Fall 1998

Genetic Variation within and among Populations of Florida Burrowing Owls (*Athene cunicularia floridana*)

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by

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11/19/98
Date

Dedication

To Daniel, Esta and Kimberly

Three Excellent and Happy Scholar

Acknowledgments

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Finally, and most importantly, thank you to my wonderful husband, Trey, and our self-sufficient and delightful children, Daniel, Esta, and Kimerbly. I love you all so very much. Thank you for keeping our home so happy and loving!

p.s. Mom and Dad……you are the greatest parents in the world! I hope you know how much we all love you!

Abstract

Genetic Variation Within and Among Populations of

Florida Burrowing Owls (*Athene cunicularia floridana)*

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This study employed DNA Polymerase Chain Reaction (PCR) and Single-Stranded Conformation Polymorphisms (SSCP) on a mitochondrial control region target to assess population structure and possible gene flow in the Florida Burrowing Owl (*Athene cunicularia floridana*), a Florida Species of Special Concern. Although widespread in Florida, Burrowing Owls occur at low densities in semi-isolated populations that are susceptible to extirpation from human development and the demographic consequences of small population size. To better manage these populations, there is a need for data on the population genetic structure of the Florida Burrowing Owl. Therefore, I acquired DNA from Burrowing Owls from stable peninsular populations (Miami, Cape Coral, and Tampa), outlying populations in north-central Florida (Suwannee, Madison, and Gilchrist counties), and the western panhandle region (Eglin AFB), and, as an out group, populations of *Athene cunicularia hypugea* (Western Burrowing Owls).

Variation among 73 individuals for a 250 base pair locus in Domain I of the control region assayed as nine genotypes. Results indicated that the frequency of these genotypes varied significantly among six populations of *A.* c. *floridana* ($G = 77.7$, $P <$

0.001). No significant variation between *floridana* and *hypugea* was detected, but there was evidence of differentiation among Miami, Gulf coast birds (Tampa and Cape Coral), and the birds of the northern and panhandle populations. Genotypes were consistent with sibling and mother-offspring relationships for the maternally-inherited marker, although some questions regarding the repeatability of the SSCP assay arose.

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Introduction

Listed as a Species of Special Concern by the Florida Game and Fresh Water Fish Commission (1990), the Florida Burrowing Owl has been the focus of several recent studies conducted, coordinated, and /or supported by that commission. Information on habitat, breeding biology, population size and trends, dispersal, and survival indicates this subspecies has been both threatened and, in some ways, aided by the intrusion of humans into its range in the past 50 years (Millsap and Bear 1997). Historically associated with the prairies of south-central Florida, *floridana* now occurs regularly in agricultural and suburban areas, and such developments as airports, industrial parks, golf courses, and military bases. While benefiting from habitat creation, as wetlands and forests have been converted to open, managed land use, populations have been shown to be vulnerable, with locally flourishing populations collapsing suddenly (Millsap 1993). Florida Burrowing Owls appear to benefit from land development up to a point but decline rapidly when development becomes more intense. Wesemann and Rowe (1987) suggested the owls prefer areas with developed lots accounting for 25%-75% of the total land available. Densities declined with either less or more development.

Because populations are locally unstable, a statewide census is difficult. The best available estimate suggests the population is between 3,000 and 10,000 pairs (Millsap 1993). The Florida Burrowing Owl occurs primarily as semi-isolated populations in peninsular Florida, the Florida Keys, and in the Bahama Islands. Recently established,

isolated breeding colonies occur in several locations in north-central Florida and on Eglin Air Force Base in the western panhandle region.

The origin of outlying populations is unclear. The Florida Burrowing Owl is non-migratory, and Millsap and Bear (1997) found a mean natal dispersal distance of only 1355 m for females and 511 m for males. It is possible that isolated populations (e.g., north-central Florida and Eglin) might each have resulted from an unusually long-distance dispersal of a single breeding pair from other *floridana* populations. Alternatively, though less likely, colonies could have been founded by migratory western birds. *S. c. hypugaea* is listed as accidental in the east from New Hampshire and Ontario south to Virginia (American Ornithologists' Union 1998:301). These northern areas of Florida had little appropriate habitat for Burrowing Owls until recent times, so the possibility of the owls having gone undetected is unlikely. A thorough census of the northern counties is made more difficult by the apparent instability of these small colonies. Several recent north-Florida populations have disappeared after only a few breeding seasons, and populations may escape notice if they do not persist for several years. A Jacksonville population that persisted for five or six years was recently extirpated, and owls nested as far north as Bainbridge, Georgia for several seasons (B.A. Millsap, personal observation) (Fig. 1).

The combination of a small statewide population, susceptibility to human activity, and frequent appearance and disappearance of new populations suggest that a study of the

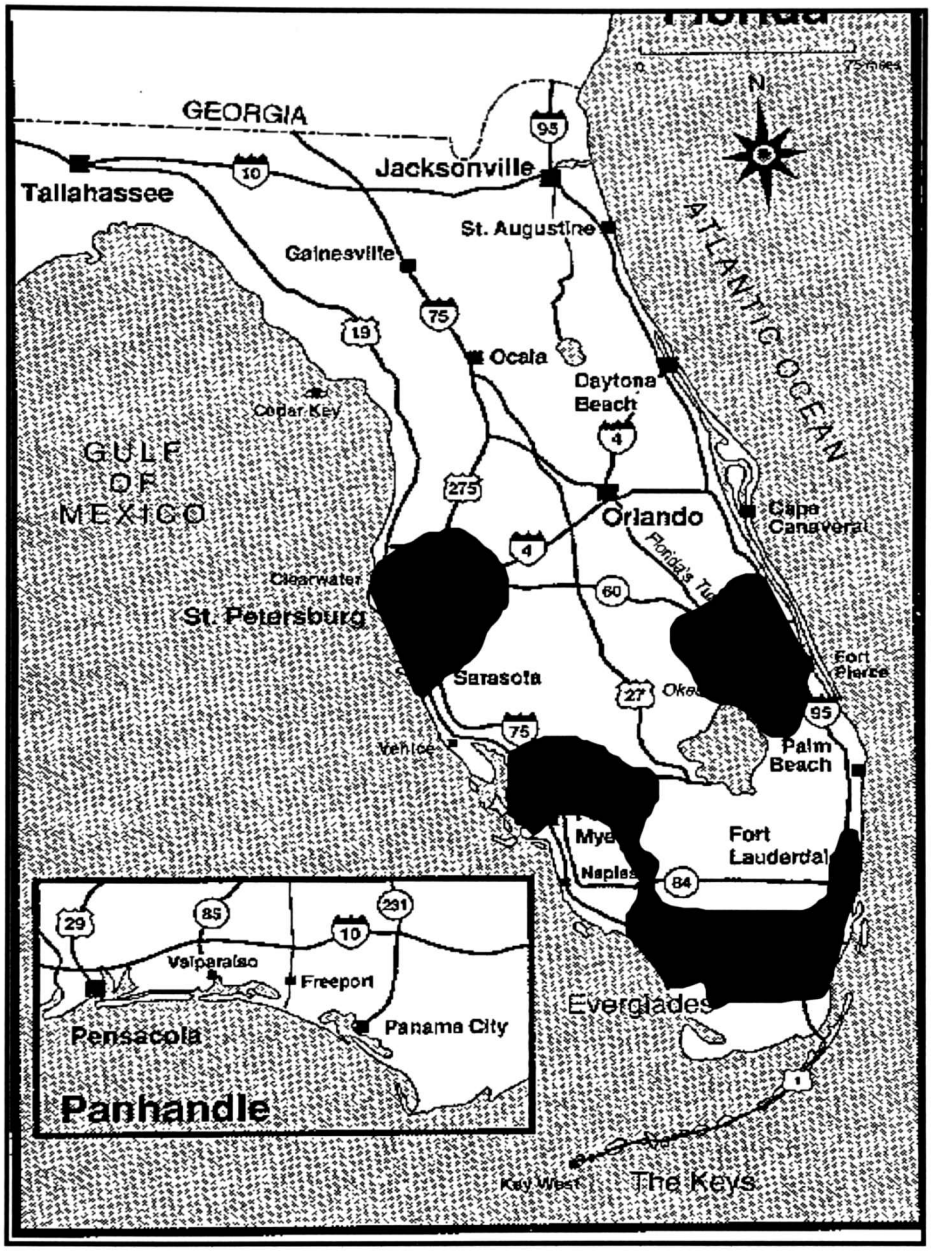


Figure 1. General distribution of Florida Burrowing Owls. Dark areas indicate regions with a Burrowing Owl density of 1-5 owls for every 40 km surveyed (based on Price et al. 1995:92).

genetic structure of Florida Burrowing Owls might be useful to guide future conservation and management decisions concerning this species. Although Desmond (1997) analyzed genetic differentiation of subspecies of Burrowing Owls, her study provided no insight into population-level questions within the Florida subspecies.

Project Objectives

My project was initiated to examine the genetic variation within and among populations of Florida Burrowing Owls. I addressed three broad questions relating to the genetic structure of Burrowing Owl populations. First, I sought information regarding subspecies differentiation between *floridana* and *hypugea*. Second, I was interested in the overall variation and in the genetic structure of *floridana* populations. Finally, I hoped the research might identify the possible origins of outlying populations of *floridana*. To meet these objectives, I gathered information in two areas: the natural history of Burrowing Owls and the molecular techniques that would best produce the desired data.

Natural History of Florida Burrowing Owls

Florida Burrowing Owls are small owls, averaging 222 mm in height, with a wingspan of approximately 530 mm (Eckert 1987:163-176.). The owls have long legs, suitable for scratching out burrows, and have bright, lemon-colored eyes. Burrowing

Owls make their nests in burrows, which they excavate themselves, or adapt from the excavations of armadillos or gopher tortoises. They prefer loose, sandy soil and open habitat, from which they can observe potential predators. Florida Burrowing Owls live in small colonies, generally two to five pairs (Eckert 1987), and extra-pair copulations and shared feeding of juveniles among nests appear to be common (Millsap and Bear 1997).

Burrowing Owls are most active at dawn and dusk, but remain active throughout the day, searching for prey. Studies of western populations of Burrowing Owls indicate that small vertebrates make up the majority of the biomass of the diets. Invertebrates such as crickets, beetles, and crustaceans are the major food items by number, although they represent a small portion of the total biomass. During nesting season, the invertebrate proportion increases substantially (Haug et al. 1993).

There is little sexual dimorphism within the species. Males tend to be slightly larger (unusual among raptor species) and, during the nesting period, males become lighter in color because the females spend more time in the burrow (Thomsen 1971, Butts 1973, Martin 1973, Millsap and Bear 1990). Florida Burrowing Owls generally lay between five and six eggs, and males and females share incubation and provisioning duties. Millsap and Bear (in press) found the mean number of young fledged to be 2.2 in the stable, peninsular populations.

The most common causes of death among Florida Burrowing Owls are collision with vehicles, and predation by domestic animals and other birds of prey (Millsap and Bear, in

press). Death within collapsed burrows is difficult to survey, but is probably considerable during wet weather or where burrows are close to new development (Millsap and Bear, in press). Egg loss to raccoons, opossums, skunks, snakes, and armadillos is common (Eckert 1987).

Taxonomically, *floridana* is considered a subspecies along with seventeen other subspecies, of the Burrowing Owl (*Athene cunicularia*). The genus *Athene* is based on karyotypic evidence (Schmutz and Moker 1991) and, based on DNA-DNA hybridization evidence, might actually qualify as a family (Sibley and Ahlquist 1990:402-411). Desmond (1997) recently compared molecular markers for subspecies from North, Central, and South America and from the West Indies. Desmond found a distinct split between owls from North and South America, estimated to have occurred 2 million years ago when the two continents were connected by the isthmian land bridge. Desmond's results indicate a North American origin for the species, with a subsequent dispersal to South America.

Historical distribution in Florida may have originated with a dispersal of western owls during the early to mid-Pleistocene glacial periods, with isolation occurring for the past 20,000 years (Webb 1990). However, lack of pronounced morphological differentiation might suggest a more recent colonization event. Early reports of colonies are generally from the central peninsula plains and the Gulf coast lowlands (Ridgeway 1914, Bent 1938). A northward range expansion appears to have followed the post-war development of the state, as swamps were filled and forests cleared.

Florida Burrowing Owls exhibit behavioral traits that differentiate them from their western counterparts. Most notably, *floridana* is non-migratory and generally excavate their own burrows. *Hypugea* depend upon fossorial mammals to excavate and maintain burrows in ground that is otherwise too hard for the owls to break open. Western Burrowing Owls live in large colonies in close association with thriving small mammal communities. This association appears to reduce predation on owls, as the mammals provide an alternative prey source for predators that the birds and mammals have in common. *Floridana* also nest in colonies, but these colonies are extremely fragmented, even where owl density is comparatively high. This fragmentation is probably made possible by the freedom from dependence upon prairie dog towns and ground squirrel communities. Fragmentation may also serve as a defense against predation.

This relative self-reliance allows for dispersal of birds to new locations, without prior colonization or the presence of fossorial mammals. Because of recorded instances of parent-offspring mating (B.A. Millsap, pers. observ.), it is possible that a single mated pair could establish an isolated colony in a new location.

Selecting a Genetic Marker and a Molecular Method

Intraspecific studies such as this one require a high level of variability in the target molecule or genomic region. Studies of birds have shown that protein (allozyme/isozyme) divergence can be limited in populations that show meaningful

mitochondrial and nuclear DNA variability (Barrowclough et al. 1985). For this reason, nucleic acid analysis is more appropriate for avian investigations at lower taxonomic levels. Important recent studies using modern nucleic acid techniques to address questions in avian ecology and evolution include studies of Red-cockaded Woodpeckers (DNA profiles and RAPD, Haig et al. 1993, Haig et al. 1994a, Haig et al. 1994b), Red-winged Blackbirds (RFLP, Ball et al. 1988), cranes (RFLP and mt DNA sequences, Krajewski and Fetzner 1994, Snowbank and Krajewski 1995, Wood and Krajewski 1996), Song Sparrows (RFLP, Zink et al. 1991), swiftlets (mt DNA sequences, Lee, et al. 1996), Dunlin (mt DNA sequences, Wenink et al. 1993) as well as other avian species.

Because the first direct sequencing of any portion of the Burrowing Owl genome had recently been completed on the mitochondrial control region and cytochrome b genes (Desmond 1997), I elected to target a portion of the mitochondrial control region (displacement or D loop), using primers designed from this sequence data. Mitochondrial DNA is frequently preferred for genetic studies, because it is maternally recombining, has a simple, closed structure, and evolves 5 - 10 times faster than nuclear DNA (Baker and Marshall 1997). The mitochondrial control region is known to evolve rapidly in numerous vertebrates and is generally considered an excellent target for studies at the species level or below. This non-coding portion of mitochondrial DNA was found to have a rate of substitution in humans between 2.8 (Cann et al. 1984) and 5 (Aquadro and Greenberg 1983) times faster than the rate of the rest of the mitochondrial genome.

The mitochondrial gene order in birds is different than it is in other vertebrates (Desjardins and Morais 1990, 1991, Ramirez et al. 1993, Quinn and Wilson 1993), with the regions tRNA Phe and tRNA Glu flanking the control region. The control region itself is generally described in three domains. Domains I and III are highly variable, and flank the highly conserved, central Domain II.

While the 1000 - 1250 bp control region has yielded informative markers in studies of Snow Geese (*Chen caerulescens*) (Quinn 1992), Dunlins (*Calidris alpina*) (Wenink et al. 1993, 1996), Grey-crowned Babblers (*Pomatostomus temporalis*) (Edwards 1993 a,b) and Red Knots (*Calidris canutus*) (Baker et al. 1994), it is not uniformly polymorphic within all avian species (Baker et al. 1994). No work at this locus besides Desmond (1997) has been published for the Strigiformes.

Numerous molecular techniques exist to address phylogenetic questions such as intraspecific variation and genetic distance. An appropriate method had to be selected from among several options, with its utility in a particular research situation balanced against its scientific and economic limitations. There were six methods that deserved consideration as possible methods for quantifying genetic differentiation among Burrowing Owl populations.

DNA-DNA hybridization. - DNA-DNA hybridization involves combining the double-stranded DNA of two species, denaturing these strands and then allowing the single strands to re-associate. The extent to which single strands from different species will re-associate provides an indication of their level of divergence. This technique is

inexpensive, easily automated, and useful for estimating relationships between species or higher taxa. However, the effective limits of resolution depend on the degree of divergence (Werman 1996). It is almost certain that populations of Florida Burrowing Owls would not have diverged from one another to an extent measurable by this method (Werman et al. 1996).

Restriction fragment length polymorphisms (RFLP). - Also called restriction site analysis, RFLP relies on known restriction endonucleases isolated from bacteria to digest DNA at constant positions within specific recognition sequences. A particular allele is cleaved into a reproducible array of fragments. Base substitutions and short insertion/deletions are reflected as changes in the fragment patterns. This method assumes that fragments of the same length are of the same sequence (homologous). Substitutions and insertions/deletions of 2-3 base pairs between the recognition sites will not be apparent. This method screens only the 4-6 base pairs of the specific code recognized by the endonuclease, and may fail to recognize variation for that reason (Dowling et al. 1996).

Randomly amplified polymorphic DNA (RAPD). - RAPD bases its analysis on the efficiency of PCR amplification for a short, random primer. RAPD uses randomly selected genes that may or may not show variability at low taxonomic levels. Because dominant allelic expression in the double strand hides recessive alleles, researchers are required to use RFLP on RAPD products to answer heterozygosity questions. Although

RAPD is inexpensive, it has shown poor repeatability and is easily contaminated by any form of DNA (bacterial, viral, etc.) allowed to enter the sample (Palumbi 1996).

Microsatellite markers. - The use of microsatellite markers to digest hypervariable sections (short tandem repeats) of DNA leads to a DNA fingerprint. This fingerprinting has the potential to be unique to each individual sampled. Unfortunately, this method requires an initially large blood sample for the development of a genomic library in order to design primers specific for the species under consideration. In this case, analysis by this method would be prohibitively time consuming and expensive (Dowling et al 1996).

DNA sequences. - Direct sequencing of a specific, variable region requires four basic steps. The target region must be isolated, and then amplified. The purified DNA is then sequenced and homologous sequences are aligned for analysis. Sequencing is a popular method for analysis of intraspecific genetic variation, although there is a trade-off between the complete information of one or two loci against the more general information on numerous loci offered by other methods. Sequencing requires the use of radioactive labeling materials and is also extremely time-consuming. For this study, only 2-3 individuals from each population could be analyzed, reducing the power of our statistical analysis. Sequencing is an appropriate diagnostic follow-up to some other, more efficient screening technique (Hillis et al. 1996).

Single-stranded conformation polymorphisms (SSCP). - The method of analyzing single-stranded conformation polymorphisms (SSCP) was selected as the most applicable and sensitive molecular technique for addressing this population-level molecular

question. SSCP is a recently developed technique for screening DNA sequences, and has proven to be sensitive at even the single base pair level. It is relatively simple and inexpensive, and its sensitivity makes it particularly useful for comparisons between and among populations (Girman 1996). One report of an avian species analyzed by this method appears in the literature (Friesen et al. 1997). Friesen et al. (1997) developed a successful assay of four nuclear intron loci between isolated subspecies and also between populations of a single subspecies of Marbled Murrelet (*Brachyramphus marmoratus)*. The method has previously proven sensitive between closely related arthropod and helminth species, between breeds in swine and horses, between populations of African wild dogs, and in numerous other animal applications (Hiss et al. 1994, Itagaki et al. 1995, Takeda et al. 1995, Marklund et al. 1995, Girman 1996).

SSCP is emerging as a favored technique for DNA variation screening at the subspecies and population level. The technique is more sensitive than the RAPD method and also allows the researcher to specifically target highly variable regions of the genome. Once identified, a region is isolated, amplified, and then denatured to a single strand. By maintaining this single strand at cool temperatures, the fragment folds into a conformation unique to its length and sequence. When strands from several individuals are run together by electrophoresis in a cool non-denaturing polyacrylimide gel, each unique conformation will travel a different distance. Base-pair substitutions, insertions, and deletions in the region are apparent. Because the fragment under investigation is approximately 300 base pairs, this method has the potential to detect more variability

than RFLP. Identification of a genomic region that consistently shows insertion/deletion mutation for the species is the time-consuming step in this process. Repeatability is good for SSCP, providing that specific parameters such as gel conditions, temperature, and run time are strictly controlled (Dowling et al. 1996).

In summary, I decided to target Domain I of the mitochondrial control region, using single-stranded conformational polymorphisms to assay for genetic variation among populations of Florida Burrowing Owls. My objective was to address questions of population origin, to assess genetic structure and genetic variation within and among populations, and to compare *floridana* to *hypugea* of the western United States.

Materials and Methods

Sample Collection

Blood samples were collected during two consecutive nesting seasons, May - July of 1997 and 1998. Samples were drawn from owls in five regions: Tampa (n = 11), Cape Coral (n = 11), Eglin Air Force Base (n = 9), Miami (n =24), Marathon Key (n =5), and Gilchrist, Madison and Suwanee counties of north-central Florida (n = 6), with the help and supervision of Florida Game and Fresh Water Fish Commission personnel (Fig. 2). We conducted sampling in accordance with the American Ornithologists' Union guidelines (Oring et al. 1988). Approximately 0.20 ml of blood was collected in sterile vials containing 0.50 ml of a lysing buffer solution (Tris-HCL, EDTA, NaCl, H_2O, SDS instructions from Mr. Tom Mullins, lab of Dr. Susan Haig, personal communication). We trapped owls by their feet at their burrows with noose carpets (Bub 1991:200-202). The traps at each burrow was continuously supervised. Field workers weighed, banded, measured (wing chord and rectrices), visually inspected, bled, and released each owl within 10 minutes of capture. Bleeding was by veinipuncture of the brachial vein with a 26-gauge needle (method outlined by Haig et al. 1993). We collected blood in capillary tubes and aspirated (by rubber bulb) the blood into vials of lysing buffer. Vials were taken back to the lab and frozen at -20°C. Further samples were solicited and received

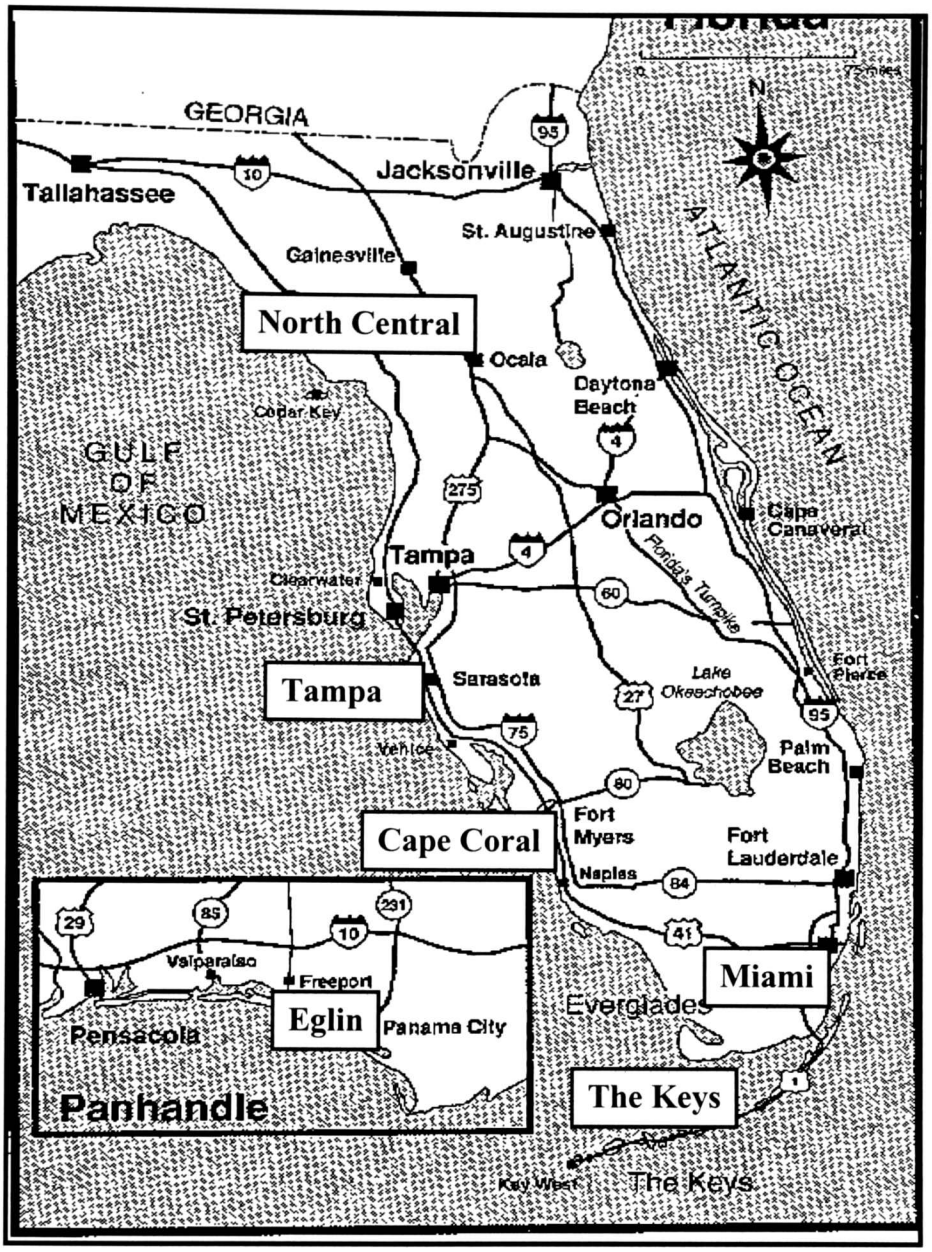


Figure 2. General location of the six sampled populations of Florida Burrowing Owls, 1997-1998. Sampled areas (Marathon Key, Miami, Cape Coral, Tampa, the North- central counties, and Eglin AFB) are shown in boxes.

from a 1993-1994 study of Miami-area owls (Desmond 1997). An additional five samples of Western Burrowing Owls (*Athene cunicularia hypugaea*) were also contributed by Dr. Martha Desmond (two samples from California, two from New Mexico and one from Nebraska).

DNA Isolation

Except where 1993-1994 and western samples had been previously extracted, DNA extraction was by United States Biochemical, Inc. (USB) kit, or by 2% Hexadecyltrimethylammonium Bromide (CTAB) extraction buffer. Blood quantities were relatively small; the USB kit proved to be more effective and was used exclusively after the early extractions. By following the procedure of the kits, the blood cell lysate was prepared and nuclease activity was inhibited. Next, the lysate was bound to a DNA extraction matrix. Then, the aqueous solution, which contains the nucleic acids, was separated from the organic phase by centrifugation. Lastly, the extracted DNA was precipitated with alcohol and eluted with 50 µl Tris-HCl (10 mM) buffer. Total genomic DNA/RNA was successfully extracted from all samples.

PCR Amplification and Gel Electrophoresis

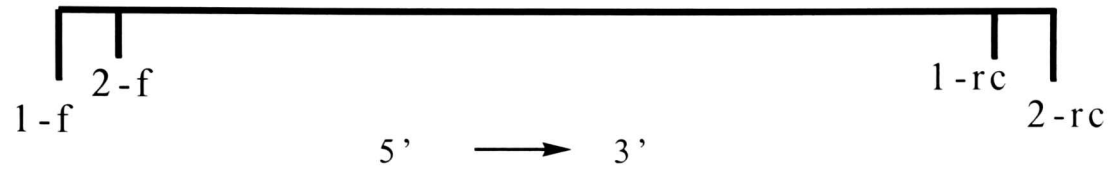
A primer-pair was designed to amplify by PCR an approximately 290 base pair stretch of the highly variable Domain I of the mitochondrial control region. This portion

in Domain I of the mitochondrial control region proved difficult to amplify, but my early tests showed it contained a sufficient amount of variation at the population level. Optimal conditions proved to be: dH_2O 25 µl, 10x Buffer (1X) 2.5 µl, MgCl (2.5 mM) 2.5 µl, dNTPs (200 µM) 0.5 ul each, primer forward and reverse complimentary (0.2 µM) 0.5 µl each, Taq enzyme 0.125 µl, and 0.5 µl template per 25µl. Two minutes at 80°C, was followed by 40 cycles of 94°C - 35", 60°C - 35", 72°C - 60" for each amplification. After numerous attempts, only approximately 50% of the individuals amplified successfully. Each PCR product was evaluated for success using 1% argarose gel electrophoresis.

A nested PCR process proved more successful, eventually amplifying 98% of the individuals. A primer pair was designed to attach just "inside" the first pair, so that approximately 20 bp were removed from the segment (Fig. 3). By amplifying each sample with the outside pair of primers, followed by a re-amplification under the same conditions using the inside pair of primers, successful amplification of 77 of 79 birds was achieved.

Single-stranded Conformation Polymorphism Electrophoresis

PCR products for each sample were run through a 0.75 mm, 7% non-denaturing polyacrylamide gel, with glycerol. Gels were run in a 0.5X TBE buffer, using a Bio-Rad Protein II xi Cell with cooling system. The electrophoresis apparatus was allowed to cool for 60 minutes. Gels were run in an ice bath to reduce "smiling," (the propensity of



Ctr-1.f: GAA TGC AAT CCT TAA TGT ATA ATT G

Ctr-1.rc: CCA TTC AAG GTA TGT ATT CAA G

Ctr-2.f: GTA CAT TAA GTT ATA TAT CCC ATA

Ctr-2.rc: TTC ACG GAA GGA CAA TTA A

Figure 3. The sequence of the primer pairs used for PCR amplification of the Burrowing Owl mitochondrial control region. A two-step PCR amplification proved the most effective. Ctr-1f and Ctr-2rc were used in step one. The product of this amplification served as the template for step two, which employed the Ctr-2f and Ctr-1rc primers.

outside samples to run more slowly than samples loaded in interior wells) because no cold room was available. PCR product was denatured for 5 minutes in a thermocycler at 98°C, with1.5µl PCR product in 1 µl of a 95 % formamide solution. Single-strand conformations were fixed by plunging samples directly from the thermocycler into ice water. Two µl of each denatured sample were loaded after 5 minutes, directly from the ice to the electrophoresis gel. A 12°C running temperature proved optimal, at constant millivolts = 22 for approximately 14 hours. Gels were removed and stained for 10 minutes using SYBR Gold Nucleic Acid stain, and were visualized by 300 nm ultraviolet trans-illumination. Bands were scored while illuminated. Gels were photographed and scoring confirmed against the photograph.

Sequencing

Five samples of PCR-amplified product and one whole *floridana* bird were sent to The University of Georgia (UGA) sequencing lab for direct sequencing. Bill Nelson at UGA extracted purified mitochondrial DNA from the whole bird and amplified that template under identical PCR conditions.

Data Analysis

Genotypes were scored by the appearance of bands. Frequency (N) of each genotype was compared among populations using a G-test. Cluster analysis (based on Euclidean distances) was used to estimate genetic distance between populations.

Results

Seventy-seven samples were eventually amplified by PCR. These samples were produced in small quantities, due to the expense of frequent failures, making concentration of the samples impracticable. Successful amplifications were determined by ultra-violet trans-illumination (Fig. 4). Seventy-three samples were successfully assayed by SSCP. Gel results were visualized by ultra-violet trans-illumination (Fig. 5). I identified nine different genotypes (Fig. 6).

The majority of individuals (85%) were of genotypes 1, 2, and 3. Eight percent of the individuals were of genotype 4, while only a single individual showed each of the remaining five genotypes. Known siblings and female parent/offspring pairs consistently scored the same genotype, as would be expected for a mitochondrial marker. A single individual that was amplified as two samples and run separately on the SSCP, also scored the same genotype. Two adult males exhibiting a similarly malformed foot also shared a genotype for this marker, further indicating a close genetic relationship (Fig. 6).

The frequencies of alternative genotypes varied among populations ($G = 77.7$, $P < 0.001$). Genotypes 1, 2, and 3 were widespread in four or more populations, with genotype 4 appearing in three populations, and genotypes 5-9 appearing only in a single

population each. Within-population variation appeared to be greatest for the Miami population, but this may have been a product of the larger sample size (Table 1).

Cluster analysis showing percent similarity among populations (based on genotype frequency) indicate a grouping of populations with Miami the most distant from the other six populations. Eglin was also somewhat isolated, the Keys were quite similar to *hypugea* of the west, the northern populations (Suwanee, Madison, Gilchrist, and Eglin) were somewhat similar to the Keys and *hypugea*, and the Cape Coral birds were similar to the Tampa population (Fig. 7).

I sought sequence data to support the genotypes indicated by SSCP, but these data proved unattainable. Of the five samples sent for sequencing, only four could be sequenced. Samples sequenced represented three genotypes indicated by the SSCP process. Sequence data produced by the forward primer contained a large proportion of "N," or indeterminate nucleotide bases. Data generated by the reverse complimentary primer were more complete, but the regions near the beginning and end of the fragment still contained too many "N" bases for analysis. The four samples that were amplified were approximately 285 base-pairs long and showed identical sequences from position 76[1] through 270 position, with an indeterminate amount of variation at each end. Sequence data generated from the whole bird (pure mitochondrial template) was much more complete for the end regions, but differed considerably from the matching sequence region of the other four individuals.

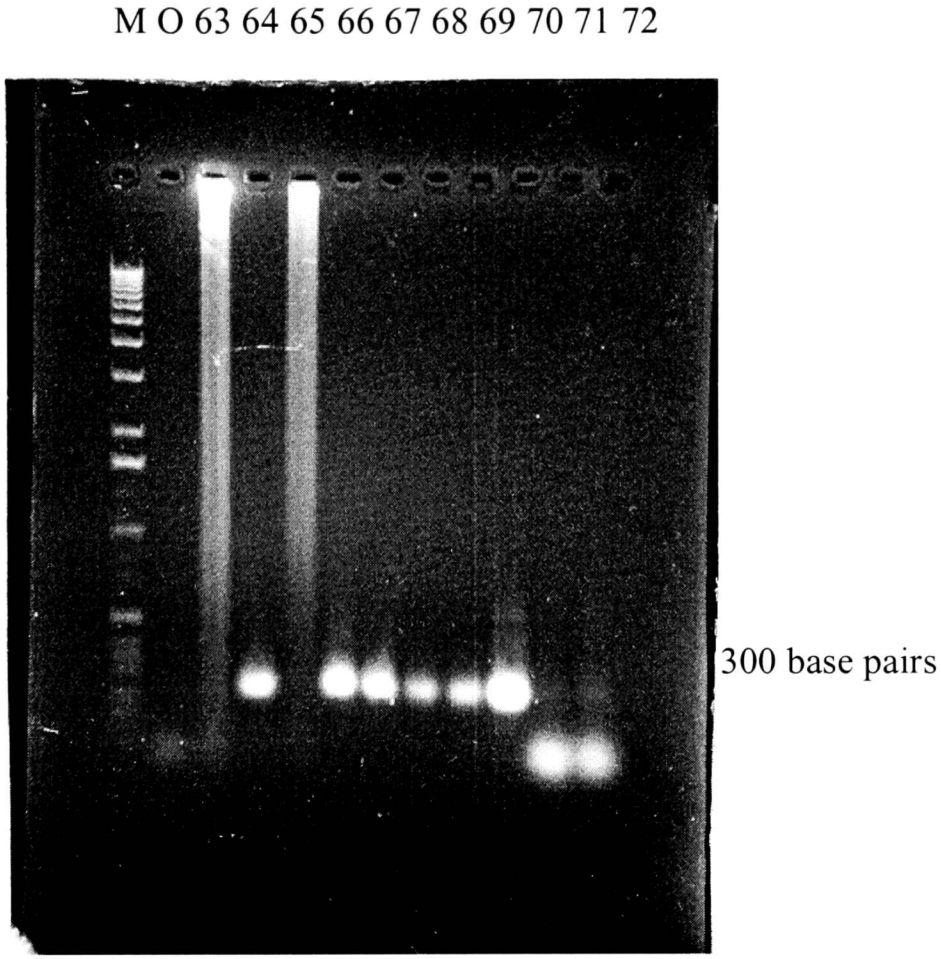


Figure 4. An example of six successful PCR amplifications of the mitochondrial control region target. Bright bands near the 300 base pair location indicate successful amplification of the target region. The smears and empty wells indicate failure for this run. Numbers indicate individual birds, O = negative control, M = 1kb ladder.

67 66 65 64 63 62 61 60 59 58 M 57

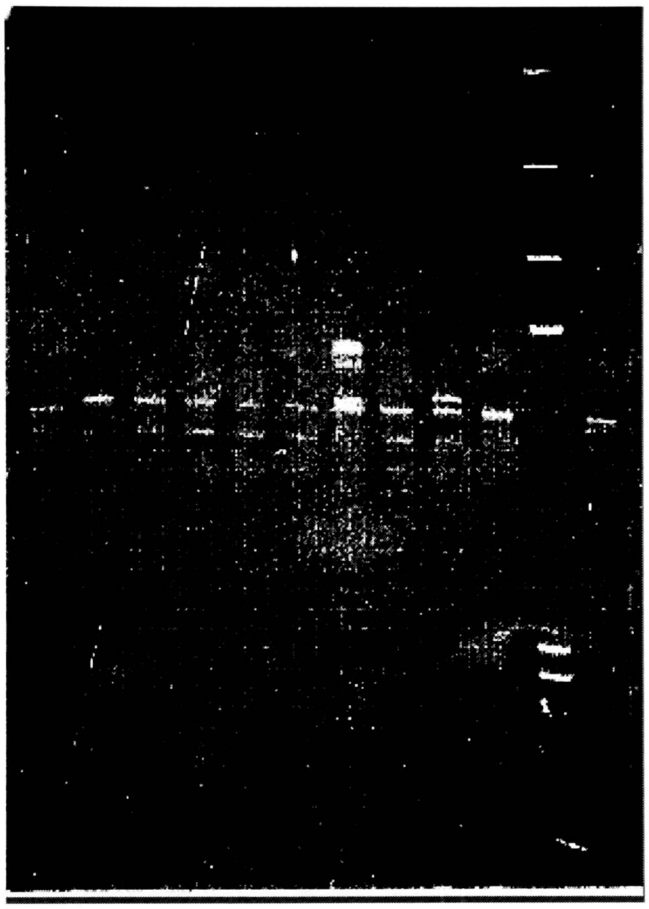


Figure 5. An example of successful SSCP gel electrophoresis. Three different genotypes are indicated in this photo; for individuals 59, 61, and the other (same genotype) individuals. Numbers indicate individual birds, M = 1kb ladder.

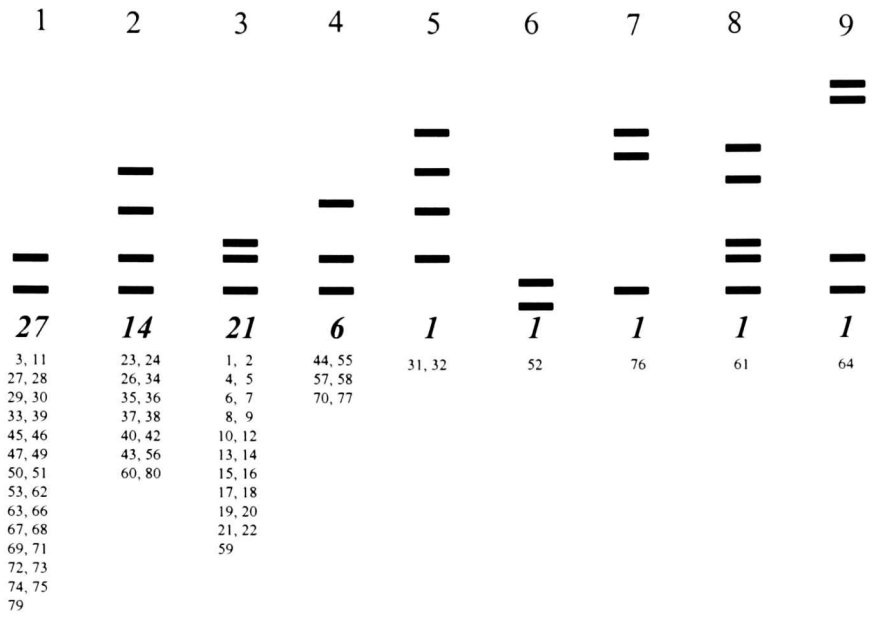


Figure 6. The nine identified genotypes are shown, with the individuals grouped by genotype. Patterns illustrate genotypes shown by SSCP. Genotype number appears at the top, total number of individuals showing this genotype appears at the bottom in italics. The individuals listed in columns exhibited that genotype. Family relationships include: Siblings - 10 & 12 and 28 & 29. A mother and three of her offspring - 71, 72, 73 and 74. Male adults who show a similar foot malformation -4 & 5. The same individual (split sample) - 31 & 32.

Table 1. Frequency of SSCP genotypes of Florida Burrowing Owls among populations and an out group of western Burrowing Owls.

	Population						
Genotype	West	Keys	Miami	Cape Coral	Tampa	N. Central	Eglin
	N = 5	N = 5	N = 24	N = 11	N = 11	N = 6	N = 9
1	0.20	0.40	0.54		0.18	0.17	0.89
2	0.80	0.40	0.125	0.27		0.17	
3			0.04	0.73	0.82	0.33	
4			0.17			0.17	0.11
5		0.20					
6			0.04				
7						0.17	
8			0.04				
9			0.04				

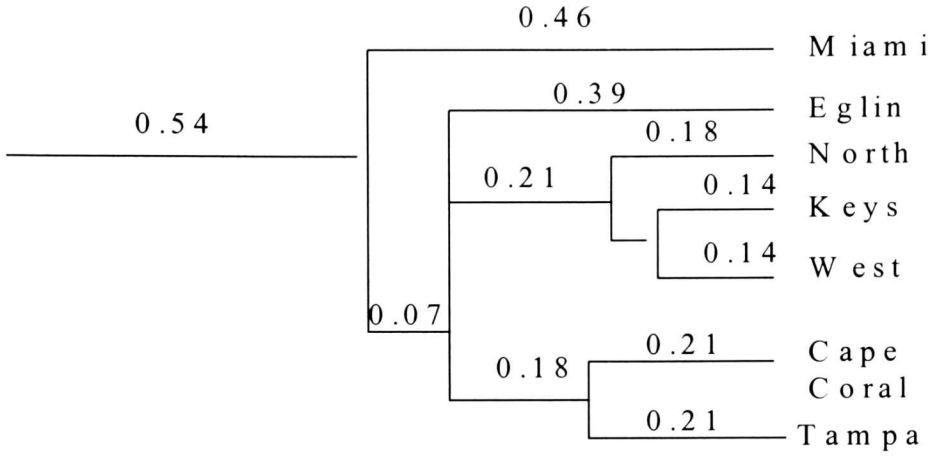


Figure 7. Cluster analysis showing percent similarity of populations of Florida Burrowing Owls, based on genotype frequency as indicated by SSCP.

Discussion

The Florida Burrowing Owl is a Species of Special Concern (FGFWF 1990) whose semi-isolated populations can be benefited, and also threatened, by development and other human activities. Data on genetic population structure are important to assess accurately this subspecies' current status and future prospects. My results indicate that at least some of these populations are genetically distinct based on the frequency of alternative mtDNA genotypes. There are two particularly interesting aspects to this variation.

First, variation revealed by SSCP appears to predate the split between eastern and western subspecies, because *floridana* is not genetically dissimilar from *hypugea*. Thus, the mtDNA variation observed in my study seems to be "older" than the 20,000 years of the generally accepted subspecies split. An alternative possibility is that *floridana* is of more recent origin than previouslythought or *floridana* is subject to ongoing immigration from *hypugea.* The second result is the rather clear split into 3 subgroups of *floridana* populations: Miami, the Gulf coast populations of Cape Coral and Tampa, and the populations of Eglin, the northern counties, and the Keys. This study has identified groups of populations with distinctive SSCP genotype frequencies. It may be important to consider this structure in efforts to manage and conserve populations of Florida Burrowing Owls, thus preserving *floridana's* current level of genetic variation.

Examples of heritable phenotypic variation, such as the recent spread of chocolate-colored irides within the Cape Coral population, highlight the importance of protecting population-level variation. While there is significant genetic structure among populations, the predominate genotypes are present, at least in small proportions, in many populations. If parent-offspring mating is common, and outlying populations can be established by a single mated pair, then the origin of outlying populations remains unclear.

Previous analyses of mtDNA variation indicate that levels of genetic differentiation among populations are often strongly linked to a species' behavioral characteristics. For example, Dunlins (*Calidris alpina*) exhibit genotypes that are highly structured geographically. These long-distance migrants show high natal-site philopatry in breeding females and populations are fragmented in their arctic refugia (Wenink et al. 1993). Well-dispersed species with continuous populations and low breeding site philopatry, such as Red-winged Blackbirds (*Agelaius phoeniceus*) (Ball et al. 1988), show little or no geographic structure within or among populations. Red-cockaded Woodpeckers (*Picoides borealis*), a species with fragmented populations, were found to have intermediate levels of geographic structure to their populations (Haig 1994). The only work to date using SSCP to address population-level variation found geographic structuring in Marbled Murrelets within North America, and variation consistent with species distinction between Asian and North American subspecies (Friesen 1997).

Florida Burrowing Owls are non-migratory and show high breeding site fidelity, and their populations vary from highly isolated to continuous but fragmented, therefore it is not surprising that I detected significant differentiation among populations. The genotype frequencies indicated by this study do suggest a population structure to *floridana* that approximates geographic distribution. The majority of birds on the Keys and in the Miami area were of genotypes 1 and 2. The birds along the Gulf coast of Florida, Cape Coral and Tampa were more likely to be genotype 3.

Three adjacent counties (Suwannee, Gilchrist and Madison) were loosely grouped as North Central Florida. The two Suwannee County birds, the most western of the northern counties, were both of genotype 3, suggesting a possible origin along the Gulf coast. The lone Madison County individual showed a unique genotype. The three individuals from Gilchrist county were of genotypes 1, 2 and 4, suggesting that this population is more similar to Miami than to the Gulf coast populations. It would seem likely that birds disperse from Miami up the central spine of the peninsula. In that case, birds sampled from the Lake Okeechobee region should also be similar to Miami and to Gilchrist County.

The birds of Eglin Air Force Base were 89% genotype 1. This population shows a strong founder effect due, at least in part, to a sampling bias, with a mother and three offspring taken in the sample. Because Genotype 1 is present in all but the Cape Coral populations, the origin of the Eglin birds is not strongly indicated by this study. Five

samples collected from *hypugea* scored as either genotype 1 or 2, so an origin from the Western Burrowing Owl cannot be ruled out for the Eglin population.

The greater variation in genotypes seen in the Miami population may indicate this as an older population, and the more recently established populations of the panhandle and the northern counties as young populations showing a strong founder effect. This result is compatible with the historical dispersal of Burrowing Owls from south to north in the state, and also with observed tendencies toward low natal dispersal distances and close-relationship mating. Small sample sizes in the outlying populations make this determination unclear, however, as the Miami variation may be a product only of a large sample size.

Variation within the *floridana* subspecies seems reasonably consistent with other avian species. I found 9 genotypes in 73 individuals. While no variation was found in Spotted Owls (the only Strigiform yet evaluated), at 23 loci for 107 individuals, the hyper-variable control region was not assayed (Barrowclough and Gutierrez 1990). Dunlins exhibited 35 genotypes in 73 individuals for mtDNA across a huge geographic range (Wenink et al. 1993), Red-winged Blackbirds had 34 genotypes in 127 individuals (Ball et al. 1988), Blue Tits showed nine genotypes in 25 individuals (Taberlet 1992), Redpoll Finches showed 17 haplotypes in 31 individuals surveyed (Seutin et al. 1995), Greenfinches were found to have 18 genotypes in 194 individuals, and 26 genotypes were apparent in 81 Lesser Snow Geese (Quinn 1992).

While the SSCP data indicates genetic variation between individuals and significant genetic structure to *floridana* populations, numerous concerns must be addressed. SSCP has been shown to be highly repeatable in previous studies, but its consistency was ambiguous in this study. Eighteen samples were re-run as a test of repeatability under consistent SSCP conditions. Three samples showed a banding pattern identical to that of their first run. Fifteen samples scored as different genotypes. Thirteen of these samples showed an absence of one or two bands, but did not have a directly contradicting band, and were scored under the genotype showing the most bands. This policy was the most conservative, and did not affect the major conclusions of the study. A low concentration of PCR product in the samples is the probable cause for "missing" bands. Only two samples showed contradictory patterns and remained at odds with their first run. PCR products could not be concentrated because of the great difficulty with which these products were amplified at all.

The inability of the UGA lab to generate clean sequence data, particularly in the areas near the primers, as well as the extreme difficulty encountered during amplification, suggests that the primer fit was not accurate. The primers were designed based upon Martha Desmond's (1997) sequencing. These were interior primers, targeting a rapidly-mutating area of the genome. It is likely that individual variation at the primer location created a poor fit for the primer pairs.

Desmond (1997) sequenced the control region for Western and Florida Burrowing Owls, and found no variation between any individuals at a 771 bp locus. This was an

unexpected result, given the high degree of variation generally observed in vertebrate control regions. A possible explanation proposed by Desmond, and a finding confirmed by others (Lopez et al. 1997, Schneider-Broussard and Neigel 1997, Collulra and Steward 1995), is the incidence of mitochondrial copies being inserted into the nuclear genome. A primer designed for a mitochondrial target might instead amplify a more slowly evolving nuclear copy of the true target, confounding phylogenetic analysis.

Due to a lack of variation in her amplified products, Desmond (1997) suspected that she had amplified a nuclear copy of the mitochondrial control region. It is also known now that some owl species (Spotted Owl, G. Barrowclough, personal communication) have two control regions in their mitochondrial DNA. It is unknown whether Burrowing Owls fall into this category as well. The large number of base pair differences between the sequence data from the pure mitochondrial amplifications and my amplifications indicates that I may have also amplified a nuclear copy. However, the sequence data for the pure mitochondrial amplification does not align with Desmond's sequences.

Difficulty in amplifying products was first assumed to be due to a lack of mitochondrial DNA in the extracted whole genome. Red blood cells do not contain mitochondria, but in birds, they do contain nuclei. Therefore, there was a great deal more nuclear DNA to "confuse" the primer during the amplification process. There was a considerable amount of randomness in the amplifications, as successful amplification

appeared to depend upon the primer locating its mitochondrial target during one of the early cycles in the PCR process. After seeing the sequence results, however, variation in the primer region itself was probably more responsible for the difficulty in amplification.

The results of this study suggest some interesting patterns in the geographic distribution of genotypes. Problems with amplification handicapped the study, but further investigation is certainly warranted. Future conservation management practices for this species will require a better understanding of gene flow, the origin of new populations, and the level of variation within stable populations.

The ideal next step is to sequence directly and map the entire mitochondrial genome for *Athene cunicularia floridana* using primers designed from other species to target conserved regions. This process is both time consuming and expensive. However, once the genome is mapped, a rapidly evolving region, such as the early portion of the control region can be targeted with a more conserved primer. Amplification should run smoothly with a consistently recognized primer, and by concentrating the PCR products, the SSCP procedure should be both unambiguous and repeatable.

A less expensive, intermediate solution is to sequence approximately 2000 base pairs of the mitochondrial genome, including much of the control region, using conserved primers from other avian species. This method would still be expected to yield a primer pair that correctly amplifies the mitochondrial control region, providing the control

region is located in Burrowing Owls in roughly the same location that it is in most avian species. The question of a vestigial control region could not be addressed, and some uncertainty would continue to exist as to the exact origin of amplified products, but by concentrating the PCR products, the SSCP procedure should also become both unambiguous and repeatable.

Any future work should concentrate on increasing sample sizes, particularly in the small, outlying populations. Acquiring samples from the Lake Okeechobee population should also be given a high priority.

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Appendix A. Collection Data for Individual Burrowing Owls included in this study.

Bird #	USFWS Band	Age (a)	Sex (b)	Known Relationships	Location
1	120406113	A	F		Live Oak, Dowling Park, Jeff Warden Pasture, 21317 124th Place
2	120406114	A	F		Live Oak, Dowling Park, Jeff Warden Pasture, 21317 124th Place
3	1204066115	A	F	Mated Pair, with 4	Tampa, McDill Air Force Base
4	1204066116	A	M	Mated Pair, with 4 Malformed Foot	Tampa, McDill Air Force Base
5	1204066117	A	M	Malformed Foot	Tampa, McDill Air Force Base
6	1204066118	A	M		Tampa, McDill Air Force Base
7	1204066119	A	F	Mated Pair, with 8	Tampa, Riverview
8	1204066120	A	M	Mated Pair, with 7	Tampa, Riverview
9	1204066121	A	F		Tampa, Tropical Acres- Unit 1
10	1204066122	J	U	Sibling, with 12	Tampa, Tropical Acres- Unit 2
11	1204066123	A	M		Tampa, Tropical Acres- Unit 2
12	1204066124	J	U	Sibling, with 10	Tampa, Tropical Acres- Unit 2
13	1204066126	A	F		Tampa, Tropical Acres- Unit 3
14	1204066127	J	U		Cape Coral, Unit 1
15	1204066128	J	U		Cape Coral, Unit 2
16	1204066129	A	M	Mated Pair, with 17	Cape Coral, Unit 2
17	1204066130	A	F	Mated Pair, with 16 Dark Eyes	Cape Coral, Unit 2
18	1204066131	A	M		Cape Coral, Unit 1
19	1204066132	A	M		Cape Coral, Unit 4
20	1204066133	A	F		Cape Coral, Unit 5
21	1204066134	J	U		Cape Coral, Unit 8
22	1204066135	A	F		Cape Coral, Unit 8
23	1204066136	A	F	Mated Pair, with 24	Cape Coral, Unit 9
24	1204066137	A	M	Mated Pair, with 23	Cape Coral, Unit 2
25	1204066138	A	F	Parent to 26	Cape Coral, Unit 2

Appendix A. Collection Data for Individual Burrowing Owls (cont.)

26	1204066139	J	U	Offspring of 25	Cape Coral, Unit 2
27	61450101	A	F		Eglin Air Force Base
28	61450103	J	U	Sibling with 29	Eglin Air Force Base
29	61450102	J	U	sibling with 28	Eglin Air Force Base
30	61478141	A	M		Marathon Key, Key Colony Beach golf course
31	61478143	A	F	Same individual as 32 Rehab. Center	Marathon Key, Florida Keys Wild Bird Rehab. Center
32	61478143	A	F	Same individual as 31 Rehab. Center	Marathon Key, Florida Keys Wild Bird Rehab. Center
33		A	U	Rehab. Center	Marathon Key, Florida Keys Wild Bird Rehab. Center
34	61478144	A	M		Marathon Key, Sombrero Beach
35	61478145	A	U		Marathon Key, Colony Beach Golf Course
36					California
37					Nebraska
38					California
39					New Mexico
40					Nebraska
43		A	U		Miami, Dade County, 6105 SW 125 Street
44		A	U		Miami, unknown rehab. Davie area
45	120417091	J	U		Miami, Dade County, Ivanhoe #3
46	120417100	J	U		Miami, Dade County, Ivanhoe #3
47	120417083	A	F		Miami, Dade County, Davie #13
48	61459646	J	U		Miami, Dade County, Davie #13
49	61459647	J	U		Miami, Dade County, Davie #13
50	61459648	J	U		Miami, Miami International Airport #8
51	61459650	J	U		Miami, Dade County, Davie #6
52	61459652	J	U		Miami, Dade County, Davie #8
53	61459657	J	U		Miami, Miami International Airport #9
54	61459659	A	U		Miami, Dade County, Rock Creek #1
55		A	U		Miami, Dade County, 13280 Stirling Rd.
56		A	U		Miami, Dade County, Davie, 14041 SW 20 St.

Appendix A. Collection Data for Individual Burrowing Owls (cont.)

57	61459661	A	U		Miami, Dade County, Davie, 7610 Stirling Road
58	61459662	A	U		Miami, Dade County, Davie, 940 Lexington Ave.
59	61459663	A	U		Miami, Dade County, Davie, 6741 NW 27 Ave.
60	61459664	A	U		Miami, Dade County, 13280 Stirling Road - Sunshine Ranches
61	61459670	J	U		Miami, Dade County, Rolling Oaks
62	61459673	J	U		Miami, Dade County, Rolling Oaks
63	61459674	J	U		Miami, Dade County, Rolling Oaks
64	61459675	J	U		Miami, Dade County, Davie, Majestic Groves
65	61459677	J	U		Miami, Dade County, Davie, Majestic Groves
66	61459679	J	U		Miami, Dade County, Opalocka Airport
67	61459681	J	U		Miami, Dade County, Opalocka Airport
68		J	U		Miami, Miami International Airport #3
69	61459682	J	U		Miami, Dade County, Opalocka Airport
70	61450104	A	F		Eglin Air Force Base
71	61450105	A	F	Mother of 73, 74 and 75	Eglin Air Force Base
72	61450106	A	M		Eglin Air Force Base
73	61450107	J	U	sibling with 74 and 75	Eglin Air Force Base
74	61450108	J	U	sibling with 73 and 75	Eglin Air Force Base
75	61450109	J	U	sibling with 73 and 74	Eglin Air Force Base
76	61484559	J	U		Madison County, 3 km. east of Lee, FL
77	61484561	J	U		Gilchrist County, 8 km SW of High Springs, FL
78	61484575	A	F		Gilchrist County, 8 km SW of High Springs, FL
79	61484556	J	U		Gilchrist County, 8 km SW of High Springs, FL
80	61484571	A	F		Gilchrist County, 8 km SW of High Springs, FL
81	61484570	A	F		Gilchrist County, 8 km SW of High Springs, FL

"Unit" designation corresponds to FGFWFC survey locations.

(a) J - Juvenile birds (in their first year), A - Adult birds, after hatching year

(b) M - male, F - Female, U - Sex unknown

Appendix B.

Sequence of Amplification from Purified Mitochondrial DNA

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  1 NNTNNNNTTN TTNTC TGAAA GCNCATTTGG CGCCATTAGT CATTTNTCAC
 51 GCTCT ATCTT ACTACAAGGA AAGCTGCTTG TCCAGCTGCC CCCTACTCAA
101 CCCCT TTTAT CCCT CCCTAT CCCACTGGTT TGCTCCCTTC AGACATCTCA
151 TCCCTGGCAT CTCT TTCATG TCCTATCTGG GGTCCCCAAA TCCCCTCCCT
201 TGAATACATA CCTT GAATGG AC
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Sequence of Amplification from Whole Genomic DNA (Individual Bird # 80)

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  1 NNTTNAGTTT NANNTTNNAN NNNANNTTAN GNNTNGCNTT CAATTATACT
 51 TTAAGGATTG CNTTCAATTG TACATTAAGT TATATATTCCC ATAATACATA
101 ATATATGTAC TATACACATA TAATGTATGC ATTATATTAA TCAGTATATA
151 AACAGACATA CCTCATATCC ACATTTCTAC TTTCAAGGAA TAATAGAGCA
201 ATGAAGGCTG GAATAACTAC ACTACTTGTA CTAAACCTAT AACTAACAAT
251 ACTACTGTAA ATGGTATATT GCTGAANNAT TGATTNANAG ANAANNNNAN
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