



Note

Genetic Assessment of the Red Squirrel in Illinois: Immigrants or Exotics?

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ABSTRACT Novel populations pose unusual challenges for wildlife managers because knowledge regarding the source of these populations is essential to develop sound management approaches. One example that illustrates the complexity of this issue is the small population of red squirrels (*Tamiasciurus hudsonicus*) identified in northeastern Illinois in the 1970s. To elucidate the source of the red squirrel population in Illinois, we examined both contemporary and less recent patterns of genetic structure using nuclear microsatellite loci and mitochondrial DNA. Analyses revealed the Illinois subpopulation was primarily comprised of descendants of immigrants from Indiana, but there was also evidence of a translocation of squirrels from Minnesota. We recommend continued protection for the red squirrel in Illinois due to its restricted geographic range, small population size, and status as a native population. © 2011 The Wildlife Society.

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Anthropogenic land use changes in the central United States have caused local extinction of many species, but occasionally a species is identified in an area where it was previously extirpated. These novel populations pose exceptional challenges to managers if the origin of the population is uncertain. For example, the specific management approach to a novel population often depends on the status of the species as either non-native or native. To determine this status, generalizations can be made based on historical accounts of a species in a region, but these records are rarely definitive because they are often based on sporadic field observations. Genetic methods offer a quantitative alternative (e.g. Eldridge et al. 2001, Scribner et al. 2003, Epps et al. 2010) and thus can inform management approaches to recently recolonized populations.

The North American red squirrel (*Tamiasciurus hudsonicus*) is common throughout much of its geographic range, which extends from the forested areas of Alaska and Canada into the Rocky Mountains, the Great Lakes Region, and the Appalachian Mountains. Despite its association with coniferous forests, red squirrels are also known to occur in the heavily fragmented landscape of the central United States. The pine plantations, fencerows, and mixed forest patches characteristic of this region are considered marginal habitat for the species (Yahner 2003). Yet, red squirrels have persisted in these marginal habitats in Indiana and Ohio since

the 20th century (Lyon 1936). More recently, red squirrels were identified in Illinois (circa 1977) near Iroquois County State Wildlife Area (ICSWA) in the northeastern part of the state (Fig. 1). Speculation about the source of the Illinois subpopulation of red squirrels has persisted since its identification, prompting 4 competing scenarios. First, the translocation scenario is based on anecdotal accounts that indicate red squirrels from Minnesota may have been released in the 1970s near ICSWA (Hoffmeister 1989) and remained isolated. Second, the immigration scenario holds red squirrels may have dispersed into Illinois naturally from Indiana. Third, the translocation and immigration scenario proposes red squirrels from Minnesota may have been released in the 1970s, but were later supplemented by dispersal from Indiana. Indeed, this hypothesis is supported by a previous study based on skull morphometrics that found both the subspecies associated with Minnesota (*T. hudsonicus minnesota*) and subspecies associated with Indiana (*T. hudsonicus loquax*) present in Illinois (Brown and Edwards 1985). Further support for both immigration scenarios is provided by historical accounts that indicate the expansion of red squirrels into the Willow Slough Fish and Wildlife Area in Indiana (WSFWA), approximately 2 km east of ICSWA, occurred in the 1970s (Whitaker and Mumford 2009). Finally, red squirrels may have immigrated from another adjoining state or could have been translocated from any other state within their geographic range, but this is unlikely (Beatty 2008).

Modern genetic techniques allow questions regarding the genetic composition of Illinois' red squirrels to be examined at finer levels of biological resolution than skull morphometrics. An ideal marker system would possess sufficient variation to

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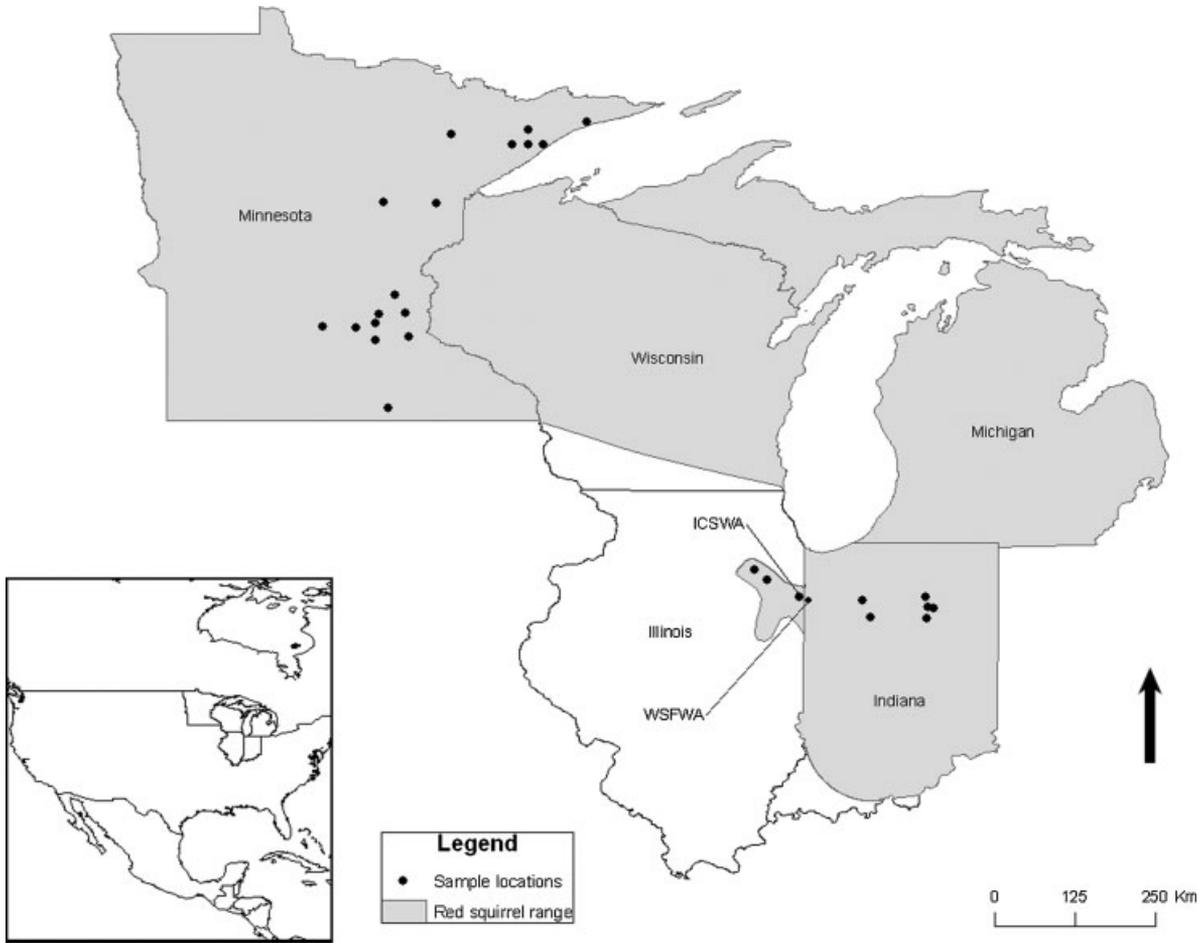


Figure 1. Approximate range of the red squirrel in the central United States (Hanson 2008, Whitaker and Mumford 2009) and sample locations for 154 red squirrels collected from 2006 to 2007. The Iroquois County Wildlife Area (ICSWA) and Willow Slough Fish and Wildlife Area in Indiana (WSFWA) are shown for reference.

permit fine scale resolution of subpopulations. Arbogast et al. (2001) and Wilson et al. (2005) demonstrated the utility of genetic techniques for investigating phylogeographic structure in red squirrels using mitochondrial DNA (mtDNA), but these studies examined relationships among red squirrel populations across geographic barriers, particularly mountain ranges. Thus, our objective was to determine the geographic source of the red squirrel subpopulation in Illinois using mtDNA to examine less recent patterns of genetic structure and highly variable nuclear microsatellite markers to evaluate contemporary patterns of genetic structure.

STUDY AREA

The ICSWA encompassed 1,004 ha in northeastern Illinois, 130 km south of the city of Chicago, Illinois, USA. The site was dominated by deciduous forest, primarily composed of oak (*Quercus* spp.), but also contained substantial amounts of coniferous forest, prairie, marsh, and oak savannah. The upper Wabash River basin in north-central Indiana was composed of highly fragmented oak-hickory (*Carya* spp.)-maple (*Acer* spp.) forest within an agricultural matrix, and contiguous forest tracts were restricted to major drainages and floodplains (Moore and Swihart 2005). Samples from

Minnesota came from a broader range of habitats, ranging from mixed conifer-deciduous forests in the northern portion of the state to deciduous forest in the south.

METHODS

In Illinois, tissue samples were taken with a 2-mm disposable biopsy ear punch from 52 anesthetized live-trapped individuals as part of a telemetry study conducted in the summers of 2006 and 2007 (Hanson 2008). Handling and sampling protocols were approved by Eastern Illinois University's Animal Care and Use Committee (permit EIU06010). Researchers, trappers, and hunters provided tissue samples from tails and ears of harvested squirrels in Indiana and Minnesota, which represented the possible source subpopulations. Spatial coordinates were recorded for each sample when possible, but for most samples outside Illinois, only the county of origin was known. Samples were initially preserved in 100% ethyl alcohol or desiccant beads and transferred to an ultra-low freezer (-80°C) for long-term storage. We extracted whole genomic DNA using the DNeasy Tissue Kit (Qiagen, Valencia, CA) following the protocol provided by the manufacturer or a modified ammonium acetate protocol (Latch et al. 2008).

Mitochondrial DNA analysis included 154 individuals, including 52 from Illinois, 50 from Indiana, and 52 from Minnesota. We amplified a 274-base pair (bp) section in the hypervariable domain III of the non-coding control region (D-loop) of the mitochondrial genome with polymerase chain reaction (PCR) using the primers OSU5020L and OSU5020H (Wilson et al. 2005). Each 25- μ l reaction included 10–50 ng of genomic DNA, 2.5 mM MgCl₂, 1.5 μ M of each primer, 0.08 mM of each dNTP (Qiagen), and 0.5 units of *Taq* DNA polymerase (Qiagen) in 1 \times PCR buffer (Promega, Madison, WI). The PCR profile was 94° C for 3 min followed by 35 cycles of 94° C for 45 s, 54° C for 30 s, and 72° C for 1 min with final elongation of 72° C for 10 min. We electrophoresed PCR products through a 1% agarose gel, stained the gel with ethidium bromide, and evaluated products with ultraviolet light. Successful samples were purified with the Wizard PCR Prep DNA Purification System (Promega) and sequenced on an ABI 3730XL with Big Dye 3.1 cycle sequencing chemistry (Applied Biosystems, Foster City, CA) at the Purdue University Genomics Core Center. Samples were sequenced with both the forward and reverse primers. We manually edited all sequences with the computer program SEQUENCHER v4.7 (Gene Codes Corp., Ann Arbor, MI) and aligned them in ClustalX (Thompson et al. 1997) with the default multiple sequence alignment parameters. As basic measures of genetic variation, we calculated nucleotide diversity (π) and haplotype diversity (h) in Arlequin v.2.0 (Schneider et al. 2000) by state subpopulation.

Sample sizes for microsatellite DNA analyses slightly differed compared to mtDNA analyses. Specifically, we removed 4 Indiana samples from the microsatellite analysis because these samples contained low signal strengths due to reduced DNA concentration. Furthermore, we received an additional 4 samples from Minnesota after sequencing had been completed but before genotyping had commenced. Thus, we generated microsatellite genotypes from 154 red squirrel tissue samples, including 52 from Illinois, 46 from Indiana, and 56 from Minnesota. We amplified 6 unlinked loci (Gunn et al. 2005) separately using PCR. Each 10- μ l PCR amplification included 10–50 ng of genomic DNA, 2.5 mM MgCl₂, 0.6 μ M of each primer, 0.08 mM of dNTPs, and 0.2 units of *Taq* DNA polymerase in 1 \times PCR buffer (Promega). The PCR profile was 94° C for 2 min followed by 30 cycles of 94° C for 1 min, a locus-specific annealing temperature (between 58° C and 64° C) for 30 s, and 72° C for 30 s with a final elongation of 72° C for 10 min (Gunn et al. 2005). We labeled each forward primer with the D4 WellRED fluorescent tag (Sigma-Aldrich, St. Louis, MO) and electrophoresed products through a high-resolution polyacrylamide gel on a Beckman CEQ 8000 (Beckman-Coulter, Fullerton, CA). We determined allele sizes manually using the Fragment Analysis v.2.3.4 software (Beckman-Coulter). We re-amplified and electrophoresed samples with low signal intensity until we observed a satisfactory signal. To quantify error, we re-amplified 25 samples for each locus and genotyped them

without knowledge of the initial genotype of the sample. We used the computer program CONVERT v.1.2 (Glaubitz 2004) to format data for input into the various software packages.

We calculated microsatellite genetic diversity measures in GDA v.1.1 (Lewis and Zaykin 2001), which included observed and expected heterozygosities, average number of alleles per locus, and total number of unique alleles within each state subpopulation and globally. We calculated allelic richness per locus and state subpopulation in FSTAT v.2.9.3 (Goudet 1995). We calculated Weir and Cockerham's (1984) estimate of F_{IS} for each locus and globally and quantified deviations from Hardy–Weinberg equilibrium with a Monte Carlo method (Guo and Thompson 1992) based on 10,000 permutations in SPAGeDi (Hardy and Vekemans 2002). To obtain F_{IS} and associated P -values for all subpopulations, we implemented this same procedure in FSTAT based on 10,000 randomizations. We controlled for increased Type I error due to multiple comparisons with a Bonferroni correction.

We considered multiple scenarios regarding the origin of the Illinois red squirrel subpopulation, and to evaluate the mtDNA data impartially, we set our expectations a priori for each scenario. Thus, we considered all possible patterns of shared haplotypes among all subpopulations and linked these potential patterns with each scenario. First, the translocation scenario would be supported if the Illinois subpopulation contained mtDNA haplotypes found exclusively in Minnesota and not found in Indiana. Second, in the immigration scenario, only haplotypes unique to Indiana would be found in the Illinois population. Third, the translocation and immigration scenario would be supported if the Illinois subpopulation contained separate, distinct haplotypes found in both Minnesota and Indiana. Finally, if the Illinois subpopulation were to contain haplotypes common to both sources or not found in either source, then the results would be inconclusive and warrant further investigation of other possible sources.

We analyzed the microsatellite data with no a priori assumptions about geographic or genetic structure. To determine the total number of populations within our dataset, we performed a burn-in of 30,000 followed by 100,000 iterations of the Markov Chain Monte Carlo (MCMC) 10 separate times for $K = 1$ –10 in STRUCTURE (Pritchard et al. 2000). We determined the length of the burn-in by following the guidelines of Pritchard et al. (2007); we examined outputs of the program for various parameters and observed when the values stabilized (i.e., F_{ST} , log-likelihood). We applied an admixture model with correlated allele frequencies among subpopulations (Falush et al. 2003), and used the ΔK method of Evanno et al. (2005) to determine the number of clusters. Once we determined the value of K , we assigned individuals to a cluster if the mean Q -vector over 10 runs was ≥ 0.90 . Longer runs with a burn-in of 300,000 followed by 1,000,000 iterations of the MCMC did not affect the results. We investigated the geographic composition of each cluster to elucidate the source of the red squirrel subpopulation in Illinois.

We then performed 2 separate assignment analyses with Indiana and Minnesota designated as the putative source populations a priori. In these analyses, we assumed all Indiana individuals comprised one subpopulation, all Minnesota individuals comprised another, and all Illinois squirrels were unknowns. The first assignment test used a Bayesian approach implemented in STRUCTURE, which required the parameterization of 2 priors in the model; MIGRPRIOR (v) represents the probability that an individual has an immigrant ancestor and GENSBACK (G) represents the number of generations v includes. We utilized the default value $G = 2$ and set v to 0.1 to avoid extreme values (see Pritchard et al. 2000). We initially set the number of clusters in the dataset to $K = 2$, but we ran a subsequent analysis at $K = 3$ to minimize forced assignments. We performed a burn-in of 30,000 followed by 100,000 repetitions of the MCMC 10 times to obtain a mean Q -vector for each Illinois individual that estimated the proportion of each individual's genome inherited from each potential source (the admixture model). We unambiguously assigned individuals to a source subpopulation if the mean Q -vector was ≥ 0.90 .

The second assignment test also utilized a Bayesian method (Rannala and Mountain 1997) that estimated allele frequencies for the given subpopulations and, for an unknown individual, computed the likelihood its genotype would occur in each of the given state subpopulations. To obtain individual assignment probabilities for this procedure, we implemented a Monte Carlo resampling procedure outlined in Paetkau et al. (2004) with 10,000 simulated individuals in GENECLASS2 (Piry et al. 2004). We assigned an individual to the subpopulation if the probability of assignment was ≥ 0.90 . We chose this value for all analyses for consistent interpretation of the results and to minimize the error associated with assigning individuals to a source with simply a higher probability or Q -vector.

In the microsatellite analyses, the translocation scenario would be supported if all Illinois individuals either self-assigned to Illinois or assigned to Minnesota. We considered self-assignment to Illinois as support of the translocation scenario due to the number of generations passed since the initial translocation event, thus accounting for the possibility that the Illinois subpopulation may have diverged from its parental population. Second, in the immigration scenario, Illinois individuals should all either self-assign to Illinois or assign to Indiana. Third, the translocation and immigration scenario would be supported if the Illinois subpopulation contained individuals that assigned to both Indiana and

Minnesota subpopulations. In this scenario, most Illinois individuals should assign to Minnesota because of the time since translocation and the distinct possibility that immigration from Indiana is on-going. Finally, if all Illinois individuals were to self-assign, then the results would be inconclusive.

RESULTS

Alignment of a 274-bp section of the mitochondrial control region revealed 27 distinct haplotypes in 154 red squirrels with 34 variable sites with 30 transitions. Nucleotide diversity and haplotype diversity were lowest in Illinois (Table 1). We found 6 haplotypes in Illinois, 13 in Indiana, and 16 in Minnesota. No haplotypes were shared among the 3 states, but Minnesota and Indiana shared 2 haplotypes not found in Illinois. Illinois shared 1 of 6 haplotypes with Minnesota and 5 of 6 haplotypes with Indiana, lending limited support to the translocation and immigration scenario (Fig. 2).

In the microsatellite analyses, missing data accounted for 1.5% of the total sample of 154 red squirrels, and error rates were $< 4\%$ for all loci. The number of alleles per locus ranged from 10 to 15 and the average number of alleles per locus was 12.83. Mean expected heterozygosity across the 3 subpopulations was 0.78 and mean observed heterozygosity was 0.70 (Table 1). Analyses performed on the entire sample indicated a significant deviation from Hardy-Weinberg equilibrium globally attributed to disequilibrium at 3 of 6 loci ($\alpha = 0.008$; $P_{\text{Thu03}} = 0.002$; $P_{\text{Thu21}} = 0.009$; $P_{\text{Thu25}} = 0.205$; $P_{\text{Thu33}} < 0.001$; $P_{\text{Thu37}} = 0.027$; $P_{\text{Thu42}} < 0.001$), indicative of substructure within the dataset.

The analysis to determine the total number of populations in STRUCTURE indicated $K = 2$ based on the ΔK method of Evanno et al. (2005). In this analysis, the program did not use any information a priori to group individuals and thus offers an unbiased technique to elucidate the source of the Illinois red squirrel subpopulation. Cluster 1 was dominated by the Minnesota and Illinois individuals. In contrast, cluster 2 was dominated by the Indiana subpopulation, but also contained a significant number of Illinois individuals (Fig. 3). These results clearly lend support to the translocation and immigration scenario because all Illinois individuals either assigned to a cluster with Minnesota individuals, assigned to a cluster with Indiana individuals, or were not assigned to either cluster. Additionally, this indicates the Indiana and Minnesota subpopulations are differentiated, albeit at a low level.

The initial assignment analysis in STRUCTURE in which we defined putative source populations a priori forced the

Table 1. Estimates of genetic diversity for 154 red squirrels from Illinois, Indiana, and Minnesota, USA, sampled in 2006 and 2007. We provide expected heterozygosity (H_E), observed heterozygosity (H_O), mean number of alleles (A), allelic richness (A_C), number of unique alleles (A_U), F_{IS} values, nucleotide diversity (π), and haplotype diversity (h) for each state subpopulation.

Subpopulation	n	H_E	H_O	A	A_C	A_U	F_{IS}	π	h
Illinois	52	0.76	0.71	8.86	8.65	1	0.06	0.01	0.66
Indiana	46	0.79	0.71	10.5	10.42	4	0.10 ^a	0.02	0.89
Minnesota	56	0.78	0.68	11.33	10.80	11	0.133 ^a	0.01	0.84

^a Significant heterozygote deficit at the Bonferroni corrected $\alpha = 0.01$.

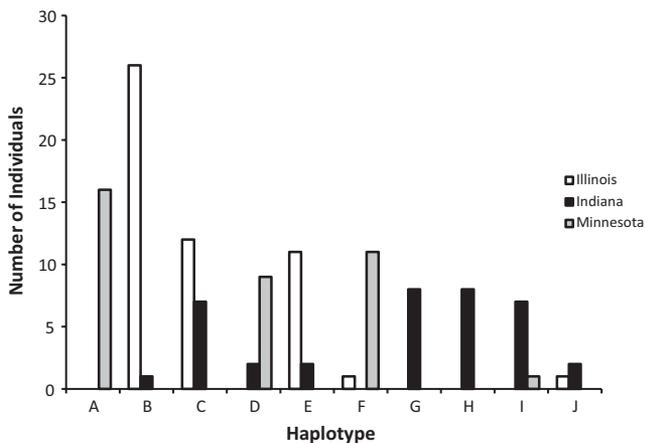


Figure 2. Haplotype frequency distribution for a 274-base pair section of the mitochondrial control region in a sample of 154 red squirrels from Illinois, Indiana, and Minnesota, USA from 2006 to 2007. This figure represents the 10 most common haplotypes in the sample of 27 total haplotypes. All subsequent haplotypes were only found in one state.

algorithm to assign unknown individuals (Illinois) to one of the 2 given sources (Indiana or Minnesota). Illinois individuals were assigned as follows: Minnesota, 21; Indiana, 12; not assigned, 19. The analysis in STRUCTURE in which we fixed K at 3 provided a reservoir for ambiguous individuals. In this analysis, individuals were assigned as follows: Minnesota, 0; Indiana, 0; Extra Subpopulation, 4; not assigned, 48. This indicated the initial analysis in STRUCTURE ($K = 2$) may have contained forced assignments. The GENECLASS2 analysis resulted in the assignment of one individual to Indiana and zero individuals to Minnesota with the remaining individuals not assigned ($Q < 0.9$).

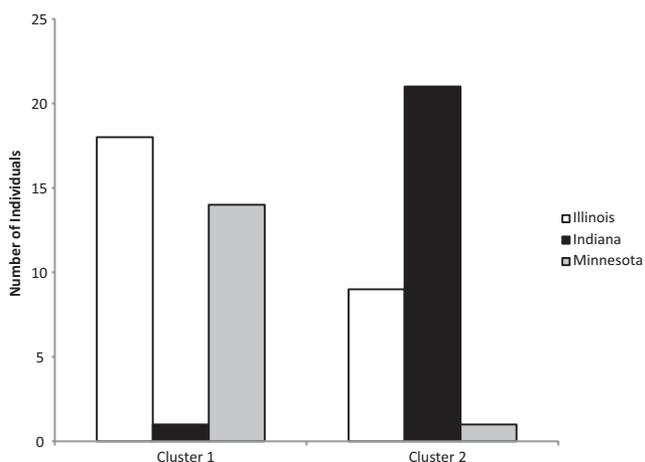


Figure 3. Geographic composition of 2 subpopulations generated in the computer program STRUCTURE with no population information provided a priori. The sample included 154 red squirrels from Illinois, Indiana, and Minnesota, USA collected in 2006–2007. Cluster 1 was primarily composed of Minnesota individuals and Illinois squirrels but also contained 1 Indiana squirrel. Cluster 2 was primarily composed of Indiana and Illinois individuals but also contained 1 Minnesota squirrel.

DISCUSSION

The initial analysis in STRUCTURE in which we made no assumptions a priori regarding putative source populations revealed 2 clusters that roughly corresponded to the Indiana and Minnesota subpopulations with Illinois individuals partitioned into both clusters (Fig. 3). Although this result strongly supports the translocation and immigration scenario, it does contrast with our expectation that most Illinois individuals would have assigned to Indiana assuming contemporary or recent immigration. Indeed, individuals with Indiana ancestry are more likely to be detected than individuals with Minnesota ancestry due to the geographic proximity of the study area to Indiana (2 km) and the possibility that immigration from Indiana has occurred in the recent past. Thus, these results indicate that the translocation event likely involved a substantial number of individuals, and dispersal rates from Indiana into Illinois were low. In contrast, we expected high rates of dispersal due to geographic proximity would have expunged any genetic evidence of a small Minnesota translocation that occurred in the early 1970s.

In the traditional assignment tests in which we delineated putative source populations a priori, we expected roughly the same proportion of individuals initially assigned to Minnesota and Indiana in the $K = 2$ analysis to move into the reservoir subpopulation in the $K = 3$ analysis. In contrast, the vast majority of individuals were not assigned to a source in the $K = 3$ analysis, indicating assignments in the $K = 2$ analysis to Indiana and Minnesota were weak, possibly due to the delineation of putative source populations a priori. However, the weak assignments and high number of individuals not assigned to a source may be the result of reduced power to detect individuals with Minnesota ancestry due to the number of generations passed since translocation and the result of a range expansion of red squirrels from Indiana. Occasional, long-distance dispersers may immigrate into an area and establish an isolated, pocket subpopulation, and the pocket subpopulation may quickly develop significant differentiation from the main population due to high levels of inbreeding (Ibrahim et al. 1996).

Peripheral populations within a species are more likely to contain less genetic variation than central portions of the population due to reduced gene flow, small population size, and founder effects (Lesica and Allendorf 1995). The red squirrel is a recent colonist of the central hardwood region, and Indiana is the southern edge of the range of the red squirrel in North America. In contrast, this species was considered abundant in Minnesota in 1892 (Herrick 1892) and was likely present at the time of European settlement due to the abundance of suitable habitat in the northern portion of the state. Thus, the presence of Indiana haplotypes within the Illinois subpopulation along with one haplotype from Minnesota strongly supports the immigration scenarios, but also provides limited evidence for a Minnesota translocation. However, these results considered in conjunction with the results from microsatellite DNA strongly support a Minnesota

translocation that was subsequently supplemented with immigration from Indiana.

Historical records also support the immigration aspect of the translocation and immigration scenario. 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 WSFWA when it was established in the 1950s but were documented on the site in 1971 (Whitaker and Mumford 2009). By 1977 they were distributed around the area, primarily in pine plantations (Whitaker and Mumford 2009). In this same year, the first red squirrels were confirmed in Illinois (Hoffmeister 1989). The precise routes of immigration are unknown, but Whitaker and Mumford (2009) 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 those found along the Kankakee and Iroquois Rivers, as pathways for dispersal.

Management Implications

Our results will inform future management decisions regarding the red squirrel in Illinois. Presently, the red squirrel is listed as a species in need of conservation by the Illinois Department of Natural Resources and is protected (Illinois Department of Natural Resources 2005) due to its restricted geographic range and apparent dependence on rare habitat (i.e., coniferous forests). A result that found the present Illinois subpopulation to be exclusively composed of descendants of translocated individuals from Minnesota would have warranted the removal of the red squirrel from the list because it may have been considered a non-native species. In contrast, we found the current subpopulation to be a combination of descendants of squirrels from Minnesota and Indiana, and thus, we recommend continued protection for the red squirrel due to its restricted geographic range, small population size, and status as a native species.

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