FINE-SCALE SPATIAL GENETIC STRUCTURE IN THE BROWN-HEADED NUTHATCH (*Sitta pusilla*)

By

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To my parents and six beloved brothers and sisters

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Determining the spatial genetic structure of declining species is an important goal for many management and conservation programs. Cooperative breeding birds are expected to exhibit spatial genetic structure over small geographic distances due to restricted dispersal and natal philopatry. The brown-headed nuthatch (*Sitta pusilla*) is a cooperative breeding bird endemic to the pine forests of the southeastern United States. Increasing conservation awareness for this species is attributed to ongoing range-wide population declines resulting from habitat loss, degradation, and fragmentation. Prior to this study no molecular genetic work had been performed for the brown-headed nuthatch, but genetic information is needed in order to assist management recommendations regarding this imperiled species.

Eight hypervariable microsatellite markers specific to the brown-headed nuthatch were used to examine patterns of fine-scale spatial genetic structure in this species. Analysis of 70 individuals from a single population revealed an average of 17 alleles per locus (range 11-24), an average observed heterozygosity of 0.69 (range 0.39-0.87), and an average polymorphic information content of 0.83 (range 0.66-0.94). Spatial genetic autocorrelation analysis using five of the microsatellite markers revealed that fine-scale spatial genetic structure exists in the brown-headed nuthatch. Significantly positive spatial autocorrelation was detected only in males when

male auxiliary adults were included and was not found in females. This is most likely due to the majority of auxiliary adults in this species being second-year males that assist the nest of at least one parent and thus exhibit natal philopatry. However, the difference between the geographic distances separating pairs of related males versus females was not statistically different, suggesting that both sexes may be dispersing similar distances from the natal territory overall. It is anticipated that the microsatellite markers developed for this research will continue to be a useful tool for population genetic studies on the brown-headed nuthatch. In addition, it is hoped that the information pertaining to fine-scale spatial genetic structure in the brown-headed nuthatch will provide valuable baseline information for management agencies and others concerned with the conservation of this imperiled species.

CHAPTER 1
INTRODUCTION

The brown-headed nuthatch (*Sitta pusilla*) is a small, non-migratory cooperatively breeding passerine endemic to the pine forests of the southeastern United States. This species historically occurred throughout much of Florida; however, it is now largely absent from most counties near and southeast of Lake Okeechobee (Withgott & Smith 1998 and references therein). Increasing conservation awareness for this species arises from ongoing population declines throughout their entire range (Sauer *et al.* 2005). Based on these population trends, the brown-headed nuthatch has been designated a species of management concern (Carter *et al.* 1998). Population declines have been attributed to habitat fragmentation, loss, and degradation (Withgott & Smith 1998). Habitat alteration, including fire suppression and landscape fragmentation, has caused a 97% decline in the area of longleaf pine ecosystems, making them among the most imperiled ecosystems in the United States (Noss *et al.* 1995). Jackson (1988) suggested that as these forests undergo further fragmentation, brown-headed nuthatch populations will continue to decline.

Cooperative breeding is an unusual mating system in birds in which more than two individuals of a species contribute to raising the young (Brown 1987). In brown-headed nuthatches, territory groups consist of mature nonbreeders ("helpers-at-the-nest" or "auxiliaries") that help protect and rear the young, but are presumably not parents of those offspring. In a recent study of the brown-headed nuthatch, Cox & Slater (2007) reported the percentage of territories with more than two adults averaged 10-32% among sites and years. The majority of groups with ≥ 2 adults consisted of a breeding pair and an auxiliary male which was presumably related to at least one breeding adult. Auxiliary females were less common, but some were found in their natal territories. Stacey & Ligon (1987) suggest that cooperative breeding in birds may

occur when species have a limited, unusual resource that selects for offspring that remain in the natal territory near that resource. As such, these species may have limited dispersal and require specialized habitats that make them particularly sensitive to habitat degradation (see also Walters *et al.* 2004).

Typical of cooperatively breeding birds, long-distance dispersal in the brown-headed nuthatch, if it occurs at all, is likely infrequent and/or limited in range (Withgott & Smith 1998). Cox & Slater (2007) noted that breeding pairs maintain long-term pair bonds and frequently excavated nests within 100m of nests used the previous year. In addition, mean dispersal distance of first-year males was generally less than two territories (<300m). Dispersal distances of females remain unknown, though typically females disperse farther than males in birds. This limited dispersal is likely to increase the susceptibility of this species to habitat fragmentation by reducing gene flow between populations and making it unlikely that individuals will disperse to recolonize distant fragments upon extirpation (Withgott & Smith 1998).

Brown-headed nuthatches have very specialized habitat requirements and rarely venture from pine-dominated forests (Withgott & Smith 1998). Preferred habitat consists of mature pine forests with open understory maintained by regular fires and retention of snags (*i.e*. standing dead trees) suitable for the excavation of cavity nests. Fire suppression increases the invasion of hardwoods and slows the creation of snags, degrading optimal habitat for this species (Withgott & Smith 1998). Wilson & Watts (1999) found a negative correlation between nuthatch abundance and canopy cover, hardwood density, and basal area of hardwoods. In addition, they found that nuthatches were over three times as likely to be found in areas containing snags than those without snags. The dependence of this species on pine forests with suitable understory and

fire regimes may make it an excellent indicator species for the health of the limited amount of remaining southeastern pine forests (Withgott & Smith 1998).

An additional factor affecting the overall status of brown-headed nuthatch populations is the reduction in effective population size due to the presence of non-breeding auxiliaries at nests, especially in populations exhibiting high rates of cooperative breeding. Within small, fragmented populations, the restricted number of breeding individuals can reduce the effective population size below the level expected for a typical, non-cooperatively breeding species. A reduction in effective population size may increase the loss of genetic variation due to deviations from non-random mating (Sugg *et al.* 1996). Empirical studies in other declining species, for example the greater prairie chicken (*Tympanuchus cupido*), have demonstrated that loss of genetic variation is correlated with reduced fitness, such as reduced hatching success (Westemeier *et al.* 1998), which further threatens population health.

In cooperatively breeding birds where adult offspring remain in their natal territory, the opportunity for inbreeding may be substantial (McRae & Amos 1999 and references therein), which can further threaten the genetic health of populations. In one instance, a female brown-headed nuthatch was documented assisting her parents during her first year, but was then involved in an incestuous mating that followed the disappearance of her mother prior to the next breeding season (J. Cox, unpublished data). Thus, cooperatively breeding birds may be particularly dependent upon gene flow to overcome the negative genetic consequences of inbreeding. Habitat fragmentation may inhibit dispersal among populations, especially for species with limited dispersal and extreme habitat specialization (Boone & Rhodes 1996), as seen in the brown-headed nuthatch. Fragmentation may prevent sufficient gene flow to rescue

populations from inbreeding depression and its associated affects on fitness (Daniels & Walters 2000).

An increasing number of studies are using molecular techniques to examine patterns of genetic variation within and among populations of threatened species (Waser & Strobeck 1998). These types of studies can reveal the degree of relatedness among individuals within populations and the degree of genetic structure among populations, thereby facilitating the inference of dispersal rates. Peterson (1992) concluded that social systems with high degrees of philopatry, as seen in many cooperative breeding birds, facilitate rapid differentiation over short spatial scales resulting in populations that have little within population genetic variation but are quite genetically distinct from one another. Low genetic variation within populations in cooperatively breeding birds may have long-term consequences for evolutionary processes, such as an inability to adapt to environmental responses and the onset of inbreeding depression (McDonald *et al.* 1999). Despite the declining status of the brown-headed nuthatch, there is little research on this species, including an absence of published molecular work, which is needed for proper management and conservation of remaining fragmented populations.

My specific research objectives are: (1) To isolate and characterize polymorphic microsatellite markers for the brown-headed nuthatch; and (2) To elucidate the fine-scale spatial genetic structure within a population of brown-headed nuthatches. It is anticipated that this research will greatly increase our knowledge of this poorly studied species of conservation interest and will also provide preliminary information for conservation organizations and others interested in management of this species.

CHAPTER 2
ISOLATION AND CHARACTERIZATION OF EIGHT POLYMORPHIC MICROSATELLITE MARKERS FOR THE BROWN-HEADED NUTHATCH (*Sitta pusilla*)

Eight highly polymorphic microsatellite loci were isolated and characterized for the brown-headed nuthatch (*Sitta pusilla*). Analysis of individuals from a single population revealed an average of 17 alleles per locus (range 11-24), an average observed heterozygosity of 0.69 (range 0.39-0.87) and an average polymorphic information content of 0.83 (range 0.66-0.94). We anticipate that these microsatellite markers will be a useful tool for population genetic studies on the brown-headed nuthatch.

The brown-headed nuthatch (*Sitta pusilla*) is a small passerine bird endemic to pine forests of the southeastern United States. This species has been undergoing significant range-wide population declines resulting from habitat loss, degradation, and fragmentation (Withgott & Smith 1998). The brown-headed nuthatch's cooperative-breeding mating system, restricted dispersal, natal philopatry, and ecological specialization may increase susceptibility to habitat alteration, which has also been suggested for other cooperative-breeding birds (Walters *et al*. 2004). Despite declining population trends, there has not been any molecular work published at this time on the brown-headed nuthatch. We developed these microsatellite loci to analyze the genetic mating system and population genetic structure of this species.

We constructed an enriched $(CA)_n$ microsatellite library using protocols from the University of Florida Interdisciplinary Center for Biotechnology Research Molecular Markers Workshop (Brazeau & Clark 2005), some of which were modified from Kandpal *et al.* (1994). Genomic DNA was isolated using the PUREGENE® DNA Purification Kit (Biozym, Hess. Oldendorf, Germany) from two individuals sampled at Tall Timbers Research Station (TTRS) in Leon County, Florida. Approximately 5µg of genomic DNA from each individual was combined and digested with *Sau*3AI restriction enzyme and size selected for fragments greater

than 400bp using Chroma Spin® columns (Clonetech Laboratories). Size-fractionated genomic DNA was ligated to *Sau*3AI linkers. Excess linkers were removed using Chroma Spin® columns before amplification of the recombinant fragments by Polymerase Chain Reaction (PCR) using the free linker oligonucleotide. Enrichment for $(CA)_n$ repeats was completed by hybridizing the fragments to a biotinylated $(CA)_{15}$TATAAGATA probe. The biotinylated products were bound to an Avidin matrix (VECTREX® Avidin D, Vector Laboratories), allowing for the removal of fragments that did not hybridize to the biotinylated probe. A second PCR amplification of fragments enriched for $(CA)_n$ repeats was performed and the Phototope® -Star Chemiluminescent Detection Kit (New England Biolabs) was used to test for successful selection of hybridized DNA fragments by performing a dot blot.

PCR products of the enriched microsatellite library were directly ligated to a plasmid vector (pCR® 2.1-TOPO® vector; Invitrogen) used to transform *Escherichia coli* (One Shot™ TOP 10 cells, Invitrogen). Colony lifts were screened using the $(CA)_n$ probe and the chemiluminescent detection kit. Forty-five colonies with strong hybridization signals were sequenced on an ABI 377 PRISM automated sequencer (Applied Biosystems) to confirm the presence of microsatellite-containing repeats.

Primer pairs complementary to the microsatellite-flanking sequence were designed for 24 of the 45 clones using the software package PRIMER 3 (Rozen & Skaletsky 1998). We optimized primer pairs and tested for polymorphism using 10 individuals from TTRS. Among 24 primer pairs designed, eight produced consistent amplification of polymorphic loci. Optimized PCR conditions consisted of 1X PCR buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 U *Taq* polymerase (New England BioLabs), 0.3 μM of the forward and reverse primer, and 8 ng of genomic DNA in a 10 μL reaction. Locus-specific optimized PCR

conditions for the eight polymorphic loci can be found in Table 2-1. All PCRs began with 95°C (5 min); 35 cycles of 95° (60s), primer-specific annealing and elongation conditions (Table 2-1); and a final extension at 72°C (30 min). We added a GTTT sequence to the 5' end of three primers and GT to a single primer to facilitate the non-templated addition of adenosine by *Taq* polymerase, commonly referred to as 'pigtailing' (Brownstein *et al.* 1996). Allele sizes were determined using the MegaBACE 1000 DNA Sequencer (Amersham, Sunnyvale, CA) and raw data was analyzed using GeneMarker® v.1.5 (SoftGenetics LLC, State College, PA).

We genotyped approximately 20-60 individuals from TTRS for each of the eight polymorphic microsatellite loci using the PCR conditions previously described. Characteristics of each primer pair are presented in Table 2. The average number of alleles per locus is 17 (range 11–24). Total exclusion probabilities for the first and second parent (0.999318 and 0.999982), expected and observed heterozygosities, polymorphic information content, and null allele frequency estimates were calculated using CERVUS 2.0 (Marshall *et al.* 1998). Deviations from Hardy-Weinberg equilibrium (HWE) and tests for linkage disequilibrium were tested using a Markov chain method provided in GENEPOP version 3.4 (Raymond & Rousset 1995).

Three of the eight loci (Table 2-2) significantly deviated from HWE following sequential Bonferroni correction (Rice 1989). The deviation from HWE for two of the loci, *Spu*36A and *Spu*L4-30, may reflect the small sample sizes for these highly polymorphic loci, although further testing is needed to confirm this. In addition, departures from HWE for the three loci may also indicate heterozygote deficit consistent with the presence of null alleles. No evidence for linkage disequilibrium ($p < 0.05$) was found between loci. Overall, these microsatellite loci are highly variable and should be valuable tools for studying many biological aspects of the brown-headed nuthatch.

Table 2-1. PCR optimization conditions for the eight microsatellite loci developed for the brown-headed nuthatch (*Sitta pusilla*).

Locus	**Annealing**	**Elongation**	**Final Mg^{2+} (mM)**	**Betaine (1.25mM)**
*Spu*L5-6	60°/ 40s	72°/ 60s	2.5	-
*Spu*A6	65°/ 10s	72°/ 10s	1.5	-
*Spu*E19	66°/ 40s	72°/ 60s	3	-
*Spu*L4-31	66°/ 40s	72°/ 60s	2.5	-
*Spu*L4-3	66°/ 30s	72°/ 45s	3	+
*Spu*L6-16	60°/ 5s	72°/ 25s	2.5	+
*Spu*36A	68°/ 5s	72°/ 45s	3	+
*Spu*L4-30	58°/ 5s	72°/ 25s	2.5	+

Table 2-2. Characterization of eight polymorphic microsatellite loci from the brown-headed nuthatch (*Sitta pusilla*) collected in Leon County, Florida.

Locus	Primer sequence (5'-3')	GenBank accession number	Fluorescent dye	Clonal repeat motif	Size range (bp)	*n*	*k*	H_O	H_E	PIC
*Spu*L5-6	F: CTTCCTTGTGCATGGTTGAA R: TCTACTGTCCCACGGGTAAAA	EF474467	HEX	$(GT)_{27}$	229-295	61	24	0.852	0.908	0.894
*Spu*A6	F: ACCTCTAGCCTTGCTTGCAG R: GCGAAGAATAGCAGGTTTGG	EF474468	FAM	$(AC)_{11}$	190-232	51	13	0.627	0.682	0.655
*Spu*E19	F: TCCTGTGAGAGCAGCAAGAA R: CTGGCATCAAAGGAAAGCAT	EF474469	HEX	$(GT)_{10}$	234-328	60	15	0.683	0.733	0.704
*Spu*L4-31*	F: CCCCAAACCCAACTCTGTTA R: TGCATTGGTTCATTACTAGATGCT	EF474470	FAM	$(GT)_{15}$	271-319	52	11	0.654	0.813	0.778
*Spu*L4-3	F: AGTCAGCACATGGAACCACA R: **GTTT**AAACCCAGCAAACATTCCAC	EF474472	HEX	$(GT)_{27}$	360-406	56	19	0.786	0.869	0.850
*Spu*L6-16	F: AGGCTCCCTGTGTAGGTGTG R: **GTTT**ATCCTTCAGGTGGGTGACTG	EF474473	FAM	$(GT)_{27}$	304-356	39	20	0.872	0.922	0.904
*Spu*36A*	F: ACAGAGGAAGCCACCAGAGA R: **GTTT**GAAGGGGCATCTCTTCTTCC	EF474474	HEX	$(GT)_{33}$	332-412	23	22	0.391	0.962	0.938
*Spu*L4-30*	F: ATGCACTGGGTTCCTGTGTT R: **GT**TTGTTCACATTGCTGGAAAGG	EF474475	FAM	$(GT)_{29}$	262-306	27	17	0.630	0.926	0.902

Bolded bases indicate 'pigtail' addition; (*n*), number of individuals genotyped at each locus; (*k*), number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content. *Indicates locus with significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction.

CHAPTER 3
FINE-SCALE SPATIAL GENETIC STRUCTURE IN THE BROWN-HEADED NUTHATCH (*Sitta pusilla*)

Understanding patterns of spatial genetic structure is important for the management and conservation of many species. Fine-scale genetic structure may be found in species exhibiting restricted dispersal and natal philopatry, as seen in many cooperative breeding birds. In this study, spatial autocorrelation analysis using five microsatellite loci was performed to examine patterns of fine-scale spatial genetic structure in the brown-headed nuthatch (*Sitta pusilla*). The brown-headed nuthatch is a cooperative breeding bird that is currently experiencing population declines attributed to habitat loss and fragmentation. Significantly positive autocorrelation was only detected in males when auxiliary adults were included and was not found in females. Contrary to expectations, the difference between the geographic distances separating pairs of related males versus females was not statistically different, suggesting that both sexes may be dispersing similar distances from the natal territory overall. This study is the first to describe the fine-scale spatial genetic structure in the brown-headed nuthatch and is important to the conservation and management of this declining species. Long-term management efforts of the brown-headed nuthatch should consider maintaining adequate genetic variation within small, potentially isolated populations, although additional studies are needed to further elucidate spatial genetic patterns in this species.

Introduction

Determining the spatial genetic structure within and among populations is important for the conservation and management of many declining species (Caizergues *et al.* 2003; Laikre *et al.* 2005; Johnson & Dunn 2006). Information regarding the genetic structure can provide important insights into behavioral and ecological processes including dispersal pattens, local adaptation, and the effects of landscape features on gene flow (Piertney *et al.* 1999; Bittner &

King 2003; Manel *et al.* 2003). Most studies to date that have analyzed population genetic structure have focused on the landscape level, often sampling individuals from multiple populations throughout the range of a species (Castric *et al.* 2001; Veit *et al.* 2005; Eggert *et al.* 2006; Sonstebo *et al.* 2007). However, there has recently been a substantial increase in the number of studies focusing on fine-scale genetic structure (*i.e.* within populations) and the underlying factors most likely generating these within-population spatial patterns (Brouat *et al.* 2003; Peakall *et al.* 2003; Hazlitt *et al.* 2004; Comer *et al*. 2005; Kitchen *et al.* 2005; Nussey *et al.* 2005; Zamudio & Wieczorek 2007). Assessing microgeographic spatial genetic structure within populations is useful for investigating limited and sex-biased dispersal, social behavior, mating systems, and barriers to gene flow within populations (Peakall *et al.* 2003; Double *et al.* 2005).

Many cooperative breeding species are expected to exhibit microspatial genetic structuring due to restricted dispersal and high levels of natal philopatry (Walters *et al.* 2004; Woxvold *et al.* 2006), yet relatively few studies have analyzed the presence of fine-scale genetic structure in cooperative breeding birds (although see Painter *et al.* 2000; Double *et al.* 2005; Temple *et al.* 2006; Woxvold *et al.* 2006). In addition to these characteristics, some cooperative breeding birds also exhibit habitat specialization and have a large number of non-breeding adults that function as helpers (Walters *et al.* 2004). These traits may facilitate genetic structure over small geographic distances (Peakall *et al.* 2003; Hazlitt *et al*. 2004; Double *et al.* 2005). In many cooperative breeding birds, natal philopatry is sex-biased towards males while females typically disperse over longer distances (Greenwood 1980; Koenig *et al.* 1992; Walters *et al.* 2004). Sex-biased dispersal may result in demes of related individuals of the philopatric sex (Hazlitt *et al.* 2004; Sugg *et al.* 1996). The limited number of studies that have examined fine-scale genetic

structure in cooperative breeding birds all detected microgeographic genetic structure over small distances (*i.e.* within a few territories), with more pronounced patterns of spatial genetic structure in the philopatric males (Painter *et al.* 2000; Double *et al.* 2005; Temple *et al.* 2006; Woxvold *et al.* 2006).

In this study, I examined patterns of fine-scale spatial genetic structure in the cooperative breeding brown-headed nuthatch (*Sitta pusilla*). The brown-headed nuthatch is a small (~10 g), non-migratory, cavity-nesting passerine endemic to the longleaf pine ecosystems of the southeastern United States (Withgott & Smith 1998). The percentage of breeding territories containing one or more auxiliary adults has been documented to vary from 10-32% (Cox & Slater 2007). Most groups containing more than two adults consist of a breeding pair and a second-year auxiliary male who is related to at least one breeding adult (Cox & Slater 2007). Breeding pairs maintain long-term pair bonds and are highly sedentary once a territory is established-- frequently excavating nests within 100m of nests used the previous year (Cox & Slater 2007). Natal philopatry appears to be heavily male-biased based on field observations, although female helpers have been documented assisting at the natal territory (Cox & Slater 2007). Most dispersing second-year males establish territories within 300m of territories held by their parents, which is generally the nearest neighbor to the natal territory (Cox & Slater 2007). On the other hand, average dispersal distance from the limited number of recaptured females (n= 6) is 1240m (J. Cox, unpublished data).

Increasing conservation awareness for the brown-headed nuthatch has resulted from range-wide population declines (Sauer *et al.* 2005), which have been attributed to habitat loss, fragmentation, and degradation (Withgott & Smith 1998). This species possesses many of the distinctive traits seen in other cooperative breeding birds that have been suggested to increase

susceptibility to habitat fragmentation (Walters *et al.* 2004) and generate fine-scale spatial genetic structure (Woxvold *et al.* 2006). Especially relevant traits for the brown-headed nuthatch include the presence of non-breeding adults that function as helpers, extreme philopatry, limited dispersal, and ecological specialization (Withgott & Smith 1998). Despite ongoing population declines in this species and the prediction that populations will continue to decline as pine forests become further fragmented (Jackson 1988), there is little research on the brown-headed nuthatch, including an absence of molecular work prior to this study. Furthermore, it is currently unknown to what extent male-biased natal philopatry and limited dispersal promote spatial genetic structure in this species.

This study combines spatial autocorrelation procedures and hypervariable microsatellite markers to examine fine-scale patterns of genetic structure in the brown-headed nuthatch. The use of spatial autocorrelation to elucidate microspatial genetic structure may be especially useful for species that exhibit natal philopatry and restricted dispersal, both of which are characteristics that may generate fine-scale nonrandom genetic patterns (Double *et al.* 2005). My research objectives were (1) to determine if genetic autocorrelation is affected by geographic distance, (2) to compare genetic autocorrelation between males and females, and (3) to examine the influence of auxiliary adults on genetic autocorrelation. I expected to find higher genetic autocorrelation at small geographic distances versus large distances accompanied by a steady decrease in r due to the overall sedentary behavior and restricted dispersal described for the brown-headed nuthatch. I also predicted that males would exhibit stronger positive autocorrelation than females due to male-biased natal philopatry in the brown-headed nuthatch. Lastly, I predicted that the inclusion of auxiliary adults would strengthen genetic autocorrelation due to presumed relatedness between breeding and auxiliary adults.

Methods

Study Site

Fieldwork for this study was conducted at Tall Timbers Research Station (TTRS) in Leon County, Florida, USA (Fig. 3-1). TTRS encompasses approximately 1,630ha dominated by upland pine habitats consisting primarily of loblolly (*Pinus taeda*) and shortleaf pines (*P. eichnata)*; although some of the area is not suitable habitat for brown-headed nuthatches (*e.g.* water bodies, hardwood hammocks). The breeding activities of brown-headed nuthatch groups within TTRS have been monitored by J. Cox since 2001, although genetic sampling was sufficient to analyze population genetic structure only for 2006. Sampling of adult individuals occurred from mid-February through May 2006, although sampling was not exhaustive over the entire study area.

Sample Collection

A total of 70 adult individuals comprising 36 territories (~ 670 ha) were sampled during the spring of 2006. For groups containing more than two adults, behavioral observations (*e.g.* dominance, incubation, and copulation), and in some instances banding records from previous years, were used to differentiate between breeding and auxiliary adults. Individuals were captured using mist nets placed near nests, which has been found to disrupt <1% of nesting attempts for this species (J. Cox, unpublished data). All nesting locations were geographically referenced with Universal Transverse Mercator (UTM) coordinates using a hand-held global positioning system; nest locations were assumed to represent the center of each territory. Birds were fitted with color bands and an aluminum United States Fish and Wildlife Service (USFWS) band. A blood sample (20-40 μL) was taken from the brachial vein of each bird and stored in 1mL of lysis buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M EDTA, 0.01 M NaCl, 1% SDS). Genomic DNA was extracted using a PUREGENE® DNA Purification Kit (Biozym, Hess. Oldendorf,

Germany) and the sex of each individual was determined using the molecular sexing method described by Fridolfsson & Ellegren (1999).

Microsatellite Genotyping

Five polymorphic microsatellite loci developed specifically for the brown-headed nuthatch were used to obtain a multilocus genotype for each individual (Table 3-1). Briefly, microsatellite loci were amplified via polymerase chain reaction (PCR) in 10μL reaction volumes containing the following: 1X PCR buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 U *Taq* polymerase (New England BioLabs), 0.3 μM of the reverse and fluorescently labeled forward primer, and 8ng of genomic DNA (see Chapter 2 for further details). Magnesium concentrations varied per locus (Table 3-1). The PCR additive betaine (1.25 mM) was used for a single locus, *Spu*L4-3, to help mitigate the presence of microsatellite stutter. All PCR amplifications began with 95°C (5 min); 35 cycles of 95° (60s), primer-specific annealing and elongation conditions; and a final extension at 72°C (30 min). A MegaBACE 1000 DNA Sequencer (Amersham, Sunnyvale, CA) produced raw data and alleles were sized using GeneMarker® v.1.5 (SoftGenetics LLC, State College, PA).

Genetic Variation

Exact tests for deviations from Hardy-Weinberg equilibrium (HWE) and tests for linkage disequilibrium were tested using a Markov chain method with 5,000 iterations implemented in GENEPOP version 3.4 (Raymond & Rousset 1995). When performing multiple comparisons, sequential Bonferroni corrections were used to reduce global Type I error (Rice 1989). Average number of alleles, observed and expected heterozygosity, mean proportion of individuals genotyped, and tests for null alleles were calculated using CERVUS 2.0 (Marshall *et al.* 1998).

Spatial Autocorrelation

To examine fine-scale genetic structure in the brown-headed nuthatch, spatial autocorrelation analysis was performed using GenAlEx, version 6 (Peakall & Smouse 2006) to describe the genetic structure over the entire study area. Unlike classical spatial autocorrelation analysis, which is implemented one locus at a time, GenAlEx6 uses a multivariate approach that strengthens the spatial signal by reducing random (locus to locus) noise (Smouse & Peakall 1999). The program generates an autocorrelation coefficient *r*, which provides a measurement of the pairwise genetic similarity of individuals whose geographic separation falls within a specified distance class. Individuals were classified into four categories: (1) All Individuals; (2) All Males (includes auxiliary males); (3) Dominant Males (breeding males only); and (4) Females.

Spatial autocorrelation was performed using automatically increasing geographic distance classes for a given user-specified base distance class. This method uses a cumulative sampling strategy so that each subsequent distance interval includes all pairwise comparisons from previous intervals. I specified a base distance class size of 100m for 15 runs so that the first distance interval would calculate *r* based on all pairwise comparisons within a distance of 0-100m, the second analysis for 0-200m, and so on until the last run (*i.e.* 0-1500m) was completed. This base distance class was chosen because 4 of the 36 (11%) territories used in this study had a sampled nearest neighboring territory 100m. Analyses were also performed using a 200m base distance class because previous research on the brown-headed nuthatch reported that the average distance between nearest neighboring territories at TTRS was 198.5m (SD = 90.7) (Cox & Slater 2007).

Statistical significance was tested in GenAlEx6 using random permutation of *r*. Permutation was repeated 1000 times to generate the 25^{th} and 975^{th} values used to define the

95% confidence intervals around the null hypothesis of $r = 0$. Significant spatial genetic structuring was declared when r exceeded the 95% confidence intervals. The autocorrelation coefficients were plotted as a function of geographic distance and visualized graphically in spatial genetic autocorrelograms.

Relatedness by Distance

In addition to spatial autocorrelation, I also used another approach to further investigate fine-scale spatial genetic structure of brown-headed nuthatches. This was performed because sampling of territories at the study site was not exhaustive and spatial autocorrelation procedures, which only analyze individuals falling within user-specified distance classes, may not capture the true spatial genetic structure. I used the program ML-RELATE (Kalinowski *et al.* 2006) to calculate maximum likelihood estimates of relatedness (r) (see Blouin 2003 for a review) for all pairwise comparisons for *Dominant Males* and *Females* separately. Although autocorrelation coefficients such as r are closely correlated with estimates of relatedness (Bank *et al.* 2005), they are not a direct estimate of genealogical relationships among individuals (Temple *et al.* 2006). Pairs of individuals within each sex exhibiting r-values greater than or equal to 0.50 were selected and the geographic distances separating these individuals were recorded. The statistical software R (R Development Core Team 2005) was used to perform a 1-tailed Wilcoxon rank sum test between these distances to assess whether a statistically significant difference existed between the average distance separating related males versus related females. In addition, Microsoft Excel was used to perform an F-test to determine whether a statistically significant difference existed between the variances in male and female distances.

Results

Genetic Variation

I sampled individuals from 36 territories: a breeding male and female were obtained for 20 territories; a breeding pair and a single male helper were obtained for 5 territories; a breeding pair and two male helpers were obtained for 1 territory; a single female was obtained for 7 territories; a single male was obtained for 3 territories; and both a dominant and auxiliary male were obtained for a single territory. Molecular sexing confirmed that all 8 auxiliary adults were males. There were prior field data for only three of the auxiliary adults, which revealed that all three helpers were banded the previous year as nestlings at the nest of the male they were currently helping when sampled. This suggests that these three individuals are offspring of the breeding male in which they were assisting, although formal parentage analysis is needed to confirm this.

Table 3-1 describes the five microsatellite loci used in this study, all of which exhibited high levels of polymorphism (average observed heterozygosity = 0.7146). A single locus (*Spu*L4-31) significantly deviated from HWE, which may be due to the presence of null alleles (null frequency estimate = 0.1033). None of the other loci showed evidence for null alleles. Linkage disequilibrium was not detected for any of the loci ($P>0.01$). The number of alleles per locus ranged from 11-24, with an average of 16 alleles per locus. The mean proportion of individuals that were genotyped at all five loci was 0.929. Reasons for missing genotypes include repeated PCR failure and ambiguous microsatellite products due to the presence of stutter bands.

Spatial Autocorrelation

Spatial autocorrelation analysis detected the predicted pattern of higher genetic autocorrelation at small geographic distances followed by a steady decrease in *r* for *All Males*

(Fig. 3-2 B), although no other sampling category revealed this pattern (Fig. 3-2). However, a weaker, albeit non-significant, pattern was found in the *All Individuals* analysis (Fig. 3-2 A), which also includes auxiliary adults. These results suggest that increasing geographic distance does affect the spatial signal of fine-scale genetic structure in brown-headed nuthatches and that the presence of auxiliary males increases the strength of positive genetic autocorrelation at small distances. Contrary to expectations, the predicted pattern was not seen in *Dominant Males* (Fig. 3-2 C), despite male-biased natal philopatry in this species. Interestingly, *Females* exhibited negative *r*-values at the two smallest distance intervals, whereas *Dominant Males* revealed positive values. Although these results are not statistically significant, they suggest that females may be less related at smaller distances than are males, which is supported once auxiliary males are included in the analyses. The autocorrelation analysis for *All Males* revealed significant positive genetic autocorrelation until approximately 1300m. Similar results for all categories were seen when analyses were performed using the 200m base distance class (results not shown).

Relatedness by Distance

A total of 19 pairwise comparisons for males and 31 pairwise comparisons for females exhibited maximum likelihood estimates of relatedness greater than or equal to 0.50. The average geographic distance between pairs of selected males was 1,585m (range = 193-2423m) and for females was 1,780m (range = 192-3799m). The 1-tailed Wilcoxon rank sum test returned a p-value of 0.393, suggesting that there is not a statistically significant difference in the geographic distances separating related males and females provided the dataset used in this study. In addition, there was substantial overlap in the 95% upper and lower confidence intervals around the average for males and females, thereby revealing a weak effect size and possible lack of biological significance (Fig. 3-3). The F-test reveled that the variances in the distances separating pairs of related males and females were not statistically different (p=0.338).

Discussion

Prior field data for the brown-headed nuthatch show that males exhibit high rates of natal philopatry and typically disperse over very short distances (Cox & Slater 2007). In contrast, females have been documented less frequently as helpers and are thought to disperse much larger distances than males (Cox & Slater 2007). This pattern of sex-biased dispersal would be expected to generate microspatial genetic structure among males with little or no pattern in females (Peakall *et al.* 2003). The spatial autocorrelation analyses presented in this study revealed that all males exhibited significantly positive genetic autocorrelation at small distances, whereas significance was not detected in females. However, significance in males was attributed to the inclusion of helping individuals, which are rare in females (J. Cox, unpublished data) and were not included in this study. Other studies on cooperative breeding birds also detected stronger positive genetic autocorrelation when auxiliary adults were included in the analyses (Double *et al.* 2005; Temple *et al.* 2006). Upon exclusion of auxiliary adults however, spatial autocorrelation did not detect significance in males, which is in contrast to field observations documenting shorter dispersal distances in males (Cox & Slater 2007).

In spatial autocorrelation, the distance class size at which r is no longer significantly positive approximates the extent of detectable positive genetic structure (Peakall *et al.* 2003). This distance is similar to the 'genetic neighborhood' size of a population (Wright 1943; Golenberg 1987). In this study, autocorrelation revealed significantly positive genetic structure among all males extending beyond six average territory widths (*i.e.* 1300m). Based on field observations of the brown-headed nuthatch, Cox & Slater (2007) reported that the average dispersal distance for second-year males dispersing equal to or greater than two territories away from the natal territory was 1,358m. Interestingly, results from both spatial autocorrelation and

field observations suggest that the average distance of gene flow (*i.e.* genetic neighborhood size) in male brown-headed nuthatches is approximately 1300m.

The analyses performed using maximum likelihood estimates of relatedness suggest that the average distance separating related *dominant* males was approximately 1,600m, which is only slightly higher (*i.e.* 300m) than the estimates obtained from mark-recapture data and spatial autocorrelation for all males (*i.e.* helpers included). Although the autocorrelation procedure did not detect significance in dominant males, this approach includes all individuals within a user-specified distance class, which may or may not be related. On the other hand, the relatedness by distance analysis disregards distance classes and only looks at those pairs of individuals exhibiting high levels of relatedness and then determines the geographic distance separating that particular pair. Despite the different approaches of these two procedures, both analyses suggest that many male brown-headed nuthatches exhibit high leves of relatedness within a 1-2km geographic distance interval, which closely approximates the previous estimate of Cox & Slater (2007).

Contrary to expectations, the average distance separating related females was only about 200m greater than for dominant males. This finding contradicts the assumption that males disperse significantly shorter distances than females; however, the range of dispersal distances in the dataset for both sexes suggests that at least some females may be dispersing larger distances than males (*i.e.* >1km). Interestingly, there was a greater number of pairwise comparisons of females (n=31) than males (n=19) that showed a relatedness estimate greater than or equal to 0.50. Tenable biological conclusions cannot be made from this finding because territory sampling was incomplete at the study site; however, this data suggest that females may not be

dispersing substantially larger distances than males as previously thought, although additional studies are needed to validate the findings in this study.

Populations undergoing restricted gene flow and an absence of selection are expected to be characterized by positive spatial genetic autocorrelation at small distances, subsequently declining through zero and becoming negative (Peakall *et al.* 2003). I predicted that spatial autocorrelation would be higher at small geographic distances accompanied by a steady decrease in *r* due to the restricted dispersal and overall sedentary lifestyle described for the brown-headed nuthatch (Withgott & Smith 1998). However, significance for this pattern was only found in *All Males*, although it was also observed, albeit non-significant, in *All Individuals*. Both of these sampling categories included male helpers, which further illustrates the impact that auxiliary males have on generating positive genetic autocorrelation at small distances in the brown-headed nuthatch. Other studies employing spatial autocorrelation on cooperative breeding birds did however find the predicted pattern for all individuals (Double *et al.* 2005; Temple *et al.* 2006), although these studies were based on larger sample sizes.

The information presented in this paper provides the first genetic study on brown-headed nuthatches and indicates that genetics needs to be considered in conservation and management decisions. The results suggest that cooperative breeding and the retention of putatively related auxiliary adults on the natal territory may facilitate high levels of relatedness at small geographic distances. However, a recent study reported that only 10-32% of groups exhibit cooperative breeding in the brown-headed nuthatch (Cox & Slater 2007); thus, philopatric offspring may not have a large overall effect on fine-scale spatial genetic structure in this species, as suggested by the autocorrelation analyses performed on dominant males only (*i.e.* those without helpers).

The relatedness by distance analyses suggest that both sexes may be dispersing small distances on average (*i.e.* <2km), although this study was limited to a single study site and is therefore unable to assess long-distance dispersal. Nonetheless, if males are dispersing shorter distances than females, then populations of the brown-headed nuthatch may be particularly dependent upon female-mediated gene flow for the introduction of novel alleles and maintenance of genetic variation within populations. If both sexes are dispersing only limited distances, as has been suggested elsewhere (Withgott & Smith 1998), then populations of the brown-headed nuthatch may require habitat corridors (*e.g.* pine plantations) to facilitate among-population dispersal in fragmented landscapes. Limited dispersal combined with sociality and ecological specialization, as seen in the brown-headed nuthatch, has been suggested to increase sensitivity to habitat fragmentation (Henle *et al.* 2004). Moreover, habitat fragmentation has already been documented to restrict dispersal in some other cooperative breeding species that also exhibit limited dispersal (Breininger 1999; Cooper & Walters 2002).

Restricted dispersal can reduce gene flow both within and among populations and lead to increased levels of relatedness within populations (Painter *et al.* 2000). Increased relatedness among social groups may increase the occurrence of inbreeding, which may be a particular concern in cooperative breeders where dispersal distances are usually short and relatedness is typically high (Koenig & Haydock 2004). Although inbreeding has been shown to reduce fitness in some cooperative breeding birds (Brown & Brown 1998; Daniels & Walters 2000), incest has been documented only twice in the brown-headed nuthatch (Fleetwood 1946; J. Cox, unpublished data). On the other hand, the analyses in this study revealed that many individuals exhibited high pairwise relatedness with members of the same sex over small geographic distances. In light of this finding, long-term management efforts of the brown-headed nuthatch

should consider maintaining sufficient genetic variation within small, potentially isolated populations.

In conclusion, this study provided a preliminary assessment of spatial genetic structure in the brown-headed nuthatch. Follow-up studies are needed that combine additional genetic and demographic information to enable a better understanding of the complex interplay between demography and spatial genetic structure in this declining species. In addition, further research is needed to elucidate the genetic mating system of this cooperative breeding bird, and also needed are studies that analyze the population genetic structure among populations to examine dispersal patterns at the landscape level. The latter will be especially important for management and conservation of the brown-headed nuthatch given the prediction that populations of this species will continue to become further isolated and fragmented as habitat destruction continues (Jackson 1988).

A

B

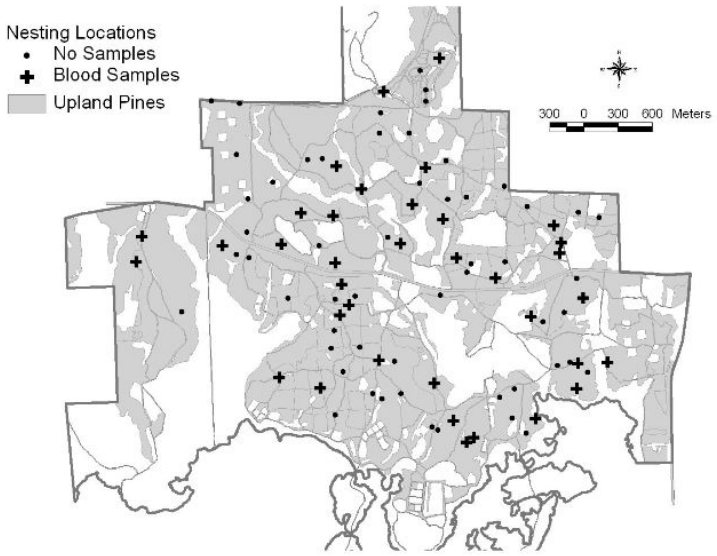
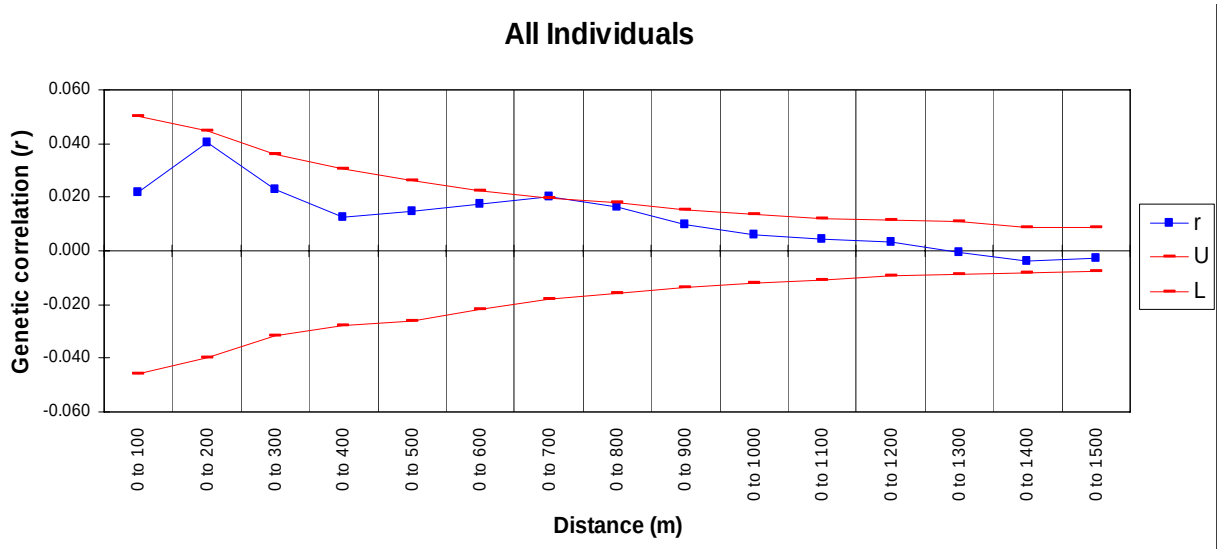
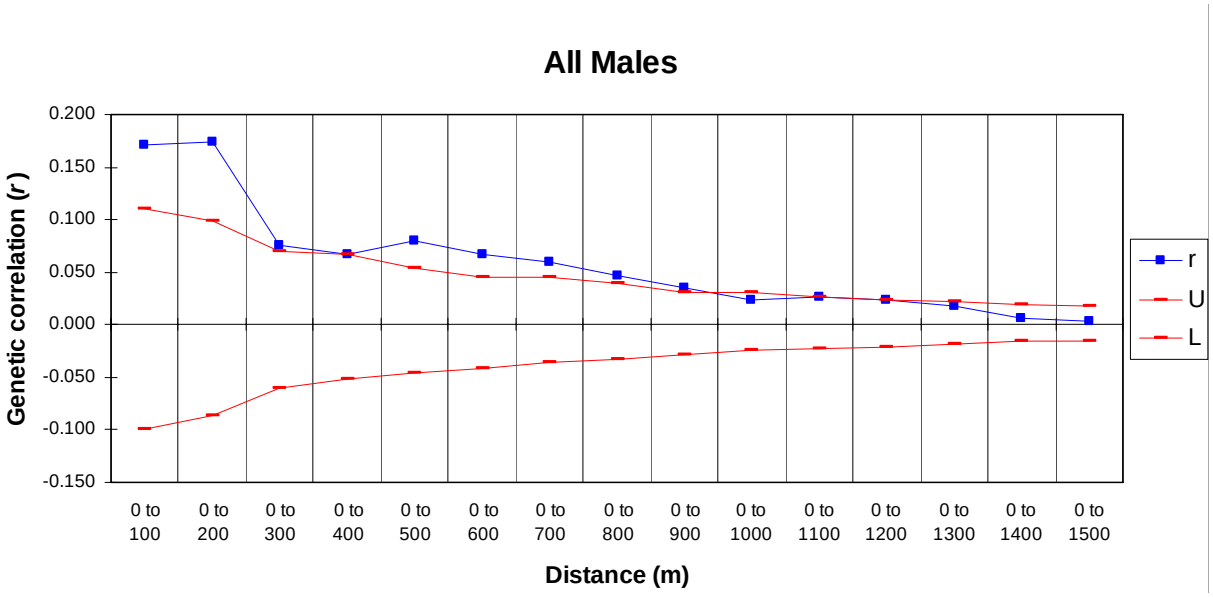


Figure 3-1. Maps showing the sampling site used in this study. A) Leon County, Florida, USA, which is where Tall Timbers Research Station (TTRS) is located (30.66 N, 84.22 W). B) + denotes individual territory locations (*n*= 36) at TTRS that were used in this study.

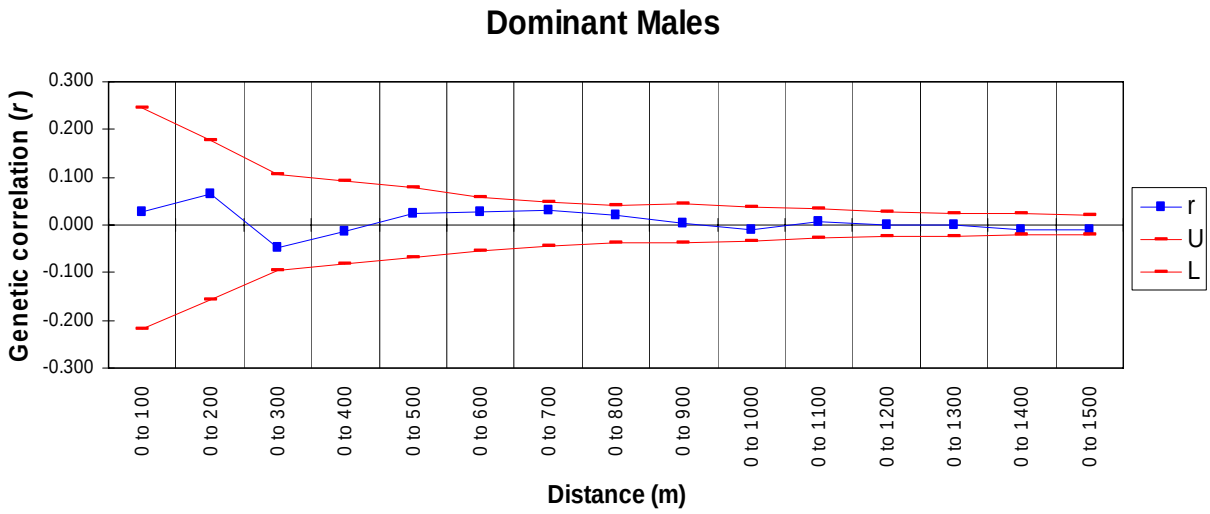
A


All Individuals
Genetic correlation (r)
0.060
0.040
0.020
0.000
-0.020
-0.040
-0.060
0 to 100
0 to 200
0 to 300
0 to 400
0 to 500
0 to 600
0 to 700
0 to 800
0 to 900
0 to 1000
0 to 1100
0 to 1200
0 to 1300
0 to 1400
0 to 1500
Distance (m)
r
U
L

B


All Males
Genetic correlation (r)
0.200
0.150
0.100
0.050
0.000
-0.050
-0.100
-0.150
0 to 100
0 to 200
0 to 300
0 to 400
0 to 500
0 to 600
0 to 700
0 to 800
0 to 900
0 to 1000
0 to 1100
0 to 1200
0 to 1300
0 to 1400
0 to 1500
Distance (m)
r
U
L

C



D

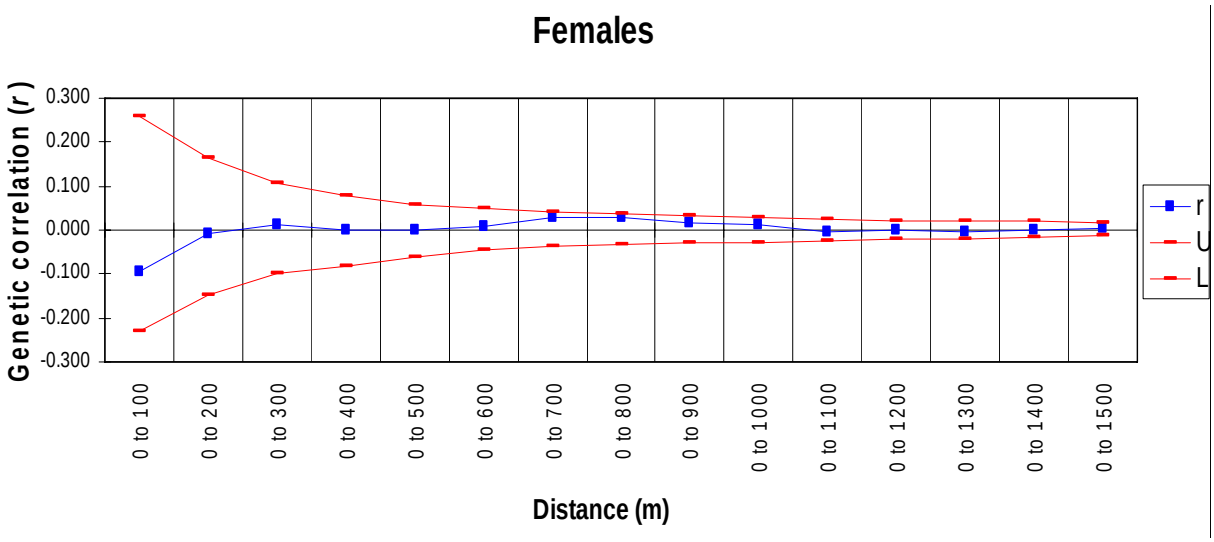


Figure 3-2. Graph showing the influence of different class sizes on genetic autocorrelation. The permuted 95% confidence interval (red lines) is shown. A) *All Individuals* (*n*= 70). B) *All males* (*n*= 37). C) *Dominant Males* (*n*= 29). D) *Females* (*n*= 33).

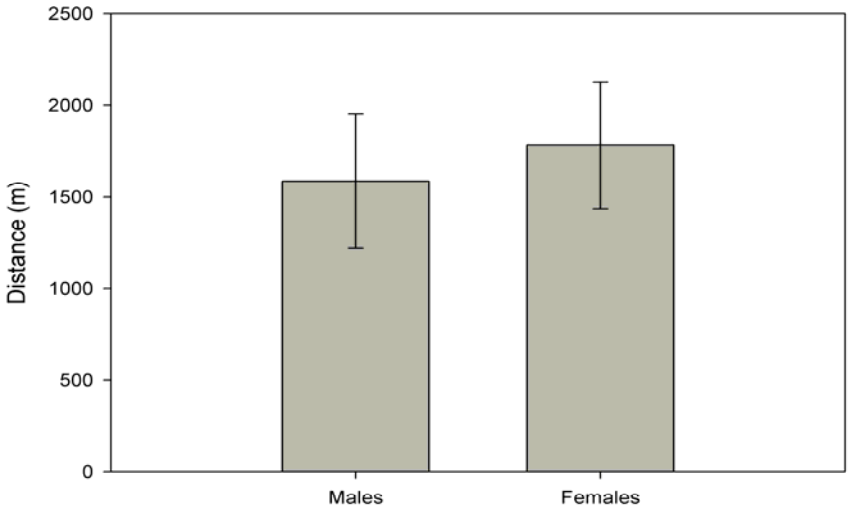


Figure 3-3. Graph showing the average geographic distance in meters separating pairs of males and females ($n_{females}$=31; n_{males}=19) from TTRS that exhibit maximum likelihood estimates of relatedness equal to or greater than 0.50. Bars represent the 95% upper and lower confidence intervals around the mean.

Table 3-1. Characterization of five polymorphic microsatellite loci used in this study. These data are from the 70 individuals sampled from TTRS during the spring of 2006.

Locus	Repeat Motif	Size range (bp)	GenBank accession number (primers)	Final Mg2+(mM)	n	k	H_O	H_E
*Spu*L5-6	$(GT)_{27}$	229-295	EF474467	2.5	69	24	0.841	0.910
*Spu*L4-31*	$(GT)_{15}$	271-319	EF474470	2.5	60	11	0.667	0.825
*Spu*E19	$(GT)_{10}$	234-328	EF474469	3.0	68	15	0.706	0.745
*Spu*A6	$(AC)_{11}$	190-232	EF474468	-	64	13	0.625	0.684
*Spu*L4-3	$(GT)_{27}$	360-406	EF474472	3.0	64	20	0.734	0.877

(*n*), number of individuals genotyped at each locus; (*k*), number of alleles at each locus; H_O, observed heterozygosity; H_E, expected heterozygosity. * Indicates locus with significant deviation from Hardy-Weinberg equilibrium after sequential Bonferroni correction.

CHAPTER 4
CONCLUSIONS

The brown-headed nuthatch exhibits distinctive traits typical of many cooperative breeding birds including limited dispersal, natal philopatry, and habitat specialization (Walters *et al.* 2004). It has been suggested that these characteristics may render cooperative breeding species unusually vulnerable to habitat loss, degradation, and fragmentation (Walters *et al.* 2004). In addition, many cooperatively breeding species are expected to exhibit microspatial structuring due to restricted dispersal and natal philopatry, yet relatively few studies to date have analyzed fine-scale genetic structure in cooperative breeding birds (Double *et al.* 2005; Temple *et al.* 2006; Woxvold *et al.* 2006). For my thesis research, I constructed species-specific polymorphic microsatellite markers to examine patterns of fine-scale spatial genetic structure in the brown-headed nuthatch.

There is no published genetic data for the brown-headed nuthatch prior to the research presented in this thesis. The first portion of my thesis research consisted of developing eight microsatellite loci specific to the brown-headed nuthatch in order to subsequently analyze population genetic structure in this species (Chapter 2). All eight microsatellite markers exhibited extremely high levels of polymorphism (Table 2-2), though only five could be scored consistently enough to be used to examine fine-scale spatial genetic structuring.

For the second part of my thesis research, I used the microsatellite markers to examine patterns of microspatial genetic structure in the brown-headed nuthatch (Chapter 3). As predicted based on male-biased natal philopatry in this species, genetic autocorrelation was more pronounced in males versus females, and became even stronger when male auxiliary individuals were included. These results are similar to the few other studies that have used spatial autocorrelation to examine fine-scale spatial genetic structure in cooperative breeding birds

(Double *et al.* 2005; Temple *et al.* 2006). Although formal kinship analyses using genetics have not been conducted, the results in this study were attributed to putative relatedness between the auxiliary males and the breeding males in which they were assisting. Overall, the spatial autocorrelation results were not as strong as expected, but nonetheless revealed that fine-scale spatial genetic structure exists in the brown-headed nuthatch.

Follow-up studies on the brown-headed nuthatch are needed to address many additional aspects of this species biology. Included are further studies employing spatial autocorrelation, but with larger sample sizes and all eight microsatellite markers. Ongoing collection of demographic data on brown-headed nuthatches from the study site used in this research is needed for additional spatial autocorrelation studies in order to allow a better understanding of the complex interplay between demography and spatial genetic structure. In addition, future studies are also needed to examine the genetic mating system of the brown-headed nuthatch. Knowing whether breeding pairs are strictly monogamous or if extra-pair fertilizations are occurring will help to further understand spatial genetic structure in this cooperative breeding species.

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BIOGRAPHICAL SKETCH

Sarah Haas was born and raised in Corpus Christi, Texas. In May of 2004, she received a Bachelor of Science degree, *magna cum laude*, in biology with a minor in chemistry from Texas State University in San Marcos, Texas. In August of 2004, Sarah began graduate school in the Zoology Department at the University of Florida working under the supervision of Dr. Rebecca Kimball and Dr. Edward Braun. Sarah's thesis research was on the population genetics of the cooperative breeding brown-headed nuthatch (*Sitta pusilla*). In addition, Sarah was the lead investigator in a conservation genetics project on the Florida snail kite (*R.s.plumbeus*). After graduation, Sarah moved to Washington D.C. to spend some time working in environmental policy and other human-dimension aspects of conservation because of her desire to participate in threatened and endangered species conservation while also being actively involved with public outreach and education on environmental issues.