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RESEARCH ARTICLE

Severe reduction in genetic variation in a montane isolate: the endangered Mount Graham red squirrel (*Tamiasciurus hudsonicus grahamensis*)

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Abstract The Mount Graham red squirrel (*Tamiasciurus hudsonicus grahamensis*; MGRS) is endemic to the Pinaleño Mountains of Arizona at the southernmost extent of the species' range. The MGRS was listed as federally endangered in 1987, and is currently at high risk of extinction due to declining population size and increasing threats. Here we present a genetic assessment of the MGRS using eight nuclear DNA microsatellite markers and a 472 bp fragment of the mitochondrial cytochrome b gene. We analyzed 34 MGRS individuals and an additional 66 red squirrels from the nearby White Mountains, Arizona (*T. h. mogollonensis*). Both nuclear and mitochondrial DNA analyses revealed an extreme reduction in measures of genetic diversity relative to conspecifics from the White Mountains, suggesting that the MGRS has either experienced multiple bottlenecks, or a single long-term bottleneck. Additionally, we found a high degree of relatedness (mean $= 0.75 \pm 0.18$) between individual MGRS. Our study implies that the MGRS may lack the genetic variation required to respond to a changing environment. This is especially important considering this region of the southwest United States is expected to experience profound effects from global climate change. The reduced genetic variability together with the high relatedness coefficients should be taken into account when constructing a captive population to minimize loss of the remaining genetic variation.

Keywords Endangered species · Conservation · Effective population size · Inbreeding · Arizona

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Introduction

Analysis of the factors that contribute to extinction is central to the conservation and management of endangered and threatened populations (Lande 1998). These populations become increasingly vulnerable to demographic stochasticity, environmental variation, random genetic effects, and extreme events like natural catastrophes (Shaffer 1981). Genetic effects are important because endangered species have small and/or declining population sizes, which result in an exponential loss of genetic diversity and an increased frequency of inbreeding (Frankham 2005). The loss of genetic diversity may jeopardize the ability for a species to respond to environmental changes (Frankel 1970, 1974), and inbreeding can reduce fitness through increased expression of deleterious traits (inbreeding depression; see Hedrick and Kalinowski 2000 for a review). Therefore, it is important for managers to evaluate endangered species from a genetic perspective to facilitate recovery and preserve remaining evolutionary potential.

North American red squirrels (*Tamiasciurus hudsonicus*) are small (<300 g), diurnal squirrels found in boreal, mixed conifer, and deciduous forests throughout much of North America. Their distribution includes the northeastern

United States, west through Canada and Alaska, and south along the Rocky Mountains to Arizona and New Mexico (Hall 1981). At the southernmost extent of the species' range exists an isolated population of a subspecies, the Mount Graham red squirrel (*T. h. grahamensis*; MGRS). The MGRS is restricted to high-elevation coniferous forests of the Pinaleño Mountains of southeastern Arizona (Hall 1981; Brown 1984). Although Allen (1894) originally described the MGRS as a unique subspecies due to pelage differences and geographic isolation, both Hall (1981) and Hoffmeister (1986) acknowledged that morphology alone does not strongly differentiate it from its nearest conspecific, *T. h. mogollonensis*. The MGRS can be distinguished through vocalizations from *T. h. lychnuchus* of New Mexico, and its most common vocalization is different from that of *T. h. mogollonensis* (Yamamoto et al. 2001). The MGRS can also be differentiated from other red squirrels using mitochondrial restriction fragment length polymorphisms (RFLP; Riddle et al. 1992) and protein electrophoresis (Sullivan and Yates 1995). Unfortunately, the two most recent phylogenetic works on North American red squirrels did not include the MGRS (Arbogast et al. 2001; Wilson et al. 2005).

In 1987, the MGRS was listed as an endangered species because its range and habitat had been reduced, and remaining habitat was threatened by anthropogenic factors, fire, insect outbreaks, and possible competition with the introduced Abert's squirrel (U. S. Fish and Wildlife Service 1987; 1993; reviewed by Sanderson and Koprowski 2009). Historical accounts suggested that the MGRS was abundant (Mearns 1907; Hoffmeister 1986), but by the mid 1900's the population declined and was believed to be extirpated (Minckley 1968). Recent survey information indicates that the population has remained below 300 individuals since Fall of 2001 (U. S. Fish and Wildlife Service 2011). In response to the declining population size and increasing threats, a thorough genetic assessment employing both nuclear and mitochondrial DNA markers is necessary to develop a conservation strategy for this species.

In this study we used nuclear (microsatellites) and mitochondrial DNA markers to assess the level and distribution of genetic diversity in the MGRS. To better evaluate these measures, we included a population of its geographically nearest conspecific, *T. h. mogollonensis,* for comparison. Additionally, managers are investigating the possibility of establishing a captive population of the MGRS (U. S. Fish and Wildlife Service 2011). A genetic analysis is necessary to identify the appropriate individuals to remove, to pair for breeding, and to release while acknowledging any population substructure and minimizing genetic loss (Lacy 1994; Ballou and Lacy 1995; Ballou et al. 2010). We discuss our results in the context of establishing a captive breeding program in addition to the implications for conservation and recovery of the MGRS.

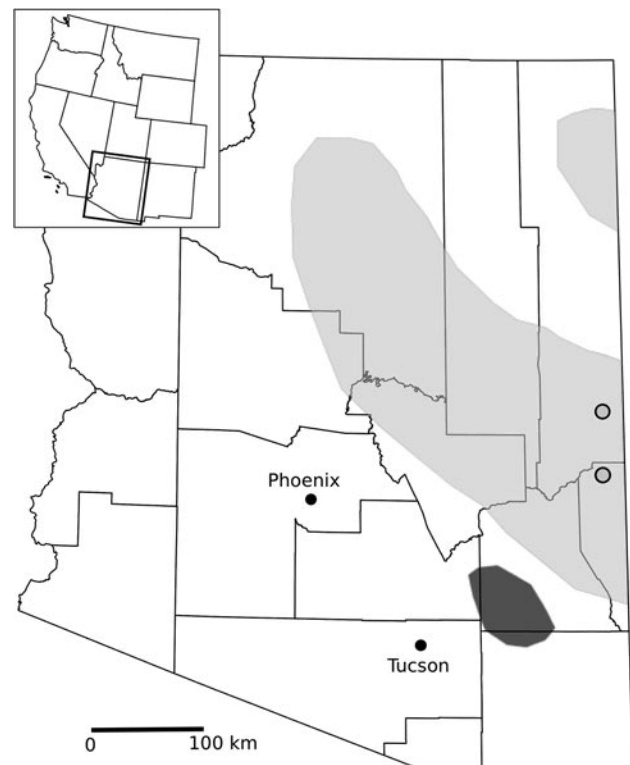

Fig. 1 Map of the sampling area. The *light grey-shaded* region indicates the distribution of *T. h. mogollonensis*, and the *dark grey-shaded* region represents the Pinaleño Mountains and the restricted range of *T. h. grahamensis*. The *open circles* represent the two sampling locations for the WMRS. (Map adapted from http://en.wikipedia.org/wiki/File:USA_Arizona_location_map.svg under the Creative Commons Attribution-Share Alike 3.0 Unported license)

Materials and methods

Sample collection and DNA extraction

We analyzed 100 red squirrel samples collected by the University of Arizona's MGRS Monitoring Program from September 2002 through November 2006. The squirrels were collected from the Pinaleño Mountains (*T. h. grahamensis*; MGRS; $n = 34$) and from two sites in the White Mountains (*T. h. mogollonensis*; WMRS; $n = 66$) of Arizona (Fig. 1). We selected either a small piece of ear tissue or 10–20 plucked hairs from live or deceased specimens and stored them at -20 °C. The use of >10 plucked hairs has provided reliable genotype estimates for microsatellites (Goossens et al. 1998). Live specimens were ear-tagged with unique numbered monel steel tags (model 1005-1, National Band & Tag Co., Newport, KY, USA) prior to release to avoid resampling of individuals. We extracted

DNA using the Qiagen DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol.

Microsatellite amplification and analysis

We amplified eight microsatellite loci developed for *T. hudsonicus* and reported by Gunn et al. (2005) for each individual (Th08, Th14, Th21, Th23, Th25, Th33, Th41, and Th49). Fluorescent labeling was performed using the method detailed by Schuelke (2000). All PCR reactions contained 2 µl DNA template, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.0 µM fluorescently labeled M13 primer (6-FAM, NED, or VIC), 0.1 µM forward primer, 1.0 µM reverse primer, 1 unit of AmpliTaq Gold® polymerase (Applied Biosystems, Foster City, CA, USA), 1X PCR buffer, and water in a total volume of 10 µl. Thermocycler conditions consisted of an initial denaturation at 94 °C for 2 min, 5 cycles (94 °C for 1 min, 64 °C for 30 s, 72 °C for 30 s), and 30 cycles (94 °C for 1 min, 55 °C for 30 s, 72 °C for 30 s). All fragment lengths were resolved on the same ABI3730 DNA Analyzer at the University of Arizona Genetics Core (UAGC; http://uagc.arl.arizona.edu) and alleles were scored using GENOTYPER v2.1 (Applied Biosystems). We subsequently re-amplified all ambiguous samples until a genotype could be determined or we did not assign a genotype.

We classified raw allele sizes into bins using FLEXIBIN v2 (Amos et al. 2007). We checked each locus in each population for scoring errors due to stutter, allele dropout, and null alleles using MICROCHECKER v2.2.3 (van Oosterhout et al. 2004). We performed exact tests for significant deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) between loci using a Markov chain method with a dememorization of 10,000 steps and 100 batches of 10,000 iterations each in GENEPOP v4 (Rousset 2008). We adjusted the nominal *p* value for multiple comparisons with a Bonferroni correction. We calculated general indices of genetic diversity, including number of alleles, allelic richness, and observed and expected heterozygosities using FSTAT v2.9.3.2 (Goudet 2001). We examined levels of inbreeding by computing the excess or deficiency in heterozygosity in individuals relative to the subpopulation (F_{IS}). We tested F_{IS} computations for a statistical difference from zero using 1,000 permutations in FSTAT.

We used a Bayesian clustering algorithm implemented in STRUCTURE v2.3.2.1 (Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009) to determine the number of clusters (populations) without any a priori knowledge of population substructure. The estimated number of populations (K) is given as the value K where the probability of the data, $\Pr(X \mid K)$, reaches a plateau. We tested for $K = 1$–8 with ten iterations of each possible K. We assumed admixture and correlated allele frequencies and ran each analysis for 100,000 burn-in generations and 500,000 generations thereafter. For our most probable number of populations, K, we combined the ten replicates using the full-search algorithm in the software CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and plotted the results using the software DISTRUCT v1.1 (Rosenberg 2004). We calculated effective population sizes using an approximate Bayesian method implemented in ONESAMP (Tallmon et al. 2008) using 50,000 iterations and the four loci that were polymorphic in all populations (Th08, Th14, Th23, Th41). Confidence intervals were assessed using the posterior distribution. We tested for presence of a population bottleneck using the software BOTTLENECK v1.2.02 (Cornuet and Luikart 1996; Piry et al. 1999) implementing a two-phased model of mutation and assessed significance using a Wilcoxon's ranked test. We also used the mode-shift indicator in BOTTLENECK as qualitative evidence for a recent (within a few dozen generations) bottleneck event (Luikart et al. 1998). Pairwise relatedness was estimated in COANCESTRY v1.0.0 (Wang 2011) using the method of Lynch and Ritland (1999) and 1,000 bootstrap replications to examine differences between populations.

Mitochondrial DNA amplification and analysis

We amplified a 472 bp fragment of the mitochondrial cytochrome b gene (cytb) in each individual using primers mcb398 and mcb869 (Verma and Singh 2003). The PCR reaction mix consisted of 3.0 µl DNA template, 0.1 µM forward and reverse primer, 0.2 mM each dNTP, 1.0 mM $MgCl_2$, 0.01 mg BSA, 1X PCR buffer, 0.5 units *Taq* polymerase (Qiagen), and water to a final volume of 20 µl. Thermocycling conditions included an initial denaturation step at 95 °C for 10 min, followed by 35 cycles (95 °C for 45 s, 52 °C for 1 min, 72 °C for 2 min) and a final extension step at 72 °C for 10 min. All PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced in both forward and reverse directions on an ABI3730 DNA Analyzer at the UAGC. All sequences were assembled, had primer bases removed, and were visually inspected for errors in SEQUENCHER 4.9 (Gene Codes Corp., Ann Arbor, MI, USA).

We used the software ARLEQUIN v3.5 (Excoffier and Lischer 2010) to organize the mitochondrial DNA (mtDNA) sequences into haplotypes and to calculate haplotype diversity (h) and nucleotide diversity (π). We inferred the phylogenetic relationships between cytb haplotypes using statistical parsimony in TCS v1.21 (Clement et al. 2000) with a probability of parsimony cutoff value of 0.95. Sequences from each of the recovered haplotypes were deposited into Genbank (Accession numbers KC306932-KC306944).

Table 1 Summary of microsatellite data analysis for red squirrels (*Tamiasciurus hudsonicus*) in the White (WMRS: *T. h. mogollonensis*) and the Pinaleño (MGRS: *T. h. grahamensis*) Mountains, Arizona

Locus	n	A	A_R	H_O	H_E	F_{IS}*
MGRS						
Th8	34	3	2.941	0.265	0.329	0.173
Th14	33	2	2	0.242	0.216	−0.123
Th21	34	1	1	0.000	0.000	N/A
Th23	34	2	2	0.206	0.234	0.118
Th25	32	1	1	0.000	0.000	N/A
Th33	34	1	1	0.000	0.000	N/A
Th41	34	2	2	0.147	0.138	−0.065
Th49	32	1	1	0.000	0.000	N/A
Mean	33.375	1.625	1.618	0.108	0.115	0.052
WMRS						
Th8	66	13	12.484	0.924	0.903	−0.023
Th14	66	14	12.757	0.848	0.901	0.059
Th21	66	5	4.485	0.621	0.618	−0.006
Th23	66	6	5.899	0.682	0.745	0.085
Th25	66	4	3.861	0.182	0.224	0.189
Th33	66	5	4.737	0.697	0.730	0.045
Th41	64	7	6.871	0.844	0.816	−0.034
Th49	63	6	5.996	0.698	0.719	0.029
Mean	65.375	7.500	7.136	0.687	0.707	0.028

The columns indicate *n* number of successful genotypes, *A* number of alleles, A_R allelic richness, H_O observed heterozygosity, H_E expected heterozygosity, and F_{IS} inbreeding coefficient

* F_{IS} values did not differ significantly from zero after 1,000 permutations

Results

Microsatellite analyses

Eight microsatellite loci were amplified and scored for 100 individuals (MGRS = 34, WMRS = 66). All eight loci were polymorphic in WMRS, whereas only four loci (50 %, Thu21, Thu25, Thu33, and Thu49) were polymorphic in MGRS. Results from MICROCHECKER indicated that no scoring errors, allele dropout, or null alleles were present. After Bonferroni correction, no loci in either population deviated significantly from HWE, and no significant LD was detected.

We calculated several indices of genetic variability and compared them between the populations (Table 1). Genetic diversity as indicated by the number of alleles, allelic richness, and observed heterozygosity was consistently four to six times greater in WMRS relative to MGRS and significant in all cases using a Welch's t-test ($p < 0.001$). We found a majority of alleles (61.5 %) in MGRS to occur at high frequency (>0.8), whereas low frequency alleles were characteristic of WMRS (Fig. 2).

We used a Bayesian inference method of population clustering implemented in STRUCTURE to quantify an individual's proportion of membership in each of K populations. The Pr(X | *K*) reached a maximum when two populations were assumed. One population consisted entirely of MGRS individuals and the other of WMRS individuals (Fig. 3). Three WMRS individuals, however, did share a moderate amount of ancestry (37 ± 0.002, 19 ± 0.001, and 30 ± 0.001 %) with MGRS. Examination of additional values of K > 2 revealed no evidence of substructure within MGRS (data not shown).

Mean estimates of effective population size were 10.4 (95 % CI 6.0–18.4) in MGRS and 48.8 (95 % CI 37.3–63.7) in WMRS. We were unable to detect a population bottleneck in both MGRS and WMRS (Wilcoxon's ranked test: $p = 1.0$ and 0.098, respectively) although the allele frequency distribution in MGRS did have a shifted mode (Fig. 2). The average pairwise relatedness among MGRS individuals was 0.75 ± 0.18, significantly greater ($p < 0.001$) than that observed in WMRS (mean = −0.01 ± 0.34: Fig. 4).

Mitochondrial DNA analyses

After removing primer bases, the final cytb amplicon length was 421 bp. Mitochondrial cytb sequence data also indicated similar reductions in measures of genetic variation for MGRS relative to WMRS. Only a single haplotype existed in MGRS ($h = 0$), whereas we found 10 haplotypes in WMRS ($h = 0.849 \pm 0.019$). Nucleotide diversity (π) per locus was zero in MGRS and 3.186 ± 1.668 in WMRS. The single MGRS haplotype was not shared with WMRS. The relationships between haplotypes using statistical parsimony indicated the MGRS haplotype (haplotype A) was at least two mutational steps different from the nearest WMRS haplotypes (haplotypes F, H, and D; Fig. 5), but within the range of differences observed between WMRS haplotypes (maximum of seven steps between haplotypes B and E; Fig. 5).

Discussion

The MGRS has experienced both recent population declines and an increase in threats that may severely impact its long-term viability (U. S. Fish and Wildlife Service 1987, 1993, reviewed by Sanderson and Koprowski 2009). These forces may also have profound effects on the amount and distribution of genetic diversity (Koprowski and Steidl 2009). In this study, we analyzed both nuclear and mitochondrial DNA markers to assess the genetic status of the MGRS.

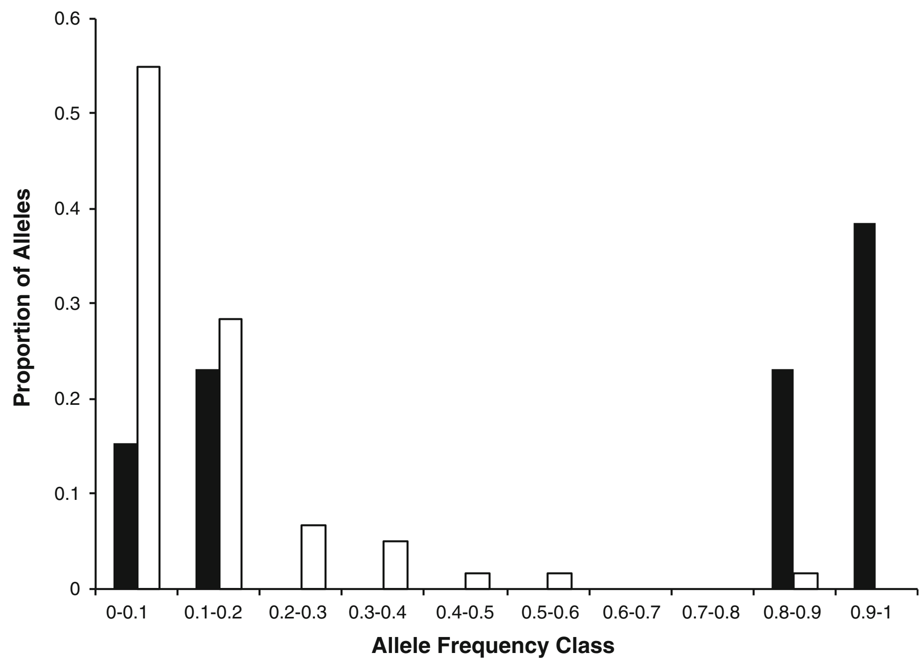

Fig. 2 Allele frequency distribution for alleles at eight microsatellite loci. The *black bars* indicate alleles in MGRS (*T. h. grahamensis*) – shifted mode—and the *white bars* indicate alleles in WMRS—non-shifted mode

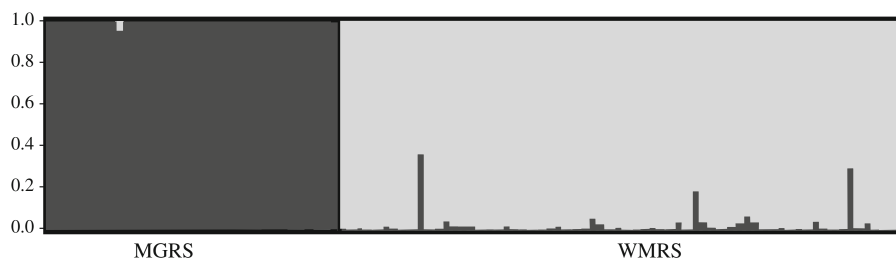

Fig. 3 Results of the STRUCTURE analysis for red squirrels (*Tamiasciurus hudsonicus*) in the Pinaleño (MGRS: *T. h. grahamensis*) and White (WMRS: *T. h. mogollonensis*) Mountains, Arizona. Each *vertical bar* represents a different individual, while the *color* of each *vertical bar* represents the proportion of ancestry from each of K = 2 populations (indicated by the *light grey* and *dark grey colors*)

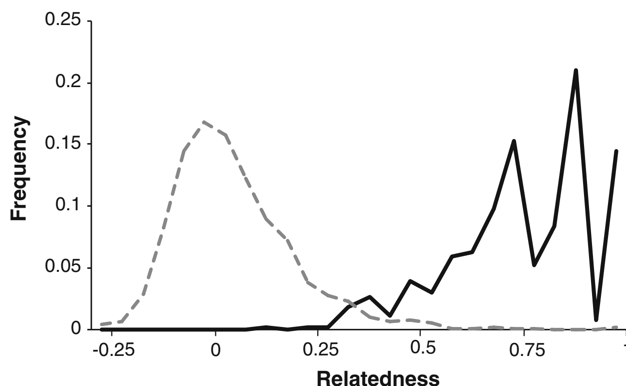

Fig. 4 Frequency histogram of the pairwise relatedness values for each population calculated using the method of Lynch and Ritland (1999) for red squirrels (*Tamiasciurus hudsonicus*) in the Pinaleño (MGRS; *black line*; *T. h. grahamensis*) and White (WMRS; *grey-dashed line*; *T. h. mogollonensis*) Mountains, Arizona

Genetic variability

For WMRS, measures of genetic variation were consistent with that reported in other North American red squirrels using the same loci (Gunn et al. 2005; Beatty et al. 2011; Kiesow et al. 2012), and heterozygosity was similar to the mean reported for sciurids (0.62, Garner et al. 2005). However, for MGRS, we uncovered a remarkable reduction of both nuclear and mitochondrial genetic diversity. We observed only four polymorphic nuclear loci, similar to Sullivan and Yates (1995) who reported no variation in the 26 allozymes examined. For mitochondrial DNA we found only a single haplotype in MGRS, consistent with the lack of variation in mitochondrial RFLPs reported by Riddle et al. (1992).

Such a drastic decrease in genetic variation is likely the result of many factors. For instance, populations at the

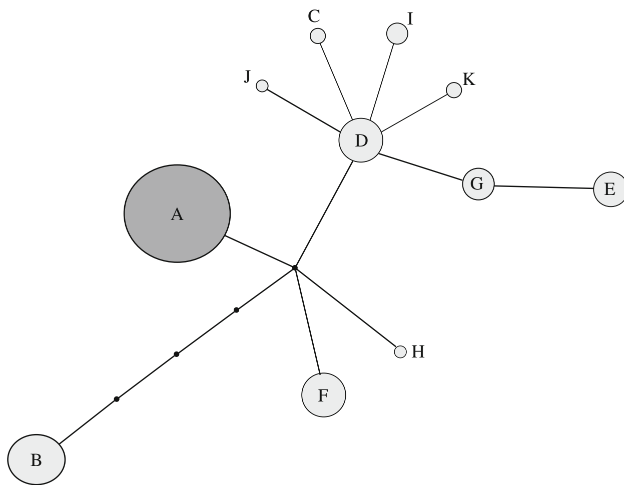

Fig. 5 A maximum parsimony network indicating the phylogenetic relationships between the recovered cytb haplotypes for red squirrels (*Tamiasciurus hudsonicus*) in the Pinaleño (MGRS; *dark grey*; *T. h. grahamensis*) and White (WMRS; *light grey*; *T. h. mogollonensis*) Mountains, Arizona. Each *circle* represents a haplotype (A–K), with size proportional to the number of haplotypes sampled. *Lines* indicate a single mutational step, and small *black circles* reflect hypothetical haplotypes not recovered in the study

periphery of a species' range, like the MGRS, often have lower genetic variation than the core population (Cassel and Tammaru 2003; Schwartz et al. 2003; Eckert et al. 2008; Huang et al. 2009). Additionally, a founder's effect resulting from early Holocene colonization of coniferous mountaintops in southeast Arizona and southwest New Mexico followed by local extinctions with the exception of those in the Pinaleño Mountains (Harris 1990; Sullivan and Yates 1995) may have reduced genetic variation. Similar histories have been suggested for other more northerly portions of the range (Wilson et al. 2005; Chavez et al. 2011). Finally, a dynamic demographic history that includes multiple population bottlenecks, and long-term small population size reduces genetic diversity. We were unable to detect a historical bottleneck from the genetic data, despite probable bottleneck events that include a devastating fire in 1685 that destroyed much of the forest in the Pinaleño Mountains (Grissino-Mayer et al. 1995) and a near extinction in the mid 1900's (Minckley 1968). We did detect a recent population bottleneck, possibly the result of a series of fires and insect outbreaks from 1996 to 2002 that reduced census population estimates by more than half (U. S. Fish and Wildlife Service 2011).

Another striking result is the high pairwise estimates of relatedness between MGRS individuals. We chose the method of Lynch and Ritland (1999), a robust estimator that outperforms many other methods in unstructured populations and can be calculated for biallelic loci (Lynch and Ritland 1999; Oliehoek et al. 2006; Toro et al. 2003). Despite HWE and evidence for random mating ($F_{IS} \sim 0$), a MGRS is, on average, related to a high degree (mean = 0.75 ± 0.18) to any other member of the population. This suggests that mating in the MGRS is random, but, due to their low levels of variation and small population size, individuals often encounter mates that are genetically similar.

Population structure

Analyses of population structure using nuclear DNA markers revealed a strict pattern of allele frequency differentiation between MGRS and its nearest conspecific. A few WMRS individuals maintained some shared ancestry with MGRS, possibly due to a residual effect of their common origin during the late Pleistocene (Harris 1990; Sullivan and Yates 1995), or the result of immigration and subsequent gene flow from MGRS into WMRS. We found no evidence of gene flow from WMRS into MGRS. Within MGRS, we did not detect the presence of subpopulations. This suggests that MGRS has maintained at least some level of gene flow across different regions of the Pinaleño Mountains, but more extensive sampling is required to substantiate this result.

We also assessed genetic structure using mitochondrial DNA, because matrilinear patterns of genetic structure may be different than that reported from nuclear loci if sex-biased dispersal exists (see Handley and Perrin 2007 for a review). Additionally, hypervariable loci, i.e. microsatellites, may overestimate differentiation after large reductions in population size (Hedrick 1999). We found a single cytb haplotype in MGRS that was not shared with WMRS. This demonstrates a similar degree of isolation and subsequent genetic drift as observed in the microsatellite results.

Conservation implications

The maintenance of genetic diversity is an important factor in conservation because it is directly correlated with fitness and evolutionary potential (Allendorf and Ryman 2002; Reed and Frankham 2003). In the MGRS, evidence of reduced fitness measured by decreased litter size and survivorship relative to conspecifics has been reported (Rushton et al. 2006; Munroe et al. 2009; Zugmeyer and Koprowski 2009). Additionally, many ecosystems in the southwestern U.S., similar to the Pinaleño Mountains, are particularly vulnerable to climate change and will likely experience increased drought, insect outbreaks, and fires (Archer and Predick 2008; Allen et al. 2010). These factors render the MGRS at a high risk of extinction, and management strategies should be designed to mitigate this risk.

Management strategies for a taxon with a single population are limited and include: increasing the population

size, establishing additional populations to avoid catastrophes to the single extant population, maximizing the reproductive rate, and insulating from environmental change (Frankham et al. 2002). Our results do not provide evidence that the MGRS is a distinct, phylogenetic unit but rather a population with extensive allele frequency differentiation from its nearest conspecific, likely the result of isolation and extensive genetic drift. Therefore, increasing genetic diversity through the translocation of individuals from WMRS remains a possibility. Such introductions have been successful in other isolated and/or inbred populations (Westemeier et al. 1998; Madsen et al. 1999; Johnson et al. 2010; Olson et al. 2012). However, isolated and rare populations, like the MGRS, may contain a suite of locally adaptive variation that can be negatively impacted through outbreeding with maladaptive individuals (Lande and Shannon 1996; Tufto 2001). Although behaviors such as vocalizations (Yamamoto et al. 2001), home range size (Koprowski et al. 2008), caching (Angell 2009) and nest use (Leonard and Koprowski 2009) differ between Mt. Graham and other subspecies, we do not know if these represent adaptations or simply behavioral plasticity. Nonetheless, future genetic work using additional nuclear loci, such as a genome-wide set of single nucleotide polymorphisms and gene sequences, will be necessary to investigate the taxonomic status of the MGRS.

Current management actions include a proposal to establish a captive population from 16 founders (U. S. Fish and Wildlife Service 2010, 2011). The removal and pairing of individuals to establish a captive population should consider the high coefficients of relatedness we observed to minimize the loss of remaining genetic variation. Finally, because populations can adapt to captivity in as little as one generation (Christie et al. 2012), we believe that preservation of tissue (i.e. gametes, embryos, other tissue) in genome resource banks is prudent. The preservation of this material can extend the generation interval and provide genomic material for future studies (Johnston and Lacy 1995).

Conclusions

Our results demonstrated that the MGRS persists despite incredibly low levels of genetic variation. However, due to demographic, environmental, and genetic concerns, the long-term persistence of the MGRS is in jeopardy. Although some taxa have maintained small population sizes and/or high levels of inbreeding for long periods of time (see Craig 1994 for examples), many such taxa are extinct. Therefore, informed management actions for the MGRS that incorporate genetics in addition to demographics, natural history, and the environment will be most effective. Finally, studies employing genome-wide markers or sequencing may be useful for identifying important adaptive and detrimental variation that can be managed accordingly (Kohn et al. 2006; Ouborg et al. 2010).

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