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Fine-scale Spatial Genetic Structure in the Cooperatively Breeding Brown-headed Nuthatch (*Sitta pusilla*)

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Abstract - Many cooperatively breeding birds exhibit fine-scale spatial genetic structure as a result of restricted dispersal and habitat specialization. *Sitta pusilla* (Brown-headed Nuthatch) is a cooperatively breeding bird restricted to mature pine-dominated forests of the southeastern United States and has been undergoing population declines across most of its range. We used five polymorphic microsatellite loci developed for this species to examine fine-scale spatial genetic structure within a site in northern Florida as well as broader genetic structure among this site and two other sites (a second in northern Florida and one in southern Georgia). Spatial autocorrelation analyses within the more densely sampled site detected positive spatial genetic autocorrelation up to 1300 m in males when auxiliary males were included, but no autocorrelation was found in females or in males when auxiliary males were excluded. At the broader scale, we found small but significant genetic differentiation among all three populations, including two sites that were separated by less than 40 km of suitable habitat. Our results suggest that both sexes of the Brown-headed Nuthatch exhibit limited dispersal, with philopatric male auxiliaries contributing to more pronounced genetic structure over small geographic distances compared to females. Our sampled populations were in a region where much suitable habitat remains, yet we still observed limited dispersal. This finding suggests that in more fragmented regions, populations may become isolated and at risk of extinction.

Introduction

Cooperative breeding in birds may occur when species have a limited resource that selects for offspring that remain in the natal territory near that resource (Stacey and Ligon 1987). As such, these species may exhibit restricted dispersal and habitat specialization, both of which may make them particularly sensitive to habitat loss, fragmentation, and degradation by hindering migration to distant habitat patches (Walters et al. 2004). These characteristics may also facilitate the formation of spatial genetic structure among populations as well as fine-scale genetic structure within subpopulations (Woxvold et al. 2006), thereby influencing patterns of genetic relatedness over microgeographic scales. In cooperatively breeding birds, males often inherit their natal territory or breed in neighboring territories (Greenwood 1980, Koenig et al. 1992), which may result in related demes of philopatric

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males, with less spatial genetic structure in the dispersing females (Double et al. 2005, Painter et al. 2000, Temple et al. 2006, Woxvold et al. 2006), although there are exceptions to this pattern (Beck et al. 2008).

Sitta pusilla (Latham) (Brown-headed Nuthatch) is a small (≈10 g), non-migratory, cooperatively breeding passerine restricted to mature pine-dominated forests of the southeastern United States (Withgott and Smith 1998). The percentage of breeding territories in Florida containing one or more auxiliary adults has been documented to vary from 10–32% among sites and years (Cox and Slater 2007). Most groups containing more than two adults consist of a breeding pair and a second-year (i.e., hatched the previous breeding season) auxiliary male that is related to at least one breeding adult (Cox and Slater 2007), though a few groups have been shown to have up to three auxiliary males (J.A. Cox, unpubl. data). Breeding pairs maintain long-term pair bonds and are highly sedentary after territory establishment, frequently excavating nests within 100 m of nests used the previous year (Cox and Slater 2007). The average distance between nearest neighboring nests in north Florida is approximately 198.5 m (SD = 90.7) (Cox and Slater 2007). Field observations suggest natal philopatry is heavily male-biased, although female helpers have been documented (Cox and Slater 2007). Most observed dispersal for males occurs within 300 m of the natal territory, which is generally the nearest neighboring territory (Cox and Slater 2007). In contrast, median dispersal for the limited number of recaptured females (n = 8) is 1450 m (J.A Cox, unpubl. data).

These characteristics of Brown-headed Nuthatches—territory site fidelity, natal philopatry, limited dispersal, and habitat specialization (Cox and Slater 2007, Lloyd and Slater 2007, Withgott and Smith 1998)—are typical of other cooperative breeding birds and have been suggested to increase susceptibility to habitat degradation and lead to fine-scale spatial genetic structure (Walters et al. 2004, Woxvold et al. 2006). Long-term population declines throughout the range of Brown-headed Nuthatches, attributed to human development, fire suppression, and logging, have led to increased conservation concern for this species (Sauer et al. 2005, USFWS 2002, Withgott and Smith 1998). Despite ongoing population declines and the prediction that populations will continue to decline as forests become further fragmented (Jackson 1988), there remains little research on the Brown-headed Nuthatch.

Molecular assessments that examine spatial genetic structure within and among populations could be useful for the conservation and management of the Brown-headed Nuthatch by providing a greater understanding about levels of genetic variability, dispersal patterns, and the probability that populations may become isolated and eventually go extinct. In this study, we examined fine-scale spatial genetic structure in the Brown-headed Nuthatch using recently developed microsatellite markers for this species (Haas et al. 2009). Samples were collected within a single well-studied site

in northern Florida to address fine-scale genetic structure, and compared with two other sampling areas in northern Florida and southern Georgia to examine broader-scale spatial genetic structure.

Field-Site Description

Two sampling sites were located in northern Florida (Tall Timbers Research Station in Leon County [TTRS; n = 70], and Osceola National Forest in Baker County [ONF; n = 16]) and one site in southern Georgia (Pebble Hill Plantation in Grady County [PHP; n = 17]) (Fig. 1). TTRS encompasses 1630 ha and is dominated by upland pine habitats consisting primarily of *Pinus taeda* L. (Loblolly Pine) and *P. echinata* P. Mill (Shortleaf Pine). PHP consists of 1214 ha and has a mix of mature *P. palustris* P. Mill (Longleaf Pine) and pine habitats similar to TTRS, while ONF encompasses 63,631 ha and is dominated by pine flatwoods and cypress-hardwood swamps. Sampling at each site was conducted from February through May of 2006 using mist-netting procedures described in Cox and Slater (2007).

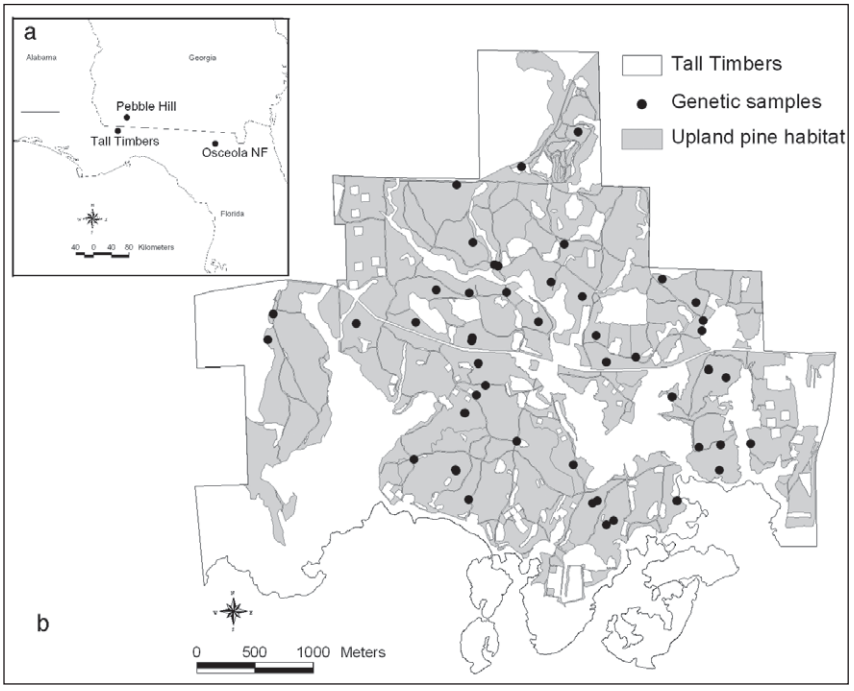

Figure 1. Maps showing the sampling sites used in this study. (a - inset) The three sampling localities used in this study, including Tall Timbers Research Station (TTRS, n = 70, lat/long: 30˚39´N, 84˚12´W), Pebble Hill (PHP, n = 17, lat/long: 30˚45´N, 84˚07´W), and Osceola National Forest (ONF, n = 16, lat/long: 30˚19´N, 82˚21´W); (b) The spatial configuration of sampled territories (n = 36, 40% of known territories during the two years of study) at TTRS.

Methods

Sample collection

Adult birds from all three field sites were mist-netted, sampled for blood (20–40 µL), banded, and released in the same location. The TTRS samples were used for spatial autocorrelation analysis and consisted of 70 birds from 36 territories, which represented approximately 40% of known territories on TTRS during the study. Of these 70 individuals, 33 were females and 37 were males, with the latter including eight auxiliary males. Only birds from TTRS were color-banded, as these individuals have been monitored at TTRS since 2001; monitoring methods can be found in Cox and Slater (2007). Breeding status (i.e., breeder versus auxiliary) of individuals within groups from TTRS that contained more than two adults was determined using behavioral observations (e.g., dominance, incubation, and copulation) and information from previous breeding seasons if available. The breeding status of adults at PHP and ONF was unknown, and sampled birds at these sites were fitted with a single federal band. Sampling 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 for spatial autocorrelation analysis. Blood samples collected in the field were stored in 1 mL of lysis buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M EDTA, 0.01 M NaCl, 1% SDS).

DNA extraction, PCR amplification, and genotyping

Genomic DNA was extracted using a PUREGENE® DNA Purification Kit (Biozym, Hess. Oldendorf, Germany), and molecular sexing for this sexually monomorphic species was performed following procedures outlined in Fridolfsson and Ellegren (1999). We used five polymorphic di-nucleotide microsatellite markers specific to the Brown-headed Nuthatch to genotype all individuals used in this study: *Spu*L5-6, *Spu*A6, *Spu*E19, *Spu*L4-31, and *Spu*L4-3 (Haas et al. 2009). The microsatellites were amplified by PCR, with each 10-uL reaction volume consisting of 1X PCR buffer (10mM Tris-HCl, 50mM KCl, 1.5mM MgCl2), 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. Magnesium concentrations and cycling conditions can be found in Haas et al. (2009). Allele sizes were determined using a MegaBACE 1000 DNA Sequencer (Amersham, Sunnyvale, CA), and raw data were analyzed using GeneMarker® v.1.5 (SoftGenetics LLC, State College, PA).

Statistical analyses

Genetic analyses included exact tests for departures from Hardy-Weinberg equilibrium (HWE) using a Markov chain method with 5000 iterations in GENEPOP, version 3.4 (Raymond and Rousset 1995). GENEPOP was also used to evaluate linkage disequilibrium within each sampling area. Auxiliary adults were excluded from these analyses because relatedness between auxiliary and breeding adults could bias results. 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 presence of null alleles were calculated using CERVUS, version 2.0 (Marshall et al. 1998).

Spatial autocorrelation analysis was performed using GenAlEx, version 6 (Peakall and Smouse 2006) to examine fine-scale spatial genetic structure within TTRS. GenAlEx 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. We specified a base distance class size of 100 m for 15 runs so that the first distance interval would calculate *r* based on all pairwise comparisons within a distance of 0–100 m, the second analysis for 0–200 m, and so on until the last run (i.e., 0–1500 m) was completed. This base distance class was chosen because it was the smallest distance interval that still encompassed multiple territories; four of the 36 (11.1%) territories from TTRS had sampled nearest neighboring territories within this distance. Autocorrelation coefficients were calculated for four sampling categories: (1) all individuals; (2) all males (includes auxiliary males); (3) dominant males (breeding males only); and (4) females. These non-independent categories were chosen in order to explore the effects of sex and the presence of auxiliary adults on patterns of fine-scale genetic relatedness. We did not analyze auxiliary males as a separate sampling category due to the small sample size obtained for these individuals ($n = 8$). Statistical significance ($P \leq 0.05$) was tested in GenAlEx6 using 1000 random permutations.

We used ML-RELATE (Kalinowski et al. 2006) to estimate relatedness for pairs of individual dominant males and females separately within TTRS. This program calculates maximum likelihood estimates of relatedness (*r*) from co-dominant genetic data. Geographic distances separating pairwise comparisons where $r \geq 0.50$ were recorded for each sex. The statistical software R (R Development Core Team 2008) was then used to perform a one-tailed Wilcoxon rank sum test to assess whether the average geographic distance separating related males differed significantly from that of related females and a Levene's test of homogeneity of variances was performed to determine whether differences existed in the variation between male and female distances. This additional approach for investigating fine-scale spatial genetic structure was performed because spatial autocorrelation procedures, which analyze all individuals located within user-specified distance classes, may not entirely capture the underlying spatial genetic structure if sampling is not exhaustive, as was the case at TTRS. We also estimated relatedness for pairs of putatively related auxiliary and dominant individuals at territories where helpers occurred.

We used F-statistics (Wright 1951) to assess genetic differentiation among the three sampling sites. We calculated both global and pairwise F-statistics using approaches in Weir and Cockerham (1984), which corrects

for sample size variation among sampling units. We tested for genetic differentiation in GENEPOP using Markov chain parameters that included a dememorization number of 5000, 500 batches, and 5000 iterations per batch (Guo and Thompson 1992). Global F_{ST} greater than zero indicates greater subdivision of genetic variance among groups than within groups, while pairwise F_{ST} estimates genetic differentiation between specific sampling localities.

Results

Genetic variation

The five polymorphic loci had an average observed heterozygosity of 0.73; the average number of alleles per locus was 19.40 (range = 12–28) (Table 1). Excluding auxiliaries, one locus (*Spu*L4-31, $P = 0.0026$) within TTRS and one locus (*Spu*L4-3, $P = 0.0016$) within ONF deviated from HWE following Bonferroni correction for multiple tests. These deviations most likely arose from the presence of null alleles specific to these sampling areas (null allele frequency estimates: *Spu*L4-31 = 0.10 in TTRS, *Spu*L4-3 = 0.14 in ONF). None of the remaining loci showed evidence for null alleles. Linkage disequilibrium was not detected within population samples ($P > 0.05$), and the average proportion of individuals genotyped at all five loci was 0.94.

Spatial genetic structure

Of the 70 individuals sampled from TTRS ($n = 33$ females, $n = 37$ males), eight of these were auxiliary males associated with seven of the 36 (19.4%) sampled territories. No female helpers were identified. Prior field data available for two of these auxiliary individuals indicated that both were banded the previous year as nestlings at the nest of the male they were currently assisting (J.A. Cox, unpubl. data), and relatedness estimates ($r \geq 0.50$) confirmed parent-offspring relationships for these two pairs. Prior field data were also available for one additional auxiliary individual that was sampled as an adult helping at the same nest the year before; however, relatedness estimates between this individual and the dominant male revealed an absence of genetic relatedness ($r = 0.00$). Of the remaining five auxiliary males, in

Table 1. Allelic diversity of Brown-headed Nuthatches from three sampling sites (TTRS, ONF, PHP) using five polymorphic microsatellite markers. k = number of alleles (number of unique alleles in parentheses); H_O = observed heterozygosity.

	*Spu*A6		*Spu*E19		*Spu*L5-6		*Spu*L4-31		*Spu*L4-3	
Locus	k	H_O	k	H_O	k	H_O	k	H_O	k	H_O
TTRS ($n = 70$)	13 (5)	0.63	15 (7)	0.71	24 (6)	0.84	11 (2)	0.67*	20 (5)	0.73
ONF ($n = 16$)	10 (2)	0.75	5 (0)	0.63	13 (3)	0.75	7 (1)	0.88	12 (3)	0.67*
PHP ($n = 17$)	9 (1)	0.73	8 (1)	0.94	14 (1)	0.82	8 (0)	0.82	15 (1)	0.75
Combined ($n = 95$)	17	0.66	16	0.72	28	0.83	12	0.73	24	0.76*

*Deviation from Hardy Weinberg Equilibrium (auxiliary individuals were omitted from HWE calculations).

which previous field data were unavailable, only a single individual exhibited parent-offspring relatedness estimates with the dominant male, while the other four auxiliaries had estimates of approximately $r = 0.00$.

Spatial autocorrelation analyses revealed that the all males category exhibited significant positive genetic autocorrelation at all distance intervals until 1300 m (range of significance for each distance class from 0–1300 m: $P = 0.002$ to $P = 0.043$), except for 0–1000 m ($P = 0.066$), with higher autocorrelation detected at small geographic distances followed by a decrease in genetic relatedness as a function of geographic distance (Fig. 2a). A similar, although non-significant, pattern was found in the all individuals category (Fig. 2b), which also included females and auxiliary males. Dominant males (Fig. 2c) and females (Fig. 2d) did not exhibit significant spatial genetic autocorrelation nor demonstrate a pattern of decreasing autocorrelation as geographic distance increased, suggesting that auxiliary males were driving the observed spatial genetic patterns.

Using ML-RELATE, a total of 19 pairwise comparisons among the sampled dominant males ($n = 29$) and 31 pairwise comparisons among the sampled females ($n = 33$) from TTRS were observed that exhibited relatedness $r \geq 0.50$. The average distance separating related pairs of dominant males (1585 m) and pairs of related females (1780 m) did not differ significantly ($P = 0.40$). The test of homogeneity of variances revealed that the variance in the distances separating pairs of related males and females was not statistically different ($P = 0.32$). However, the range of distances separating males (193–2423 m) was narrower than that for females (192–3799 m), and a test of homogeneity of variances using only the top 50% of the distance values revealed significant differences ($P = 0.02$) between the sexes (Fig 3).

The within-population results suggest there can be longer-range dispersal, but it is likely limited. Consistent with this, examination across populations resulted in small but significant genetic structure ($F_{ST} = 0.01$, $P < 0.001$) among all three sampled locations. Tests of genetic differentiation for pairwise estimates of F_{ST} among the three sites were also statistically significant (F_{ST} range = 0.01–0.02; $P < 0.01$). We did not compare pairwise F_{ST} for males and females separately due to the smaller sample sizes at PHP and ONF.

Discussion

Prior field data from TTRS based on color-band re-sighting of Brown-headed Nuthatches suggest that males exhibit high rates of natal philopatry and typically disperse short distances, while females help parents less frequently and disperse greater distances than males (Cox and Slater 2007). In this study, we sampled seven territories (19.4%) at TTRS that included auxiliary males, which is in accordance with previous estimates of 10–32% of breeding territories containing one or more auxiliary adults (Cox and Slater 2007). The observation of unrelated auxiliaries has been previously documented in Brown-headed Nuthatches, in which adult males provided

assistance at neighboring nests following the failure of their own nests or inability to acquire their own territory (i.e., facultative helping; Cox and Slater 2007). In one of these cases in our study, the auxiliary was related to the female but not the dominant male, and may have resulted from an extra-pair copulation. For the others, we have insufficient data to determine whether

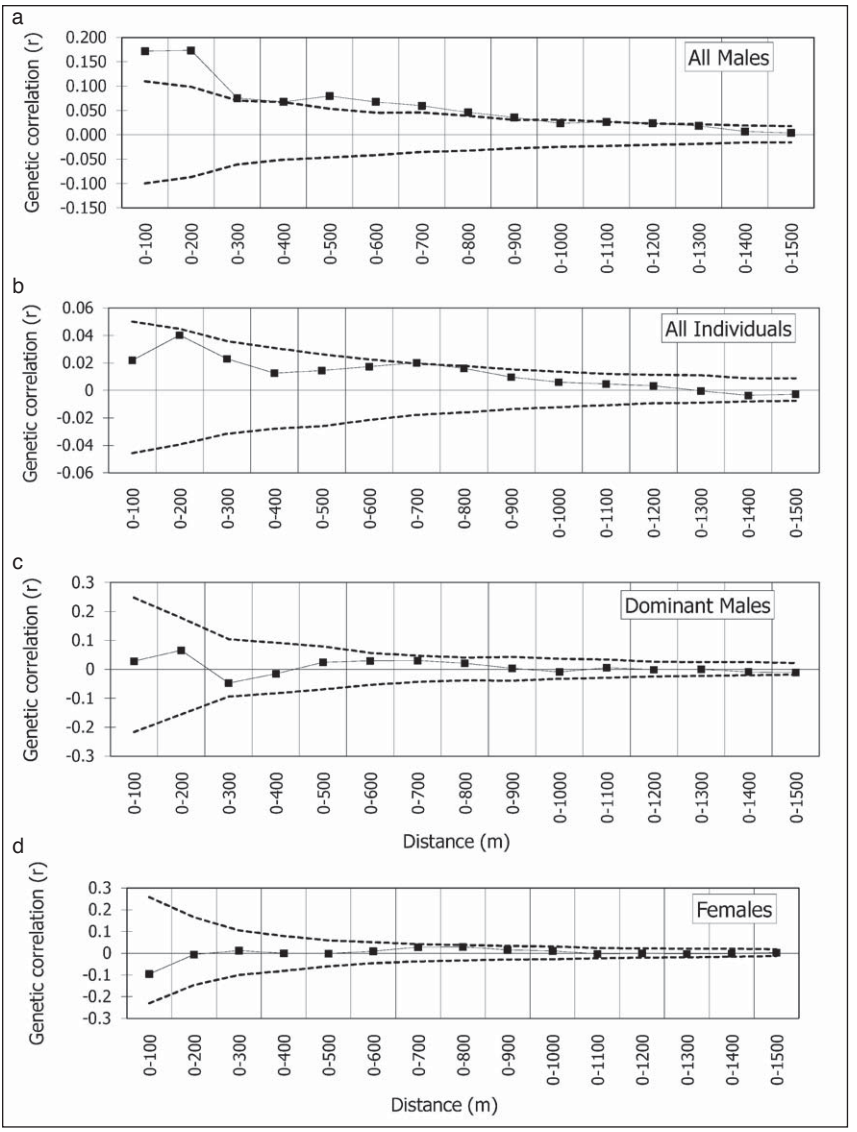

Figure 2. Genetic autocorrelation (*r*) across geographic distances. The permuted 95% confidence interval is shown (dashed lines represent the 25th and 975th limits). Significant spatial genetic structure occurs when *r* exceeded the confidence intervals. (A) all males ($n = 37$), (B) all individuals ($n = 70$), (C) dominant males ($n = 29$), and (D) females ($n = 33$).

these are unrelated individuals assisting a nest or offspring from previous years resulting from extra-pair fertilizations.

This pattern of male-biased natal philopatry and limited dispersal should lead to greater fine-scale spatial genetic structure in the philopatric sex, with less structure in the dispersing sex (Peakall et al. 2003). Spatial autocorrelation analysis revealed significant fine-scale genetic structure for all males, but not just the dominant males. The difference is likely due to related auxillaries within natal territories, to unrelated auxillaries that are likely extra-pair offspring sired by neighboring males, and possibly from offspring of neighboring territories that dispersed to become auxillaries. Other studies implementing spatial autocorrelation analyses for assessing fine-scale spatial genetic structure in cooperatively breeding birds also detected stronger positive autocorrelation in the dispersal-restricted sex when auxiliary adults were included in the analyses, and attributed these patterns to auxiliary individuals being related to dominant individuals (Double et al. 2005, Temple et al. 2006).

In spatial autocorrelation, the distance class at which genetic autocorrelation is no longer significantly positive approximates the extent of

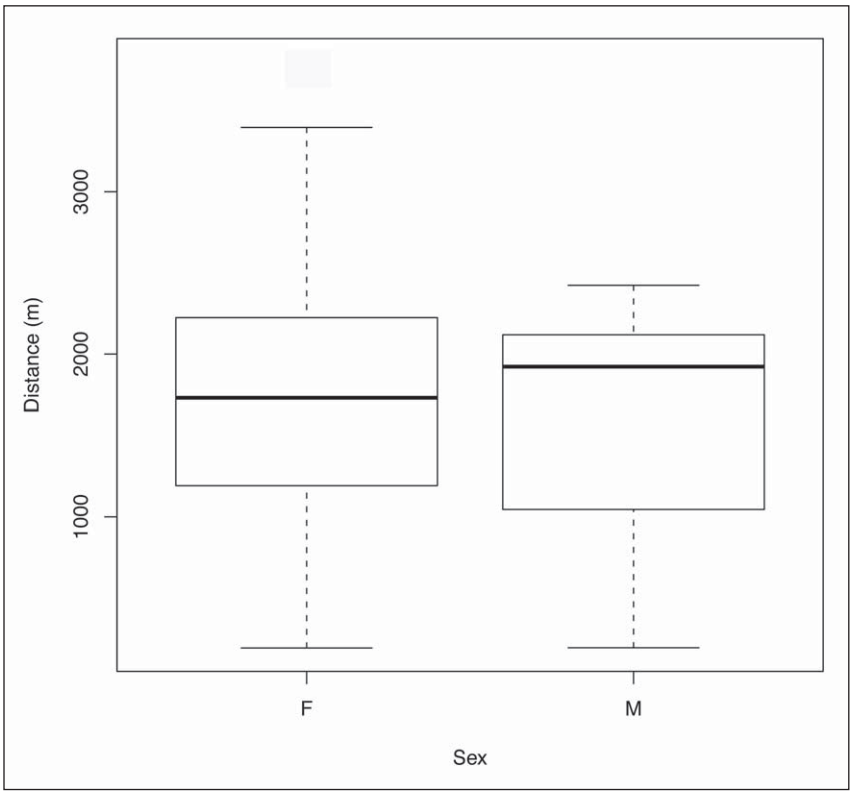

Figure 3. Boxplot illustrating the geographic distances (in meters) separating pairwise comparisons of related ($r \geq 0.50$) individuals for each sex (F = female, M = male).

detectable positive genetic structure (Peakall et al. 2003), and is similar to the "genetic neighborhood" of a population (Golenberg 1987, Wright 1946). In many cooperatively breeding species, these neighborhoods are characterized by high genetic relatedness in the philopatric sex (Daniels and Walters 2000, Hegner and Emlen 1987). Spatial autocorrelation revealed significant positive genetic structure among all males extending beyond six average territory widths (1300 m, average territory width 200 m), excluding a single distance class (0–1000 m) that was not significant. This estimate is similar to field observations, which indicate that the average dispersal distance for second-year males dispersing more than two territories from the natal territory is 1358 m (Cox and Slater 2007). Pairwise estimates of relatedness suggested that the average geographic distance separating related males (excluding auxiliaries) at TTRS was approximately 1600 m, only slightly higher than the genetic neighborhood estimate of 1300 m for all males using spatial autocorrelation. Although the autocorrelation analysis did not detect statistical significance in dominant males for any of the distance classes, spatial autocorrelation takes into account all sampled individuals within a user-specified distance class regardless of genealogical relationship. Not all territories at TTRS could be sampled, and it is possible that denser sampling might reveal positive spatial autocorrelation in both all males and the subset dominant males, as was detected in *Malurus cyaneus* (Ellis) (Superb Fairy-wren) and *Ramphocinclus brachyurus* (Vieillot) (White-breasted Thrasher) (Double et al. 2005, Temple et al. 2006). Despite differing methodologies, both approaches suggest that natal dispersal and genetic neighborhoods of male Brown-headed Nuthatches occur within one to two kilometers of the natal territory.

Contrary to field-based expectations of greater female dispersal, the average geographic distance found between related pairs of females was only 200 m greater than that for related dominant males. This finding might suggest that females are not dispersing substantially greater distances than males, in contrast to the low mark-recapture success of females at TTRS (Cox and Slater 2007). However, there was a statistically significant difference in the variance of distances separating pairs of males and females when only the top 50% of observations were used, and the maximum distance between related females is much larger (1.4 km, or approximately seven territory widths) than that between males. These findings suggest that females may account for most long-distance dispersal events and are likely important for maintaining genetic exchange among neighboring and potentially fragmented populations. Moreover, the negative, albeit statistically non-significant, spatial autocorrelation detected in females at the smallest distance intervals could reflect the propensity of females to disperse greater distances from the natal territory as compared to males, as suggested by field observations (Cox and Slater 2007; J.A. Cox, unpubl. data). Given the high variance in distance between related females, it may

require much larger sample sizes for females to detect greater female dispersal using spatial autocorrelation.

Supporting the limited size (<2 km) of male genetic neighborhoods observed at TTRS, analysis of broader-scale genetic structure among the three sampling localities suggested genetic differentiation can take place over small geographic distances in the Brown-headed Nuthatch. Samples from TTRS and PHP were separated by less than 40 km of suitable habitat, yet still exhibited small but significant genetic differentiation. McDonald et al. (1999) reported that genetic differentiation among populations of cooperatively breeding *Aphelocoma coerulescens* (Bosc) (Florida Scrub-jay) was three times higher than in its nonsocial sister species, *Aphelocoma californica* (Vigors) (Western Scrub-Jay). The authors attributed this finding to differences in the ecology of the Florida Scrub-Jay, including the highly sedentary lifestyle and habitat specialization of this cooperatively breeding species, features also shared by Brown-headed Nuthatches.

Conservation and management implications

Molecular genetics approaches are useful for assessing levels of relatedness among individuals within small and potentially isolated populations as well as for inferring patterns of gene flow both within and among populations (Allendorf and Luikart 2007). Such information can be valuable for management objectives that seek to preserve the genetic health of threatened species and may help to prevent the need for more drastic management actions such as "genetic rescue", in which translocations of individuals are needed to maintain adequate levels of genetic variation in a population (Tallmon et al. 2004). This preservation of within-population genetic diversity may be particularly important for cooperatively breeding species, such as the Brown-headed Nuthatch, since these species often exhibit sedentary lifestyles, natal philopatry, and restricted dispersal (Walters et al. 2004, Woxvold et al. 2006).

This paper provides the first assessment of genetic structure in the cooperatively breeding Brown-headed Nuthatch. It has been suggested that this species seldom ventures from pine-dominated forests due to their specialized habitat requirements and limited dispersal (Cox and Slater 2007, Lloyd and Slater 2007, Wilson and Watts 1999, Withgott and Smith 1998), which has led to concerns that individuals will be unlikely to recolonize distant fragments upon local extirpation (Withgott and Smith 1998). Our results, which demonstrate genetic differentiation among geographically close populations, are consistent with this suggestion. However, the differences we found are small, and the data from TTRS suggest that some individuals are likely to disperse longer distances. Thus, at least in areas where sufficient suitable habitat remains, there may be sufficient gene flow to prevent excess inbreeding and facilitate recolonization of extirpated populations if necessary.

Additional molecular genetic studies of the Brown-headed Nuthatch that analyze spatial genetic structure in relation to specific landscape features such as habitat fragmentation (e.g., landscape genetics; Manel et al. 2003), as well as studies that enable a better understanding of their genetic mating system will be important to fully understand how to best conserve and manage this little-studied species. These areas of research will be especially important given the prediction that populations of Brown-headed Nuthatches will continue to become further isolated as habitat fragmentation of southeastern pine forests proceeds (Jackson 1988).

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