MOLECULAR GENETIC ASSESSMENT OF POPULATION STRUCTURE, PATERNITY, AND SEX RATIOS FOR THE REDDISH EGRET

THESIS

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ABSTRACT

MOLECULAR GENETIC ASSESSMENT OF POPULATION STRUCTURE, PATERNITY, AND SEX RATIOS FOR REDDISH EGRETS

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We examined distantly isolated colonies of Reddish Egret to determine global population genetic structure. We analyzed 13 polymorphic satellites and used nine of them to accomplish seven goals: 1) to assess range wide population differentiation among Reddish Egret (*Egretta rufescens*) populations, 2) identify extent of gene flow and immigration among populations, 3) determine any historical occurrence of bottlenecks, 4) assess genetic differentiation between color morphs, 5) clarify subspecies status of *E. r. dickeyi,* a completely dark morph population located in and around Baja, Mexico, 6) assess paternity of nest mates, 7) determine offspring sex ratio. We collected blood samples of 244 nestlings, each from a separate nest, from colonies in Texas, Baja California, Bahamas, and Florida. Genetic differentiation was dramatic (global Fst = .257) throughout the Reddish Egret's range extending from Baja California, Mexico to Great Inagua, Bahamas. Differentiation occurred between 3 distinct regions but not

between colonies/islands within regions. Genetic diversity (Alleles per locus, and heterozygosity) is less in Baja, Mexico and Great Inagua populations than the Texas/Florida population due to minimal immigration between regions and historical population reductions. Dark and white color morphs when present within the same region showed little to no differentiation. Patterns of recent population bottlenecks are evident in each of the 3 regional populations. With evidence of limited gene flow in addition to low genetic diversity and prospects of habitat loss we recommend that Reddish Egrets be managed as 3 distinct or evolutionary significant units (Baja, Texas/Florida, and Inagua). Furthermore, our results do not refute the current subspecies status of *E. r .dickeyi.* In nests where all offspring were sampled, possible multiple paternity was identified 15.4% of the time. Offspring sex ratio did not deviate significantly from expected.

CHAPTER I

INTRODUCTION

Conservation Biology

Conservation biology is a relatively recent field that takes a multidisciplinary approach to address concerns over species, communities, and ecosystems that are perturbed by pervasive anthropogenic influence (Soule 1985). It is often called a crisis discipline in that swift action is required even when not all of the necessary information is available; inaction would be more harmful (Groom et al. 2006). Conservation draws on the tools and knowledge from other fields such as ecology, evolution, population biology, molecular genetics, biogeography, spatial ecology, policy, and law among many others to ultimately promote biodiversity due to its importance to the long term viability of entire systems. The World Conservation Union (IUCN) recognizes and suggests the conservation of three important components of biodiversity: genetic diversity, species diversity, and ecosystem diversity (McNeely 1990). Conservation biology deals with the threat of extinction that can be divided into two paradigms: the small population paradigm and the declining population paradigm (Caughley 1994). Those organisms with small or declining numbers are at the greatest risk of losing the battle for survival and going extinct due to increasing human pressures such as habitat destruction, fragmentation, economic overexploitation, introduced species, and pollution (Groom et al. 2006). Conservation biology recognizes the strain that exponential human growth has put on many organisms and ecosystems, and the intrinsic and economic value of

biodiversity and aims to mitigate and minimize the current rapid loss. Molecular techniques that allow for the assessment of genetic diversity represent a significant tool for conservation biologist in evaluating the viability of small or declining populations or organisms though genetics should not be used in singularity when managing such populations (Lande 1988).

Population genetics is the study of genetic variation within and among populations and it has a very logical application to conservation biology and species of concern (Conner & Hartl 2004). Genetic variation is important to a species' ability to adapt and evolve when faced with changes in the environment. The Fundamental Theorem of Natural Selection states that the rate of evolutionary change in a population is proportional to the amount of genetic diversity available (Fisher 1930). Often a species does not occur as a single panmictic population, but rather as several subdivided populations with each experiencing the heterogeneities of the various geographic locations (Conner & Hartl 2004). Geographic separation of populations when accompanied by a lack of immigration by individuals or gene flow to other populations can lead to genetic differentiation (Erlich & Raven 1969; Coyne 2004). Gene flow can act as a homogenizing force among separated populations, allowing disjunct populations to share alleles. In the absence of geographic barriers to gene flow, distance between populations in a species that occupies an area greater than the dispersal distance tendencies of the organism, may result in isolation of populations leading to differentiation (Wright 1943).

Genetics of Small Populations

Inbreeding is of major concern within small populations as it can lead to an increase in homozygosity or a decline in genetic variation associated with heterozygosity. Inbreeding is defined as a situation where the likelihood of mating with a related individual is greater than expected by random chance (Conner & Hartl 2004). The danger of inbreeding for a small population comes in the form of reduced fitness due to the loss of heterozygosity and the increased frequency of deleterious alleles; this is known as inbreeding depression (Reed & Frankham 2003). In an already small population a decline in fitness can lead to extinction (Saccheri et al. 1998). Inbreeding depression is shown to significantly impact survival, reproduction, birth weight, resistance to disease, predation and environmental stress in bird and mammal populations (Keller & Waller 2002). It can also decrease metabolic efficiency, disease resistance, growth rate, and reproductive physiology (Gilpin & Soule 1986). In a small isolated population of Greater Prairie Chickens, genetic diversity in addition to fitness as measured by fertility, hatching success, had significantly declined and were lower than nearby larger populations until a strategy to introduce individuals from other nearby populations was enacted (Westemeier 1998). The positive benefit of small inbred populations gaining individuals from larger populations has been shown in other species (Madsen 1999); fitness has been restored in inbred populations through human-induced gene flow (see review by Keller & Waller 2002). Gene flow is an important factor and if the population is small but still receives dispersing individuals from other populations the negative fitness impacts can be avoided.

Genetic drift is a relatively weak force of evolution but given small enough populations, the impact can be substantial. Drift is a change in gene frequencies over

time due to chance. Each offspring receives one copy or allele for each gene from each parent and it is purely chance that the other parental allele was not passed on to the next generation. Within a small isolated population the likelihood of an allele being lost completely or conversely, fixed, is much higher given selection is not acting on that particular loci (Rich et al. 1979). This fixation of alleles equates to increased homozygosity and the associated impacts of decreased genetic variation.

The extreme decrease in population size or a population bottleneck completely remove alleles (due to low frequency) from the population or significantly change allele frequencies of the population compared to before the bottleneck event. Once the population is small it becomes susceptible to the detrimental effects of inbreeding and genetic drift. A rapid decline in the number of individuals can lead to a decline in genetic diversity represented by number of alleles per locus and heterozygosity but this loss in genetic variation can be mitigated by a quick population recovery (Nei 1975; Allendorf 1986; Coates 1992). If a population fails to rebound and remains small after a decline, it becomes more vulnerable to the forces of genetic drift and inbreeding (Ellstrand & Elam 1993; Allendorf, 1986; Frankham 1995). Ultimately though, a bottleneck can lead to a loss in evolutionary potential and should be avoided (Frankham et al. 1999). In small populations the relationship between bottlenecks, inbreeding depression, and genetic drift acting in concert with demographic and environmental factors can create a positive feedback loop that can create what is known as an "extinction vortex" whereby any stochastic event associated with the factors above including a natural catastrophe may cause a population to go extinct (Gilpin & Soule 1986; Soule & Simberloff 1986).

Neutral versus Adaptive variation

Often a major concern with small or declining populations is genetic variation because of the adaptive potential it provides in the face of environment change. Most population genetic studies work with neutral genetic. Adaptive traits, variation under selection, are determined through quantitative genetic studies and neutral and adaptive variation cannot be directly linked (Holderegger 2006). Reed and Frankham (2003) found that the correlation between measures of population genetic diversity and population fitness was significant though it only explained 19% of the variation. In an earlier meta-analysis by Reed and Frankham (2001) they explored the relationship between molecular genetic variation and quantitative genetic variation to discover that the correlation was weak ($r = 0.217$) and concluded that the predictive value of molecular genetic variation was limited in relation to quantitative variation. Examination of the relationship between neutral genetic differentiation (F_{st}) and quantitative genetic differentiation (Q_{st}) suggests that there exists a significant correlation ($r = 0.75$) between the two measures of differentiation (Merilä & Crnokrak 2001). Though others have found this relationship to be weak as well ($r = 0.36$) explaining only 14% of the variation (McKay & Latta 2002). Though neutral genetic markers are ideal for the study of gene flow and differentiation, one should be cautious of any strong predictions about adaptive variation and evolutionary potential.

Units of Conservation

Population genetics can inform management decisions and conservation planning by distinguishing populations within a species that have unique evolutionary trajectories due to reproductive isolation, current geographic separation, and locally adapted

phenotypic traits (Conner & Hartl 2004). Such populations should be recognized as evolutionarily significant units (ESU). The development of the idea and term originated at a conference of zoo biologists in regard to the issue regarding conservation of species that had multiple unique subspecies. The term evolutionary significant unit would later become an attempt to articulate what exactly a "distinct population segment" is as mentioned in the definition of "species" in the Endangered Species Act (Groom et al. 2006). A large debate has played out over the last twenty plus years over criteria used to define an ESU (Ryder 1986; Waples 1991; Dizon et al. 1992; Moritz 1994; Vogler & DeSalle 1994; Pennock & Dimmick 1997; Dimmick 1999; Crandall et al. 2000, Frazer & Bernatchez 2001), though the goal to preserve biodiversity at an organizational level below that of the species has remained consistent with each definition. By protecting the diversity at a level below that of the species, we are ultimately protecting the building blocks (genetic variation) of, and the processes of, evolution in natural populations. Though some definitions are stricter than others, several authors recommend the use of genetics as a basis for recognizing an ESU and moreover recognize that in support of genetic data should be ecological data (life history, morphology, and behavior) and that ESU recognition may vary from case to case (Crandall et al. 2000, Moritz 2002).

Study Species

Description

The Reddish Egret, *Egretta rufescens*, is a medium-sized dimorphic heron weighing approximately 700 to 850 g and standing 70 to 80 cm tall with females slightly smaller than males. Each sex can display either the dark or white color morph. The dark morph displays a dark neutral grey body and shaggy cinnamon lanceolate plumes on the

crown, nape, neck and upper breast. The white morph has similar lanceolate plumes on the head and neck but all plumage is white. Both color morphs also have a distinct bi-colored bill with pink at the base and black on the distal half. Lores around the eyes are a distinctive turquoise blue during courtship and pair formation but return to flesh color during breeding. The back and sides of the legs are slate blue but fade to black after nesting.

Range and Taxonomy

The Reddish Egret (*Egretta rufescens*), with it's narrow habitat requirements, has an estimated global population of 5000-7000 adults (Paul 1991; Green 2006). The geographic distribution of *E. rufescens* is restricted by the availability of foraging habitat comprised of broad, saline coastal flats, ideal for its active foraging behavior (Paul 1991). The Reddish Egret is a year round resident along the coastlines (Fig. 1) of Texas, Florida, the Pacific and Gulf Coasts of Mexico, and the Bahamas (Cook 1913; Paul 1991). Three subspecies are recognized: *E. r. rufescens, E. r. dickeyi,* and *E. r. colorata* are suggested to represent populations in Texas/Florida/Caribbean, Baja California, and the Yucatan/Belize respectively though the validity of these forms are not established and they appear to be weakly differentiated morphologically (Lowther & Paul 2002). *E. r. rufescens* represents populations in Laguna Madre of Tamaulipas, Mexico and Texas, as well as the gulf coast states of the U.S. and the Caribbean *E. r. dickeyi* exhibits decidedly darker head and neck plumage than the nominate race (*E. r. rufescens)* ranging from cameo brown to chocolate of Ridgeway's color standards (Lowther & Paul 2002). Blake (1977) suggests that *E. r. colorata* exhibits slightly paler neck and ornamental plumes and a slightly larger body size though it is more likely that these differences

represent seasonal changes rather than geographical variation (Paul 1991). Blake also suggests that *E. r. colorata* possibly averages slightly larger in size than the nominate race (*E. r. rufescens)* occurring on the U.S. Gulf Coast (1977). *E. r. dickeyi* is said to be comprised completely of the dark morph of the bird while the population in the Great Inagua, Bahamas, *E. r. rufescens*, is unique from the others in that it is comprised of primarily (92%) the white morph (Allen 1955). Locations in-between such as Chiapas, Quintana Roo, Texas, and Florida vary in the percentage of white morphs but most tend to have a majority of the dark morph (Lowther & Paul 2002).

Dispersal and Movement

The capacity for dispersal in *E. rufescens* is understood generally from banding data that was collected in Texas over a 60 year period from 1923-1983 (Telfair & Swepston 1987). Most individuals recovered through recapture or resighting were found within Texas or Louisiana though a good portion were recovered in various places including Southern Mexico on the Pacific side, Yucatan, Eastern Mexico on the Gulf side, and Central America (Telfair & Swepston 1987). No individuals were ever seen or captured in Baja California, Florida, or the Caribbean. In Texas, post-breeding Reddish Egrets display a slight northward movement June through September and then a subsequent southward movement possibly as far as Central America if they move at all, based on banding data (Paul 1991). Nearly all of the banded individuals recovered were juveniles leaving questions about adult dispersal and whether juveniles return to natal area or remain in locations they disperse to. The degree of gene flow among separated populations of Reddish Egret globally appears to be limited but quantification of gene

flow rates may be important for conservation efforts as populations with low diversity and no immigration may warrant special protection.

History

Current populations in Texas and Florida are considered "threatened" and a "species of special concern," respectively, due to near extinction during plume hunting at the turn of the 20th century and current threats such as habitat loss (Lowther & Paul 2002). Threatened species are those species which the Texas Parks and Wildlife Commission has determined are likely to become endangered in the future (TPWD). The plume trade nearly extirpated Reddish Egrets from Florida as no Reddish Egrets were reported in Florida until 1937 (Powell et al. 1989). The population in Texas made an apparent recovery with estimates of 2000 pairs on Green Island in 1921 (Pemberton 1922) and 3206 pairs found in all of Texas on a survey in 1939 (Paul 1991); but numbers have since declined with current estimates at 900 to 950 breeding pairs (Green 2006). Florida, where Reddish Egrets were once considered common or abundant in many areas (Audubon 1843; Gambel 1848; Maynard 1881; Scott 1887) has seemingly not recovered with current estimates at 250 to 300 breeding pairs (Green 2006). Such dramatic declines in population size can be cause for concern, as the decline may lead to a loss of genetic diversity associated with a population bottleneck and potential future inbreeding. Estimates for the entire Baja California region of western Mexico are approximately 500 pairs while estimates for Great Inagua, Bahamas, which is likely the stronghold for a relatively unknown Caribbean population, is approximately only 200 breeding pairs but likely less than 100 breeding pairs based on recent surveys (Green 2006; Green & Hill unpubl. data).

Plumage Dimorphism

While assessments of differentiation within species are usually reserved for distinct populations and/or sub-populations, the unique dimorphic plumage of *Egretta rufescens* warrants an examination of the genetic differentiation between the two color morphs. The Great White Heron, originally described as a distinct species, is now considered to be a white morph of a polymorphic Great Blue Heron subspecies, *Ardea herodias occidentalis*. In the Florida Keys, where the ranges of the white and dark morph of subspecies *A. h. occidentalis* overlap, McGuire (2001) found that pairs more often mated assortatively though the few mixed pairs that did occur were enough to prevent differentiation between populations of the white and blue morph of the subspecies (*A. h. occidentalis)* in Florida Bay. Kondrashov (1998) suggested that assortative mating may divide a population even in the absence of natural selection but notes that there are strict modes by which sympatric speciation can occur in the absence of selection. Both color morphs of the Reddish Egrets are attracted differentially to their own plumage coloration during flock formation with all individuals of both morphs landing at like-plumaged decoys (Green & Leberg 2005). Although, the courting of the opposite color morph has been observed (Audubon 1843; Pemberton 1922). The hypothesis that color polymorphisms are linked to alternative strategies is supported by the finding that a majority of known cases of species polymorphisms are associated with reproductive parameters and behavioral, life-history, and physiological traits, which can all have a genetic basis (Roulin 2004). The exploitation of alternative habitat with respect to coloration and disruptive selection may maintain polymorphism in the population (Roulin 2004; Galeotti et al. 2003). In *Egretta rufescens,* it has been shown that color morphs

may alter foraging tactics based on their degree of crypsis to prey (Green 2005). Species specific information with regard to color dimorphism of Reddish Egret suggests that the two morphs may vary in more than just color and that a genetic assessment of the differentiation between morphs is justified.

Habitat

Egretta rufescens is a coastal wading bird that occurs predominantly in the Southeast US (mainly Texas and Florida) as well as on the Pacific and Gulf Coasts of Mexico and the Bahamas. They are also known to be present on a few Caribbean islands and parts of Central and South America but little data exists as to the evidence of breeding (Green 2006). Breeding Reddish Egrets were recently documented in Cuba (Denis et al. 1999) and have also been confirmed in Bonaire, Lesser Antilles (Voous 1983; Paul 1991), and Belize (Scott & Carbonell 1986). Foraging habitat is considered to dictate the highly coastal nature of the Reddish Egret (Paul 1991). Habitat used for foraging typically consists of shallow, barren coastal flats. In Texas, foraging spots include wind tidal flats and alluvial overwash zones of barrier islands while in Florida they may forage at open banks and ponds inside mangrove keys, intertidal flats along the Florida peninsula, "cat-eye ponds", and occasionally open beaches and reefs (Voous 1983; Paul 1991; Lowther & Paul 2002,). Based on nestling regurgitations in Laguna Madre, TX, the nestling diet was made up of sheepshead minnow (*Cyprinodon variegates*; 83%), longnose killifish (*Fundulus similis*; 9%), pinfish (*Lagodon rhomboids*; 5%); striped mullet (*Mugil cephalus*) and lady fish (*Elops saurus*) also (McMurry 1971; Simersky 1971; Holderby & Green unpubl. data 2009). The diet of nestlings at Aransas Bay, TX consisted of sheepshead minnow (*Cyprinodon variegates*; 69.6%), mullet

(*Mugil cephalus*, *M. curema*; 10.6%), pinfish (*Lagodon rhomboids*; 5.4%), longnose killifish (*Fundulus similis*; 4.3%), and tidewater silverside (*Menidia peninsulae*; 3.3%) as well as 11 other fish species at lower frequencies, 1 shrimp (*Penaeus aztecus*), and 1 crab. Regurgitated pellets collected from nestlings in Florida Bay, Fl consisted of sheepshead minnow (*Cyprinodon variegates*; 53.6%), sailfin molly (*Poecilia latipinna*; 13.5%),goldspotted killifish (*Floridichthys carpio*; 10.6%), marsh killifish (*Fundulus confluentus*; 8.3%) as well as 28 other fish species at lower frequencies, 1 shrimp (*Penaeus duorarum*), and 2 insects (Paul 1991).

Nesting and Reproduction

In Florida, the Reddish Egret typically nests in red mangrove (Rhizophora *mangle*), black mangrove (*Avicennia germinans*), and Brazilian pepper (*Schinus terebinthifolius*) located on mangrove keys and dredge material islands. In Texas, nests occur on natural or artificial dredge islands in a variety of low vegetation including bisbirinda *(Castela erecta texana)*, Spanish Dagger (*Yucca treculeana*), prickly pear cactus (*Opuntia engelmannii*), sea oxeye (*Borrichia frutescens*), sea purslane (*Sesuvium portulacastrum*), goldenrod (*Solidago sp)*, ragweed (*Ambrosia sp.)*, or even on bare sand (Lowther & Paul 2002).

Nesting attempts may be initiated in almost any month in south Florida but most breeding occurs during 2 primary pulses: November to February and February to May. Among colonies along the Florida peninsula, breeding occurs primarily from February to June. In Texas, nesting primarily occurs between March and June. Though not well studied, it has been speculated from observation that most nesting in the Bahamas occurs probably between November and May (Spaans 1974; Voous 1983; Green & Hill unpubl.

data). Clutch size is usually three to four eggs, averaging 3.12 in Texas (McMurry 1971) and 2.75 in Florida (Paul 1996).

The goal of this research is to gain insight into the amount of genetic diversity and the degree of dispersal and differentiation among global populations of the Reddish Egret. This population genetic study will also examine recent historical population declines and subspecies designations. Understanding the mechanisms that contribute to the genetic structure of the species can inform future decisions concerning the recovery and success of this vulnerable waterbird.

Specific Objectives

1. Identify the degree of population differentiation among global population of Reddish Egrets.
2. Identify the extent of gene flow among populations of Reddish Egret.
3. Determine occurrence (if any) of historical bottlenecks range wide.
4. Clarify the subspecies status of *E.r. dickeyi*.
5. Assess genetic differentiation between the dark and white morph of the Reddish Egret.

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CHAPTER II

POPULATION GENETIC STRUCTURE OF THE REDDISH EGRET

Introduction

The World Conservation Union (IUCN) has recommended as a goal, the conservation of genetic diversity as one of three components for the maintenance of biodiversity (McNeely 1990). Genetic variation provides the raw material for evolution in the face of environmental change. Anthropogenic influences may require organisms to respond much quicker to a more rapidly changing environment, and without necessary genetic variation species with small populations may face extinction risk exacerbated by the population genetics of small population size (Groom et al. 2006). In addition to demographic and environmental uncertainties populations of threatened or endangered organisms are subject to genetic consequences as well as a result of their small population size (Mills 2007). Genetic drift in small populations is powerful in its ability to remove variation from the gene pool (Rich et al. 1979). Another genetic threat to small populations comes in the form of decreased fitness due to inbreeding depression (Hedrick & Kalinowski 2000). In an already small population, a decline in fitness can lead to extinction (Saccheri et al. 1998). The extreme decrease in population size or a population bottleneck also has the ability to reduce variation by completely removing alleles (due to low frequency) from the population or significantly changing allele frequencies of the population Once the population is small it becomes susceptible to the detrimental effects of inbreeding and genetic drift. A rapid decline in the number of

individuals can lead to a decline in genetic diversity represented by number of alleles per locus and heterozygosity but this loss in genetic variation can be mitigated by a quick population recovery (Nei 1975; Allendorf 1986; Coates 1992). If a population fails to rebound and remains small after a decline, it becomes more vulnerable to the forces of genetic drift and inbreeding (Ellstrand & Elam 1993; Allendorf, 1986; Frankham 1995).

Studies of population structure evaluate how genetic variation is partitioned across a species' range and the ecological factors that contribute to genetic structure. Often a species does not occur as a single panmictic population, but rather as several subdivided populations with each experiencing the heterogeneities of the various geographic locations. Population genetics can inform management decisions and conservation planning through the ability to distinguish populations within a species that have unique evolutionary trajectories. Geographic separation of populations when accompanied by a lack of immigration by individuals or gene flow to other populations can lead to population differentiation, the first stage of allopatric speciation (Coyne & Orr 2004). Physical barriers such as geographical features of the landscape can prevent movement of individuals between populations (Hayes & Sewlal 2004) and lead to differentiation (Caizergues 2003). The vagility of organisms like birds allows them to overcome many of the physical barriers compared to other verterbrates (Avise 1996; Crochet 2000). In the absence of geographic barriers, distance between populations in a species that occupies an area greater than the dispersal distance tendencies of the organism, may result in isolation of populations (Wright 1943; Slatkin 1993). Areas of unsuitable habitat that create significant distance between isolated populations can lead to differentiation (Duffie et al. 2009). Behavioral barriers to dispersal such as philopatry

can be sufficient to prevent gene flow and promote genetic differentiation in birds (Avise 1996). Philopatry is present in many island nesting seabirds with individuals breeding in natal areas as opposed to dispersing (Frederickson & Peterson 1999; Friesen et al. 2007).

The Reddish Egret (*Egretta rufescens*), with it's narrow habitat requirements and limited distribution, has an estimated global population of 5000-7000 adults (Paul 1991; Green 2006). *E. rufescens* is a plumage dimorphic coastal wading bird that is a year round resident predominantly in the Southeast US (mainly Texas and Florida) as well as on the Pacific and Gulf Coasts of Mexico and the Bahamas (Cook 1913; Paul 1991). Color morphs consist of dark, slate colored individuals and all white individuals common to both sexes.

Three subspecies are recognized: *E. r. rufescens* is the nominate race representing populations in Laguna Tamaulipas, Mexico, Bahamas and the gulf coast states of the U.S while *E. r. dickeyi* and *E. r. colorata* are suggested to represent populations in Baja California region and the Yucatan respectively though the validity of these forms are not established and they appear to be weakly differentiated morphologically (Lowther & Paul 2002). The largest concentration of *E. r. rufescens* is in Texas having an estimated 900 - 950 breeding pairs. Reddish Egrets in this region consist of both color morphs. *E. r. dickeyi* is comprised completely of the dark morph of the bird while the population in the Great Inagua, Bahamas is unique from the others in that it is comprised of primarily (92%) the white morph (Allen 1955). Blake (1977) suggests that *E. r. colorata* exhibits slightly paler neck and ornamental plumes and a slightly larger body size though it is more likely that these differences represent seasonal changes rather than geographical variation (Paul 1991).

Current populations in Texas and Florida are considered "threatened" and a "species of special concern" respectively due to near extinction during the plume trade at the turn of the 20th century and current threats such as habitat loss (Lowther & Paul 2002). The plume trade potentially extirpated Reddish Egrets from Florida no Reddish Egrets were observed until 1937 (Powell et al. 1989). The population in Texas made an apparent recovery from hunting exploits but is currently below historic highs due to recent anthropogenic impacts (Pemberton 1922; Paul 1991; Green 2006). Florida which had many areas where Reddish Egrets were once considered common or abundant (Audubon 1843; Gambel 1848; Maynard 1881; Scott 1887) has seemingly not recovered with current estimates at 250 to 300 breeding pairs (Green 2006).

The unique dimorphic plumage of *Egretta rufescens* also warrants an examination of the genetic differentiation between the two color morphs. Kondrashov (1998) suggests that assortative mating may divide a population even in the absence of natural selection. Both color morphs of the Reddish Egrets are attracted differentially to their own plumage coloration during flock formation with all individuals of both morphs landing at like-plumaged decoys (Green & Leberg 2005). Although, the courting of the opposite color morph has been observed (Audubon 1843; Pemberton 1922). In *Egretta rufescens,* it has also been shown that color morphs may differ in foraging behaviors based on their degree of crypsis to prey (Green 2005). The hypothesis that color polymorphisms are linked to alternative strategies is supported by the finding that a majority of known cases of species polymorphisms are associated with reproductive parameters and behavioral, life-history, and physiological traits, which can all have a genetic basis (Roulin 2004). The two

morphs may vary in more than just color suggesting that a genetic assessment of the differentiation between morphs is justified.

In this study I used 9 polymorphic microsatellite markers to assess the genetic diversity and the genetic structure of reddish egret populations throughout most of its range. Disjunct populations, subspecies designations, varying color morph frequencies and the residential nature suggests differentiation across the range. I predict that differentiation will be present between *E. r. rufescens* and *E. r. dickeyi* due to distance and dispersal tendencies. Within *E. r. rufescens* distance will be a barrier to gene flow between Texas and Bahamas populations. I am also expecting no differentiation between dark and white morph individuals within the same population. Historic impacts of plume trade will show evidence of genetic bottleneck in Texas.

Materials and Methods

Field Sampling

We collected 8-37 genetic samples from each of 9 breeding colonies in April – July 2006, March – July 2007, March – July 2008, January 2009, and March 2009 for a total of 223 individuals. Sampling locations (Fig. 2) spanned from Baja Sur, Mexico, the Texas/Mexico Gulf coast, and the Bahamas (Table 2). Blood was obtained from the brachial vein of nestlings or fledglings during the breeding season using a 25 gauge needle. We drew approximately 4 µl of blood from the bird into a capillary tube and then deposited the blood into a vial containing 600 µl of cell lysis solution (Puregene, Gentra Systems). Blood was typically collected from only 1 individual per nest to reduce the possibility of similar genotypes from siblings being incorporated in the analysis. If blood

was collected from more than one individual from a nest, then an individual from that nest was chosen randomly for inclusion in the analysis.

Laboratory Methods

We performed DNA extraction using Puregene DNA isolation protocol for avian whole blood. We screened 13 microsatellite loci primers and found 12 that could be successfully amplified in all 223 samples: Er21, Er22, Er41, Er42, Er43, Er51, Er23, Er44, Er31, Er45, Er24, Er46 using methods as follows. We carried out PCR reactions using a BIO-RAD PTC-100 thermocylcer in a volume of 20 µl under a standard protocol: initial denaturation at 94 ^{o}C for 3 min, 35 cycles of 94 ^{o}C for 40 s, primer specific annealing temperature for 40 s, 72 ^{o}C for 30 s, and a final extension at 72 ^{o}C for 4 min. WellRed dye (Sigma Proligo) labeled PCR products for all loci ran through a Beckman Coulter CEQ 8800 DNA sequencer for microsatellite detection and scoring using a CEQ DNA 600 size standard.

Statistical Analysis

We assessed standard measures of genetic variation for 9 colonies at 9 loci including gene diversity (Nei 1973 and 1987), number of alleles, allelic richness, and observed heterozygosity. Nei's gene diversity (H_e), allelic richness, and number of alleles were calculated using FSTAT (Goudet 1995; 2001) with allelic richness being adjusted for sample size due to the variability of samples collected from each population (El Mousadik & Petit 1996). ARLEQUIN version 3.1 (Excoffier & Schneider 2005) was used to test for deviations from Hardy-Weinberg expectations (Guo & Thompson 1992) and linkage disequilibrium. In populations where a deficiency in heterozygosity was present, we used the program MICROCHECKER to determine whether deviations from

Hardy Weinberg expectations were due to the presence of null alleles (Oosterhout et al. 2004). We measured population differentiation using the Raymond and Rousset (1995a) exact text performed in GENEPOP (Raymond & Rousset 1995b).

I assessed the genetic structure across the entire range of the Reddish Egret by calculating pairwise F_{ST} and R_{ST} for each pair of sampled colonies using ARLEQUIN (Excoffier & Schneider 2005). F_{ST} and R_{ST} are measures of interpopulation differentiation with F_{ST} being calculated using allele frequencies (Weir & Cockerham 1984) without regard to a mutational model and R_{ST} being calculated using the sum of squared number repeat differences following the stepwise mutational model (Slatkin 1995). 16,000 permutations were used to test the significance of covariance components and fixation indices. I performed a hierarchical analysis of genetic structure in ARLEQUIN (Excoffier & Schneider 2005) using AMOVA to test against the null hypothesis that variation is partitioned according to subspecies designations. AMOVAs examined colonies were placed into 2 groups to fit subspecies divisions and 3 groups to based on distance between regions.

Program STRUCTURE 2.3.1, which uses a Bayesian clustering method to assign individuals to the appropriate population, was used to assess the most probable number of groups or populations without bias from existing subspecies designations or geographical distribution (Pritchard 2000). We tested k values (1- 9) 5 times each, using a burn-in time of 10,000 proceeded by 50,000 iterations to attain a mean value of the likelihood of each k. The most likely number of populations, k, we assessed by observing Δk, an *ad hoc* statistic associated to the degree of change in mean log probability between subsequent values of K (Evanno 2005). The program was executed using the no

admixture model and the independent allele frequencies option, which are most appropriate for discrete populations with allele frequencies expected to be reasonably distinct (Pritchard et al. 2009).

To test the hypothesis of no differentiation between color morphs, pairwise Fst values were calculated between red morphs in Baja California, red morphs in Texas/Mexico Gulf, white morphs in Texas/Mexico Gulf, and white morphs in Great Inagua, Bahamas. Program STRUCTURE is also used to identify true populations and how color morph corresponds to predicted k.

Using the Isolation by distance (IBD) web service (Bohonak 2002; Jensen et al. 2005), we examined the relationship between genetic distance and geographic distance (Slatkin 1993) to see if there exists a correlation between the two as expected if genetic differentiation among colonies was structured according to an isolation by distance model. The presence of a positive relationship can indicate a lack of gene flow between distant populations and/or the low dispersal tendencies of a species. The program IBD uses Reduced Major Axis (RMA) regression to estimate the slope and intercept of the relationship in addition to a Mantel test (Mantel 1967) to determine the significance of isolation by distance relationship. We analyzed the relationship between genetic distance, using measures of pairwise F_{ST} values, and geographic distance, using pairwise straight line Euclidean distances, both untransformed and log transformed, with significance being assessed using 20,000 randomizations.

To test the hypothesis of recent population bottlenecks due to the plume trade impacts early in the 20th century, we used program BOTTLENECK 1.2 to determine whether populations had experienced a recent population reduction (Cornuet & Luikart

1996; Piry et al. 1999). We used both the Stepwise Mutation Model (SMM) and the Two Phase Model (TPM) to perform the Wilcoxon 1-tailed test which is most powerful when using less than 20 loci (Piry et al. 1999). The SMM is recommended for testing microsatellite data but the TPM may be more appropriate (Luikart & Cornuet, 1998; DiRienzo et al. 1994). As recommended by Piry et al. (1999), when using the Two Phase Model (TPM) we incorporated 95% single step mutations into the TPM with a variance of 12 and performing 16000 iterations.

Results

Hardy-Weinberg, Linkage Disequilibrium and Genetic Diversity

Twenty-one of 108 loci-colony combinations showed significant departures from Hardy-Weinberg expectations before Bonferroni corrections for multiple comparisons. Three of these combinations were associated with significant excess heterozygosity. After correction, 9 of 108 loci-colony combinations exhibited a departure from HWE ($P < 0.00046$, adjusted critical value). No single locus deviated from HW expectations in all 9 populations. Twenty-four of 594 loci-pair-colony combinations and 1 of 66 loci-pair combinations across all populations showed linkage disequilibrium before correction for multiple comparisons. Upon Bonferroni correction, no significant linkage between loci occurred within colonies or across all colonies ($P < 0.00076$, adjusted critical value). Using MICROCHECKER, 14 of 108 loci-colony pairings analyzed exhibited signs of the possible presence of null alleles with 12 of the possible nulls within three loci. To be conservative in our analyses we removed loci Er41, Er42, and Er24 which contained most of the possible nulls. All further analyses used 9 loci: Er21, Er22, Er43, Er51, Er23, Er44, Er31, Er45, and Er46.

The number of alleles per locus ranged from 2 to 12 and allelic richness and gene diversity were significantly different across all colonies ($\chi^2 = 35.24$, $p < 0.001$, Table 2; $\chi^2 = 29.79$, $p < 0.001$, Table 3). Observed heterozygosity differed significantly across all colonies ($\chi^2 = 22.71$, $p < 0.004$) with the mean ranging from 0.38 to 0.51 while expected heterozygosity was also significantly different among populations ($\chi^2 = 29.81$, $p < 0.001$) with the mean ranging from 0.41 to 0.61 (Table 4).

Population Structure

Exact tests for population differentiation found significant differences in allele frequencies for 20 of 36 pairwise comparisons. Estimates of F_{ST} and R_{ST} revealed significant differentiation in 20 out of 36 pairwise population comparisons. F_{ST} values ranged from -0.009 to 0.404 and R_{ST} estimates ranged from -0.012 to 0.694 (Table 5). Global F_{ST} value estimated over all populations and loci was 0.155 ($p < 0.0005$) while our global R_{ST} estimates were 0.199 ($p < 0.0005$). Comparisons of sampling colonies within Texas/Mexico Gulf populations showed no signs of significant differentiation. Baja California and Great Inagua, Bahamas populations exhibited large significant differentiation from Texas/Mexico Gulf populations as well as between each other. Results of the AMOVA suggest that differentiation is greatest among regions and not between populations within regions (Table 9, 10)

Bayesian clustering analysis, as performed by program STRUCTURE (Pritchard 2000), gave the strongest support to a structure that recognized 3 distinct genetic units (Fig. 3, 4). Approximation of ΔK (Evanno et al. 2005), identified a peak value at $K = 3$ (Fig. 5). Populations on Isla Piedra and Isla Concha in Baja California, Mexico formed a

single cluster and all Texas/Mexico Gulf populations formed a second cluster, while the population in Great Inagua, Bahamas formed the last unique cluster.

No structure was identified between dark morphs and white morphs within Texas as little to no differentiation was observed (F_{ST} = 0.002, Fig 6) and between color morphs globally differentiation aligned with geographic population (Fig. 6). This is also supported by previous results in structure which did not support clusters K = 4 – 9.

Isolation by distance and Bottleneck

Genetic distance was significantly positively correlated with both log transformed (r^2 = 0.792, p = 0.002; Fig. 6) and untransformed geographic distance (r^2 = 0.919, p = 0.002; Fig.7) indicating the genetic distance between distant regions was less similar and genetic distance between colonies close together within a region were more similar. Using program BOTTLENECK, we detected no evidence for a recent population reduction in any of the populations (p > 0.05).

Discussion

The results of this study suggest that genetic structure is most pronounced at the regional level and not between islands within regions. F_{ST} and R_{ST} values from Baja California, Mexico and Great Inagua, Bahamas were large and significant when compared with each other and/or Texas/Mexico Gulf populations and Mantel tests identified geographic distance as a dispersal barrier. Results from program STRUCTURE also suggest the existence of 3 major regions of distinct genetic units. A previous study using mtDNA to look at population structure of Reddish Egrets along the Texas coast found no structuring among 16 colonies in conjunction with the results we found in Texas using microsatellites (Bates et al. 2009).

Despite the vagility of the Reddish Egret there seems to be some degree of at least regional philopatry. Due to the unique habitat, specifically foraging habitat that Reddish Egrets prefer, there is presumably not a continuous line of suitable coastal habitat. This leads to many disjunct colonies that are most often associated with coastal lagoons or otherwise open, calm, shallow saltwater habitats and may play a large role in the resulting population structure. The Reddish Egret is considered a "weakly" migratory species based on adult birds remaining in and around breeding areas throughout the year; some minimal northward movement occurs after breeding in both Texas and Baja (Cooke 1913; Lowther & Paul 2002; Green, unpubl banding data). Long distance dispersal appears to be rare and present mainly in juveniles (Bent 1926; Telfair & Swepston 1987; Paul 1991). The genetic evidence from pairwise Fst and AMOVA suggest that a regional philopatric behavior exists and little migration takes place among regions. A large debate has played out over the last twenty years over the criteria used to define an ESU (Ryder 1986; Waples 1991; Dizon et al. 1992; Moritz 1994; Vogler & DeSalle 1994, Pennock & Dimmick 1997; Dimmick 1999; Crandall et al. 2000; Frazer & Bernatchez 2001), though the goal to preserve biodiversity at an organizational level below that of the species has remained consistent with each definition. Though some definitions are stricter than others, several authors recommend the use of genetics as a basis for recognizing an ESU and moreover recognize that in support of genetic data should be ecological data (life history, morphology, and behavior) and that ESU recognition may vary from case to case (Crandall et al. 2000; Moritz 2002). These distinct regions should be treated as 3 ESU's due to the high degree of differentiation between them and the geographical isolation that

prevents gene flow among them though it will be interesting to find out how colonies in Florida compare.

The history of plume hunting with regards to the Reddish Egret is best known to have occurred in Florida and Texas with both populations being affected significantly. The Florida population was nearly extirpated and the population in Texas has since recovered, but still remains below the highest estimates in the historical records (Paul 1991). Little to nothing is known of historical populations in places other than Texas and Florida including Baja California, Mexico and Great Inagua, Bahamas. A population that experiences a dramatic decline in size can undergo a loss of genetic variation (Nei 1975) as has occurred in the greater prairie chicken (Bouzat et al. 1998; Bellinger et al. 2003). Program BOTTLENECK failed to detect evidence of any recent genetic bottleneck in any of the populations in this study. Bates et al. (2009) did not detect reduced genetic diversity in Reddish Egrets from Texas but did find signals of a population expansion indicating a possible recovery from a previous low. Initial impacts of a genetic bottleneck include the loss of unique alleles, but a population can preserve genetic diversity if recovery occurs quickly (Allendorf 1986; Coates 1992). If a population fails to rebound and remains small after a decline, it becomes more vulnerable to the forces of genetic drift and inbreeding (Allendorf 1986; Ellstrand and Elam 1993; Frankham 1995). These results suggest the population in Texas recovered quickly enough to mitigate the impacts of a severe population reduction though current anthropogenic impacts may be limiting them from reaching historic population levels.

Differentiation between the two color morphs was non existent within the Texas/Mexico gulf region and differentiation that was present was due to regional factors

of isolation. Dark and white individuals within Texas/Mexico are more similar to each other than they are to like colored individuals of the other regions. Although many of the mating events between Reddish Egrets appear to be assortative based on color morph, courtship between opposite color morphs has been observed and extra pair copulations are not uncommon for other herons and egrets (Gladstone 1979; Ramo 1993). Gene flow between color morphs through mixed morph mating events though not common occurs with enough frequency to prevent differentiation between the morphs (Wright 1930; Mills & Allendorf 1996). It is very likely that enough mixed morph mating events take place whether as typical pair bond or as an extra pair copulation event to prevent any differentiation from occurring between color morphs within the same population (Hill Thesis CH IV). As it is the case that a pair of dark morphs may have white offspring (Green & Hill unpubl. data), the potential for imprinting upon the offspring and attraction to like individuals may also be a factor promoting mating between different morphs (Immelman 1972; Slagsvold et al. 2002).

Our results indicate 3 evolutionarily distinct population units that support the subspecies *E.r. dickeyi* and propose a new unique population within *E. r. rufescens* occurring in Great Inagua, Bahamas. The lack of suitable habitat and philopatric tendencies has created geographic isolation between populations of a plumage dimorphic waterbird that is a species of concern. Small populations sizes in each of the regions puts the Reddish Egret at risk to stochastic demographic and environmental events in the short term and loss of genetic variation in the long term. I advocate the preservation of all Reddish Egret breeding colonies and foraging habitat available. The Reddish Egret is the rarest and least studied heron in North America and this research reveals the importance

of focusing conservation efforts on regional populations and the protection of any and all breeding sites for Reddish Egrets.

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CHAPTER III

TWELVE POLYMORPHIC MICROSATELLITES FOR THE REDDISH EGRET

The Reddish Egret (*Egretta rufescens*), with it's narrow habitat requirements and limited distribution, has an estimated global population of 5000-7000 adults (Paul 1991; Green 2006). It remains the rarest heron in North America, with populations still recovering from the plume trade that nearly extirpated the species from Florida and Texas in the early 20th century. As a coastal species, it is also subjected to the many impacts associated with anthropogenic influence including habitat alteration, commercial ventures, recreational use, and pollution among others. The plumage dimorphic egret has disjunct populations from Baja California, Mexico to Great Inagua Bahamas with dispersal, structure, and genetic diversity within and among the populations mostly unknown. Previous work with mtDNA for Reddish Egrets has shown no evidence for structure among colonies on the Texas coast (Bates et al. 2009). Highly polymorphic neutral markers like microsatellites are highly useful for answering ecological and population biology related questions (Selkoe & Toonan 2006).

Genomic DNA was obtained by drawing 4 µl of blood from the brachial vein in the wing of nestling egrets; all nestlings were returned to the nest immediately after sample collection. We performed DNA extraction using Puregene (Qiagen) DNA isolation protocol for avian whole blood. Approximately 100 µg samples from 13 individuals from different breeding colonies were sent to Genetic Identification Services (GIS, Chatsworth, CA, www.genetic-id-services.com) for development of 8

microsatellite enriched libraries of motifs CA, AAC, ATG, CATC, GA, AAG, TACA and TAGA. Microsatellite containing clones were sequenced using the ET Terminator Cycle Sequencing Kit (Amersham Biosciences) on an Applied BioSystems 377 DNA Sequencer. We then designed 78 PCR primers for the flanking regions for appropriate microsatellite sequences using DesignerPCR, version 1.03 (Research Genetics, Inc.). PCR amplifications were carried out in total volume of 10 µl containing 1 µl of 10X reaction buffer, 2 mM MgCl2, 0.2mM of each DNTP, 0.3 µM of forward and reverse primers, 0.25 U taq polymerase, and approx 0.2 ng/µl DNA template under the following PCR conditions: 3 min at 94 °C; followed by 35 cycles of 40 sec at 94 °C, 40 sec at 55 °C to 57 °C, 30 sec at 72 °C: followed by a final extension of 4 min at 72 °C; then storage at 4 °C. From the 78 primers developed, 13 primers were successfully amplified in 13 samples.

The 13 primer pairs were then optimized for PCR conditions using 8 individuals taken from different breeding colonies. One set of primers was excluded because it failed to amplify consistently across samples. The twelve remaining primers had the 5' end of the forward primer fluorescently labeled with WellRED dye (Sigma-Proligo) for allele scoring carried out on a Beckman Coulter CEQ 8800. Arlequin 3.0 (Excoffier 2005) was used to assess the deviation from Hardy-Weinberg and linkage disequilibrium in 31 individual nestlings from different nest on a single breeding colony located in the Laguna Madre in Texas (East Flat Spoils Island). We used the program MICROCHECKER to determine weather deviations from Hardy Weinberg expectations were due to the presence of null alleles (Oosterhout et al. 2004).

The number of alleles per locus varied from 2 to 10 with a mean of 4.33 (Table 5). A deficiency in observed heterozygosity was found in 4 of the 12 primers (Er41, Er42, Er23, Er24) before bonferroni correction but in only 2 after correction (Er41, Er23). Observed heterozygosity ranged from 0.03 to 0.90 while expected heterozygosity ranged from 0.03 to 0.88 (Table 7). MICROCHECKER (Oosterhout et al. 2004) detected the possibility of null alleles at only one locus (Er41). No linkage was found between pairs of loci after bonferroni correction. The microsatellite primers described here are expected to be useful in studies concerning population structure and gene flow in addition to unknown aspects of Reddish Egret ecology.

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CHAPTER IV

MULTIPLE PATERNITY AND OFFSPRING SEX RATIO IN REDDISH EGRETS

Introduction

The availability of molecular tools to study the ecology and population biology has greatly advanced our ability to answer difficult questions concerning a species' biology. In addition to the most common uses of molecular tools to assess structure and genetic diversity among populations, they have also shown to be useful in assessing relatedness and paternity of individuals (Queller & Goodnight 1989; Griffith et al. 2002). Extra pair copulation (EPC) occurs when an individual already having formed a pair bond with a mate, copulates with another individual other than its breeding mate (Westneat et al. 1990). EPC can lead to clutches of offspring with multiple sires. Multiple paternity occurs when two males each successfully mate with the same female and fertilize eggs producing a single brood with more than one sire. Multiple paternity has been observed in many different taxa (Avise 1994) though for many years it was thought that most bird species were monogamous (Lack 1968). Behavioral observations demonstrating the occurrence of EPCs and the ability to genotype individuals with molecular tools has provided evidence of multiple paternity within avian clutches (Gladstone 1979; Westneat 1990; Griffith et al. 2002). Møller and Birkhead (1993) suggest that the degree of sociality is tightly linked with the frequency of extra-pair paternity due to the density of nests (but see Westneat & Sherman 1997). Many colonial

nesting birds such as those in the order Ciconiiformes have exhibited extra-pair copulation and multiple paternity (Gladstone 1979; McGillikan 1983; Fredrick 1987; Ramo 1993; Krebs et al. 2004; Wei et al. 2005; Minõ et al 2009). One important aspect of multiple paternity lies in the fact that this phenomenon can create a greater effective population size (Suggs & Chesser 1994; Pearse & Anderson 2009) which is of special concern with regard to small and/or declining populations.

Genetic tools can also be used for sexing organisms whose sex may be difficult to determine visually or without invasive techniques. The sex of an organism is important for ecological, conservation, and breeding studies. Many adult bird species show no significant sexual dimorphism between males and females. Sexing individuals with molecular techniques can provide a quick and accurate method for determining an individual's sex (Lessels & Mateman 1998; Griffiths et al. 1998; Fridolfsson & Ellegren 1999). The implications of an altered offspring sex ratio differing from a 1:1 ratio can be suggestive of the female condition in relation to habitat quality or male fitness (Svennson & Nilsson 1996; Nager et al. 1999; Kolliker et al. 1999).

The Reddish Egret (*Egretta rufescens*), with it's narrow habitat requirements and limited distribution, has an estimated global population of 5000-7000 adults (Paul 1991; Green 2006). It remains the rarest heron in North America, with populations still recovering from the plume trade that nearly extirpated the species from Florida and Texas in the early 20th century; the species is considered "threatened" and a "species of special concern" in Texas and Florida respectively (Lowther & Paul 2002). As a coastal species it is also subjected to the many impacts associated with anthropogenic influence, including habitat alteration, disturbance from commercial and recreational use, and

pollution among others. Several gaps in the knowledge of the ecology of this species exist in part due to its rarity. Multiple paternity and offspring sex ratio are two areas where information is lacking that could contribute to the conservation and management of this species. Reddish Egrets are thought to be monogamous but several other birds in the family Ardeidae have shown to engage in extra pair copulations (Ramo 1993; Krebs et al. 2004; Wei et al. 2005). In this study we used 9 polymorphic microsatellite loci to examine multiple paternity of reddish egret nest mates as well as determine the offspring sex ratios.

Materials and Methods

Field Sampling

We collected genetic samples from 212 individual nestlings in 8 breeding colonies in April – July 2006, March – July 2007, March – July 2008. Sampling locations spanned the Texas/Mexico Gulf coast in the Laguna Madre of Texas and Tamaulipas, Mexico (Table 8). Blood was obtained from the brachial vein of nestlings or fledglings during the breeding season using a 25 gauge needle to puncture the vein. We drew approximately 4 µl of blood from the bird using a capillary tube and then plunged into a vial containing 600 µl of cell lysis solution (Puregene, Gentra Systems). In 38 nests across the 8 colonies, we collected blood samples from the entire clutch to assess paternity.

Laboratory Methods

We performed DNA extraction using Puregene (Qiagen) DNA isolation protocol for avian whole blood. We screened 13 microsatellite loci primers and found 12 that could be successfully amplified. Of those 12, 3 were removed for the possible presence

of null alleles which can easily impact paternity analysis (Jones 2005). We carried out PCR reactions using a BIO-RAD PTC-100 thermocylcer in a volume of 20 µl under a standard protocol: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 40 s, 56 °C (+/- 2 °C) for 40 s, 72 °C for 30 s, and a final extension at 72 °C for 4 min. WellRed dye labeled (Sigma Genosys) PCR products for all loci were ran through a Beckman Coulter CEQ 8800 DNA sequencer for microsatellite detection and scoring using a CEQ DNA 600 size standard.

Individuals were sexed using primers 2250F and 2718R as proposed by Fridolfsson & Ellegren (1999). PCR reactions followed the methods of Fridolfsson & Ellegren (1999) but quantities were adjusted to 50 µl volumes to accommodate the BIO-RAD PTC-100 thermocylcer. The thermal sequence of the thermocycler was modified from the Fridolfsson & Ellegren (1999) method by removing the touchdown sequence with the protocol as follows: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Products were then run on a 2% agarose gel for manual scoring. As females in birds are the heterogametic sex, females will have one more additional band than males.

Statistical Analysis

Arlequin version 3.1 (Excoffier & Schneider 2005) was used to examine deviations from Hardy-Weinberg expectations (Guo & Thompson 1992) and linkage disequilibrium. Laguna Vista Spoils was grouped with Green Island and Site 5 was grouped with Rabbit Island for Hardy-Weinberg analysis to increase the sample size and because of close spatial proximity. In populations where a deficiency in heterozygosity was present, we used the program MICROCHECKER to determine whether deviations

from Hardy Weinberg expectations were due to the presence of null alleles (Oosterhout et al. 2004). GERUD 2.0 (Jones 2005) was used to assess the minimum number of sires in a complete clutch when maternal genotype is unknown by reconstructing the maternal genotype from the progeny genotypes.

Offspring sex ratios were calculated for individuals within complete clutches and across all sampling sites. The G test for Goodness of fit was used to compare offspring sex ratio with expected 1:1 sex ratio.

Results

Twelve of 72 loci-population combinations showed significant departures from Hardy-Weinberg expectations before Bonferroni corrections for multiple comparisons. One of these combinations was associated with significant excess heterozygosity. After correction, 4 of 72 loci-population combinations exhibited a departure from HWE ($P < 0.00069$, adjusted critical value). No single locus deviated from HW expectations in all 9 populations. Seventeen of 396 loci-pair-population combinations and 0 of 66 loci pair combinations across all populations showed linkage disequilibrium before correction for multiple comparisons. Upon sequential Bonferroni correction, no significant linkage between loci occurred within populations ($P < 0.00012$, adjusted critical values). Using MICROCHECKER, 9 of 72 loci-population pairings analyzed exhibited signs of the possible presence of null alleles with all 9 of the possible nulls within three loci. To be conservative in our analysis we removed the 3 loci with most of the possible null alleles, so all further analyses will use 9 of the loci. Output from GERUD 2.0 (Jones 2005) suggested that 6 out of 39 (15.4%) clutches had a minimum of two fathers contributing to offspring genotypes. Nests where blood was taken from all nestlings varied from 2 to 4

individuals depending on the number of eggs layed, hatched or nestlings that survived. Among complete nests that had 3 or more nestlings sampled, 6 out of 22 (27.3%) nests showed evidence for multiple paternity. Among complete nests where only 2 nestlings were able to be sampled 0 of 15 nests showed evidence of multiple paternity.

Sex ratio among nests where all individuals from the nest were sampled was 48 males to 57 females. The frequency of males (45.7%) is not significantly different from the expected 1:1 sex ratio ($G = 0.772$; $p = 0.379$). Sex ratio among all 212 individuals sampled across the Texas/Mexico coast was 97 males and 115 females. The frequency of males (45.7%) is also not significantly different than the expected offspring sex ratio ($G = 1.53$; $p = 0.216$).

Discussion

Despite presumed monogamy (Lowether & Paul 2002), multiple paternity occurred in ~16% of all Reddish Egret nests examined. It is likely that this number is conservative and the actual percent of nests with multiple paternity may be even higher. We failed to detect multiple paternity in nests with only two nestlings, presumably due to the likelihood that it is not difficult to reconstruct only 1 maternal and 1 paternal genotype to match offspring genotypes. Even if a multiple loci had 4 different alleles, the combination could still possibly come from only two adults. The actual percentage of multiple paternity nests may be closer to the estimated 27.3% from nests with 3 nestlings sampled. Whether these extra pair copulation events leading to multiple paternity are occurring between other mated males or unmated males needs to be examined to understand the impact on effective population size. In a paternity study with Roseate Spoonbills (*Platalea ajaja*), another colonial nesting waterbird, the occurrence of half

siblings in clutches indicated multiple sires were present in 12 of 28 (42.8%) nests from a natural population (Minõ et al. 2009). In Chinese Egrets (*Egretta eulophotes*) attempted EPCs were successful 77% of the time (Wei et al. 2005). Extra-pair copulations may be more common place among Reddish Egrets than recognized as evidenced by other members of family Ardiedae (Gladstone 1979; Wei et al. 2005).

The offspring sex ratio did not significantly deviate from the expected 1:1 ratio suggesting that none of the potential mechanisms for sex ratio adjustment are occurring within Reddish Egrets (Krackow 1995). The use of molecular tools did allow the investigation of otherwise difficult to ascertain aspects of Reddish Egret biology and ecology. There is opportunity for further study of extra-pair copulation behavior with respect to frequency of EPC events, mate guarding (Wei et al. 2005), the relationship of nest density to EPC behavior and as the Reddish Egret is a plumage dimorphic bird, to also determine any differences in EPC behavior in relation to color morph. For a bird that is generally thought of as a vulnerable species, there are many areas of the Reddish Egret ecology with little information available, so we call for a greater effort in the study of Reddish Egret biology using a multidisciplinary approaches and tools.

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APPENDIX

Figures & Tables

Figure 1. Reddish Egret Range

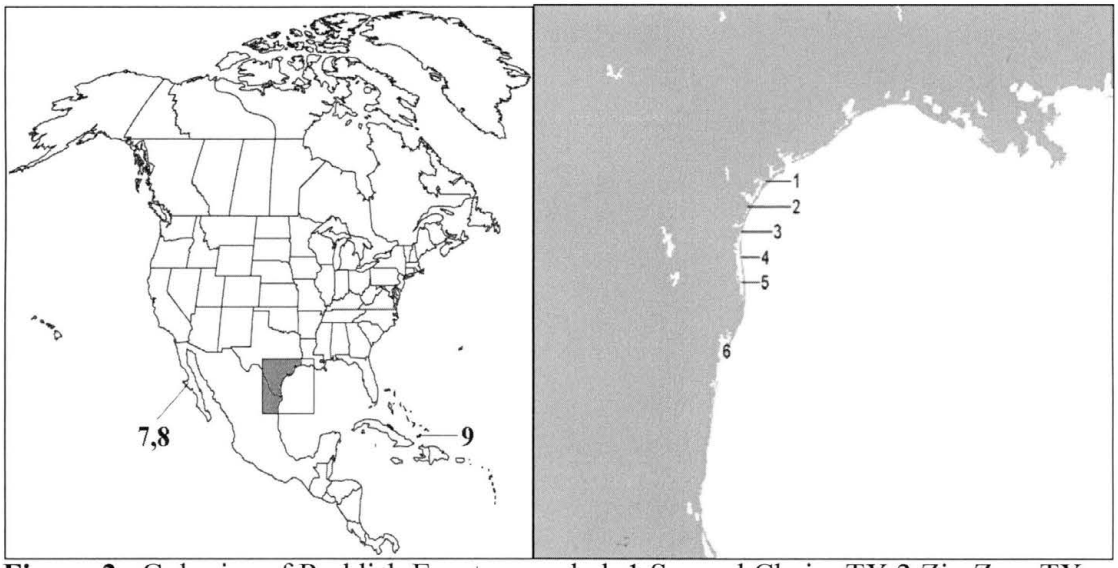

Figure 2. Colonies of Reddish Egrets sampled: 1.Second Chain, TX 2.Zig Zag, TX 3. Rabbit Island, TX 4.East Flat Spoils, TX 5.Green Island, TX 6. Queso Island, Mexico 7. Isla Concha, Baja California 8. Isla Piedra, Baja California 9. Lake Rosa, Great Inagua, Bahamas.

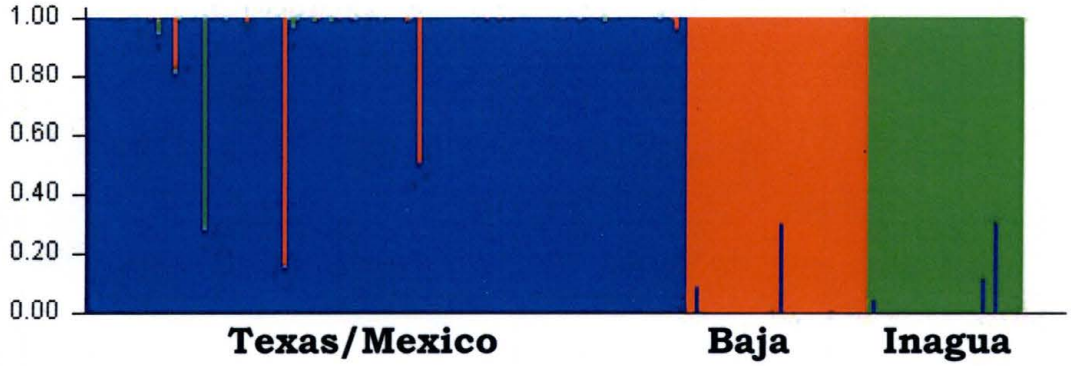

Figure 3. K = 3 clusters as performed by STRUCTURE

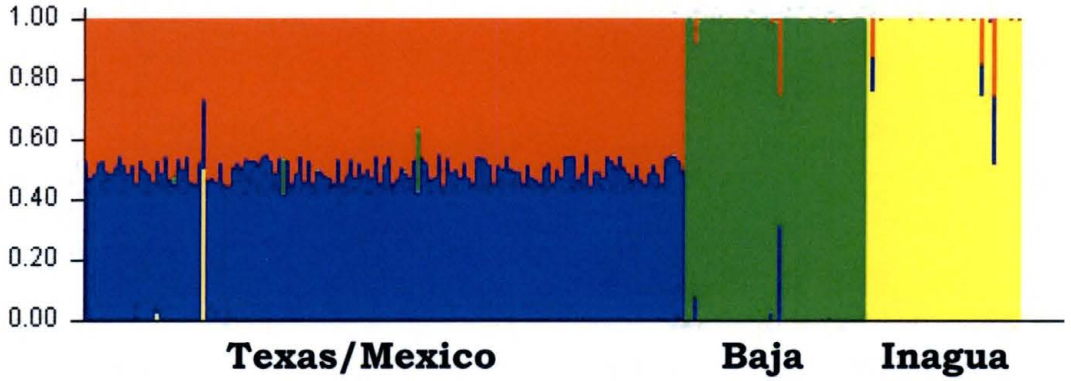

Figure 4. K = 4 clusters as performed by STRUCTURE

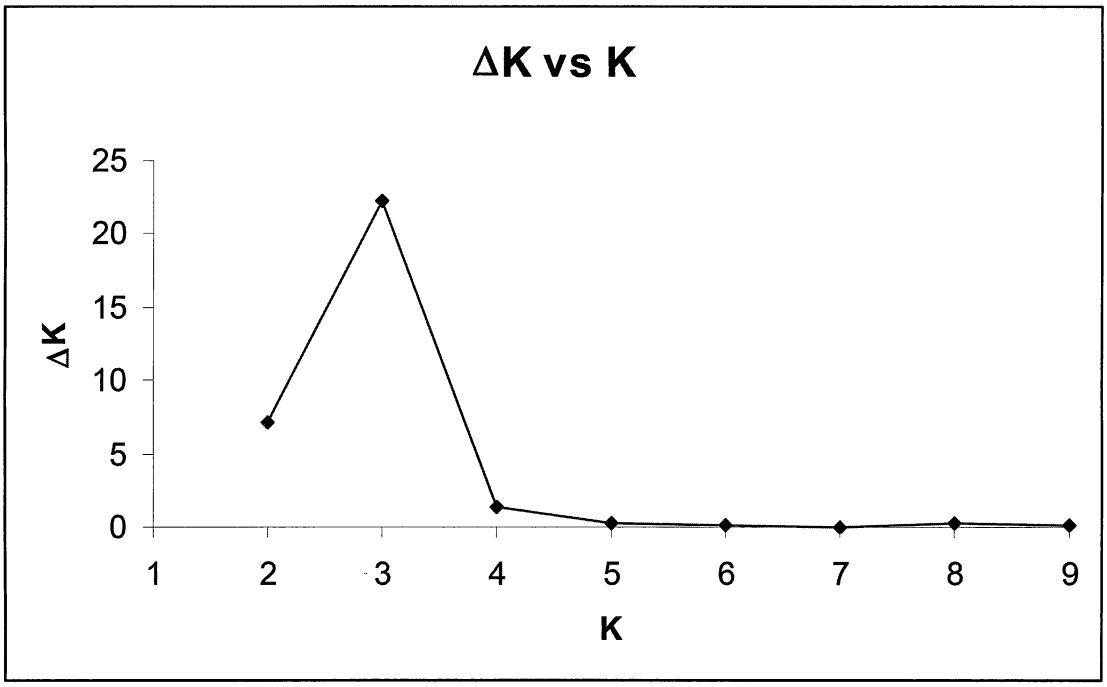

Figure 5. ΔK vs K

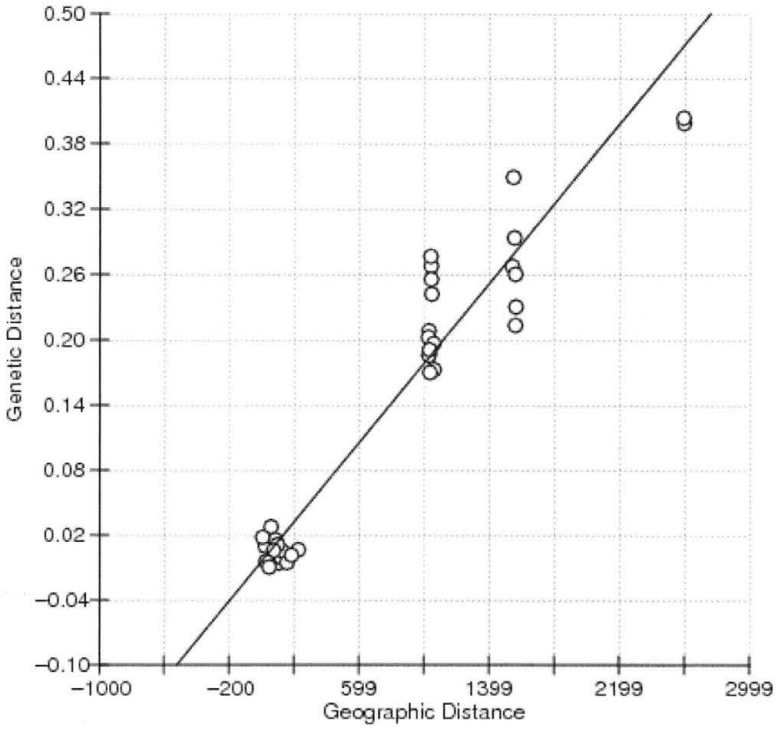

Figure 6. Scatter plot of genetic distance vs. geographic distance (km) for all pairs of populations with RMA regression line.

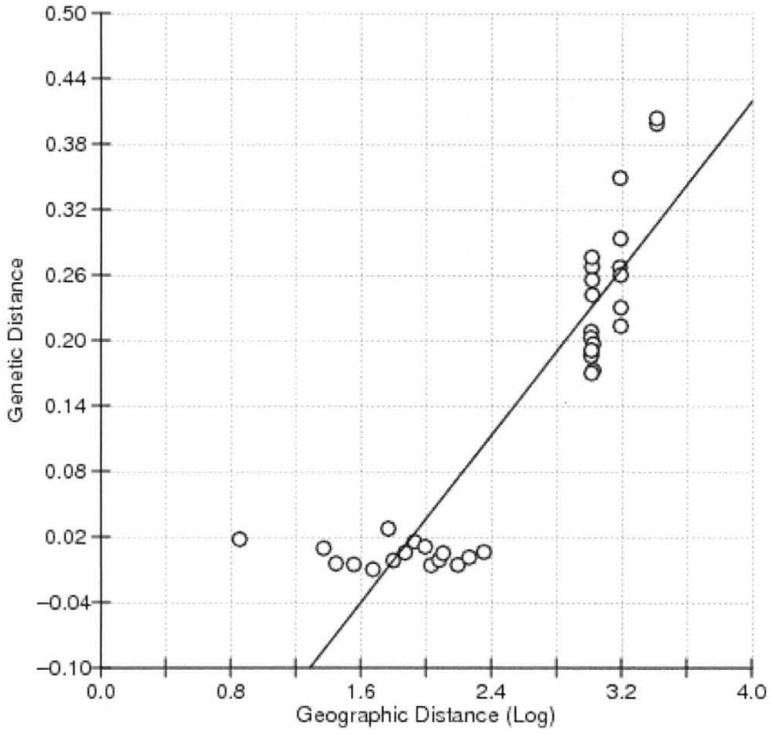

Figure 7. Scatter plot of genetic distance vs. log geographic distance (km) for all pairs of populations with RMA regression line

Table 1. Sampling locations and sample sizes

Location	Sample Site	Abbreviation	Lat/Long	Sample Size
Lower Laguna Madre, Texas	East Flat Spoils	EFS	26°43'20.49N 97°25'38.80W	31
Lower Laguna Madre, Texas	Green Island	GI	26°23'31.07N 97°19'27.03W	13
Upper Laguna Madre, Texas	Rabbit Island	RI	27°14'47.73N 97°24'51.24W	32
Ayers Bay, Texas	Second Chain	SC	28°11'34.48N 96°48'52.10W	20
Upper Laguna Madre, Texas	Zig Zag Island	ZZ	27°37'52.47N 97°15'47.02W	32
Laguna Madre de Tamaulipas, Mexico	Queso Island	QI	25°19'05.10N 97°27'00.90W	15
Laguna de Ojo, Baja Sur, Mexico	Isla Concha	CI	27°49'32.67N 114°14'02.25W	8
Laguna de Ojo, Baja Sur, Mexico	Isla Piedra	PI	27°42'10.51N 114°09'29.61W	35
Great Inagua, Bahamas	Lake Rosa	LR	21°03'12.44N 73°24'46.00W	37

Table 2 .Allelic Richness per locus/population

	EFS	GI	RI	SC	ZZ	QI	CI	PI	LR	Overall
A106	1.453	1.862	1.440	1.400	1.585	1.000	1.000	1.000	1.000	1.309
E213	4.498	3.614	4.273	4.279	3.686	4.581	3.000	3.031	3.711	4.154
H224	2.788	2.996	2.440	2.884	3.025	2.533	2.000	2.000	1.997	2.597
E202	7.585	7.196	7.552	7.816	7.259	6.593	3.000	2.890	4.530	7.138
E114	3.811	2.615	3.194	3.934	3.275	2.000	2.000	2.405	1.716	3.619
H222	2.710	2.615	2.690	2.400	2.000	2.910	2.000	1.918	1.998	2.367
B108	1.258	1.862	1.775	1.400	2.086	1.000	2.000	1.998	1.000	1.801
H113	2.000	2.000	2.000	2.000	2.000	2.000	1.000	1.000	1.000	1.999
H201	4.827	3.800	3.970	4.273	4.295	3.600	2.000	3.063	2.764	4.203
AVG	3.437	3.173	3.259	3.376	3.246	2.913	2	2.145	2.191	

Table 3 Gene Diversity per locus/population

	EFS	GI	RI	SC	ZZ	QI	CI	PI	LR
A106	0.063	0.147	0.061	0.050	0.091	0.000	0.000	0.000	0.000
E213	0.741	0.689	0.736	0.738	0.699	0.757	0.563	0.570	0.710
H224	0.582	0.631	0.515	0.601	0.562	0.543	0.464	0.458	0.400
E202	0.882	0.869	0.881	0.879	0.857	0.838	0.563	0.590	0.727
E114	0.627	0.468	0.594	0.567	0.578	0.514	0.250	0.459	0.129
H222	0.509	0.458	0.498	0.497	0.468	0.533	0.536	0.228	0.413
B108	0.032	0.147	0.147	0.050	0.201	0.000	0.321	0.416	0.000
H113	0.506	0.481	0.506	0.511	0.508	0.476	0.000	0.000	0.000
H201	0.687	0.404	0.649	0.684	0.685	0.562	0.125	0.369	0.426

Table 4. Number of alleles, allele size range, observed heterozygosity (Ho), expected heterozygosity (He) for 9 microsatellite loci in 9 Reddish Egret colonies

Primer	EFS	GI	RI	SC	ZZ	QI	CI	PI	LR	Overall
A106										
# Alleles	2	2	2	2	2	1	1	1	1	2
Range	141-147	141-147	141-147	141-147	141-147	147	147	147	147	141-147
H_o	06	15	06	05	09	0 0	0 0	0 0	0 0	05
H_e	06	15	06	05	09	0 0	0 0	0 0	0 0	
E213										
# Alleles	6	4	5	5	5	5	3	4	4	7
Range	201-211	203-209	203-211	201-209	201-209	201-211	201-205	199-203	203-209	199-211
H_o	81	92	81	90	69	93	1 0	77	89	86
H_e	74	70	74	74	70	76	59	57	71	
H224										
# Alleles	3	3	3	3	4	3	2	2	2	4
Range	144-152	144-152	144-152	144-152	140-152	144-152	144-148	144-148	144-148	140-152
H_o	48	46	44	50	44	73	38	40	38	47
H_e	58	62	51	60	56	55	46	46	39	
E202										
# Alleles	10	8	11	10	9	8	3	3	6	12
Range	278-333	293-328	278-333	278-343	278-328	278-328	298-313	298-308	278-333	278-343
H_o	90	92	94	90	84	93	1 0	49	76	85
H_e	88	87	88	88	86	84	59	59	73	
E114										
# Alleles	6	3	5	5	4	2	2	3	2	7
Range	182-206	182-206	182-206	182-206	182-206	182-200	200-202	196-202	182-206	182-206
H_o	58	38	47	50	53	53	00	11	03	35
H_e	63	46	59	57	58	51	23	45	13	
H222										
# Alleles	4	3	4	3	2	3	2	2	2	5
Range	190-206	190-206	190-206	190-206	198-206	190-206	198-206	198-206	198-206	190-206
H_o	42	62	53	55	47	60	38	20	35	46
H_e	51	46	50	50	47	53	53	23	41	
H113										
# Alleles	2	2	2	2	2	2	1	1	1	2
Range	180-188	180-188	180-188	180-188	180-188	180-188	188	188	188	180-188
H_o	48	23	59	40	50	60	0 0	0 0	0 0	31
H_e	51	47	51	51	51	48	0 0	0 0	0 0	
H201										
# Alleles	6	5	5	5	5	5	2	4	4	6
Range	130-166	130-166	142-166	142-166	142-166	142-166	154-158	154-166	154-166	130-166
H_o	61	46	63	60	72	67	13	40	38	51
H_e	69	41	65	68	69	57	13	37	43	
Over all loci										
Avg alleles/locus	4 56	3 56	4 33	4 11	4 00	4	2 27	2 86	3 33	
H_o	49	48	51	49	50	71	46	38	46	
H_e	51	48	51	51	52	61	41	44	47	

Table 5. Estimates of population differentiation

	EFS	GI	RI	SC	ZZ	QI	CI	PI	LR
EFS	-	0 01053	-0 00491	-0 00524	-0 00101	-0 00178	0 19015	0 20921	0 21360
GI	-0 00341	-	0 02752	0 00551	0 01620	0 00720	0 26583	0 27807	0 34891
RI	-0 00113	0 00970	-	0 00573	-0 00417	-0 00704	0 18539	0 20302	0 23032
SC	0 01843	-0 01724	0 02207	-	-0 00923	0 00438	0 17243	0 19790	0 26741
ZZ	0 00394	-0 01474	0 00613	-0 01190	-	0 00048	0 17045	0 19188	0 26035
QI	0 02495	0 01554	-0 00856	0 01761	0 01449	-	0 23548	0 25589	0 29231
CI	0 24785	0 26867	0 19802	0 14616	0 01449	0 19002	-	0 02127	0 39817
PI	0 3837	0 45139	0 32195	0 29335	0 27332	0 35322	0 04813	-	0 40416
LR	0 23959	0 38128	0 19688	0 36668	0 29668	0 27905	0 59536	0 69409	-

F_{ST} is shown above diagonal, R_{ST} is shown below diagonal. Underlined values are statistically significant at $\alpha = 0.05$

Table 6. F_{ST} between color morphs

	Baja Red	TX/MEX Red	Inagua White	TX/MX White
Baja Red	-			
TX/MEX red	0.20300	-		
Inagua White	0.39241	0.22086	-	
TX/MEX White	0.19849	0.00195	0.21415	-

Table 7. Primer ID, Repeat Motif, Primer Sequence, Annealing Temperature, Size Range, Number of Alleles per locus, and Observed and Expected Heterozygosity

Primer	Motif	Primer Sequence (5¢-3¢)	T_a °C	Size (bp)	# Alleles	H_o/H_e
Er21	$GG(GT)_{11}(GC)_2$	F AGG-AAG-AGG-AGA-GGT-GAG-GT R CCA-AGA-GCA-CTT-TTC-TAT-CAA-G	56 8	141-147	2	0 06/0 06
Er22	$(CT)_{17}$	F TTC-AGA-CAG-ACT-GGA-ATC-ACA-G R AAG-TTT-ATT-GGC-AGC-AGA-TAG-C	57 3	201-211	6	0 81/0 74
Er41	$(TATC)_5TATT(TATC)6TATT(TATC)_4$	F AAT-ACG-CAT-CAA-GAC-AAA-TCA-G R TCA-TGC-CTT-CTA-TGA-CAC-TAG-G	57 7	286-298	2	*‡0 16/0 43
Er42	$(GATT)_8$	F CTC-CAG-TGC-AGA-ACT-AGA-CCT-G R CCT-GCT-GAT-TGA-AAC-ATG-AAT-T	57 8	209-221	4	0 74/0 68
Er43	$(ATCT)5ATTT(ATCT)_5$	F GCA-GGA-CCT-ACC-CTT-CAG R GTG-CCT-TTC-ACA-TCT-CAC-TC	55 7	144-152	3	0 48/0 58
Er51	$(TTCTC)_6TTNTC(TTCTC)_4$	F AAC-AGG-CTT-CCC-AGA-GTG R TGA-ACC-CTC-AGG-CAT-TTA-C	57 5	278-333	10	0 90/0 88
Er23	$(TC)_{23}$	F AGA-GGT-TTC-CCT-TTC-CTA-GAT R ATG-GCA-TTC-TGT-ATG-GGT-AG	56 5	182-206	6	*0 58/0 62
Er44	$(TAGG)_5(TAGA)_6$	F GAG-GGC-GAG-AAC-TTG-AGG R CAA-AGC-AAC-AAA-ACA-TTC-AGC	57 3	190-206	4	0 42/0 51
Er31	$(GTT)_5GCT(GTT)_3$	F GCA-AAA-GAT-TCA-AGT-CTG-ATG R TAG-TTG-GGC-AGC-ATA-ATG-ATA	57 1	292-298	2	0 03/0 03
Er45	$(TATC)_3TTTC(TATC)_5$	F AAG-GAA-ATA-ATG-GCG-ATA-GC R TCT-ACC-AGG-GCA-GTA-AAC-TAA-A	56 5	180-188	2	0 48/0 51
Er24	$(GA)_{22}$	F CAT-TTG-CTT-TAT-CCA-AGA-CCT R TAT-CCT-CAT-TTT-CCT-CAG-TGT-C	56 3	203-221	5	0 68/0 76
Er46	$(GAAG)_{17}$	F AGG-GAA-AGA-AAG-AGA-GGG-AC R TGC-TAC-CAC-TTT-GAA-ACA-GAC	56 2	130-166	6	0 61/0 69

* Significant deviation from Hardy Weinberg after correction

‡ Possible null alleles

Table 8. Sample locations and sample sizes

Location	Sample Site	Abbrev.	Lat/Long	Complete Nests	Total Indiv.
Lower Laguna Madre, Texas	East Flat Spoils	EFS	26°43'20.49N 97°25'38.80W	9	47
Lower Laguna Madre, Texas	Green Island	GI	26°23'31.07N 97°19'27.03W	2	17
Lower Laguna Madre, Texas	Laguna Vista Spoils	LGV	26°08'35.22N 97°16'40.29W	2	6
Upper Laguna Madre, Texas	Rabbit Island	RI	27°14'47.73N 97°24'51.24W	11	52
Upper Laguna Madre, Texas	Site 5	S5	27°14'07.05N 97°24'59.15W	1	8
Ayers Bay, Texas	Second Chain	SC	28°11'34.48N 96°48'52.10W	3	23
Upper Laguna Madre, Texas	Zig Zag Island	ZZ	27°37'52.47N 97°15'47.02W	9	44
Laguna Madre de Tamaulipas, Mexico	Queso Island	QI	25°19'05.10N 97°27'00.90W	2	15

Table 9. AMOVA using 3 groups

Source of Variation	d.f.	Sum of Squares	Variance components	Percentage of variation	P value
Among groups	2	143.256	0.591	22.88	**0.004**
Among populations within groups	6	14.203	0.009	0.34	0.280
Within populations	437	866.604	1.983	76.77	**0.000**
Total	445	1024.063	2.583		

Table 10. Amova using 2 groups

Source of Variation	d.f.	Sum of Squares	Variance components	Percentage of variation	P value
Among groups	1	70.76	0.400	15.37	**0.028**
Among populations within groups	7	86.70	0.222	8.51	**0.000**
Within populations	437	866.604	1.983	76.12	**0.000**
Total	445	1024.063	2.605		

VITA

Austin Evan Hill was born in Bossier City, Louisiana, on November 18, 1982 the son of Robin Evans Hill and Steven Jay Hill. Austin Graduated from Plano Senior High School, Plano, Texas, in 2001. After one year at Collin County Community College, he enrolled at Southwestern University, Georgetown, Texas in 2002. He received his Bachelor of Science degree in biology in 2005. In August 2006, Austin entered the Population and Conservation Biology graduate program at Texas State University-San Marcos.

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