mainland populations. The investigation of these hypotheses was carried out in hopes of establishing a quantitative measure of the degree of isolation of the Seahorse Key cottonmouth population to create a foundation from which future comparative studies between this island population and mainland populations might be launched. This research was also intended to create a knowledge base about this system that, when combined with current and future
morphological, physiological, and genetic comparative studies of these island and mainland snakes, has the potential to generate new hypotheses regarding the behavioral and physiological effects of habitat fragmentation in this and possibly other vertebrate species.

**Study Sites**

Three study sites (one island and two mainland) were sampled for genetic comparison between November 2000 and October 2002 (Fig. 1). The first study site was the island of Seahorse Key in the Cedar Keys National Wildlife Refuge, the second was a coastal mainland site in the Lower Suwannee National Wildlife Refuge (Levy County, FL), and the third was an interior mainland site in Paynes Prairie State Preserve (Alachua County, FL). Both mainland sites were initially selected to approximate the geographic area of Seahorse Key (0.67 ha) but required expansion beyond this size to allow collection of adequate sample sizes. Approval for animal use was obtained from the University of Florida’s Institutional Animal Care and Use Committee (IACUC; #Z074), and approval for animal collection from preserve areas was obtained from the Florida Department of Environmental Protection (#01040212) and the United States Fish and Wildlife Service (#4151502005; #4151102002).

Samples were collected throughout Seahorse Key in a rough representation of the population density variation found on the island, and were thus collected in greatest numbers from areas rich in nesting bird rookeries. The correlation between snake and nesting bird densities supports the theory that bird rookeries are the primary food source for the adult cottonmouth population. Samples collected from Gardner Point and the vegetation along the beach - areas where rookeries are common - comprised 40% and 36% of the total sample, respectively. Samples collected from the upland ridge and dock
area, where birds are less common, comprised 16% and 8%, respectively. Sample collection from Gardner Point was completed in a single day, whereas sample collection from the beach, ridge, and dock areas required numerous visits to the island. The disproportionate amount of time spent sampling Gardner Point relative to the rest of Seahorse Key probably indicates that cottonmouth population density is much greater there.

The Paynes Prairie State Preserve study site is located approximately 97 km northeast of Seahorse Key and was chosen for its close proximity to the University of Florida, large size (85 km$^2$), and for its centralized location between the eastern and western coasts of northern Florida. Cottonmouths from Paynes Prairie State Preserve were collected primarily from two distinct locations approximately 8 km apart along a riverbed. The type locality of *Agkistrodon piscivorus conanti* is located 11 km SE of Gainesville, FL (Gloyd 1969), in close proximity to samples collected from Paynes Prairie State Preserve during this study.

The Lower Suwannee National Wildlife Refuge (214 km$^2$) was selected as a study site for its coastal mainland location and close proximity to Seahorse Key. The Suwannee River, with an average flow rate of 304.7 m$^3$/s (Nordlie 1990), is a possible dispersal vector for cottonmouth snakes in that area, and thus, snakes from the Lower Suwannee National Wildlife Refuge could potentially be frequent migrants to the islands of the Cedar Keys. The Lower Suwannee National Wildlife Refuge study site is located approximately 40 km north of Seahorse Key. With the exception of three individuals found near park access roads, all cottonmouths from Lower Suwannee National Wildlife Refuge were collected along nine miles of County Road 349.
Methods

Cottonmouth samples for DNA analysis from Seahorse Key (25), Paynes Prairie State Preserve (25), and Lower Suwannee National Wildlife Refuge (22) were collected in numbers large enough to ensure an adequate sampling of each population’s genetic structure. Shed skins were collected and stored in paper when possible. Fresh tissue samples were obtained via scale clipping (when live animals were encountered) or tail tip severance (when freshly killed animals were encountered) and were saved in buffer solution of saturated NaCl, 25 mM EDTA (pH 7.5) and 20% DMSO (modified from Amos & Hoelzel 1991).

Live snakes were captured using a steel snake hook and were safely restrained within a plastic tube of appropriate diameter while scale clipping took place. A single scale of keratinized epidermis was excised from the aseptically swabbed posterior end of the animal (in accordance with IACUC permit #Z074). Styptic powder was applied to the clipped area with a moistened, cotton-tipped applicator prior to release to minimize any bleeding. No noticeable change in behavior was observed following scale clipping, and an individual snake that was spotted beneath a particular tree multiple times before capture was observed there weeks after the procedure.

Of the Seahorse Key DNA samples, 24 were collected as shed skins and one as a tail tip. Of Lower Suwannee DNA samples, 15 were collected as tail tips and seven were collected as scale clippings. Of the Paynes Prairie DNA samples, ten were collected as scale clippings, nine as tail tips, and six as shed skins. PCR techniques allow for the effective use of extremely limited quantities of DNA in genetic analyses (Busch et al. 2000), so these methods of collection yielded acceptable quantities of DNA.
DNA was isolated from shed skins using a modified CTAB protocol (Clark 1998) and from tissue samples using standard phenol/chloroform methodology (Hillis et al. 1996). Isolated DNA was then amplified using PCR. All molecular techniques were conducted in the University of Florida’s Biotechnologies for the Ecological, Evolutionary, and Conservation Sciences Laboratory in the Interdisciplinary Center for Biotechnology Research (ICBR). Isolated DNA was then amplified using PCR.

Purified cottonmouth DNA samples were digested with a 6 base-pair rare-cutter restriction enzyme, EcoRI, and a 4 base-pair common-cutter restriction enzyme, Msel. Digested DNA was preselectively amplified (Fig. 2) and then selectively amplified (Fig. 3) using three adapter-specific primer combinations (EcoRI-ACC + Msel-ACTT, EcoRI-ACC + Msel-ACAA, and EcoRI-ACC + Msel-ACTA; see Table 1). These three primer combinations were selected from sixteen available primer combinations following preliminary tests (Roark, unpublished data). Primer sets produced sufficient DNA fragments of appropriate size and number for analysis.

Selectively amplified DNA fragments were separated by electrophoresis in a 5% polyacrylamide gel. The resulting banding pattern was imaged using a Typhoon fluorescence scanner (Amersham Pharmacia Biotech, Uppsala, Sweden) in the University of Florida’s ICBR Molecular Services Core, and the presence or absence of fluorescent AFLP marker bands was scored using the default settings for FragmeNT (Version 1.1, Molecular Dynamics) software. Manual scoring of bands corrected for obvious software algorithm errors, and only bands that could be scored unambiguously were used for analyses. The highest and lowest 10% of molecular weight bands were also excluded because bands of low weight may include products from the interaction of primers alone,
and bands of great weight may be strongly influenced by differences in reaction conditions and DNA quality (Bagley et al. 2001). Band sizes were determined by the FragmeNT software based on comparison to DNA standards (Invitrogen Low DNA Mass Ladder; Invitrogen 50 bp DNA Ladder, Carlsbad, CA, USA).

**Analyses**

Bands were classified as either monomorphic (found in all samples) or polymorphic (found in some but not all samples). Polymorphic bands were binomially scored for each snake with a ‘1’ for the presence of a band and a ‘0’ for the absence of a band at each polymorphic locus. All monomorphic bands were omitted from the analyses.

Thirty-three polymorphic loci were analyzed using Tools For Population Genetics Analysis© (Version 1.3) software (Miller 1997). The software calculated population subdivision using Wright’s F-Statistic (Φst) (Weir & Cockerham 1984) and a 95% confidence interval (based on 5000 bootstrap replicates). The accumulated number of gene differences per locus (Genetic distance, D, Nei 1972), the proportion of genes that are common to both populations being compared (Genetic identity, I, Nei 1972), and Nei’s unbiased heterozygosity estimate (1978) were also calculated.

The rate of gene flow between populations was assessed using Wright’s F-Statistic to estimate the number of migrants between populations per generation (Nm). Nm was calculated based on Φst = 1/(1+4Nm) (Wright 1943, 1969). Low rates of gene flow (less than 1 individual per generation) will indicate that the differences seen between populations may be attributable to genetic drift, whereas high rates of gene flow (more than 1 individual per generation) will suggest this scenario unlikely and point to
mechanisms other than genetic drift as the source of this variation (Slatkin 1987, Avise 1994).

**Results**

Apparent differences in phenotype and behavior in Seahorse Key cottonmouths relative to mainland cottonmouths were not reflected in the assessment of genetic variation within and between populations. When the genetic relationship between the Seahorse Key and Lower Suwannee populations was assessed, $\Phi_{st} = 0.020$ with a 95% C.I. of 0.028 - 0.012, genetic identity ($I$) = 0.989, genetic distance ($D$) = 0.012, and $Nm = 12.4$. The Seahorse Key and Paynes Prairie relationship showed $\Phi_{st} = 0.009$ with a 95% C.I. of 0.017 – 0.002, $I = 0.995$, $D = 0.005$, and $Nm = 29.2$. Finally, in the Lower Suwannee and Paynes Prairie relationship, $\Phi_{st} = 0.005$ with a 95% C.I. of 0.0115 - 0.000, $I = 0.997$, $D = 0.003$, and $Nm = 48.8$. Nei’s (1978) unbiased heterozygosity estimate indicated an average heterozygosisty of 35.6% among alleles in the Seahorse Key population, 43.9% among alleles in the Lower Suwannee population, and 39.1% among alleles in the Paynes Prairie population (Table 1).

**Discussion**

The close proximity of all $\Phi_{st}$ values to zero indicates a near-complete absence of interpopulation genetic variation and suggests that significant gene flow exists between all three populations. Genetic identity values near 1 and genetic distance values near 0 indicate that nearly 100% of genes present are shared by these populations. Estimated numbers of migrants per generation range from 12.4 between Seahorse Key and Lower Suwannee to 48.8 between Paynes Prairie and Lower Suwannee, as calculated directly from $\Phi_{st}$ values. This result further suggests a minimal amount of genetic divergence between populations.
These results, however, do not necessarily mean that the Seahorse Key cottonmouth population is not somewhat isolated. Survival of harsh shifts in environmental conditions, such as those associated with isolation in a novel habitat, is believed to be largely dependant on plasticity (Bonnet et al. 1999) rather than genetic divergence in most systems. It has been theorized that differences between island and mainland populations are probably short-term responses to increased longevity and high population densities on islands rather than genetic drift or directional selection following the isolation incident (Adler and Levins 1994).

It should also be pointed out that slow mutation rates leading to small population divergences are assumed to be correlated with slow metabolic rates and relatively long life spans. Organisms with low metabolic rates and long life spans tend to have longer nucleotide regeneration times and thus may have a slower rate of molecular evolution (Martin and Palumbi 1993). As an ectothermic vertebrate with a potential life span of approximately 20 years, *A. piscivorpus* almost certainly possesses one, if not both, of these characteristics. Previous studies of intrapopulation genetic variability in cottonmouths support this potential explanation. Low levels of genetic variation have been found between populations of *A. piscivorpus piscivorpus* at the northern edge of its range in southeastern Virginia (I = 0.964; Merkle 1985), and populations of *A. piscivorpus leucostoma* located east of Memphis, TN, showed no difference in DNA content when flow cytometry was employed (Tiersch et al. 1990).

Given that previous studies have suggested limited genetic variability throughout the range of this species (Merkle 1985, Tiersch et al. 1990), trends observed within the low $\Phi_{st}$ values, small genetic distances, and high genetic identity scores should be
considered when addressing the stated hypotheses. Although gene flow appears uninterupted between populations in this study, the hypothesis that the Seahorse Key cottonmouth population is more genetically distinct from the mainland populations than these populations are from each other is supported by the finding that the genetic distances and $\Phi_{st}$ values between the Seahorse Key population and either of the mainland populations are larger than the genetic distance and $\Phi_{st}$ value between the two mainland populations. The estimated rate of migration between the mainland populations is also much higher than the estimated rate of migration between either of the two mainland populations and the Seahorse Key population. Genetic identity scores also indicate that the two mainland populations have a slightly higher percent genetic similarity to each other than either does to the Seahorse Key population.

Similarly, the hypothesis that gene flow into the Seahorse Key population is greatly reduced relative to that between mainland populations is supported by the finding that $\Phi_{st}$ value is smallest, and consequently the migration rate is greatest, between the two mainland populations when compared to the $\Phi_{st}$ values and migration rates between either mainland population and the Seahorse Key population. Also, the lowest estimated heterozygosity was found in the Seahorse Key population (35.6% versus 39.1% and 43.9%), possibly indicating reduced dispersal ability in this population relative to the mainland populations.

Although these small genetic differences may allow us to begin theorizing about general trends in the ecologies of the study populations, they are insufficient to establish a quantitative measure of the extent to which the Seahorse Key cottonmouth population is isolated. Variations in $\Phi_{st}$, genetic distance, and genetic identity values between
populations are miniscule, and bootstrapping indicates that the $\Phi_{st}$ values’ 95% C.I.s do overlap. Nei’s unbiased heterozygosity estimate may offer the most reliable quantitative measurement of the Seahorse Key cottonmouth population’s geographical isolation, and thus a genetic investigation utilizing a co-dominant marker system that allows the direct measurement of heterozygosity (i.e., microsatellites) could be beneficial.

The final goal of this research was to establish a knowledge base about the Seahorse Key system that, when combined with current and future research in this location, could assist in the generation of new hypotheses regarding the effects of habitat fragmentation in this and possibly other vertebrate species. Through this study, it has become apparent that significant genetic divergence between this island population and mainland populations has not occurred. Whether this lack of divergence exists because the Seahorse Key population is not geographically isolated, because it has not been isolated for a sufficient period of time to affect the genetic composition of its members, or because the microevolutionary processes associated with population isolation work slowly in this particular taxon can not be determined from these data.

However, the knowledge that the Seahorse Key population is not genetically distinct from mainland populations is inherently valuable. Keogh et al. (2003) suggested that a lack of genetic diversity between insular and mainland snake populations may have positive implications for species conservation because the loss of an isolated population may not significantly reduce genetic diversity within the entire species. This statement does not mean, however, that continued monitoring and management of these systems is not required, since the loss of insular populations such as the Seahorse Key cottonmouths could affect prey populations or open niches for invasive or introduced species capable of
unbalancing the entire ecosystem (Keogh et al. 2003). Furthermore, the variations that were detected in $\Phi_{st}$ values, genetic distances, genetic identities, and estimated heterozygosities between these populations occur in a manner suggesting that microevolutionary processes commonly associated with genetic isolation could be in their early stages in the Seahorse Key cottonmouth population. In light of the pattern of slight divergences demonstrated by this study, these results serve to heighten awareness of the need for a non-genetic study of the interpopulation migration rates of island and mainland cottonmouth snakes in and around the Cedar Keys National Wildlife Refuge.
Figure 1. Study sites.
1) 72°C – 2 minutes – Initial incubation
2) 94°C – 20 seconds – denaturing
3) 56°C – 30 seconds – annealing
4) 72°C – 2 minutes – extension

5) 72°C – 2 minutes – Final extension
6) 60°C – 30 minutes – Final incubation
7) 4°C – Storage

20 cycles

**Figure 2. Preselective amplification program.**

1) 94°C – 2 minutes – Initial denaturation
2) 94°C – 20 seconds - denaturation
3) 66°C – 30 seconds - annealing
4) 72°C – 2 minutes – extension

5) 94°C – 20 seconds – denaturation
6) Decrease 1°C per cycle – 30 seconds – annealing
7) 72°C - 2 minutes – extension

8) 94°C – 20 seconds – denaturation
9) 56°C – 30 seconds – annealing
10) 72°C – 2 minutes – extension

11) 60°C – 30 minutes – Final incubation
12) 4°C - Storage

9 20

**Figure 3. Selective amplification program.**
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence: 5’ – 3’</th>
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</thead>
<tbody>
<tr>
<td>EcoRI-ACC</td>
<td>GACTGCCTACCAATTTCACC</td>
</tr>
<tr>
<td>MseI-ACTT</td>
<td>GATGAGTCCTGAGTAACCTT</td>
</tr>
<tr>
<td>MseI-ACAA</td>
<td>GATGAGTCCTGAGTAACCA</td>
</tr>
<tr>
<td>MseI-ACTA</td>
<td>GATGAGTCCTGAGTAACCA</td>
</tr>
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</table>

Table 1. AFLP selective amplification primers used in study.

<table>
<thead>
<tr>
<th>Populations</th>
<th>Φst</th>
<th>C.I.</th>
<th>I</th>
<th>D</th>
<th>Nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHK-LSNWR</td>
<td>0.020</td>
<td>0.028 - 0.012</td>
<td>0.989</td>
<td>0.012</td>
<td>12.4</td>
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<tr>
<td>SHK-PP</td>
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<td>0.017-0.012</td>
<td>0.995</td>
<td>0.005</td>
<td>29.2</td>
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<tr>
<td>LSNWR-PP</td>
<td>0.005</td>
<td>0.012-0.000</td>
<td>0.997</td>
<td>0.003</td>
<td>48.4</td>
</tr>
</tbody>
</table>

Table 2. Comparison of inter-population relationships.
LIST OF REFERENCES


Burger, L. W. 1971. Genera of pitvipers (Serpentes: Crotalidae). University of Kansas Press, Lawrence, KS.


BIOGRAPHICAL SKETCH

Andrew Warner Roark was born on December 6, 1976, in Charlotte, NC, to Roger and Annette Roark. He earned the degree of Bachelor of Science in biology with a concentration in medical humanities from Davidson College, Davidson, NC, in May 1999. Following graduation, Andrew began conducting research in the Laboratory of Developmental Neurobiology, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, MD. In August 2000, he began graduate studies in the Department of Zoology, University of Florida.