CONSERVATION GENETICS OF THE RED-COCKADED WOODPECKER

by

TRAVIS I. ALSTAD

(Under the direction of Campbell J. Nairn and Robert J. Warren)

ABSTRACT

This project investigated the genetic population structure of the red-cockaded woodpecker (*Picoides borealis*), specifically as it pertains to translocations at three restoration sites and one donor population. We calculated F-statistics in an AMOVA framework using microsatellite loci and mitochondrial haplotypes to assess population differentiation and inbreeding in the red-cockaded woodpecker. The results of the study suggested that range-wide translocations can mitigate some of the detrimental effects associated with population fragmentation. However, the results also showed that there are still sufficient reasons to be concerned about the genetic health of small isolated populations of *P. borealis*. We developed both nuclear microsatellite and mitochondrial genetic markers for this study and these markers could be useful for future studies of red-cockaded woodpecker population characteristics and ecology. In addition, we developed two DNA based methods for sexing *P. borealis* that could be useful for studies of sex-ratio as well as monitoring purposes.

INDEX WORDS: Red-cockaded woodpecker, *Picoides borealis*, translocation, microsatellite, population structure, AMOVA, sex-ratio

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B.S.F.R, University of Georgia, 2007

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2010

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ACKNOWLEDGEMENTS

First, I would like to thank my parents. Their sacrifices and hard work instilled in me the desire and will to succeed in whatever venture I take on. Secondly, I would like to thank my advisors Bob Warren and Joe Nairn for their guidance, wisdom and patience over the past couple of years. They believed in me and gave me a chance to succeed, despite not being the most qualified student to ever apply for graduate school. I would like to thank Brian Shamblin who entertained my endless questions, regardless of whether or not I completely thought them through before asking. I would also like to thank Bob Cooper, Mike Conner, Larry Wood, Phil Spivey, Eric Spadgenske, and especially Jonathan Stober. Without the input of these individuals, this project would not have been possible. Lastly, I would like to thank the Warnell School of Forestry and Natural Resources, the University of Georgia, the Joseph W. Jones Ecological Research Center, Enon and Sehoy Plantations, Georgia Department of Natural Resources, Alabama Department of Natural Resources, the Department of Defense, the U.S. Forest Service, and U.S. Fish and Wildlife Service for their financial support and collaboration.

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CHAPTER 1

INTRODUCTION, LITERATURE REVIEW, OBJECTIVES AND THESIS FORMAT

Introduction

The red-cockaded woodpecker (*Picoides borealis*) population has been severely reduced due to habitat destruction. This species has very specific habitat requirements, including large mature pine trees and general absence of midstory (Jackson 1979, Ligon 1970). Currently, *P. borealis* exists in small populations scattered across the southeastern United States (USFWS 2003). Undoubtedly, this large-scale habitat fragmentation has disrupted the natural rate of gene flow that existed in a population that was once semi-continuous across its range (Schiegg and Walters 2006). Lack of gene flow between populations can cause populations to become genetically divergent from parent populations and, if the fragmentation is severe enough, can result in inbreeding depression (Keller and Waller 2002). Previous studies have shown significant population divergence in *P. borealis* (Stangel et al. 1992, Haig et al. 1994, Haig et al. 1996).

The purpose of this study was to measure inbreeding and population divergence in three restoration populations and one donor population of *P. borealis*. In addition, I was interested in what effects, if any, translocations have had in increasing genetic diversity and decreasing inbreeding. The results of this study can help biologists make more informed decisions in regards to the effects of translocations on genetic diversity and overall genetic health of populations.

Literature Review

Conservation Status

The endangered red-cockaded woodpecker (*Picoides borealis*) once inhabited a nearly continuous range from eastern Virginia to eastern Texas (Ligon 1970). The range of this species has been greatly reduced to small patches of suitable habitat (USFWS 2003). Reduction of habitat is due to an increase in short rotation silviculture in the Southeast (Wood et al 1985), large-scale clear cutting at the turn of the turn of the twentieth century (Lennartz 1983), and fire suppression (Waldrop et al 1992). *P. borealis* has highly specialized habitat requirements. It requires pine trees approximately 80 years old for cavity excavation (Jackson 1979) as well as pine stands with low-basal area and an open midstory (Ligon et al. 1986). Because of the reduction in habitat and the subsequent reduction in population size, *P. borealis* was listed as endangered in 1970 (Meier 1995). *P. borealis* population continued to decline until the early 1990s. Eventually the actions taken by USFWS and other agencies, including artificial cavity installation and intensive habitat management, began to slow the decline of *P. borealis* (Escano 1995). Today *P. borealis* continues to recover. Large-scale translocations of *P. borealis,* to reintroduce it to areas of extirpation and to augment existing small populations, are key to red-cockaded woodpecker population growth. This represents a tremendous undertaking and is an excellent example of cooperation between U.S. Fish and Wildlife Service, Department of Defense, U.S. Forest Service, state wildlife agencies and private landowners (Hamilton 2004).

Breeding Biology

Red-cockaded woodpeckers live in a spatial amalgamation of cavity trees known as a cluster. In addition to the breeding pair, the cluster can contain several helpers which are usually male offspring from previous years (Walters et al. 1988, James 1991). Red-cockaded

woodpeckers are believed to be completely monogamous and the helpers do not breed (Haig et al. 1994). They do, however, assist with cavity excavation, feeding nestlings and territory defense (Ligon 1970, Lennartz et al. 1987). Each bird in the cluster roosts in its own cavity and the breeding male's cavity, which is often the newest, is used for the nest (Ligon 1970). The unusual breeding biology of this species often results in groups of closely related individuals living in close proximity. This phenomenon can influence the genetic population structure of this species.

Population Genetics

Population fragments of red-cockaded woodpeckers range in size from a few individuals to hundreds of breeding pairs and often are separated by great distances. Habitat fragmentation has disrupted the natural gene flow that once would have mitigated inbreeding effects in *P. borealis* populations (Schiegg and Walters 2006). As inbreeding increases, genetic diversity and heterozygosity decrease, and population fragments begin to diverge genetically from each other and the parent population. Documented in many taxa, inbreeding depression arises from reproduction by related individuals and results in a loss of reproductive fitness (Charlesworth 1987, Hedrick 1994). Even at relatively low levels, inbreeding depression has increased extinction risk significantly in experimental and wild populations of many species (Jimenez et al. 1994, Bijlsma et al. 2000). Theoretical models have suggested that inbreeding in wild populations of *P. borealis* can have detrimental effects on hatchling success, recruitment and dispersal, thereby leading to an overall reduction in evolutionary fitness (Daniels and Walters 2000, Schiegg et al. 2006).

Heterozygosity and population divergence are related to inbreeding and molecular markers have been used to quantify genetic diversity of *P. borealis*. Stangel et al. (1992) used

electrophoretic allozyme analyses of samples collected from many sites from Texas to North Carolina and showed that mean-heterozygosity of *P. borealis* was correlated with population size. Smaller populations showed a reduction in heterozygosity, which indicated some divergence effect. Mean-genetic difference between population fragments was correlated to geographic distance, indicating a reduction of gene flow between fragments. Haig et al. (1996) used Random Amplified Polymorphic DNA (RAPDs) and samples from a geographically smaller range of *P. borealis* populations to show that six populations in southern Florida were genetically divergent and that divergence was correlated to geographic distance. Additionally, overall genetic variation within populations was undesirably low.

Previous studies indicate that *P. borealis* populations have experienced some degree of detrimental genetic effects because of population fragmentation (Stangel et al. 1992, Haig et al. 1994, Haig et al. 1996, Schiegg et al. 2002). These studies provide some insight into the genetic health of the endangered red-cockaded woodpecker, but additional studies are warranted. Advances in molecular techniques provide new approaches that offer advantages over previous methods that have been applied to population studies in *P. borealis*. Analyses using RAPDs and electrophoretic allozymes have a lower resolution than the methods more commonly used today and have been shown to be only weakly correlated to more advanced methods, such as Simple Sequence Repeats (SSRs; microsatellites). Additionally, RAPDs provide a high estimation of relatedness between individuals when compared to SSRs (Powell et al. 1996). For these reasons, I chose to use microsatellite genotypes and single nucleotide polymorphisms (SNPs) in mitochondrial DNA sequences for this study.

Study Area

Four study sites, consisting of one donor and three restoration populations of *P. borealis*, were used for this research project. The donor site is the Francis Marion National Forest (FMNF), located near McClellanville, South Carolina, which comprises 102,130ha of mainly open pine forest interspersed with limestone sinks and pocosins. Francis Marion is burned regularly, however, due to close proximity of the Charleston metropolitan area prescribed fire is used less frequently at this site than other sites included in this study. FMNF possesses one of the largest populations of *P. borealis* with approximately 400 breeding groups, and is a donor population that provides birds for smaller populations. This site suffered a near direct hit by hurricane Hugo in 1989. The storm leveled approximately 1/3 of all trees in the forest and 87% of active cavity trees. *P. borealis* population rebounded quickly due to implementation of artificial cavity inserts (Hooper et al 1990). The other sites in this study are restoration populations, meaning that they are currently accepting translocated birds from other larger populations and have not yet reached the current restoration target of 30 potential breeding groups.

The second site was the Joseph W. Jones Ecological Research Center at Ichauway, located in Baker County, Georgia. Ichauway comprises approximately 11,700ha of mainly longleaf pine (*Pinus palustris*) and wiregrass (*Aristada stricta*). The stand-condition at Ichauway is open with low basal area and relative absence of midstory. Ichauway is on a two year prescribed fire cycle and a large portion of the property is managed extensively for *P. borealis*. Management practices at Ichauway include artificial cavity installation and removal of nest kleptoparasites. The population of red-cockaded woodpeckers at Ichauway fluctuates between 10 and 20 breeding groups per year.

The third site studied was Silver Lake Wildlife Management Area (WMA). Formerly known as Southlands Experimental Forest, this site was once the *P. borealis* mitigation site for International Paper and was recently purchased by the Georgia Department of Natural Resources. Silver Lake WMA is approximately 3400-ha of mostly open longleaf pine interspersed with wetlands. Silver Lake WMA is the first site owned by the state of Georgia that is populated with red-cockaded woodpeckers. The population consists of approximately 20 breeding groups, but due to limited space there is little room for on site expansion.

The last population I studied occurred on the Enon and Sehoy Plantations, which are adjacent sites. Together these sites are over 10,100-ha of open grown shortleaf (*Pinus echinata*). This property has been managed extensively for game species such as northern bobwhite (*Colinus virginianus*) and for timber harvest. The property is on a one year prescribed fire regime and has recently begun accepting translocated red-cockaded woodpeckers from other sites. At the time of sampling the population consisted of only five breeding groups, but has since more than doubled due to the efforts of land managers.

Objectives and Format

The main objective of this research was to study the effects of translocations on red-cockaded woodpecker population structure, specifically how it relates to genetic diversity, inbreeding and population divergence. Additional objectives of this project were to test the effectiveness of buccal swabs as a sampling technique for *P. borealis*, develop genetic markers to be used in this project and future projects, and develop molecular methods for sex determination in *P. borealis*. This thesis was written in manuscript format. Chapter 1 is an introductory chapter which includes a literature review of red-cockaded woodpecker conservation status and genetic research. Chapter 2 describes microsatellite marker development

and testing for *P. borealis*. Chapter 3 reports the results of our study examining population structure, inbreeding, genetic diversity and population divergence. Chapter 4 outlines genetic sex identification in *P. borealis*. Chapter 5 is a concluding chapter which summarizes the project and outlines the management implications of our research.

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CHAPTER 2

NOVEL TETRANUCLEOTIDE AND PENTANUCLEOTIDE MICROSATELLITE LOCI IN THE RED-COCKADED WOODPECKER (*Picoides borealis)*

Alstad T.A., B.M. Shamblin, R.J. Warren, J.M. Stober, L.M. Conner, R.J. Cooper, C.J. Nairn. Submitted to *Conservation Genetics Resources*, June 19, 2009.

Abstract: We describe 12 polymorphic tetranucleotide and pentanucleotide loci in The red-cockaded woodpecker (*Picoides borealis*). An average of 6.25 alleles per locus were identified based on a screening of 21 individuals from the Joseph W. Jones Ecological Center in southwestern Georgia. Observed heterozygosity ranged from 0.048 to 0.952. These markers could be used for both population level studies and individual identification.

Keywords: Red-cockaded woodpecker, *Picoides borealis*, microsatellite, tetranucleotide, pentanucleotide

The red-cockaded woodpecker (*Picoides borealis*) is an endangered species native to mature pine forests of the southern United States. Large-scale habitat removal and alteration have drastically reduced *P. borealis* from a nearly continuous range to small pockets of suitable habitat (U.S. Fish and Wildlife Service 2003). Genetic studies are essential to examine the effects of population fragmentation, bottlenecks and inbreeding on this species. It is also of interest to study the consequences of translocations and other restoration efforts on the genetic diversity and viability of *P. borealis*. This paper describes the development of locus-specific primers and PCR conditions for amplification of 11 polymorphic tetranucleotide loci and 1 pentanucleotide locus in *P. borealis*. We included five additional loci that were monomorphic in our samples, but may be useful outside this study population or for inter-species utilization (Table 1).

Microsatellite loci were isolated using the protocol described by Glenn and Schable (2005). Genomic DNA was extracted from a muscle sample taken from a deceased (from natural causes) *P. borealis* specimen using a Charge Switch® magnetic bead isolation kit (Invitrogen). DNA was eluted in 80 uL and digested using *RSAI* endonuclease (New England Biolabs) at 37^{o} C for 1 hour, and 10 μL was separated on a 1% agarose gel for 1hour at 100 volts to evaluate digestion. Double-stranded SuperSNX linkers (Glenn and Schable 2005) were prepared and ligated to the digested DNA and ligation success was verified using PCR and agarose gel electrophoresis. All PCR reactions were conducted using an Applied Biosystems 9700 thermocycler. Digested DNA was hybridized to three different mixtures of biotinylated oligonucleotide probes (Integrated DNA Technologies). Mix 1: $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$. Mix 2: $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTG)_6$. Mix 3: $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$. The

hybridized DNA and biotinylated probes were captured using streptavidin coated magnetic beads (DynaBeads®, Invitrogen®). Samples were washed and enriched DNA was eluted and amplified using PCR. The enrichment was repeated once. Enriched DNA was ligated into the pCR2.1 TOPO vector and transformed into TOP 10 competent cells using the TOPO TA ® cloning kit (Invitrogen®). A total of 664 colonies was screened, purified using ExoSap-IT® (New England Biolabs), and sequenced using BigDye v3.1 (Applied Biosystems) chemistry. Sequence reactions were cleaned up using Sephadex G-100 Fine (Sigma Aldrich) and analyzed on an Applied Biosystems 3730*xl* DNA Analyzer. Trace files were trimmed, edited and assembled in Seqman® (Lasergene®), and contigs were screened using MSATCOMMANDER (Faircloth 2008). We selected 42 microsatellite-containing sequences and designed primers using Primer 3.0 (Rozen and Skaletsky 2000). PCR amplification was conducted using an untagged locus-specific primer containing a GTTT pig-tail, combined with a CAG (CAGTCGGGCGTCATCA) or M13 (GGAAACAGCTATGACCAT) tailed locus-specific primer and a fluorescently labeled CAG or M13 primer (Boutin-Ganache 2001). Fluorophores used for labeled primers included HEX, FAM (Integrated DNA Technologies) and NED (Applied Biosystems) and were scored using a ROX size standard (Applied Biosystems). Two modifications were incorporated into the original protocol. The concentration of the tailed locus-specific primer was increased four-fold to 0.2µM. In addition, the denaturing and annealing time were increased from 20s to 30s. Cycling parameters (Don et al. 1991) were 95^{o} C for 5 min, 21 cycles of 95^{o} C for 30s, highest starting T_m (65^{o} C or 63^{o} C) minus 0.5^{o} C per cycle for 30s and 72^{o} C for 1 min followed by 24 cycles of 95^{o} C for 30s, 54.5^{o} C for 30s, 72^{o} C for 1min, and a final extension of 72^{o} C for 10 min. Primer-specific initial annealing temperatures are listed in Table 1. Amplicon sizes were resolved on an Applied Biosystems 3730*xl* DNA Analyzer and

scored in GENEMAPPER (Applied Biosystems). Primer pairs for 18 microsatellite loci produced consistent amplification and were screened for polymorphisms using total genomic DNA isolated from 21 *P. borealis* individuals from the Joseph W. Jones Ecological Research Center. Twelve loci were found to be polymorphic and 5 loci were found to be monomorphic (Table 1). For the 12 polymorphic loci, GENEMAPPER genotype data were converted using GMCONVERT (Faircloth 2006) and imported into Cervus (Kalinowsky 2007) to calculate allelic richness (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and polymorphism information content (PIC). The number of alleles at each locus ranged from 2 to 10 and averaged 6.25 alleles per locus. Data were imported into GENEPOP (Raymond and Rousset 1995) to calculate deviations from Hardy-Weinberg equilibrium (HWE). One locus, *Pbr*108, differed significantly ($P<0.05$) from HWE following sequential Bonferonni correction (Rice 1989). Another locus, *Pbr*121, did not fulfill the requirements to calculate deviation from HWE in GENEPOP. No linkage disequilibrium was detected between loci. The deviation of *Pbr*108 from HWE could be due to several factors including the small sample size of the test population, the locus being under selection or non-random mating. Additionally, we hypothesize that the diverse origins of the study population could cause *Pbr*108 to deviate from HWE. Over the past 12 years, numerous birds have been translocated from larger, more stable populations to the Jones Center. Only one male existed at the Jones Center in the mid 1990s, and most birds in the population are either translocated birds or the descendants of translocated birds. Further research is necessary to test this hypothesis.

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Table 2.1 Twelve polymorphic microsatellite loci and five monomorphic loci in *P. borealis*

Locus	Primer Sequence 5' to 3'	GenBank Asc. #	TM °	A	Ho	He	PIC
*Pbr*101	F: (CAG)CAACATCTGCAATCCATGTAACTT	GQ272472	65	4	0.714	0.605	0.515
	R: (GTTT)GCTCCAAATCTTTGATTCTGTTTT						
*Pbr*104	F: (GTTT)TGCCTGTTGCAAGCTAAGAA	GQ272475	65	9	0.857	0.818	0.779
	R: (CAG)GGGAGTCTGGGTATTTGCAC						
*Pbr*106	F: (CAG)TCATCAGCCTCAAGTCCACA	GQ272476	65	10	0.952	0.847	0.805
	R: (GTTT)AGAGCTGAGGAGCCATGAGA						
*Pbr*108	F: (CAG)TGGGAGGTTCCATAAAATCAA	GQ272478	65	6	0.381	0.707	0.637
	R: (GTTT)GCACTGAACAGGAGGAAATTG						
*Pbr*110	F: (M-13)AGAGACCCATGCTGGTCAAG	GQ272479	65	10	0.857	0.87	0.833
	R: (GTTT)TATTGCCAAAGGGAATCTGC						
*Pbr*117	F: (CAG)TGCAAGTATTTCTTCCTATGGGTA	GQ272482	65	4	0.429	0.742	0.674
	R: (GTTT)GCACACACATGCACACTTAAAG						
*Pbr*121	F: (M-13)GCCTCTTCTTTCAAGCACAATT	GQ272483	65	2	0.048	0.048	0.045
	R: (GTTT)CTAGCAGAATCACTGGGCTGT						
*Pbr*127	F: (M-13)TCCATCTCTCTAGCTCTAGCTCTGT	GQ272484	63	7	0.81	0.801	0.75
	R: (GTTT)AGCAGAATCTCTTCTTCGGTGA						
*Pbr*128	F: (CAG)CAAATGCTTTGGAAACTCTCAG	GQ272485	63	6	0.714	0.655	0.602
	R: (GTTT)TTCACACCTCTGGATTGTTCAC						
*Pbr*129	F: (CAG)TCACCAGAACAAGAGGACACAG	GQ272486	63	4	0.19	0.182	0.172
	R: (GTTT)AGCAGAATCCAACTGACAACAA						
*Pbr*135	F: (CAG)ATCCCCAGTGGTGTAAGTCATC	GQ272487	63	3	0.667	0.557	0.486
	R: (GTTT)GCAGAGTTAAGGGAAGTGATGG						
*Pbr*155	F: (CAG)AGCCACCCTTCAGGTAGTTGTA	GQ272488	65	10	0.905	0.851	0.81
	R: (GTTT)AATTGAGATGCTGAGGATGCTT						
Monomorphic Markers							
*Pbr*102	F: (CAG)AAGATCATCCAAGCCCTCTG R: (GTTT)CCCTTGCCCTATCACAACAG	GQ272473	65	-	-	-	-
*Pbr*103	F: (CAG)GGTGTTGGGTGATAGGTTGG	GQ272474	65	-	-	-	-
	R: (GTTT)GCCAGTGTGATGGATATCTGC						
*Pbr*107	F: (CAG)AAGAGGGCCAATGACATCC	GQ272477	65	-	-	-	-
	R: (GTTT)GCCACACTGCTCCTGATACA						
*Pbr*112	F: (CAG)GGGAGCAGTCATTTAATAATCAG	GQ272480	65	-	-	-	-
	R: (GTTT)TGTCAGTCATCTCTTCTGGTGA						
*Pbr*115	F: (CAG)AATCACTGTGTGGGAGTGAATG	GQ272481	65	-	-	-	-
	R: (GTTT)CCCTCACTTGGTTTTCAAGTTC						

CHAPTER 3

GENETIC POPULATION STRUCTURE OF THE RED-COCKADED WOODPECKER IN DONOR AND RESTORATION POPULATIONS

Alstad T.A., B.M. Shamblin, R.J. Warren, J.M. Stober, L.M. Conner, R.J. Cooper, L.A. Wood and C.J. Nairn. To be submitted to *Conservation Genetics*.

ABSTRACT

The endangered red-cockaded woodpecker (*Picoides borealis*) has experienced severe decline and population fragmentation since at least the late 1800s. In the 1990s, programs using translocations from larger, stable populations to small, remnant populations and areas of extirpation were implemented. We examined genetic diversity in one large donor and three small restoration populations of *P. borealis* using multi-locus microsatellite genotypes and mitochondrial haplotypes in an AMOVA framework. Population differentiation (F_{ST}) was lower than reported in previous studies, which were conducted prior to large-scale translocations of *P. borealis* throughout its geographic range. We detected significant inbreeding coefficients for all populations studied. An analysis of haplotypes from the mitochondrial control region showed significant population differentiation, demonstrating the effects of drift on small, isolated populations. The results of this study suggest that translocations have had a positive effect in mitigating inbreeding and population differentiation. However, inbreeding and differentiation remain a problem due to the reduction or loss of natural mechanisms and rates of gene flow in this once widely distributed population.

Introduction

The red-cockaded woodpecker (*Picoides borealis*) is endemic to pine communities, predominantly longleaf pine (*Pinus palustris*), of the southeastern United States and was once distributed from Virginia through southern Florida and eastern Texas (Ligon 1970). Large-scale habitat loss has reduced the distribution and abundance of the endangered red-cockaded woodpecker (*Picoides borealis*) to a fraction of its former range (U.S. Fish and Wildlife Service 2003). Reduction of longleaf pine ecosystems to less than 5% of their pre-colonial distribution, extensive logging of older trees required for nesting, and fire suppression are major contributors to *P. borealis* population declines (Walters et al. 2002). Biologists have estimated as much as a 99% reduction in *P. borealis* population size (Costa and Jordan 2003).

The species was listed as endangered in 1973 and its decline continued until at least the early 1990s (James 1991). Currently, *P. borealis* inhabits small fragmented islands of suitable habitat throughout the southeastern United States. Population fragments range in size from a few individuals to a few hundred breeding pairs and often are separated by large expanses of unsuitable habitat (Conner and Rudolph 1989, Costa and Jordan 2003, U.S. Fish and Wildlife Service 2003). Habitat fragmentation can disrupt dispersal and the gene flow necessary to mitigate inbreeding effects in *P. borealis* populations. As a consequence, small isolated populations that experience few dispersal events are subject to inbreeding depression (Schiegg et al. 2002).

Red-cockaded woodpeckers live in a spatial amalgamation of cavity trees known as a cluster. In addition to the breeding pair, the cluster can contain a several helpers which are usually male offspring from previous years (Walters 1988, James 1991). Red-cockaded woodpeckers are believed to be completely monogamous and the helpers do not breed (Haig

1994a). They do, however, assist with cavity excavation, feeding nestlings and territory defense (Ligon 1970, Lennartz et al. 1987). Each bird in the cluster roosts in its own cavity and the male's cavity, which is often the newest, is used for the nest (Ligon 1970). A cluster that contains an adult male and an adult female is referred to as a potential breeding group (PBG). The unusual breeding biology of this species often results in groups of closely related individuals living in close proximity, which may influence the genetic population structure of this species.

Theoretical models have suggested that inbreeding in wild populations of *P. borealis* can have detrimental effects on hatchling success, recruitment and dispersal, thereby leading to an overall reduction in evolutionary fitness (Daniels and Walters 2000, Schiegg et al. 2006). Genetic diversity is correlated with population size, which places small populations at greater risk of losing diversity through drift than large populations (Nei 1987, Frankham 1995). Inbreeding depression has been documented in many taxa and arises from reproduction by related individuals, which can reduce reproductive fitness (Charlesworth and Charlesworth 1987, Hedrick 1994). In both experimental and wild populations of many species, even relatively low levels of inbreeding depression have increased the risk of extinction significantly (Jimenez et al. 1994, Bijlsma et al. 2000).

Previous studies indicate that *P. borealis* populations have experienced detrimental genetic effects as a result of population fragmentation (Stangel et al. 1992, Haig et al. 1994b, Haig et al. 1996, Schiegg et al. 2002). The first range-wide study of genetic diversity and structure in *P. borealis* populations examined allozymes representing 16 genetic loci (Stangel et al. 1992). This study reported a mean fixation index of $F_{ST} = 0.14$ for the 26 populations examined. Results of a subsequent analysis using random amplified polymorphic DNA (RAPD) markers were generally consistent with those from allozymes (Haig et al. 1994b). The study

indicated that genetic variance within *P. borealis* population was undesirably low with F_{ST} = 0.19 (Haig et al. 1994b), which is considerably higher than most species of birds with stable populations (Evans 1987). Subsequent RAPD analysis that included south Florida populations of *P. borealis* reported a F_{ST} of 0.21, further indicating low genetic diversity and significant levels of sub-population differentiation (Haig et al. 1996).

Considerable efforts have been directed towards reintroduction of *P. borealis* in areas of extirpation and augmentation of small populations with translocations from larger, more stable populations (Carrie et al. 1999, Costa 2003). Translocations have proven instrumental in increasing the size of small populations, but effects on the genetic population structure of *P. borealis* have not been well characterized. In general, the relationship between the practice of establishing populations through translocations and subsequent effects on genetic diversity is poorly understood (Hendrickson and Brooks 1991, Stockwell et al. 1996). Several studies of other species examining the genetic structure of populations founded by translocations have reported increased divergence and reduction in allelic diversity due to drift in founder populations compared to remnant populations (Sigg 2006, Stockwell et al. 1996).

We utilized 10 novel nuclear microsatellite markers and mitochondrial DNA (mtDNA) sequencing to examine and compare genetic diversity in and among one large remnant (donor) population and three restoration populations of different sizes that are recipients of *P. borealis* translocations. These genetic approaches may be useful in characterizing genetic diversity in extant populations and the effects of translocation on genetic structure of populations undergoing restoration.

Methods

We collected samples from one donor and three restoration populations of *P. borealis* (Figure 1). The donor population, Francis Marion National Forest, South Carolina, contains approximately 400 potential breeding groups (PBGs). Restoration populations sampled were the Joseph W. Jones Ecological Research Center at Ichauway (hereafter, Ichauway; 17 PBGs), Silver Lake Wildlife Management Area (hereafter, Silver Lake; 17 PBGs) and Enon and Sehoy Plantations (hereafter, Enon; 6 PBGs). On-site biologists collected buccal swabs opportunistically during routine banding of nestlings as part of population monitoring and recovery efforts. Adults were captured using pole nets. At Francis Marion, one nestling was randomly chosen and swabbed per nest from 55 randomly selected nests in this donor population, during April and May of 2008 and 2009. We also obtained buccal swabs from 20 adults being translocated from Francis Marion to other populations. At Ichauway, 102 individuals were sampled from 25 clusters during 2007 - 2009. At Silver Lake, 46 individuals were sampled from 14 clusters during 2008 and 2009. At Enon, 14 individuals were sampled from six clusters during 2008.

Birds were swabbed in the inside cheek using Isohelix buccal swabs (Epicentre Biotechnologies) for 15s. Swabs were air dried and stored at -20o C. Genomic DNA was extracted from buccal swabs using a Charge Switch® kit (Invitrogen®). DNA was eluted in 10 mM Tris-HCl pH 8.0 and stored at -20o C. A 1020 base pair region of the mitochondrial control region was sequenced. It was initially amplified and sequenced using universal avian primers L15710 and H1251 (Sorenson et al. 1999). The amplified region was cloned in a TOPO TA® cloning kit (Invitrogen®) and sequenced using M-13 forward and reverse primers and BigDye 3.1 (Applied Biosystems). Four specific primers DL10F, DL13R, DL596F and DL758R, (Table

1) were designed to amplify two fragments covering the 1020 bp region and tagged with M-13 primer sites for subsequent DNA sequence analysis. Primers were designed using Primer-3 (Rozen and Skaletsky 2000) and synthesized by Integrated DNA Technologies. PCR amplification was performed in 20 μL reactions containing 10 ng genomic DNA, 1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl), 100 μg/ml bovine serum albumin (New England Biolabs), 2.5 mM MgCl2 Solution, 0.5 μM of each primer, 0.25 mM dNTPs and 1.5U Amplitaq Gold (Applied Biosystems). Polymerase chain reaction (PCR) was performed on an Applied Biosystems 370 or 9700 thermocycler using the following conditions: one denaturing step at 95 o C for 5 minutes, followed by 45 cycles of 95 o C for 30 s, 51 o C for 30 s and 72 o C for 1 min, and a final extension of 72 o C for 10 min. PCR amplicons were resolved on 1% agarose gels and purified using ExoSAP-IT (New England Biolabs). The control region was sequenced using M-13 forward and reverse primers and 4 internal primers (Figure 2), BigDye 3.1 (Applied Biosystems), analyzed on an Applied Biosystems 3730*xl* data analyzer, and assembled and edited in SeqMan® (Lasergene®).

Samples were genotyped using 10 microsatellite loci designed for *P. borealis* (Alstad et al. 2009). We tagged either the forward or reverse primer with a M-13 or CAG tag to attach a fluorophore label for 3-primer PCR (Boutin-Ganache et al. 2001). PCR was conducted in a 10 μL reaction containing 10 ng genomic DNA, 1X PCR Buffer, 100 μg/ml bovine serum albumin, 2.5 mM MgCl2, 0.25 mM dNTPs, 0.5 μM untagged primer, 0.05 μM tagged primer, 0.45 μM fluorophore tag, and 0.5 U Amplitaq Gold (Applied Biosystems). Cycling parameters (Don et al. 1991) were 95 o C for 5 min, 21 cycles of 95 o C for 20 s, the highest starting TM minus 0.5o C per cycle for 30s and 72 o C for 1 min. followed by 24 cycles of 95 o C for 30 s, 54.5 o C for 30 s and 72 o C for 1 min, and a final extension of 72 o C for 10 min. Amplicons were resolved on

an Applied Biosystems 3730*xl* DNA analyzer and scored in GENEMAPPER. Genotypes were exported and converted in GMCONVERT (Faircloth 2006) for use in statistical analysis.

Statistical Analyses

Data were analyzed using two approaches. First, analyses were conducted with the individual as the sample unit. This method may have been biased due to related individuals within a cluster constituting non-independent samples. A second set of analyses also was conducted using one randomly selected individual from each family cluster.

Microsatellite genotypes from all populations were used to calculate observed heterozygosity (H_O), expected heterozygosity (H_E) and mean number of alleles per locus (A) in CERVUS (Kalinowski et al. 2007). We paired loci to test for inter-population differences in heterozygosity and mean number of alleles between populations using Wilcoxon's signed-rank tests. For all populations, differences between expected and observed heterozygosities were tested for significance using a Wilcoxon's signed-rank test. Genotypes were imported into ARLEQUIN (Excoffier et al. 2005) to conduct an analysis of molecular variance (AMOVA) and calculate fixation indices of F_{ST}, F_{IS} and F_{IT} using the sum of squared differences model (R_{ST}) method (Slatkin 1995) and tested for significance (10,100 permutations). Population-specific F_{IS} was also calculated in ARLEQUIN and tested for significance (10,100 permutations). Linkage disequilibrium (1,023 permutations) and Hardy-Weinberg Equilibrium (Markov Chain 10,000; dememorization 5,000) were measured and tested for significance in ARLEQUIN followed by *a posteriori* sequential Bonferroni correction (Rice 1989). Population structure for all four populations was tested using the Bayesian clustering algorithm STRUCTURE (5,000 Burnin; 10,000 MCMC) using an admixture model (Pritchard et al. 2000). In addition, our two most extensive data sets (Francis Marion and Ichauway) were tested in STRUCTURE using a no-

admixture model both with and without *a priori* putative population of origin data. The Francis Marion population was tested for heterozygote excess using BOTTLENECK (Cornuet and Luikart 1996).

Sequences of the mitochondrial control region were used to calculate allelic richness (AR). Haplotype data were imported into ARLEQUIN to conduct an AMOVA, calculate Φ_{ST}, and test for significance (10,100 permutations) using a genetic distance method that accounted for differences in transition and transversion rates as well as differences between purine and pyrimidine transitions (Kumar et al. 1993, Tamura & Nei 1993).

Results

We genotyped 193 individuals from the three small restoration populations and one large donor population using ten nuclear microsatellite markers. We obtained high quality sequences from a 1020 base pair region of the mitochondrial control region for 209 individuals from the same four populations. Pairs of loci in linkage disequilibrium (LD) varied between populations ranging from zero at Silver Lake to five pairs at Francis Marion (Table 2). Enon and Ichauway had one pair each in linkage disequilibrium. No pair of loci in LD appeared in more than one population. Loci differing significantly ($P < 0.05$) from Hardy-Weinberg equilibrium (HWE) also varied between populations. One locus, *Pbr108*, deviated significantly from HWE in all populations sampled and was removed from further analyses. Two loci (*Pbr104*, *Pbr106*) were out of HWE in three of the four populations sampled. Loci not in HWE ranged from 3 to 8 per population.

The number of alleles per nuclear locus, averaged across all loci, ranged from 4.1 to 7.9. Expected heterozygosity (H_E) ranged from 0.7087 at Ichauway to 0.7294 at Silver Lake (Table 3). There was no significant difference in H_E for any pair-wise population comparison. For all

populations, observed heterozygosity (H_O) was significantly lower than H_E ($P < 0.05$). Observed heterozygosity ranged from 0.4486 at Silver Lake to 0.6554 at Ichauway. Observed heterozygosity, when analyzing one individual per cluster, was significantly greater at Ichauway than Enon ($P = 0.038$), Silver Lake ($P = 0.007$), and Francis Marion ($P = 0.009$). When analyzing all individuals sampled, H_O was significantly greater at Ichauway than Silver Lake ($P = 0.005$) and Francis Marion ($P = 0.022$). Regardless of the sampling unit, all other pair-wise comparisons of H_O between populations were not statistically significant. No significant ($P <0.05$) bottleneck as measured by heterozygote excess was detected in the Francis Marion donor population.

Population-specific inbreeding coefficients (F_{IS}) ranged from 0.0134 to 0.3694 and varied based on whether data were analyzed using all individuals or one individual per cluster (Table 3). When data were analyzed with all individuals included, F_{IS} was significantly greater than zero ($P < 0.05$) for all populations except Enon. When analysis was conducted using one individual per cluster, F_{IS} was significantly greater than zero ($P < 0.05$) for all populations except Ichauway. F_{IS} was highest at Silver Lake and Francis Marion and lowest at Ichauway and Enon.

All fixation indices (Table 4) were significantly greater than zero ($P < 0.05$), regardless of whether all individuals or one individual per cluster was analyzed. Fixation indices, when including all sampled individuals, were $F_{ST} = 0.0804$, $P < 0.0001$; $F_{IS} = 0.2497$, $P < 0.0001$; and $F_{IT} = 0.3101$, $P < 0.0001$. The AMOVA partitioned genetic variance as follows: within populations = 8.04%, among individuals within populations = 22.96%, and within individuals = 69.00%. Fixation indices, when including one randomly selected individual from each cluster, were $F_{ST} = 0.0751$, $P = 0.001$; $F_{IS} = 0.2971$ $P < 0.0001$; and $F_{IT} = 0.3499$, $P < 0.0001$. The AMOVA for one randomly selected individual partitioned genetic variance as follows: within

populations = 7.50%, among individuals within populations = 27.48%, and within individuals = 65.02% (Table 4).

Analysis in STRUCTURE did not detect the presence of population structure when all populations were included. When only the two larger, more extensively sampled populations, Francis Marion and Ichauway, were included in the analysis, STRUCTURE correctly assigned 93% of individuals when a putative population of origin was included and 91.5% with no *a priori* population assignment.

Sequencing of mtDNA identified 22 unique haplotypes among the populations (Table 5). Allelic richness (NA) (Table 6) was highest at Ichauway (NA = 13) and lowest at Enon (NA = 5). Francis Marion and Silver Lake had 8 and 6 haplotypes, respectively. Haplotypic fixation indices for all individuals was φST = 0.2479 (P = 0.000), compared to φST = 0.2685 (P = 0.000) for one individual per cluster.

Discussion

Seven pairs of loci were in linkage disequilibrium throughout the sampled populations. LD can be attributed to loci being under selection, physical linkage, genetic drift or gene flow from populations with differing allele frequencies (Kimura and Ohta 1971, Ohta 1982). Because no pair of loci in LD appeared in more than one population, it is unlikely that any of the loci pairs are physically linked. A low rate of gene flow due to significant geographic isolation, lack of translocations, and subsequent random drift may have affected linkage disequilibrium in the Francis Marion population. In addition, the Francis Marion population experienced a genetic bottleneck when Hurricane Hugo struck in 1989. Prior to Hurricane Hugo, this was the second largest population of red-cockaded woodpeckers, but the storm resulted in the loss of 70% of the birds in the population (Hooper et al. 1991). In addition, 87% of active cavity trees, a resource

essential to the survival of *P. borealis*, were lost (Hooper et al 1990). Other possible reasons for LD in the study populations are high rates of gene flow (translocations) in restoration populations, inbreeding, and drift. Each of the populations had between three and eight loci that departed significantly from HWE. All deviations from HWE were due to a significant ($P < 0.05$) heterozygote deficiency. We predicted deviations from HWE in the restoration populations (Enon, Ichauway, and Silver Lake) because of small population size and potential inbreeding. Gene flow is a dominant force influencing allele frequencies and HWE in these restoration populations. The relatively high rate of translocations (>2 birds per year) combined with the low rate of emigration violates the key assumption of HWE that the effects of migration on allele frequencies is negligible.

For all populations H_O was significantly lower than H_E ($P < 0.05$). H_E did not significantly differ between populations. H_O was significantly greater at Ichauway than other populations. We detected significant inbreeding (F_{IS}) in all populations examined. Francis Marion had a moderate level of inbreeding ($F_{IS} = 0.29$ for one individual per cluster sampled). While this could be an artifact of the Hurricane Hugo bottleneck, other mechanisms may also influence measures of inbreeding in this population. In general, bottlenecks will produce a heterozygote excess (Cornuet and Luikart 1996); however, tests using BOTTLENECK showed that Francis Marion had a heterozygote deficiency. After the bottleneck, the Francis Marion population recovered quickly due to the development of artificial cavity management techniques (Hooper et al. 1991). Demographically and genetically speaking, this practice may have limited detrimental effects because bottlenecks of multiple generations cause more severe reductions in genetic diversity than single generation bottlenecks, even if the single generation bottleneck is

more severe (England et al. 2003, Freeland 2005). Therefore, inbreeding and lack of immigration may have been major contributors to F_{IS} levels in the Francis Marion population.

Ichauway had the lowest inbreeding coefficient (F_{IS} = 0.172 for one individual per cluster and F_{IS} = 0.120 for all individuals sampled). Although Ichauway had the lowest inbreeding coefficient of populations examined, it was still significant and represents moderate inbreeding. We speculate that a population of approximately 17 PBGs would have a much higher F_{IS} if it were not for the many translocations it has received over the past 10 years. The diverse origins of donor birds at Ichauway have likely contributed to the low F_{IS}.

The highest F_{IS} was recorded in Silver Lake (F_{IS} = 0.485 for one individual per cluster and F_{IS} = 0.346 for all individuals sampled). The exact translocation history of this population is unknown. However, several translocations and at least one unaided immigration have occurred at this site in recent years (Phil Spivey, personal communication). One possible explanation of the high F_{IS} at Silver Lake is the lack of available recruitment clusters. With approximately 3400 ha of suitable habitat, Silver Lake is considerably smaller in geographic area than the restoration areas at Ichauway and Enon. Therefore, dispersing red-cockaded woodpeckers at Silver Lake may be more likely to choose a cluster already inhabited by a related individual. The only instance of non-significant, population-specific F_{IS} was at Enon, when all individuals were analyzed. Interestingly, removing related individuals from the analysis caused the population-specific F_{IS} for Enon to become significant. Because of an extremely small population and an extremely small sample size, it is possible that sampling error caused the cluster method to be significant.

We found that inbreeding coefficients were similar for both small restoration and large remnant populations of red-cockaded woodpeckers. In general, small populations would be

expected to have a higher level of inbreeding due to low probability of selecting a non-related mate. Because of similar inbreeding coefficients between restoration and remnant populations, we theorize that high rates of gene flow via translocations are moderating the effects of inbreeding in these small populations. However, because of the recent bottleneck in our large donor population, and the limited scope of this study, further investigations throughout the range of the species will be necessary to better understand the relationship between translocations and inbreeding. The highly significant inbreeding coefficient ($P < 0.0001$) in the larger remnant population at Francis Marion highlights the need to establish and expand populations in a manner that will facilitate gene flow between both small and large populations.

Fixation indices were significant in all populations examined with $F_{ST} = 0.0751$ for one individual per cluster and $F_{ST} = 0.0804$ for all individuals. However, fixation indices were lower than in previous studies. Stangel et al. (1992) and Haig et al. (1994b, 1996) calculated fixation indices of $F_{ST} = 0.14$, $F_{ST} = 0.19$ and $F_{ST} = 0.21$, respectively. One study calculated the average F_{ST} for 23 species of non-endangered, widely distributed birds to be 0.05 (Evans 1987). Direct comparisons between studies are difficult due to the different types of markers utilized. However, this analysis using microsatellite loci is consistent with previous studies using allozymes and RAPDs in suggesting that fragmented *P. borealis* populations are significantly diverged.

As expected, Φ_{ST} (0.2479) was considerably higher than nuclear F_{ST}; Φ_{ST} is more sensitive to drift than nuclear F_{ST} because it is only inherited maternally and not randomly segregated. The high Φ_{ST} demonstrates that, although there has been significant progress in red-cockaded woodpecker restoration efforts, small isolated populations remain susceptible to loss of diversity through drift.

The current red-cockaded woodpecker recovery plan states that one objective of translocations is the reduction of isolation between sub-populations (USFWS 2003:94). It is possible that the reduction in F_{ST} between the early/mid 1990s and the present reflects some level of success in meeting this objective. If so, it is more likely a result of the continuing translocations facilitating gene flow rather than an actual reduction in geographic and reproductive isolation. There is still a great need to establish and expand populations in a manner that facilitates gene flow from a spatial perspective. Once translocations cease, processes associated with reproductive isolation such as genetic drift and inbreeding will continue to affect the genetic population structure of this species. This assumption is supported by studies in other species examining the effects of translocations (Stockwell et al. 1996, Sigg 2006).

There is considerable debate on the role translocations may play in disrupting the natural genetic structure of populations and reducing genetic diversity through post-translocation drift (Forbes et al. 1995, Houlden et al. 1996, Stockwell et al. 1996, Williams et al. 2000). Because translocations to *P. borealis* restoration populations have been relatively continuous since its inception, the effects of drift on restoration populations may have been minimized. We hypothesize that prior to widespread habitat fragmentation *P. borealis* had a relatively panmictic population structure. The lack of population-specific alleles (Stangel et al. 1992, Haig et al. 1994b) and the long distance dispersal ability observed in *P. borealis* (Walters et al.1988, Conner et al. 1997) are consistent with this theory. However, further studies examining remnant populations and their relationship with donor populations are needed.

Previous widespread translocations and extirpation make it unlikely that the original, range-wide genetic population structure of *P. borealis* will ever be known. Therefore, we concur with previous studies that the primary considerations for translocation decisions should be

demographic in nature. In regard to genetic population structure, the role of translocations should be to increase genetic diversity through gene flow in a manner that provides the most adaptive potential. In addition, the degree of phenotypic variation should be considered carefully. Birds in the southern portion of the range are significantly smaller than those in the northern extent of the range (Mengle and Jackson 1977); therefore, geographically proximate populations are the best candidates for donors (Haig et al. 1996).

Our study shows that adequate differentiation exists between geographically proximate populations (Enon, Silver Lake, and Ichauway) for translocations to promote allelic diversity. Logistics have been a primary factor in selecting donor populations, which has led to geographically proximate populations being selected for translocation. In addition, alternating donor and recipient sites have been employed in a manner that has varied which donor birds have been translocated to which recipient populations. This method of selecting donors has contributed towards increasing genetic diversity and reducing differentiation in isolated populations. If one of the goals of translocations is to reduce the effects of drift and inbreeding on small isolated populations, this study shows that some measure of success can be achieved. The Ichauway population demonstrates that high levels of gene flow and adequate spatial characteristics for sustained population growth can result in levels of genetic diversity and inbreeding that are similar to the large donor population at Francis Marion.

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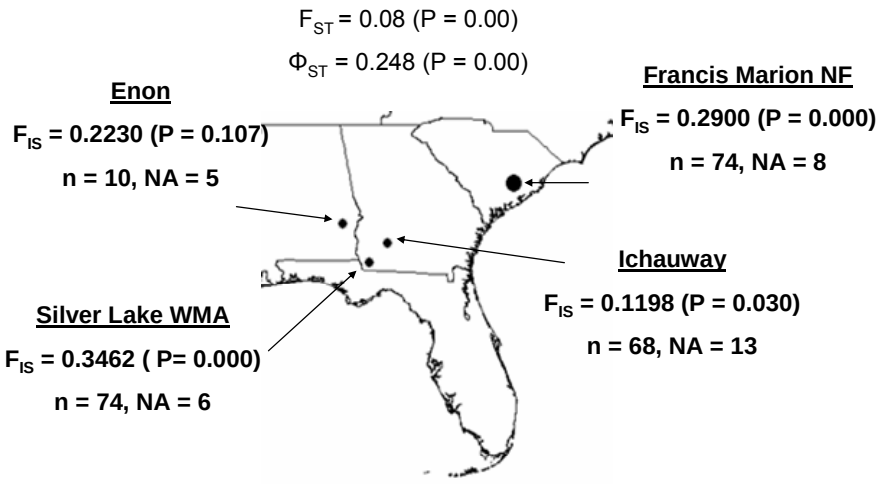

Figure 3.1 Map showing study area, population specific inbreeding coefficients (F_{IS}), number of samples (n), and mitochondrial haplotype richness (NA)

Table 3.1 Mitochondrial Primer Sequences for 1020 Bp fragment of control region in *P. borealis*

Fragment 1	Sequence 5' TO 3'
Amplification Primers	
DL-10f	CACCCCTTTATCATTATCGGCCAACTG
DL-758r	CACCCGCCGCACTTTGAAG
Sequencing Primers	
DL-4f	AGTTATGGGCCGTTACCATGGAGT
DL-4r	ACTCCATGGTAACGGCCCATAACT
Fragment 2	
Amplification Primers	
DL-13r	GTGGCAGAGGATAGTTTAGTCTAGAATGC
DL-596f	GCGCCTCTGGTTCCTATCTCAG
Sequencing Primers	
DL-1f	ATTGTATTCACCCGCCGCACTTTG
DL-1r	CAAAGTGCGGCGGGTGAATACAAT

Table 3.2 Populations sampled, pairs of loci in linkage disequilibrium (LD) and loci out of Hardy-Weinberg Equilibrium (HWE)

Population	Pairs of Loci in LD	Loci out of HWE
Enon	*Pbr104-Pbr107*	*Pbr104, Pbr106, Pbr108*
Ichauway	*Pbr135-Pbr155*	*Pbr104, Pbr108, Pbr117, Pbr128*
Francis Marion	*Pbr108-Pbr117, pbr110-Pbr117, Pbr101-127, Pbr117-Pbr127, Pbr128-Pbr135*	*Pbr106, Pbr108, Pbr 110, Pbr 117, Pbr127, Pbr135, Pbr 155*
Silver Lake	NA	*Pbr104, Pbr106, Pbr108, Pbr 110, Pbr 117, Pbr127, Pbr128, Pbr 135*

Table 3.3 Sampled populations and sampling units, number sampled (N) inbreeding coefficients (F_{IS}), (observed heterozygosity (H_O)/expected heterozygosity (H_E)) and average alleles per locus (A) of microsatellite genotypes from four populations using an AMOVA

Locus	Ichauway (n = 68)	Francis Marion (n = 74)	SL WMA (n = 41)	Enon (n =10)
Pbr101	0.647/0.592 4	0.465/0.620 4	0.400/0.542 3	0.444/0.451 3
Pbr104	0.667/0.709 10	0.662/0.537 6	0.491/0.670 8	0.000/0.567 3
Pbr106	0.866/0.820 10	0.623/0.839 10	0.563/0.842 10	0.750/0.867 6
Pbr108	0.383/0.704 8	0.407/0.803 10	0.367/0.767 8	0.250/0.867 6
Pbr110	0.791/0.872 14	0.574/0.873 15	0.571/0.888 15	0.778/0.869 7
Pbr117	0.418/0.670 5	0.328/0.703 4	0.311/0.651 4	0.375/0.758 4
Pbr127	0.619/0.781 8	0.477/0.803 7	0.419/0.859 10	0.429/0.824 5
Pbr128	0.574/0.611 6	0.657/0.762 6	0.449/0.585 4	0.800/0.668 5
Pbr135	0.545/0.496 3	0.394/0.543 4	0.396/.701 6	0.600/0.605 4
Pbr155	0.875/0.831 11	0.600/0.746 11	0.684/0.788 9	0.625/0.808 6
Mean	0.639/0.709 7.9	0.519/0.723 7.7	0.465/0.729 7.7	0.505/0.729 4.9
Cluster F_{IS} (P)	0.1718 (0.0303)	0.2900 (0.0000)	0.4854 (0.0000)	0.3833 (0.0156)
Individual F_{IS} (P)	0.1198 (0.0293)	0.2900 (0.0000)	0.3462 (0.0000)	0.2230 (0.1065)

Table 3.4 AMOVA tables and fixation indices (F_{ST}, F_{IS}, F_{IT}) calculated from microsatellite loci using sum of squared differences (R_{ST}) for both sampling methods

	AMOVA Table for One Individual Per Cluster				AMOVA Table for All Individuals Sampled			
Variation	D.F.	Sum of Squares	Variance Components	Percentage of Variation	D.F.	Sum of Squares	Variance Components	Percentage of Variation
Among Populations	3	516.313	2.5884 Va	7.5	3	840.135	2.629 Va	8.04
Among Individuals	123	5091.703	9.4814 Vb	27.48	197	7402.052	7.507 Vb	22.96
Within Populations								
Within Individuals	127	2849.000	22.4331 Vc	65.02	201	4534.5	22.560Vc	69.00
Total	253	8457.016	34.5029		401	12776.687	32.696	
	F_{ST} (P±SE) 0.0751 (0.001±0.001)	F_{IS} (P±SE) 0.2971 (0.000±0.000)	F_{IT} (P±SE) 0.3499 (0.000±0.000)			F_{ST} (P±SE) 0.0804 (0.000±0.000)	F_{IS} (P±SE) 0.2497 (0.001±0.001)	F_{IT} (P±SE) 0.3101 (0.000±0.000)

Table 3.5 Sampled populations and sampling units, allelic richness (NA) and fixation indices (ϕ_{ST}) from AMOVA using Tamura & Nei's distance, based on mitochondrial haplotype.

	N	NA
Ichauway	83	13
Enon	11	5
Silver Lake WMA	41	6
Francis Marion	74	8
Fixation Index	ϕ_{ST} (P±SE)	
Cluster	0.2479 (0.000±0.000)	
Individual	0.2685 (0.000±0.000)	

Table 3.6 Twenty-two unique haplotypes based on a 1021Bp region of mitochondrial control region from 212 individuals.

Haplotype	Polymorphic Sites																	GenBank Accession Number
	98	122	305	443	562	643	677	722	723	728	821	843	858	871	952	954	1008	
rcwA	C	A	G	G	A	A	C	G	G	G	G	T	T	T	-	T	A	GU571176
rcwB	T	A	G	G	A	A	C	G	G	G	G	T	T	T	-	T	A	GU571177
rcwC	C	A	G	G	A	A	T	G	G	G	G	C	T	T	-	T	A	GU571178
rcwD	C	A	G	G	A	A	C	G	G	G	G	T	T	T	-	A	A	GU571179
rcwE	C	A	G	G	A	A	C	G	G	G	A	T	T	T	-	T	A	GU571180
rcwF	C	A	G	A	A	A	C	G	G	G	G	T	T	T	-	T	A	GU571181
rcwG	C	A	G	A	A	A	C	G	G	G	G	T	C	T	-	T	A	GU571182
rcwH	C	A	G	G	A	A	C	G	G	A	G	T	T	T	-	T	A	GU571183
rcwI	C	A	G	G	A	A	C	G	G	G	G	T	T	T	-	T	G	GU571184
rcwJ	C	A	G	G	A	A	C	A	G	G	A	T	T	T	-	T	A	GU571185
rcwK	C	A	G	A	A	A	C	G	G	G	A	T	T	T	-	T	A	GU571186
rcwL	C	A	G	G	C	A	C	G	G	G	G	T	T	A	-	A	A	GU571187
rcwM	C	A	A	G	A	A	C	A	G	G	A	T	T	T	-	T	A	GU571188
rcwN	C	G	G	G	A	A	C	G	G	G	G	T	T	T	-	T	A	GU571189
rcwO	T	A	G	G	A	A	C	A	G	G	G	T	T	T	-	T	A	GU571190
rcwP	T	A	G	G	A	A	C	G	G	G	A	T	T	T	-	T	A	GU571191
rcwQ	C	A	G	G	A	A	C	G	A	G	G	T	T	T	-	T	A	GU571192
rcwR	C	A	G	G	A	A	C	A	G	G	G	T	T	T	-	T	A	GU571193
rcwS	C	A	G	G	A	A	C	A	G	A	G	T	T	T	-	T	A	GU571194
rcwT	T	A	G	G	A	A	C	G	A	G	G	T	T	T	-	T	A	GU571195
rcwU	C	A	G	G	A	G	C	G	G	G	G	T	T	T	-	T	A	GU571196
rcwV	T	A	G	G	A	A	C	G	G	A	G	T	T	T	T	T	A	GU571197

CHAPTER 4

MOLECULAR SEXING OF THE RED-COCKADED WOODPECKER

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Abstract

We report two methods for sexing the endangered Red-cockaded Woodpecker (*Picoides borealis*) using molecular techniques. The first method relies on a restriction site polymorphism in the homologous CHD1 genes on the avian sex chromosomes W and Z. The second method relies on PCR using a fluorescently labeled tag, the existing P2 primer and two novel *P. borealis* specific reverse primers. The former method provides an inexpensive and reliable method for laboratories that do not have access to a capillary. The latter method is a faster alternative for processing large amounts of data. These methods could be useful for studying sex-ratios in this cooperatively breeding species as well as identifying sexes for monitoring and management purposes.

Keywords: *Picoides borealis*, Red-cockaded woodpecker, sex-ratio, cooperative breeder, RFLP, fluorophore

Molecular techniques have greatly improved the ability to determine the sex of birds. Some authors estimate that 50% of the world's bird species are sexually monomorphic as adults and nearly all are monomorphic as nestlings (Griffiths et al. 1998). Because of this, many techniques have been published to sex birds using DNA-based methods including karyotyping (Fridolfsson et al. 1998), hybridization (Miyaki et al. 1997), RAPDs (Griffiths and Tawari 1993, Williams et al. 1993) and AFLPs (Griffiths and Orr 1999). In addition, a number of PCR-based assays utilizing polymorphism in the chromodomain-helicase-DNA binding protein 1 gene (CHD1) have been published (Griffiths et al. 1998, Kahn et al. 1998, Fridolfsson and Ellegren 1999). CHD1-based methods rely on amplification fragment length differences in homologous genes on the W and Z chromosomes resulting in two amplicons in females and one amplicon in males. While CHD- based assays are often termed universal they can be problematic when there exists insufficient fragment length polymorphism to be easily resolved or when a mutation occurs in the primer binding site resulting in one or both of the bands not amplifying. In addition, CHD1 based methods along with other published methods have not been tested on all species and it is likely they will not work equally well for all taxa. One species for which current CHD1 methodologies are insufficient is *P. borealis* (*Picoides borealis*). This is due to an eight base pair difference in the homologous CHD1 introns and inefficient amplification of the CHD1 fragment on the W chromosome. We report two different CHD1 assays for sexing *P. borealis*. One assay relies on an AFLP using the existing P2/P8 primers (Griffiths et al 1998). The other assay uses the P2 primer tagged with a fluorophore and two novel *P. borealis* specific reverse primers, which when resolved on a capillary, results in two amplicons for females and one for males. The latter technique can also be resolved on an agarose gel without the use of fluorophore tags.

The endangered Red-cockaded Woodpecker uses a cooperative mating system. Each breeding pair enlists "helpers", which are usually the male offspring of the breeding pair, to assist with the rearing of young. The breeding group inhabits a spatial amalgamation of cavities drilled into living pine trees referred to as a cluster (Ligon 1970, Lennartz et al 1987). The evolutionary implications of cooperatively breeding organisms are the subject of much debate (Koenig and Walters 1999). Some authors state that the sex-ratio in cooperative breeders should favor the helper sex; this is known as the repayment model (Emlen et al. 1986). It is estimated that the presence of helpers, which are mostly male, can increase productivity by 0.39 offspring per year (Heppell et al. 1994). Therefore it is logical to hypothesize that *P. borealis* sex-ratios would deviate significantly from 50:50 and favor males. However, previous studies of *P.borealis* sex ratio have produced mixed results. A significant male biased sex ratio was detected in 168 individuals sampled in South Carolina (Gowaty and Lennartz 1985). A much larger sample of 984 individuals in North Carolina produced results that did not differ significantly from zero (Walters 1990). We do not feel that these studies are exhaustive enough to draw conclusions about sex-ratio biases for an entire species across its range. Current studies on *P. borealis* fail to incorporate year to year variations in selection pressures such as local resource competition and local mate competition, that potentially influence facultative sex ratio adjustment (West et al. 2005). Because sex-ratio studies on *P. borealis* have relied on sexing after the emergence of sexually dimorphic plumage, they have ignored potentially confounding variables such as differential survival of nestlings younger than 14 days and differential hatching success between sexes. By developing a molecular sexing technique we will be able to further investigate the primary sex ratio in addition to the secondary sex ratio of *P. borealis*.

Sample Collection and DNA Isolation

Buccal swabs were collected from nestlings from 15 individuals at the Joseph W. Jones Ecological Research Center at Ichauway near Newton, Georgia and from 20 individuals at Francis Marion National Forest in South Carolina. Buccal swabs were taken when monitoring biologists banded nestlings. Birds were sexed by morphological characteristics at a later date after plumage had emerged. Birds were swabbed in the inside cheek using Isohelix® buccal swabs (Epicentre Biotechnologies) for 15s. Swabs were air dried and stored at -20o C until processed. Genomic DNA was extracted from buccal swabs using a Charge Switch® (Invitrogen®) magnetic bead isolation procedure. DNA was eluted in Tris-HCL ph 8.0 and stored at -20o C.

Restriction Fragment Length Polymorphism

Polymerase chain reaction (PCR) was run on genomic DNA using the P2 and P8 primers. Fragment amplification was performed in 20 μL reactions using 5 ng genomic DNA, 1X PCR Gold buffer (50mM KCl, 10mM Tris-HCl pH8.3, Applied Biosystems), 100 μg/ml bovine serum albumin (New England Biolabs), 2.5 mM MgCl2 Solution, 0.75 μM of each primer (P2 and P8), 0.25mM dNTPs and 1.5U Amplitaq Gold (Applied Biosystems). PCR was performed on an Applied Biosystems 9700 thermocycler using the following conditions: one denaturing step at 95 o C for 5 minutes, followed by 55 cycles of 95 o C for 30 s, 50 o C for 30 s and 72 o C for 1 min, followed by an extension of 72 o C for 10 min and a hold at 4 o C. Amplification success was verified on a 1% agarose gel at 100V for one hour. This sexing assay is supposed to produce two bands, however two bands were not visible. We cut out the band that was visible which presumably contained both homologous genes. The gel excisions were purified using a MinElute™ Gel Extraction Kit (Qiagen®) and eluted in 10 μL of elution buffer. Purified products were cloned in a TOPO TA® cloning kit (Invitrogen®) and sequenced using M-13

forward and reverse primers and BigDye 3.1 (Applied Biosystems). The resulting sequences were contiged and edited in SeqMan® (LaserGene®) and verified as a portion of the avian CHD1 gene by a BLAST of the NCBI database, GenBank. The 2 sequences were imported into SeqBuilder® (LaserGene®) and searched against the restriction enzyme library for polymorphism between males and females that would produce a sexually dimorphic pattern. A portion of the PCR product (8 μL) was digested in a 20 μL reaction containing 0.5 U *AseI* restriction endonuclease (New England Biolabs), 1X NEB Buffer 3 (100mM NaCl, 50mM Tris-HCL, 10mM MgCl2, 1mM Dithiothreitol, 100 mg/ml bovine serum albumin (New England Biolabs), and conversion buffer (35mM Tris-HCl pH 8.0, 7.5 mM $MgCl_2$ and 50mM NaCl) to optimize the salt concentration. Digestion was carried out at 37^{o} C for one hour and was verified on a 1% agarose gel by electrophoresis at 100V for 1 h. Male samples produced one band of approximately 400 bp and female samples produced 2 bands of approximately 400 bp and 200 bp (Figure 1). The sex of the individuals was identified correctly in all 15 individuals sampled at the Jones Center and in all 20 individuals sampled at Francis Marion National Forest.

Fluorescently Labeled PCR

Sequences of a portion of the *P. borealis* CHD1 gene were imported into MegAlign® (LaserGene®) and aligned using the Clustal W Method. *P. borealis* specific reverse primers that complemented the existing P2 primer were designed for both the Z chromosome (Pz260 5'-TCTGAATGCATTTTTCACAAGC-3') and W chromosome (Pw319 5'-TTTCACTGCTGGTGTTTTGG-3'). The P2 primer was tagged with a M-13 (5'-GGAAACAGCTATGACCAT-3') primer so the fluorescent label NED® (Applied Biosystems) could be attached. Amplification was performed in a 20 μL reaction containing 1X PCR Gold Buffer (50mM KCl, 10mM Tris-HCl pH 8.3, Applied Biosystems), 2.5mM MgCl2, 100 μg/ml

bovine serum albumin (New England Biolabs), 0.25mM dNTPs, 0.5µM Pz260 reverse primer, 0.5µM Pw319 reverse primer, 0.2 µM M13 tagged P2 forward primer, 0.3 µM NED (Applied Biosystems) tag and 0.5U Amplitaq Gold polymerase (Applied Biosystems). PCR was performed on an Applied Biosystems 9700 thermocycler using the following conditions: one denaturing step at 95 o C for 5 minutes, followed by 50 cycles of 95 o C for 30 s, 53 o C for 30 s and 72 o C for 1 min, followed by an extension of 72 o C for 10 min and a hold at 4 o C. Fragment sizes were resolved on an Applied Biosystems 3730*xl* DNA analyzer and scored in GENEMAPPER (Applied Biosystems). Male samples resulted in one fragment of 260bp and female samples resulted in both the male fragment and the female fragment of 319bp (Figure 2). The correct gender was identified in all 20 samples from Francis Marion National Forest. The correct gender was identified in 14 out of the 15 samples taken from The Joseph W. Jones Center. The CHD1-W gene failed to amplify in one presumptive female. The sample was re-amplified four times, each time producing both the CHD1-Z and CHD1-W bands. It is likely the false negative occurred due to the low concentration of the buccal swab derived genomic DNA used in testing.

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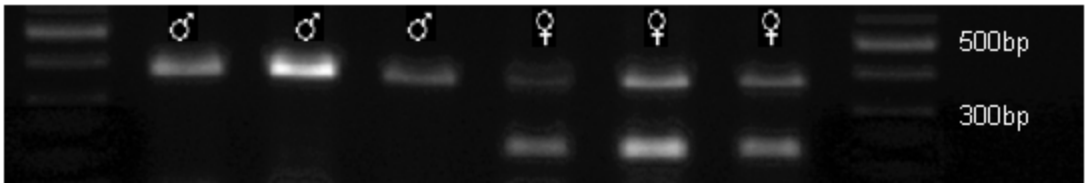

Figure 4.1 Banding pattern for three males (left) and three females (right) using AFLP sex identification on 1% agarose gel at 100V for 1 h with 2-Log ladder.

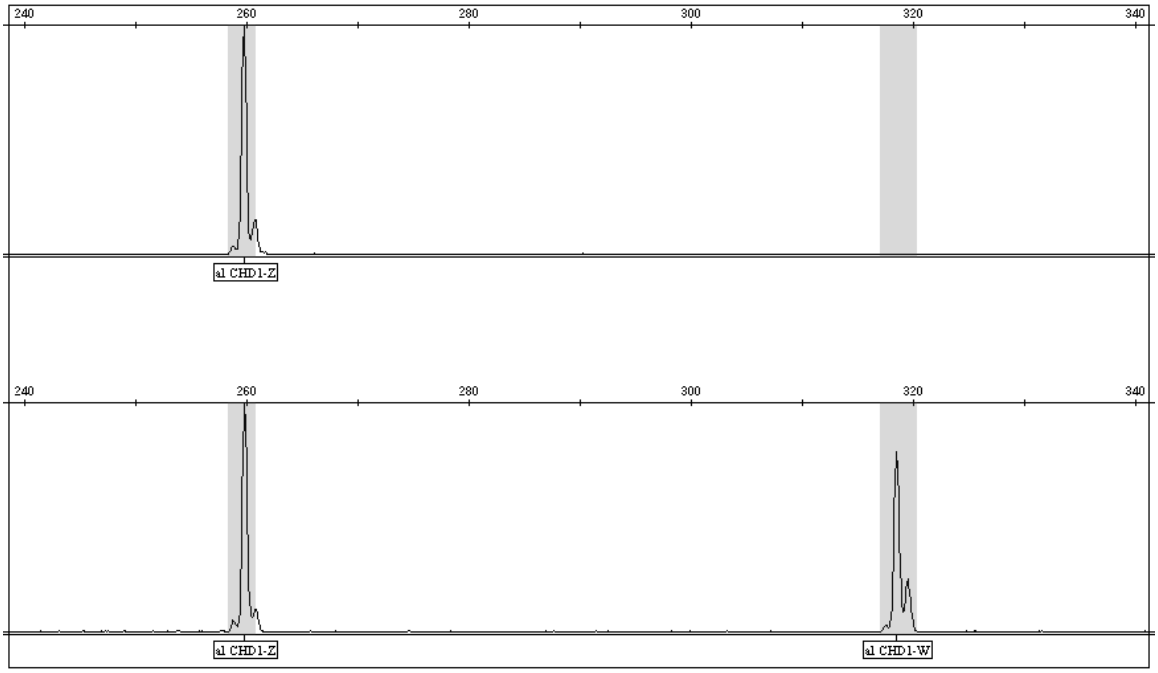

Figure 4.2 Example electropherogram of male genotype (top) and female genotype (bottom) using the P2 forward primer and Pz260 and Pw319 reverse primers.

CHAPTER 5

CONCLUSION

This project was completed to advance our understanding of the current state of red-cockaded woodpecker genetic health. Specifically, I was interested in how translocations have affected genetic diversity, population structure and inbreeding. In addition I was interested in developing genetic markers for this study and future studies, assessing the effectiveness of buccal swabs for use in genetic studies on *P. borealis* and developing an assay for determining sex of nestlings.

In order to compare genetic diversity between restoration and donor populations I collected DNA samples from four locations: one donor population, Francis Marion National Forest and three restoration populations, the Joseph W. Jones Ecological Research Center at Ichauway, Silver Lake Wildlife Management Area, and Enon/Sehoy Plantation. Individual birds were genotyped using 10 nuclear microsatellite markers (Alstad et al. 2009) and haplotyped based on a 1020 base pair region of the mitochondrial genome. Fixation indices (F_{ST}, F_{IS}, F_{IT}, Φ_{ST}) were calculated using an AMOVA in ARLEQUIN (Excoffier et al. 2005). Expected and observed heterozygosities were calculated in CERVUS (Kalinowski et al. 2007) across all microsatellite loci. Deviations from Hardy-Weinberg Equilibrium and significance of linkage disequilibrium were also measured.

My results show that F_{ST} in our group of populations, which include restoration populations, were lower than previous range-wide studies of red-cockaded woodpecker population structure (Stangel et al. 1992, Haig et al. 1994, Haig et al. 1996). Although different

markers were used, multiple studies have shown that practical comparisons can be made between F-statistics using different markers, as long as those markers conform to the expectations of Hardy-Weinberg equilibrium (Estoup et al. 1998, Ross et al. 1999, Desvignes 2001). I found that restoration populations had similar or higher levels of genetic diversity and reduced inbreeding when compared to the donor population. Several previous studies are critical of the way translocations disrupt the natural population structure of certain species (Stockwell et al. 1996, Sigg 2006). I suggest that in the case of *P. borealis*, translocations have helped to mitigate some of the detrimental effects of population fragmentation, like drift and inbreeding, primarily caused by habitat destruction.

In addition, I found that buccal swabs provide a viable alternative to more invasive sampling methods like blood sampling and feather pulls. Buccal swabbing was equally effective for adults and nestlings and genomic DNA was amplified from more than 90% of collected samples.

Overall, the results of this study are encouraging for red-cockaded woodpecker restoration across its range. I found decreased levels of population divergence in comparison with previous studies. I also found that translocations provided gene flow that reduced divergence and inbreeding. However, I still calculated significant deviation and significant inbreeding in all populations studied. Mitochondrial fixation indices were undesirably high and indicate that genetic drift still poses a threat to population health. It is my opinion that if translocations cease in small populations, natural rates of gene flow will not be high enough to counteract population differentiation. Ultimately, in order to fully restore *P. borealis* population, connectivity between fragments must be restored.

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