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A genetic assessment of the red squirrel in Illinois: Natives, immigrants, or exotics?

William Beatty
Eastern Illinois University

A GENETIC ASSESSMENT OF THE RED SQUIRREL IN ILLINOIS: NATIVES, IMMIGRANTS, OR EXOTICS?

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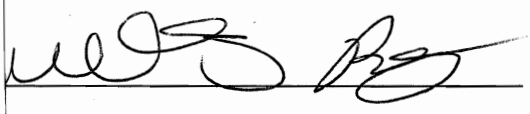

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A GENETIC ASSESSMENT OF THE RED SQUIRREL IN ILLINOIS: NATIVES, IMMIGRANTS, OR EXOTICS?

BY

WILLIAM BEATTY

THESIS

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2008

I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING
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DEDICATION

For my family, and their unwavering love and support throughout the years,

Elizabeth, Mark, and Daniel for inspiring me,

Mom, for her endearing love and affection, and

Dad, for instilling a love of the outdoors in me, and the inspiration to enter a field in conservation.

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CHAPTER 1

THE ORIGIN OF THE RED SQUIRREL POPULATION IN ILLINOIS INFERRED FROM MTDNA

ABSTRACT

A small population of red squirrels (*Tamiasciurus hudsonicus*) has existed in Iroquois County, Illinois since the 1970's. The origin of this population is unknown, but some accounts suggest they are the offspring of released red squirrels from Minnesota. Alternatively, they may be a remnant Illinois population or more recent immigrants from Indiana. Finally, this population could reflect a mixed ancestry of the above scenarios. Mitochondrial DNA (mtDNA) was utilized to determine the genetic relationships among red squirrel populations in Illinois, Indiana, Michigan, Minnesota, and Wisconsin. My results showed low levels of haplotype diversity and nucleotide diversity in the Illinois sample. Pair-wise comparisons of Φ_{ST} values indicated the present Illinois population is most similar to squirrels from Indiana. An unrooted haplotype cladogram (TCS) and haplotype frequency distribution also indicated Illinois squirrels are genetically similar to squirrels in Indiana, but some evidence of a Minnesota component is present. We conclude the present Illinois red squirrel population is primarily composed of descendents of Indiana immigrants.

INTRODUCTION

The North American red squirrel (*Tamiasciurus hudsonicus*) is common throughout much of its geographic range and the biology and ecology of this species have been studied extensively. Topics as diverse as genetics (Arbogast et al. 2001; Wilson et al. 2005), coevolution with conifers (Parchman and Benkman 2002), competition with other granivores (Benkman et al. 2001), and edge effects (Anderson and Boutin 2002) have been addressed in recent years. The present range of the red squirrel in North America encompasses much of the Rocky Mountains, Appalachian Mountains, and New England. Although red squirrels are commonly associated with coniferous forests, they are known to occur in the heavily fragmented landscape of the Midwest, including Indiana and Ohio (Steele 1998). The pine plantations, fencerows, and mixed forest patches characteristic of the Midwest are considered marginal habitat for the species (Yahner 2003). Despite this, red squirrels have persisted in areas such as Indiana throughout the 20th century (Lyon 1936).

The history and ecology of the red squirrel in Illinois are poorly understood. It likely inhabited the northern portion of the state in the early 1800s, but disappeared circa 1900 (Hoffmeister 1989). In 1977, a population of unknown origin was identified in Kankakee Co. in northeastern Illinois. Shortly thereafter, red squirrels were confirmed in Will Co. to the north and Iroquois Co. to the south (Hoffmeister 1989). Speculation regarding the geographic source of the newly discovered red squirrel population in Illinois prompted four hypotheses. First, several accounts suggest red squirrels from Minnesota were released near Iroquois Co. State Wildlife Area (ICSWA) in the 1970s establishing the present population (Hoffmeister 1989). Second, a small native

population of red squirrels may have persisted in the area through the early 1900s and went unnoticed until the 1970s. Third, as advocated by Hoffmeister (1989), red squirrels may have immigrated naturally from Lake and Newton Cos. in Indiana in the 1970s. Consistent with the immigration hypothesis is the expansion of red squirrels into the Willow Slough Fish and Wildlife Area, approximately 2 km east of ICSWA, in the 1970s (Mumford and Whitaker 1982). Finally, the current population might be a combination of the descendents of translocated squirrels from Minnesota, remnant Illinois individuals, and/or immigrants from Indiana.

In an earlier attempt to address the origin of Illinois' current red squirrel population, Brown and Edwards (1985) conducted a morphological analysis of 14 skulls from Illinois. Based on this analysis, 7 skulls were assigned to the group native to Indiana and Ohio, and 6 skulls were assigned to the type that occurs in Minnesota, Wisconsin, and northern Michigan. One skull was not assigned to either source due to missing data. Brown and Edwards (1985) offered two, mutually exclusive interpretations of the data: 1) the current population is of mixed ancestry or 2) low correlations in the analysis indicate insufficient data to discriminate between the two subspecies.

The limited geographic range, low numbers, and ambiguous origin of this population has led the Illinois Department of Natural Resources (IDNR) to list the red squirrel as a critical species in need of conservation (IDNR 2005). Recently, playback surveys were conducted throughout northeastern Illinois to delineate the current geographic range of the species in the state, and a radio-telemetry study was conducted to evaluate summer habitat use (Hanson 2008). Results suggested the range of the red squirrel has expanded in Illinois to the northwest and southwest by approximately 70 km

since 1980 (Hanson 2008). Hanson (2008) attributed the current range expansion to dispersal along the riparian corridors of the Kankakee River to the northwest and the Iroquois River to the southwest, lending support to the notion that the re-emergernce of the species in Illinois may be due to immigration from Indiana.

Modern molecular and genetic techniques allow the questions regarding the geographic origin of Illinois' red squirrels to be examined from a different perspective. Arbogast et al. (2001) and Wilson et al. (2005) demonstrated the utility of genetic techniques, examining relationships among red squirrel populations across historic, geographic barriers, particularly rivers and mountain ranges. Arbogast et al. (2001) analyzed the mitochondrial cytochrome-*b* gene in red squirrels and used parsimony analysis to suggest two distinct clades, one confined to southern Colorado, New Mexico and Arizona and another clade spanning the rest of North America. Additionally, the control region [Displacement(D)-loop] in mitochondrial DNA (mtDNA) indicated a phylogenetic split on opposite sides of the Green River in Wyoming (Wilson et al. 2005). Thus, all squirrels collected in my study represent a single clade based upon these data.

There are many characteristics that make mtDNA suitable for an analysis of genetic relationships in mammals. Mitochondrial DNA has high mutation rates compared to most nuclear DNA and does not undergo recombination. Pedigree studies have estimated mutation rates within the mitochondrial control region to range from 0.025 (Howell et al. 1996), to 0.0043 per generation (Siguroardóttir et al. 2000).

The heavily fragmented landscape of the Midwest poses a different challenge for studying the genetics of forest-dwelling taxa. While there are few topographic barriers to

gene flow, large expanses of unsuitable habitat, including agricultural fields, roads, and urban areas, may isolate red squirrel populations. For example, the current geographic range of the red squirrel indicates a disconnect between the Wisconsin and Illinois populations (Hanson 2008). Therefore, the primary objectives of this study were to: (1) elucidate the geographic origin of the current red squirrel population in Illinois utilizing mtDNA and (2) examine intraspecific genetic variation across the states of Illinois, Indiana, Michigan, Minnesota, and Wisconsin.

METHODS

A total of 229 tissue samples was collected from red squirrels inhabiting 5 Midwestern states, including 52 from Illinois, 50 from Indiana, 43 from Michigan, 52 from Minnesota, and 32 from Wisconsin (Figure 1). Samples from the Upper Peninsula (UP) of Michigan were grouped with Wisconsin squirrels in final data analysis due to geographic proximity and a preliminary analysis that indicated the Michigan UP shared more haplotypes (3) with Wisconsin than it did lower Michigan (0). The corrected totals taking into account the re-grouping are: Illinois, 52; Indiana, 50; Michigan, 24; Minnesota, 52; Wisconsin, 51. The methods and results discussed henceforth were based upon this latter grouping.

In Illinois, tissue samples were taken with a 2-mm ear punch from anesthetized live-trapped individuals as part of a concurrent telemetry study (Hanson 2008). Handling and sampling protocols followed the guidelines of the American Society of Mammalogists (Gannon et al. 2007) and were approved by Eastern Illinois University's Animal Care and Use Committee (Permit EIU06010). Researchers, trappers, and hunters provided tissue samples from harvested squirrels in all other states. Samples were

initially preserved in 100% ethyl alcohol or desiccant beads and transferred to an ultra-low freezer (-60°C) for long-term storage.

Whole genomic DNA was extracted using the DNeasy Tissue Kit following the protocol provided by the manufacturer (Qiagen, Valencia, California) or a modified ammonium acetate protocol (Latch et al. 2008). A 272 - 274 base pair section in the hypervariable domain III of the non-coding control region (D-loop) was amplified with polymerase chain reaction using the primers OSU5020L (5'-CCTTTAGCTGGCATAG GTA-3') and OSU5021H (5'-CATTATATGGAGTGGAGAA GG-3'; Wilson et al. 2005). Each 25 µl reaction included 10 – 50 ng of genomic DNA, 2.5 µl 10× buffer, 2.5 mM $MgCl_2$, 1.5 µM of each primer, 0.08 mM of each dNTP (Qiagen) and 0.5 units of *Taq* DNA polymerase (Qiagen). The PCR profile was 94°C for 3 minutes followed by 35 cycles of 94°C for 45 seconds, 54°C for 30 seconds and 72°C for 1 minute with a final elongation of 72°C for 10 minutes. PCR products (5 µl) were electrophoresed through a 1% agarose gel, stained with ethidium bromide and evaluated with ultraviolet light. Successful samples were purified with the Wizard PCR Prep DNA Purification System (Promega, Madison, Wisconsin) and sequenced on an ABI 3730XL with Big Dye 3.1 cycle sequencing chemistry (Applied Biosystems, Foster City, California) at the Purdue University Genomics Core Facility. Samples were sequenced with the forward and reverse primers. All sequences were manually edited in the computer program SEQUENCHER v.4.7 (Bromberg et al. 1995).

Sequences were aligned in CLUSTALX (Thompson et al. 1997) with the default multiple sequence alignment parameters (Gap opening, 10; gap extension, 0.2; DNA Transition Weight, 0.5). The shape parameter of the gamma distribution was calculated

based on a neighbor-joining tree constructed using the program DAMBE v.4.5.57 (Xia and Xie 2001). Nucleotide diversity (π) and haplotype diversity (h) were calculated in ARLEQUIN (v.2.0; Schneider et al. 1997) by state. An analysis of molecular variance (AMOVA; Excoffier et al. 1992) with 16000 permutations was performed in ARLEQUIN based on genetic distances among haplotypes (Tamura and Nei 1993). The AMOVA procedure examined nucleotide diversity within and among states and was used to calculate Φ-statistics between states. Significance was determined with 3000 permutations in ARLEQUIN and the false discovery rate (FDR; Benjamini and Yekutieli 2001) was utilized to obtain an experiment wide alpha level (α_{EW}) to account for multiple tests. This method offers increased statistical power compared to the conservative Bonferroni correction (Rice 1989).

To test the hypothesis of population expansion in the Indiana and Illinois populations, Fu's F_S (Fu 1997) and a mismatch distribution (Rogers and Harpending 1992) also were performed in ARLEQUIN. An unrooted haplotype cladogram was generated in TCS (v.1.21; Clement et al. 2000). A 95% connection limit was applied and gaps were considered a 5th state in the analysis. The cladogram was edited to illustrate the frequency and location of each haplotype.

RESULTS

Alignment of a 272 – 274 bp section of the mitochondrial control region revealed 43 variable sites with 36 transitions, 6 transversions, and 2 insertion/deletion events. A total of 47 haplotypes were found among 229 red squirrels sampled across a five-state region in the Midwest.

The number of haplotypes found in each state ranged from 6 in Illinois to 17 in Wisconsin (Table 1). Twenty-six haplotypes were observed in only one individual with an additional 8 haplotypes found in multiple individuals but observed in only one state. Wisconsin did not share any haplotypes with Illinois or Michigan whereas all other states shared at least one haplotype. Illinois shared three haplotypes with Michigan and one haplotype with Minnesota. Five haplotypes were shared by squirrels inhabiting adjacent states in three cases: Illinois and Indiana, Indiana and Michigan, and Wisconsin and Minnesota. The most common haplotype (A) was observed 28 times in two states, Minnesota and Wisconsin. The second most common haplotype (B) was observed in Illinois and Indiana a total of 27 times. The third most common haplotype (C) was found in 21 individuals in the states of Illinois, Indiana, and Michigan. Illinois was the only state that did not contain at least one unique haplotype. I found 4 unique haplotypes among Indiana squirrels, 6 in Michigan, 10 in Minnesota, and 14 in Wisconsin.

I observed high haplotype diversity and nucleotide diversity among Michigan squirrels, despite a small sample (Table 1). The lowest observed haplotype diversity was observed in Illinois with the second lowest nucleotide diversity (Table 1). An AMOVA revealed 72% of genetic variation was attributed to variation among individuals within states while 28% of genetic variation was attributed to differences among states. The overall Φ_{ST} was significant with 16000 permutations in ARLEQUIN ($\Phi_{ST} = 0.28, p < 0.001$), providing evidence of population substructure. All pair-wise comparisons of Φ_{ST} values showed significant differences with 3000 permutations in ARLEQUIN, except the Michigan-Indiana comparison and the Michigan-Wisconsin comparison ($\alpha_{EW} = 0.017$; Table 2). Additionally, Φ_{ST} values suggest Illinois squirrels are genetically similar to the

Indiana population while the Φ_{ST} comparison between the Illinois and Minnesota samples suggests less similarity (Table 2). The mean Φ_{ST} value for all Illinois pair-wise comparisons is the highest, indicating dissimilarity among the Illinois population and all others sampled (Table 2). Results also suggested squirrels from Indiana and lower Michigan were not significantly different, nor were those from lower Michigan and Wisconsin. The relationship between Indiana and Michigan may be attributed to geographic proximity and a lack of barriers to gene flow. The low value associated with the Illinois and Indiana comparison supports immigration as the primary source of the extant Illinois red squirrel population. Also, tests of neutrality and a mismatch distribution analysis performed in ARLEQUIN did not show any indication of population expansion.

The haplotype frequency distribution (Figure 2) revealed Illinois squirrels most frequently shared haplotypes with Indiana squirrels and to a lesser extent Michigan squirrels. Overall, 40% of Indiana squirrels shared a haplotype with at least one Illinois individual while 33% of Michigan squirrels shared a haplotype with an Illinois squirrel. Haplotype F, observed in a single individual from Illinois, was observed in 11 Minnesota squirrels, accounting for 21% of squirrels sampled from Minnesota. Haplotype J was the only haplotype observed in Illinois that was seen more frequently in Michigan than Indiana. The haplotype frequency distribution supports immigration as the primary source of the extant Illinois red squirrel population, with the one exception (haplotype F).

The unrooted haplotype cladogram revealed Illinois haplotypes are more closely associated with Indiana and Michigan haplotypes with two exceptions (Figure 3). Haplotype J, situated near the center of the cladogram, was one mutational step away

from haplotypes observed in Indiana, Michigan, Minnesota and Wisconsin but three mutational steps away from the closest Illinois haplotype. Similarly, haplotype F (observed in 11 Minnesota samples and 1 Illinois sample) was five mutational steps away from the nearest Illinois haplotype (J) but only one step away from haplotypes found in Indiana, Michigan, Minnesota, and Wisconsin. Michigan exhibited the largest difference between two specific haplotypes with 14 mutational steps separating haplotypes CC and C (Figure 3). Two haplotypes found in Wisconsin (M and JJ) were 13 mutational steps apart while Indiana, Illinois and Minnesota all had haplotypes 12 mutational steps apart. Consequently, the Φ_{ST} comparisons, haplotype frequency distribution, and unrooted haplotype cladogram all support immigration from Indiana as the primary source of the current Illinois red squirrel population.

Discussion

Origin of the Illinois Red Squirrel Population

My primary objective was to investigate the genetic composition of the small red squirrel population in Illinois to determine its geographic origin. Results indicate immigration from Indiana served as the primary source of the present Illinois red squirrel population. The Illinois-Indiana Φ_{ST} value (Table 2) was the lowest, and Illinois shared the most haplotypes with Indiana (Figure 2, Figure 3). Additionally, Illinois was the only population that did not contain a unique haplotype, suggesting a recent event likely established the present Illinois population.

Despite the strong evidence for immigration from Indiana, my data also suggest a small number of translocated squirrels from Minnesota may have contributed to the current Illinois population. The individual that shared haplotype F with 11 Minnesota

squirrels was sampled at ICSWA, only 2 km west of the Willow Slough State Fish and Wildlife Area in Indiana, but > 600 km from the nearest Minnesota sample. This, in combination with the significant difference between Minnesota and Indiana populations (Table 2), indicates haplotype F denotes a true Minnesota lineage, and not an Indiana haplotype that was not sampled. Consequently, my results suggest a mixed population, comprised primarily of descendents of immigrated individuals from Indiana with a small genetic contribution from Minnesota.

My conclusions are consistent with a prior study that used skull morphometrics to investigate the origin of the Illinois population (Brown and Edwards 1985). These authors concluded that some Illinois squirrels more closely resembled the Indiana type, whereas others resembled the Minnesota group. My genetic data suggest that Illinois squirrels are most similar to Indiana. I speculate immigration from the source population in Indiana has all but erased the genetic evidence of Minnesota ancestry.

The discovery of red squirrels in northeastern Illinois in the 1970s coincides with a general range expansion of this species in Indiana. Red squirrels were not observed on the Willow Slough Fish and Wildlife Area when it was established in the 1950s but were documented on the site in 1971 (Mumford and Whitaker 1982). By 1977 they were distributed around the area, primarily in pine plantations (Mumford and Whitaker 1982). In this same year, the first red squirrels were confirmed in Illinois along the Kankakee River near the towns of Momence and Aroma Park, and Kankakee River State Park (Hoffmeister 1989). The precise routes of immigration are unknown, but Mumford and Whitaker (1982) suggested red squirrels moved via riparian corridors in Indiana. Similarly, Hanson (2008) reported the current distribution of red squirrels in Illinois is

consistent with individuals using forested riparian corridors such as the Kankakee and Iroquois Rivers to disperse. Goheen et al. (2003) suggested the red squirrel has expanded its range in the Midwest at the expense of a competitor, the gray squirrel (*Sciurus carolinensis*). Results of that study indicated red squirrels were more successful when moving through agricultural fields than gray squirrels, leading Goheen et al. (2003) to speculate that red squirrels are less sensitive to fragmentation than gray squirrels.

The genetic variation observed in my study is comparable to a study conducted across 17 mountain ranges in Colorado, Idaho, South Dakota, Utah, and Wyoming (Wilson et al. 2005). I observed 47 haplotypes (n = 229), whereas Wilson et al. (2005) observed 35 haplotypes with a smaller sample (n = 153). In comparing the sequences from both studies, a deletion event is evident in all samples from the Midwest and in a select few individuals from the western states sampled by Wilson et al. (2005). A high number of observed haplotypes is expected in rodents (Bromham et al. 1996). The red squirrel has a short generation time (Steele 1998) allowing significant opportunities for the mutation of new haplotypes in the population.

A study examining the genetic structure of the Eurasian red squirrel (*Sciurus vulgaris*) observed 26 haplotypes in a sample of 207 squirrels (Barratt et al. 1999). Despite a high level of observed genetic differentiation, the authors did not find any significant or consistent phylogenetic results (Barratt et al. 1999). Subsequent studies of this species in Europe have found no distinct geographic pattern of haplotype distribution (Finnegan et al. 2007; Hale et al. 2004; Ogden et al. 2005). My results did not show a distinct regional pattern (Figure 3), but I did observe clear associations among squirrels in

Illinois with Indiana and lower Michigan, possibly due to the relatively small population in Illinois.

Regional Patterns of Genetic Variation

The Michigan-Wisconsin Φ_{ST} non-significant comparison may be attributed to grouping the UP samples with Wisconsin. A post-hoc, follow-up analysis in ARLEQUIN was conducted to elucidate any patterns in genetic variation among samples from the Michigan UP, lower Michigan, and Wisconsin. An analysis separating all three regions had mixed results after application of the FDR ($\alpha_{EW} = 0.028$). The UP was not different from both lower Michigan ($\Phi_{ST} = 0.01$; $p = 0.25$) and Wisconsin ($\Phi_{ST} = 0.09$; $p = 0.04$), but lower Michigan and Wisconsin were different ($\Phi_{ST} = 0.08$; $p = 0.01$). The Φ_{ST} measure of population differentiation indicates the UP is more similar to lower Michigan than it is to Wisconsin; however, the UP shared 3 haplotypes with Wisconsin and none with lower Michigan.

Subspecies accounts of the red squirrel in the region also imply similarity between the UP and lower Michigan relative to Wisconsin. *Tamiasciurus hudsonicus loquax* is believed to extend from central Illinois to the Atlantic Ocean, including the UP and lower Michigan, while *Tamiasciurus hudsonicus minnesota* ranges from central Minnesota through Wisconsin (Steele 1998). However, the previous classifications of red squirrel subspecies have been based on skull morphometrics and pelage color (Howell 1936). Additionally, taxonomic distance coefficients of the red squirrel in a morphological skull analysis in the Lake Superior region revealed between subspecies were similar to within subspecies measures (Kramm et al. 1975).

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Table 1. The number of haplotypes, haplotype diversity (h), and nucleotide diversity (π) with standard errors in each state for a 272 - 274 bp section of the mtDNA control region in a sample of 229 red squirrels.

State (n)	Haplotypes	h	π
Illinois (52)	6	0.664 ± 0.045	0.011 ± 0.006
Indiana (50)	13	0.894 ± 0.017	0.023 ± 0.012
Michigan (24)[1]	12	0.917 ± 0.032	0.021 ± 0.012
Minnesota (52)	16	0.838 ± 0.032	0.010 ± 0.006
Wisconsin (51)[2]	17	0.897 ± 0.023	0.016 ± 0.009

[1] Samples from the Michigan lower peninsula.

[2] Includes 19 samples from the Michigan Upper Peninsula.

Table 2. Lower diagonal: pair-wise comparisons of Φ_{ST} values for a 272 – 274 bp section of the mtDNA control region in a sample of 229 red squirrels from 5 Midwestern states. Mean Φ_{ST} values for each state are listed at the bottom of each column. Upper diagonal: the respective *p* values calculated in ARLEQUIN (v.2.0; Schneider et al. 1997) based on 3000 permutations. Corrections for multiple testing were performed using the false discovery rate method (FDR; Benjamini and Yekutieli 2001). Asterisks indicate comparisons that were significant ($\alpha_{EW} = 0.017$)

	Illinois	Indiana	Michigan	Minnesota	Wisconsin
Illinois	-	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Indiana	0.22*	-	0.200	< 0.001*	< 0.001*
Michigan	0.30*	0.01	-	< 0.001*	0.048
Minnesota	0.62*	0.27*	0.23*	-	< 0.001*
Wisconsin	0.43*	0.11*	0.04	0.12*	-
Mean	**0.40**	**0.15**	**0.15**	**0.31**	**0.18**

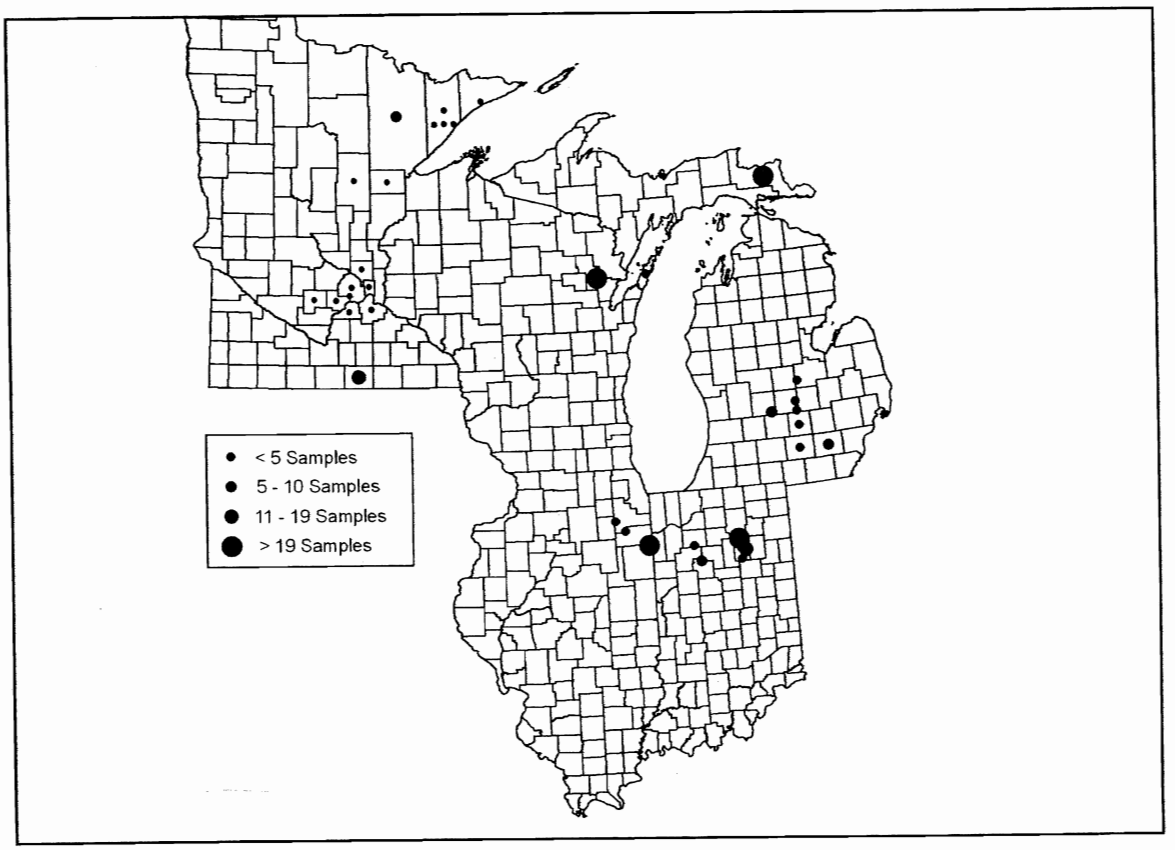

Figure 1. Sampling locations for 232 red squirrels across the Midwest. The size of the circle represents approximate numbers of samples taken from a locality.

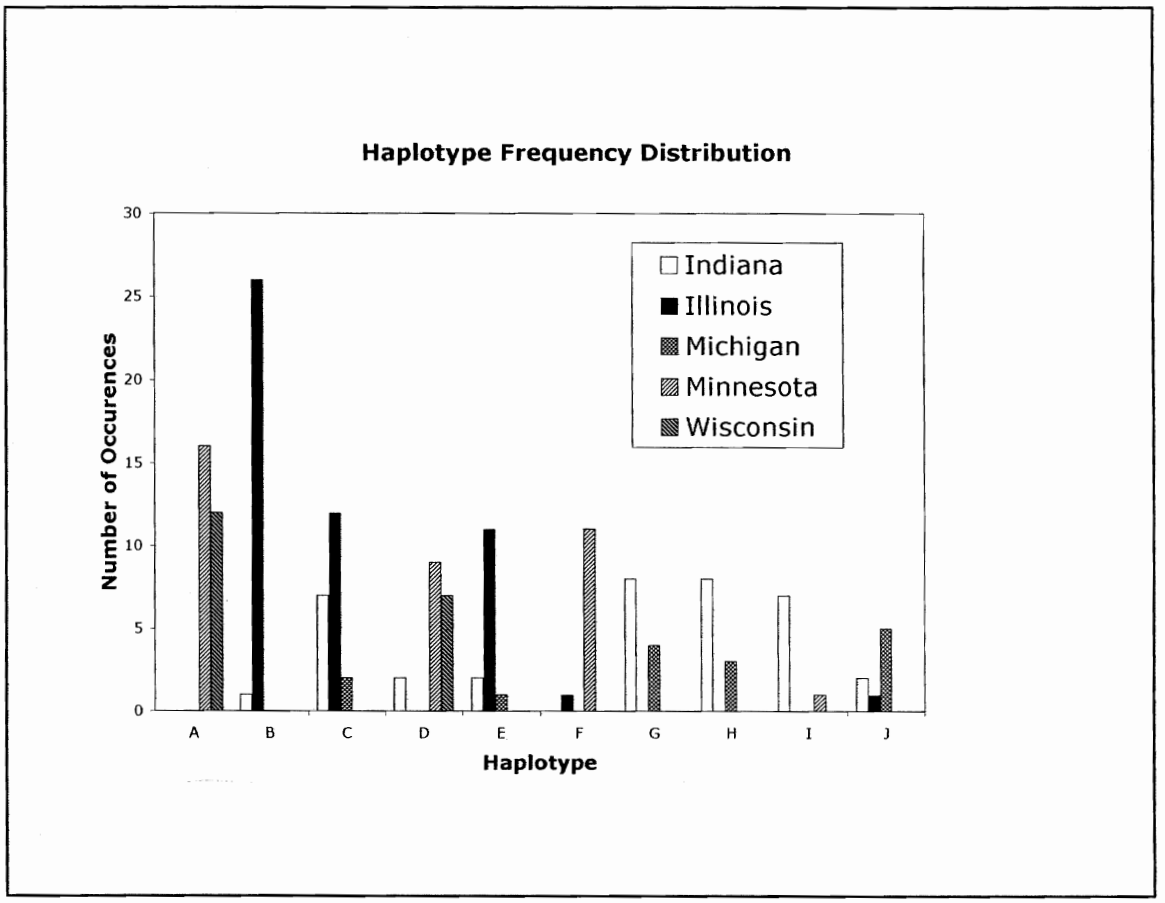

Figure 2. The haplotype frequency distribution for a 272 – 274 bp section of the mtDNA control region in a sample of 229 red squirrels. The figure shows only 10 of the 47 observed haplotypes to illustrate the distribution of the Illinois squirrels.

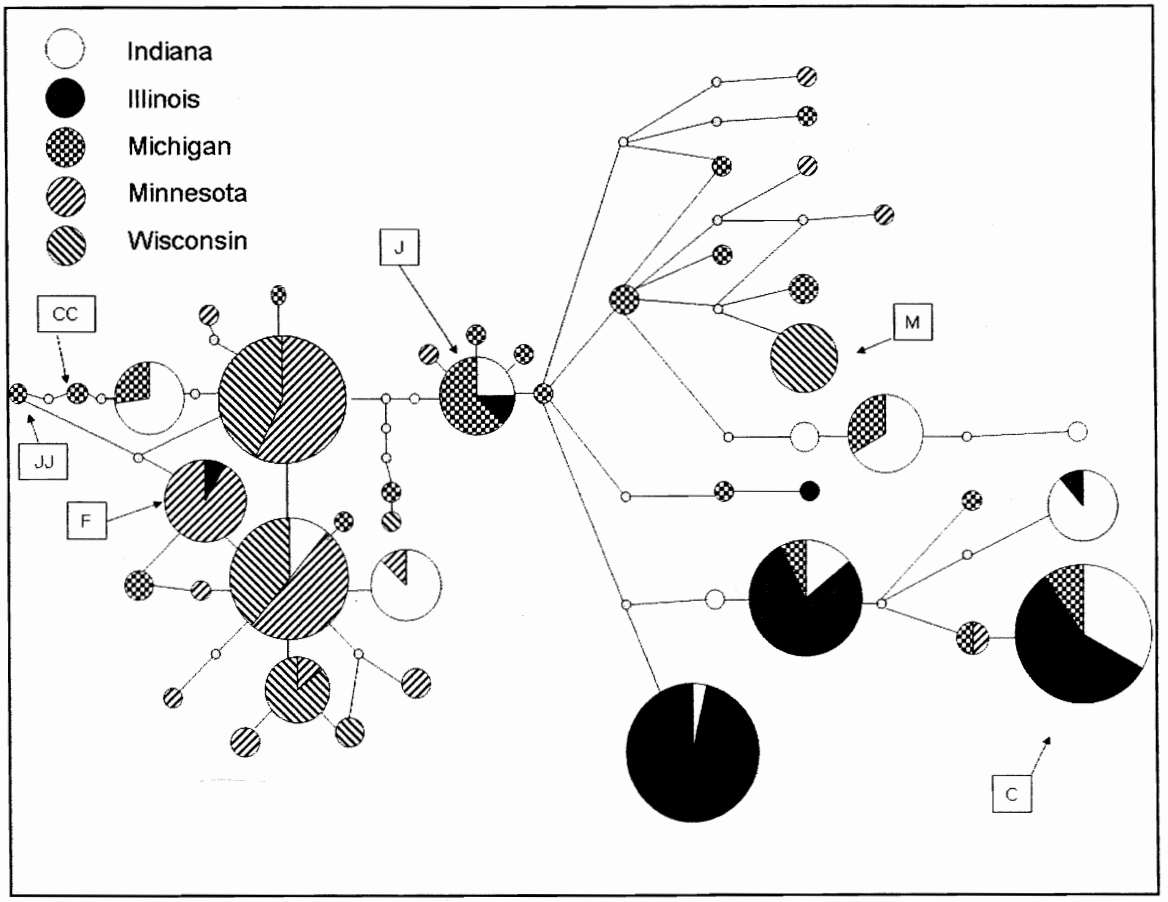

Figure 3. The unrooted haplotype cladogram for a 272 – 274 bp region of the mtDNA control region in a sample of 229 red squirrels. Each circle represents a distinct haplotype and empty circles symbolize intermediate haplotypes not observed in the sample. Haplotypes connected to one another via a line are one mutational step apart. Consequently, haplotypes closer together are more similar. Letters denote assigned haplotypes are for reference only.

CHAPTER 2

CONTEMPORARY PATTERNS OF GENETIC STRUCTURE OF RED SQUIRRELS IN THE MIDWEST

ABSTRACT

The red squirrel occurs in the heavily fragmented landscape of the lower Midwest (e.g. Illinois, Indiana), but is more commonly associated with coniferous forests of the upper Midwest (e.g. Michigan, Minnesota, Wisconsin). Six microsatellite loci were utilized to examine the genetic structure of the red squirrel in the Midwest and elucidate the source of the current Illinois population. A traditional assignment test in STRUCTURE indicated a mixed population in Illinois, composed primarily of descendents of immigrants from Indiana with a small element of Minnesota ancestry. Analyses in GENELAND indicated two populations over the entire study area, identifying Illinois as one population and squirrels from Indiana, Michigan, Minnesota, and Wisconsin as the other. Pair-wise F_{ST} and F'_{ST} values confirm a slight differentiation of the Illinois population from all others. Overall, the red squirrel population in the Midwest exhibits low levels of differentiation with high levels of variation. I speculate the minor genetic difference seen in the Illinois population is attributable to the recent range expansion of this species into Illinois during the 1970s. Furthermore, I found no genetic evidence to suggest Illinois red squirrels are either a remnant population or genetically unique. Therefore, I do not recommend that red squirrel as a candidate for the Threatened and Endangered Species List in Illinois due to its mixed ancestry.

INTRODUCTION

The North American red squirrel (*Tamiasciurus hudsonicus*) is commonly associated with coniferous forests, but occurs in the lower Midwest among a heavily fragmented agricultural landscape. Presently, Illinois is host to a small population, but the geographic origin and history of the red squirrel in Illinois is ambiguous. It was likely present in the northern portion of the state until 1900, but confirmed records from this period are sparse (Hoffmeister 1989). After nearly an 80-year absence, a population of unknown origin was confirmed in Kankakee Co. in northeastern Illinois in 1977. Red squirrels were subsequently confirmed in nearby Will and Iroquois Cos. (Hoffmeister 1989).

There are presently four hypotheses on the source of the extant Illinois red squirrel population. First, local accounts suggest red squirrels from Minnesota were released near Iroquois Co. State Wildlife Area (ICSWA) in the 1970s (Hoffmeister 1989). Second, a small population of native squirrels may have persisted undetected in the area from 1900 – 1977. Third, red squirrels may have immigrated into Illinois from Indiana as part of a general range expansion in the Midwest (Goheen et al. 2003; Hoffmeister 1989; Mumford and Whitaker 1982). Finally, the present Illinois population may be a mixed population, comprised of genetic elements from Minnesota, Indiana, or Illinois.

The Illinois Department of Natural Resources (IDNR) has designated the red squirrel as a critical species in need of conservation (IDNR 2005) based on its limited geographic range and low numbers in the state. Presently, the red squirrel is protected in

Illinois, but is not listed as a threatened or endangered species. The conclusions of this study have implications for the conservation and management of this species in Illinois. If the population is composed of descendents of translocated squirrels from Minnesota, then the population could be considered exotic, negating protection. If the population represents a remnant with a unique genetic legacy, then inclusion on the Illinois Threatened and Endangered Species List would be warranted. Finally, if the population is comprised mainly of descendents of recent immigrants from Indiana, then no management efforts would be necessary. Alternative criteria to listing of the red squirrel in Illinois include: (1) the species exhibits a restricted habitat or low population in Illinois, and/or (2) the Illinois red squirrel population is a disjunct unit.

The primary goal of this study was to apply genetic markers to determine the geographic source of the red squirrel population in Illinois. A secondary objective was to evaluate and examine the present population structure of the red squirrel across the states of Indiana, Illinois, Michigan, Minnesota, and Wisconsin. I applied microsatellite markers to achieve my objectives because of their high affinity for mutation. A traditional assignment test in which populations were defined *a priori* was utilized to achieve my primary objective, and spatially implicit and explicit models were employed to achieve the secondary objective. Considering the evidence presented in the previous chapter of this thesis that focused on mitochondrial DNA (mtDNA), and the work of Hanson (2008), I make final recommendations on the conservation status of the species in Illinois.

METHODS

Sample Collection and Laboratory Methods

I collected a total of 232 red squirrel tissue samples, including 52 from Illinois, 46 from Indiana, 43 from Michigan, 56 from Minnesota, and 35 from Wisconsin (Figure 1). I followed the protocol outlined in the previous chapter for tissue sample collection and storage. Samples from the Upper Peninsula of Michigan (UP) were grouped with Wisconsin squirrels due to geographic proximity. The re-grouped sample sizes were: Illinois, 52; Indiana, 46; Michigan, 24; Minnesota, 56; Wisconsin, 54.

Six microsatellite loci (Thu03, Thu21, Thu25, Thu33, Thu37, Thu42; Gunn et al. 2005) were amplified separately with PCR. Each 10 μl reaction included 10 – 50 ng of genomic DNA, 2 μl 5× buffer, 2.5 mM $MgCl_2$, 0.6 μM of each primer, 0.08 mM of dNTPs (Promega, Madison, Wisconsin) and 0.2 units of *Taq* DNA polymerase (Promega). The PCR profile was 94°C for 2 minutes followed by 30 cycles of 94°C for 1 minute, annealing temperature (between 58°C and 64°C) for 30 seconds and 72°C for 30 seconds with a final elongation of 72°C for 10 minutes. The forward primer was labeled with the D4 WellRED fluorescent tag (Sigma-Aldrich, St. Louis, Missouri) and products were electrophoresed through a high-resolution polyacrylamide gel on a Beckman CEQ 8000 (Beckman-Coulter, Fullerton, California). Allele sizes were determined using the Fragment Analysis v.2.3.4 software (Beckman-Coulter). Samples with low signal intensity were amplified and electrophoresed until a satisfactory signal was observed. An additional 25 unknown samples were also amplified and electrophoresed to quantify error for each locus.

Data Analysis

The computer program CONVERT (v.1.2, Glaubitz 2004) was utilized to format data for input into the various software packages. I tested for a deficiency of heterozygotes in the entire population in GENEPOP (v.4.0, Rousset 2007) for each locus and globally (Rousset and Raymond 1995). To estimate *p* values, the Markov chain method of Guo and Thompson (1992) was utilized (dememorization steps = 10000, number of batches = 500, iterations per batch = 5000). An unbiased probability of identity (PID) was also calculated to estimate the power of the loci utilized in the analyses (Paetkau et al. 1998).

The program GDA (v.1.1; Lewis and Zaykin 2001) was utilized to calculate observed and expected heterozygosity, average number of alleles, total number of unique alleles, and frequency of unique alleles for each state population. Pair-wise F_{ST} values were calculated in ARLEQUIN (v.2.0; Schneider et al. 1997) and standardized F_{ST} values (F'_{ST}) were calculated following the method of Hedrick (2005). The standardized measure (F'_{ST}) adjusts for the high level of variation observed in loci like microsatellites and ranges from 0 to 1. The traditional F_{ST} may not range from 0 to 1 for loci with high levels of polymorphism (Hedrick 2005). I tested for evidence of a genetic bottleneck in the Illinois population with the mode shift method (Luikart et al. 1998) in the program BOTTLENECK (v.1.2.02; Piry et al. 1999) for each state. The mode shift method was utilized because alternative tests (sign test, Wilcoxon test) are sensitive to significant departures from Hardy-Weinberg equilibrium (Luikart and Cornuet 1998). The false discovery rate (FDR; Benjamini and Yekutieli 2001) was utilized to obtain an experiment wide alpha level (α_{EW}) to account for multiple comparisons when appropriate (locus specific Hardy-Weinberg tests and F_{ST} pair-wise comparisons).

I used the computer program STRUCTURE (v.2.1; Pritchard et al. 2000) to test several hypotheses regarding population structure. To test the hypothesis of a remnant Illinois population, I estimated K, the number of populations, following the procedure outlined in Pritchard and Wen (2004) for $K = 1$ to $K = 10$ given all samples. In a subsequent analysis, I excluded the Illinois samples for comparative purposes. I followed the procedure outlined in Evanno et al. (2005), using ΔK as an indicator of the number of clusters within our data to provide a better estimate of K. Each run consisted of a burnin of 30,000 followed by 100,000 replicates for 10 runs at each K.

A traditional assignment test (Manel et al. 2005) was also performed in STRUCTURE with all Illinois individuals serving as unknowns. Four potential source populations were defined *a priori* (Indiana, Michigan, Minnesota, Wisconsin). In this analysis, 10 runs were performed, each consisting of a burnin of 30,000 replicates followed by 100,000 replicates of the Markov Chain Monte Carlo (MCMC). I calculated the mean Q-vector for each Illinois individual in the sample and an average for all Illinois squirrels. The maximum observed value in the mean Q vector for each individual was used for assignment to one of four source populations defined *a priori* (Indiana, Michigan, Minnesota, Wisconsin). An admixture model was applied and allele frequencies were allowed to correlate among populations (Falush et al. 2003) for all analyses performed in STRUCTURE.

GENELAND (v.2.0.12; Guillot et al. 2005b) may detect population substructure at lower levels of differentiation than STRUTURE due to its ability to include spatial information (Guillot et al. 2005a). In GENELAND I ran both spatially explicit and implicit models. The UTM coordinates of each squirrel's location were recorded when

possible and applied to the spatial models. In other cases, where only the county was known, I used the coordinates corresponding to the center of the county. In order to minimize the error associated with these coordinates, I ran spatially explicit models with uncertainty coordinates of 1 km, 10 km, 50 km and 100 km. A 1 km uncertainty coordinate was considered appropriate for samples of known location due to estimates of dispersal capabilities of juvenile red squirrels (Berteaux and Boutin 2000; Larsen and Boutin 1994). Uncertainty coordinates of 10 km, 50 km, and 100 km were considered to determine if GENELAND clustered my samples differently in these analyses and to account for the limited spatial information for some samples. The Dirichlet distribution (Pritchard et al. 2000) was used to model allele frequencies because it has been shown to perform better than the alternative F-model (Guillot et al. 2005a). I set the maximum rate of the Poisson process to 232, the number of individuals in the sample as recommended by Guillot et al. (2005a). The maximum number of nuclei in the Poisson – Voronoi tessellation was set to 696, 3 times the maximum rate of the Poisson process, as also suggested by Guillot et al. (2005a). The various uncertainty coordinates were used along with 100,000 iterations of the MCMC and a thinning of 10 for a total of 10,000 stored replicates in analyses to infer the number of populations. I ran each model 10 times to obtain an accurate estimate of K given the data. Additionally, I ran the MCMC 10 times without coordinates (Spatial Model = False) to compare results. In this analysis, the maximum number of nuclei was set to 232, the number of individuals. I did not observe a change in the results with longer runs of the MCMC (1,000,000 iterations, thinning of 10 for 100,000 stored replicates).

Results of the analyses in GENELAND with differing uncertainty levels were examined to determine the true number of populations, utilizing the mode of K as an estimate. I then performed 5 additional runs each for the uncertainty levels of 1 km, 10 km, 50 km, and 100 km with K fixed (at the mode of K as determined by previous analyses) to assign individuals to populations as recommended by Guillot et al. (2005b). In these analyses, I ran the MCMC 100,000 times, with a thinning of 10. The posterior probability of population membership for each pixel of the spatial domain (100 × 100 pixels with a burnin of 100) was also calculated and averaged over the 5 runs. Individuals were unambiguously assigned population membership if the probability of population membership was greater than 0.7.

Observed and expected heterozygosity were calculated on the inferred populations (based on GENELAND results) in addition to the average number of alleles, total number of unique alleles, and frequency of unique alleles. Levels of differentiation among the inferred populations were quantified with F_{ST} and F'_{ST}. The program BOTTLENECK was used to perform the mode shift test on the inferred populations.

RESULTS

Missing data accounted for 1.1% of the total sample of 232 red squirrels while no samples were removed from the analysis. The number of alleles per locus ranged from 11 (Thu37) to 17 (Thu42) with a mean of 13.83. Analyses performed on the entire sample indicated a significant deviation of Hardy-Weinberg Equilibrium globally ($\alpha = 0.05$; $p < 0.00$) attributed to disequilibrium at all six loci ($\alpha_{EW} = 0.02$; $p_{Thu03} < 0.00$; $p_{Thu21} < 0.00$; $p_{Thu25} = 0.01$; $p_{Thu33} < 0.00$; $p_{Thu37} = 0.01$; $p_{Thu42} = 0.01$). Expected heterozygosity levels ranged from 0.76 to 0.80 while observed heterozygosity levels ranged from 0.68 to

0.71 within each state (Table 1). Minnesota contained the highest average number of alleles per locus while Illinois contained the lowest. Similarly, Minnesota contained the most unique alleles and Illinois contained the fewest. Estimates of F_{ST} and F'_{ST} indicate low levels of population substructure (Table 2). Illinois contained the highest mean values for both of the parameters, and Michigan contained the lowest mean values. Results of the mode shift test in BOTTLENECK did not show evidence of a genetic bottleneck in any of the given state populations. The overall probability of identity, combined for all six loci was 6.75×10^{-9}.

Attempts to estimate the true K, the number of clusters, in STRUCTURE yielded ambiguous results. In the analysis that tested the hypothesis of a remnant population (Illinois samples included), the mean natural logarithm of the probability of the data [L(K)] was highest at $K = 3$, but was also comparatively high at $K = 1$ and $K = 2$ (Figure 2a). Additionally, the plot of ΔK did not illustrate a dramatic increase at any K, possibly indicating $K = 1$ (Figure 2b). The analysis that excluded Illinois samples yielded similar results, indicating $K = 1$ (data not shown). The ambiguity of results on the true K in STRUCTURE may be attributed to low levels of population substructure as supported by F_{ST} and F'_{ST} (Table 2). STRUCTURE is able to begin to detect the true K at F'_{ST} values above 0.28; values of 0.39 and above are required for STRUCTURE to assigned 97% of individuals to the correct population (Latch et al. 2006).

The results of the traditional assignment test in STRUCTURE, in which state boundaries were used to delineate potential source populations, indicate an admixed population in Illinois. The mean Q-vector for all Illinois individuals over 10 iterations was: Michigan, 0.46; Indiana, 0.26; Minnesota, 0.12; and Wisconsin, 0.08 with 30

squirrels assigned to Michigan, 16 assigned to Indiana, 6 assigned to Minnesota, and 0 assigned to Wisconsin. Most individuals were assigned unambiguously (> 0.50) to one population (Michigan, 24 of 30; Indiana 10 of 16; Minnesota, 2 of 6; Total, 40 of 52).

Initial GENELAND analyses conducted to infer the true number of populations produced a clear mode in all analyses (uncertainty coordinates of 1 km, 10 km, 50 km, and 100 km) at $K = 2$. The spatially implicit model performed in GENELAND produced a mode at $K = 2$, in contrast to the ambiguous results in STRUCTURE. The general agreement of GENELAND on the structure of my samples at the four differing uncertainty coordinates (1 km, 10 km, 50 km, 100 km) confirms that uncertainty on spatial coordinates is not a significant parameter in the model (Guillot et al. 2005a).

Figure 1 and Figure 3 outline the results of the GENELAND analysis with K fixed at 2 to determine the probability of population membership for all individuals, given uncertainty coordinates of 1 km, 10 km, 50 km, and 100 km while not considering ambiguity. Red squirrels in Illinois along with squirrels sampled from Willow Slough Fish and Wildlife Area in Indiana are grouped into one population, henceforth referred to as the "Illinois" group. All other sampled areas were considered a separate population, hence referred to as the "Midwest" group. All assignments were unambiguous ($Q > 0.7$) except one individual from Pulaski Co., Indiana, which was ambiguously assigned to the Midwest group ($Q = 0.69$).

Analyses performed on the inferred populations based on GENELAND results also indicate a low level of population differentiation. The pair-wise F_{ST} value was 0.03 while the pair-wise F'_{ST} value was 0.22. The Illinois inferred population contained only one unique allele, while the Midwest population contained a high frequency of unique

alleles compared to the state populations (Table 1). The mode shift method performed in BOTTLENECK did not show any evidence of a genetic bottleneck.

Only 2 of the 25 total runs of the MCMC to infer population membership did not group the samples as previously described above (at 1 km uncertainty and 50 km uncertainty). Instead, Michigan (lower peninsula), Indiana, and Illinois clustered as one population while the Minnesota, Wisconsin, and the UP were grouped as another. The pair-wise F_{ST} value reflects the weaker structure when given these two populations (F_{ST} = 0.01; F'_{ST} = 0.13).

Discussion

The results of the STRUCTURE and GENELAND analyses differ on the estimate of *K*, the number of populations. The spatially implicit model of the data in GENELAND indicated two populations, whereas STRUCTURE was ambiguous on the number of populations. All spatially explicit models in GENELAND (1 km, 10 km, 50 km, 100 km uncertainty) indicated $K = 2$, agreeing with the spatially implicit model in the same program, offering evidence of substructure that STRUCTURE did not identify. The discrepancy may be attributable to GENELAND initializing *K* at a uniform distribution for which the user sets K_{min} and K_{max}, making the distribution of *K* a soft prior in the model (Guillot et al. 2005a). The program infers *K* based on the mode of *K* along the MCMC. STRUCTURE simply computes the likelihood of the data given a user-defined *K*. The user then must calculate the most probable *K* based on various *ad hoc* methods as discussed previously (Evanno et al. 2005; Pritchard and Wen 2004).

Results of the traditional assignment test indicate the extant red squirrel population in Illinois is primarily descendents of immigrants from lower Michigan and

Indiana, with a small element of Minnesota ancestry. The low F_{ST} and F'_{ST} values between Indiana and Michigan reflect genetic similarity between those states, which accounts for the high number of Illinois individuals assigned to Michigan (Table 2). However, the high affinity of Illinois squirrels to Michigan squirrels may be evidence of actual immigration patterns. It has been assumed that westward dispersal of squirrels from Indiana established the extant red squirrel population in Illinois due to geographic proximity. My results suggest immigration from the northeast (Michigan) combined with dispersal from the east (Indiana), may account for the present red squirrel population in Illinois.

The recent range expansion of red squirrels in Illinois (Hanson 2008) and the lower Midwest may be impacting a competing species, the gray squirrel (*Sciurus carolinensis*; Goheen et al. 2003). Gray squirrels were documented to only inhabit the largest forest patches in a study of mammal communities in Illinois (Rosenblatt et al. 1999), and studies show a long-term decrease in gray squirrel abundance as forest cover decreases (Nixon et al. 1978). The red squirrel and gray squirrel also showed similar willingness to disperse from fencerows, but the gray squirrel had a higher risk of predation during dispersal than red squirrels (Goheen et al. 2003).

My results suggest the ability of the red squirrel to traverse unsuitable habitat has resulted in a low degree of genetic differentiation between populations in the Midwest. However, high F_{ST} and F'_{ST} means for the Illinois population indicate differentiation from all other states, and the Φ_{ST} pair-wise comparisons from the previous chapter confirm this pattern. Although I found no evidence of a founder effect in the Illinois population, differentiation of Illinois from all other sources may be attributed to a range

expansion into Illinois that was documented to occur in the 1970s. Depending on the model of dispersal, studies have shown occasional long-distance dispersers may immigrate into an area and establish an isolated, pocket population (Ibrahim et al. 1996). The pocket population may develop significant differentiation from the main population, and this trend may be observed for hundreds of generations after the main wave of expansion has reached the pocket population (Ibrahim et al. 1996). A similar scenario may have occurred in Illinois and western Indiana and historical records support this claim. Red squirrels were documented in extreme northern Indiana in the late 1800s and early 1900s, but only recently were documented in Pulaski Co., Indiana (1972) and Newton Co., Indiana (1971) along with Illinois (1977; Mumford and Whitaker 1982).

A high level of genetic variation combined with low differentiation has been documented in only a few previous studies. Simonsen et al. (1998) observed high levels of variation in mtDNA sequences and microsatellites in African buffalo (*Syncerus caffer*), but low levels of differentiation on the regional scale. High levels of differentiation at the local scale were observed in the white-footed mouse (*Peromyscus leucopus*) while there was no evidence of isolation by distance at larger geographic scales (Mossman and Waser 2001). I observed high levels of variability with low levels of differentiation at the regional scale, and no evidence of isolation by distance.

The genetic structure of arboreal squirrels has not been well studied in North America. There are limited studies of microsatellite loci in tree squirrels, and most studies of arboreal squirrels in Europe utilized mitochondrial DNA to examine genetic variation in the Eurasian red squirrel (*Sciurus vulgaris*; Finnegan et al. 2007; Barratt et al. 1999). Significant substructure has been documented in the latter species in the Italian

Alps with both mtDNA and microsatellite DNA (Trizio et al. 2005). These results are not surprising because the Eurasian red squirrel is considered a habitat specialist, adapted to montane, coniferous forests, and is expected to inhabit more isolated habitat patches with potential for limited gene flow among subpopulations. In contrast, I did not find significant substructure among North American red squirrel populations in the Midwest. This is also not surprising; previous studies indicate this species persists in deciduous, mixed and coniferous forests, and thus is not a habitat specialist (Hanson 2008; Goheen and Swihart 2005). The ability of the red squirrel to disperse across the fragmented landscape of the lower Midwest has produced little genetic variability across this region.

Conclusions

The results of this portion of my study agree with my previous analyses (Chapter 1) on the origin of the red squirrel population in Illinois. Independent evidence from both mtDNA and microsatellite DNA indicate the extant population is primarily composed of immigrants from Indiana and/or lower Michigan, but also offer evidence of a possible translocation of squirrels from Minnesota that contributed to the present population. Microsatellite analyses indicate the Illinois population may be considered separate from the rest of the Midwest. However, the absence of unique alleles in the Illinois population implies the Illinois population is not a remnant, rather the result of a range expansion from Indiana. This range expansion, combined with an apparent one-time transfer of Minnesota squirrels into the area, has produced a mixed population in Illinois, differentiated from the larger Midwestern population.

A field study suggested the red squirrel has expanded its geographic range in Illinois to the northwest and southwest by approximately 70 km since the 1980s (Hanson

2008). This range expansion, combined with my results that indicate the population is not likely a disjunct nor genetically unique population, suggest the species does not warrant listing on the Illinois Threatened and Endangered Species list.

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Table 3. Estimates of diversity measures in a sample of red squirrels (n = 232) from the Midwest based on state, and the inferred populations based on GENELAND results. Expected heterozygosity (H_e), observed heterozygosity (H_o), the average number of alleles per locus (A), and the number of unique alleles (A_u) were calculated in the program GDA. The frequency of unique alleles in population is given in parentheses.

	n	H_e	H_o	A	A_u
Given Populations					
Illinois	52	0.76	0.71	8.83	0 (0.0)
Indiana	46	0.79	0.71	10.5	2 (< 0.01)
Michigan	24	0.77	0.69	8.83	3 (0.01)
Minnesota	56	0.78	0.68	11.33	4 (0.01)
Wisconsin	54	0.80	0.68	10.83	3 (0.01)
Inferred Populations					
Illinois	54	0.76	0.72	9	1 (< 0.01)
Midwest	178	0.80	0.69	13.83	30 (0.07)

Table 4. Estimates of F_{ST} (lower diagonal) and F'_{ST} (upper diagonal) for a sample of red squirrels (n = 232) across five Midwestern states. The mean for each state is given below. Significance was calculated with 3000 permutations in the program ARLEQUIN. Corrections for multiple testing were performed using the false discovery rate method (FDR). Asterisks indicate comparisons that were significant (α_{EW} = 0.017).

	Illinois	Indiana	Michigan	Minnesota	Wisconsin
Illinois	-	0.22	0.15	0.19	0.23
Indiana	0.04*	-	0.03	0.20	0.10
Michigan	0.03*	0.01	-	0.13	0.05
Minnesota	0.04*	0.04*	0.03*	-	0.13
Wisconsin	0.04*	0.02*	0.01*	0.02*	-
Mean F_{ST}	**0.04**	**0.02**	**0.02**	**0.03**	**0.02**
Mean F'_{ST}	**0.19**	**0.14**	**0.09**	**0.16**	**0.13**

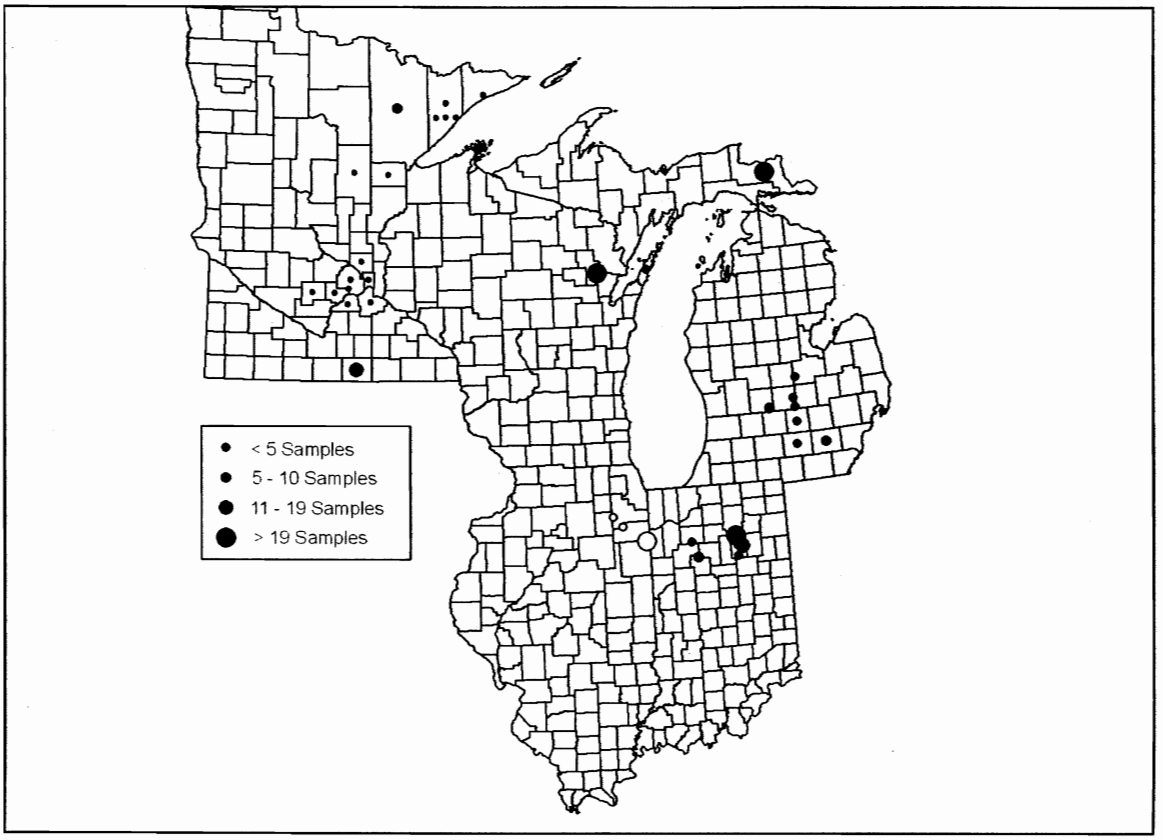

Figure 4. Sampling locations for 232 red squirrel samples in the Midwest. Size of the circles reflects the number of samples taken from the locality. Black circles represent one inferred population ("Midwest") while empty circles represent another inferred population ("Illinois") based on the GENELAND results.

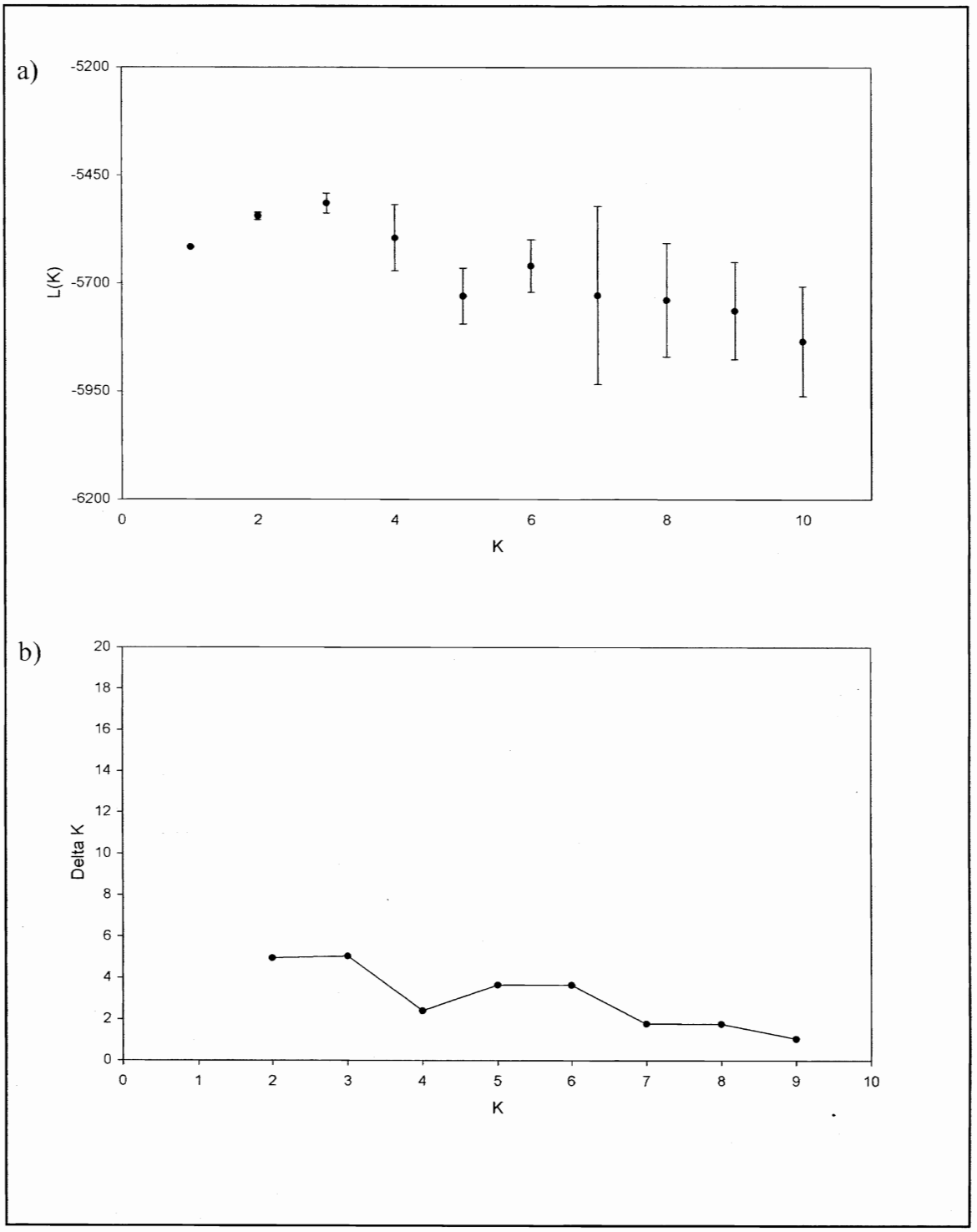

Figure 5. The mean natural logarithm of the probability of the data (a), and ΔK values (b) as calculated based on STRUCTURE results and the method outlined in Evanno et al. (2005).

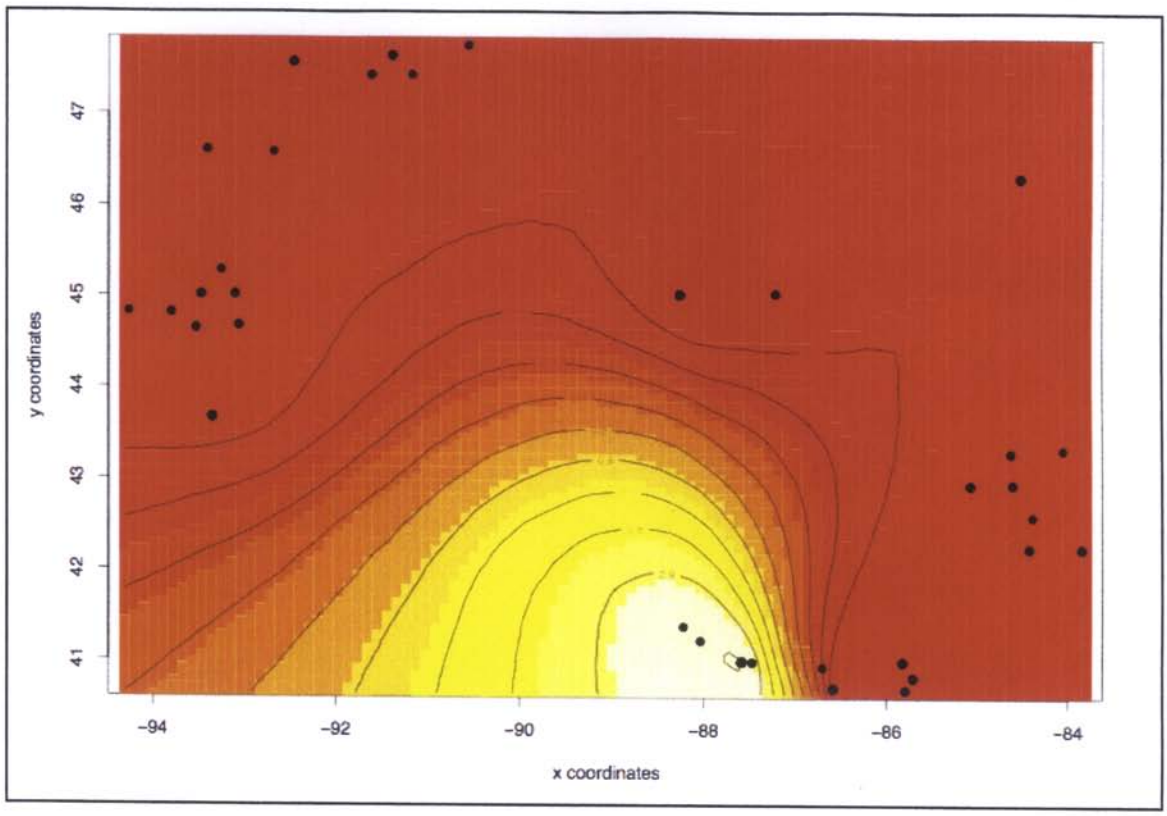

Figure 6. The probability of population membership for the region inferred by GENELAND for a sample of 232 red squirrels across a five-state region in the Midwest for $K = 2$.