

POPULATION GENETICS AND GENOTYPING FOR MARK-RECAPTURE STUDIES OF NORTHERN GOSHAWKS (*ACCIPITER GENTILIS*) ON THE KAIBAB PLATEAU, ARIZONA

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ABSTRACT.—Advances in molecular techniques have facilitated use of genetic data in demographic wild-life studies. An important first step in genetic mark-recapture is selecting markers that uniquely “mark” and reliably “recapture” individuals. Markers should be tested on reliable DNA from known individuals (blood) before being used on non-invasively sampled DNA (hair, scat, or molted feathers). To evaluate whether Northern Goshawks (*Accipiter gentilis*) can be uniquely identified by genotyping, 113 known (banded, sexed) goshawks from the Kaibab Plateau, Arizona, were genotyped using DNA from blood and five microsatellite markers and a sex-linked gene. We used mean relatedness to test whether adults in the population were related and probability of identity ($P_{(ID)} =$ probability that two random individuals from the population have the same genotype) to test the ability of multi-locus genotyping for uniquely identifying goshawks. We used genetic data to assess inbreeding and demographic data to estimate the effective population size. Sixty-nine adult goshawks were sexed correctly and genotyped. Expected heterozygosity was high ($H_E = 0.81$), and relatedness among adults was low ($r = -0.017$). All individuals sampled (69 adults, 44 nestlings) had unique five-locus genotypes, the overall probability of identity was low ($P_{(ID) unbiased} = 7.03 \times 10^{-7}$), and the observed $P_{(ID)}$ was <0.0001 . Thus, Kaibab goshawks were uniquely “marked” by genotyping. Despite a small effective population size ($N_e = 37$ individuals), goshawks on the Kaibab Plateau functioned as a large breeding population with no inbreeding ($F_{IS} = -0.001$). We hypothesized that genetic diversity is maintained by gene flow via immigration of individuals from distant forests.

KEY WORDS: *Northern Goshawk*; *Accipiter gentilis*; capture-recapture; genetic tagging; individual identification; molecular sexing; probability of identity.

GENÉTICA POBLACIONAL Y GENOTIPIFICACIÓN PARA ESTUDIOS DE MARCADO-RECAPTURA DE *ACCIPITER GENTILIS* EN KAIBAB PLATEAU, ARIZONA

RESUMEN.—Los avances en las técnicas moleculares han facilitado el uso de información genética en estudios demográficos de fauna silvestre. Un primer paso importante en estudios genéticos de marcado

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y recaptura es seleccionar marcadores que “marquen” inequívocamente y que permitan “recapturar” confiablemente a los individuos. Los marcadores deben ser probados en ADN confiable de individuos conocidos (sangre) antes de ser usados en muestreos no invasivos de ADN (pelo, excremento, o plumas mudadas). Para evaluar si los individuos de la especie (*Accipiter gentilis*) pueden ser identificados por genotipificación, estudiamos 113 gavilanes conocidos (sexados y anillados) de la meseta Kaibab, Arizona, usando ADN de la sangre y cinco marcadores microsátélites y un gen ligado al sexo. Usamos el parentesco promedio para probar si los adultos en la población estaban relacionados y la probabilidad de identidad ($P_{(ID)}$ = la probabilidad que dos individuos al azar de la población tengan el mismo genotipo) para probar la habilidad de la genotipificación mediante múltiples loci para identificar individualmente a los gavilanes. Utilizamos datos genéticos para determinar el grado de endogamia e información demográfica para estimar el tamaño efectivo de la población. Sesenta y nueve gavilanes adultos fueron correctamente sexados y genotipificados. La heterocigocidad esperada fue alta ($H_E = 0.81$) y el parentesco entre adultos fue bajo ($r = -0.017$). Todos los individuos analizados (69 adultos, 44 polluelos) tuvieron genotipos únicos con cinco loci, la probabilidad de identidad total fue baja ($P_{(ID)}^{unbiased} = 7.03 \times 10^{-7}$), y la $P_{(ID)}$ observada fue <0.0001 . Así, los gavilanes de Kaibab fueron “marcados” singularmente por genotipificación. A pesar de un tamaño efectivo de la población pequeño ($N_e = 37$ individuos), los gavilanes en el Kaibab Plateau funcionaron como una población reproductiva grande y sin endogamia ($F_{IS} = -0.001$). Nuestra hipótesis es que la diversidad genética es mantenida por flujo genético a través de la inmigración de individuos de los bosques lejanos.

[Traducción de Mauricio Cotera]

Northern Goshawks (*Accipiter gentilis*) are highly secretive and are most easily detected during the breeding season when they aggressively defend their nests and young. While their defensive behavior at nests facilitates capture-recapture studies of breeding individuals, population monitoring is difficult because individuals often forgo breeding, or their nests fail early in a breeding season. Even in years of high productivity, mark-recapture studies can be prohibitively expensive because population sampling requires large field crews and multiple nest visits to many breeding territories to capture and recapture breeding goshawks (Reynolds et al. 2005).

Because of recent improvements in molecular techniques (Haig 1998, Parker et al. 1998), genetic capture-recapture may be a viable alternative to traditional capture-recapture methods for goshawks. Collecting molted feathers requires fewer nest visits than traditional capture-recapture methods. Breeding goshawks begin an annual molt during spring (Squires and Reynolds 1997) and because they spend much of the breeding season near their nests, they drop many of their molts within their nest areas, including years when nesting attempts fail. Thus, goshawk feathers are readily collected from nest areas and may provide an efficient means to non-invasively sample their populations.

Several factors influence the success of genetic capture-recapture studies. An appropriate number of highly variable genetic markers for identifying individuals are required, and potential biases must

be identified. “Shadow effects” (lack of discrimination of individuals because of low variability or sampling too few markers) can negatively bias estimates of population abundance and positively bias estimates of survival (Mills et al. 2000). On the other hand, when more markers than necessary are used, population abundance may be overestimated and survival underestimated if genotyping errors add unique “genotypes,” and thus individuals, to population samples (Lukacs and Burnham 2005). Both biases will inflate variance and lower precision of parameter estimates (Lukacs and Burnham 2005).

Microsatellites are currently a preferred molecular marker for identifying individuals because they are easily interpreted (i.e., heterozygous genotypes are easily distinguished from homozygous genotypes), highly variable, bi-parentally inherited, and generally appear to be selectively neutral. Further, a large body of literature exists on microsatellite evolution (Jarne and Lagoda 1996, Goldstein and Pollock 1997, Estoup et al. 2002), which has facilitated the development of much statistical theory and analytical software (Hedrick 2005). However, microsatellites are expensive and time consuming to develop for each newly-studied species. Occasionally primers used to amplify microsatellite markers in one species can be used in related species (Ellegren 1992, Primmer et al. 1996, Galbusera et al. 2000, Martinez-Cruz et al. 2002).

Prior to starting a non-invasive genetic study, establishing intrapopulation genetic structure (i.e.,

levels of inbreeding and relatedness) and the frequency of null alleles (alleles that fail to amplify) is necessary for providing baselines against which feather samples can be compared (Mills et al. 2000). Likewise, it is important to establish statistical power of multi-locus genotyping for identifying individuals with an independent population sample. We present results from a pilot study where we assessed the feasibility of implementing a non-invasive genetic capture-recapture study on a population of Northern Goshawks on the Kaibab Plateau, Arizona. Before assessing the utility of molted feathers as a viable source of DNA, we established a dependable genotyping marker set using DNA derived from blood (Taberlet and Luikart 1999).

Our objectives were to: (1) screen species-specific and cross-specific (among species) microsatellite markers, (2) test a sex-linked gene in goshawks known to distinguish males and females in other raptors (Kahn et al. 1998), (3) assess the power of multi-locus genotyping to uniquely identify individuals using probability of identity analysis (P_{ID}); probability that two individuals drawn at random from the same population share the same multi-locus genotype), and (4) estimate average relatedness, inbreeding, and effective population size for the goshawk population on the Kaibab Plateau.

METHODS

Field Collection. The goshawk study population is located on the Kaibab Plateau in northern Arizona, an area that includes the North Kaibab Ranger District of the Kaibab National Forest and the North Rim of the Grand Canyon National Park (for descriptions of the study area see Reich et al. 2004, Reynolds and Joy 2005). It is a forested plateau surrounded by shrub-steppe habitat—the nearest forests being 97 km to the north, 250 km to the east, 80 km to the west, and 89 km to the south—except for a small patch of forest 18 km south on the south rim of the Grand Canyon. Sampled nests were well distributed across the study area. We captured 69 adult, breeding goshawks (1991–93, 2000–02) and 44 of their nestlings (Reynolds et al. 1994). We sexed adult goshawks using morphometrics (mass, tarsus length) and behavior. Blood was sampled from the brachial vein with 22-gauge needles and non-heparinized capillary tubes (volume \leq 0.10 ml). Blood was transferred into STE (Sodium Chloride-Tris-EDTA) buffer-filled storage tubes kept cool in insulated containers with frozen cold-packs until crews returned to the field station, where samples were subsequently frozen (-20°C). At the close of the field season, blood was transferred to and stored at -80°C at Colorado State University, Fort Collins, CO.

Laboratory Methods. We extracted DNA using QIAamp mini blood kits (Qiagen, Inc., Valencia, CA U.S.A.) following the manufacturer's protocol. To find microsatellites, we screened published and unpublished

primer sets that included microsatellites originally isolated from Northern Goshawks (Topinka and May 2004), European goshawks (*Accipiter gentilis gentilis*), Golden Eagles (*Aquila chrysaetos*), and Red Kites (*Milvus milvus*; Peck 2000). We also tested primers that amplify an intron within the avian CHD (chromo-helicase-DNA binding), which was used to determine gender in Red-tailed Hawks (*Buteo jamaicensis*) and Great Horned Owls (*Bubo virginianus*; Kahn et al. 1998). The CHD gene is located on the Z and W sex chromosomes. We expected males to be homozygous (ZZ genotype) and females to be heterozygous (ZW genotype).

We used PCR (Polymerase-Chain-Reaction) to amplify microsatellites in 25 μl reactions using 0.5 μl (AGE 1a) or 1.0 μl (all other markers) of template DNA, 2.5 μl 10 \times buffer containing 15 mM Mg_2Cl_3 (Promega Corp. Madison, WI U.S.A.; for markers AGE 1a, AGE 2 and AGE 4 an additional 3 mM Mg_2Cl_3 was added), 20 mM dNTPs, 25 pM each primer, 1U Taq polymerase, and one drop mineral oil to prevent evaporation. Negative controls (reactions that include all reagents except template DNA) were included in every set of reactions, and we used "cold start" PCR where tubes (in racks) were kept on ice to prevent premature non-specific priming. We used MJR PTC-100 thermocyclers programmed for the following protocol: denature at 94°C for 4 min, 31 cycles of denature at 94°C for 40 sec, annealing at 58°C for 40 sec, and chain extension at 72°C for 40 sec, with a final extension at 72°C for 5 min.

We used PCR to amplify the CHD sex-linked gene in 25 μl reactions using 1.0 μl template DNA and the same reaction buffer described above. The PCR protocol included an initial 5 min at 95°C denature, 11 cycles of denature at 94°C for 30 sec, annealing at 52°C for 35 sec, and chain extension at 72°C for 2.0 min, 31 cycles of denature at 92°C for 30 sec, annealing at 56°C for 35 sec, and chain extension at 72°C for 2.0 min, with a final extension at 72°C for 7 min.

We used gel electrophoresis to separate alleles. For microsatellites, we used 8% polyacrylamide (Long Ranger, Cambrex Corp., Rockland, MA U.S.A.) denaturing gels (55 cm long) that were run at 45 watts for 4–5 hr, depending on allele size. For the CHD sex-linked gene, we used single-strand-conformation-polymorphism (SSCP) methods (Hiss et al. 1994) and electrophoresed alleles on non-denaturing gels at 5 watts for 15 hr.

For microsatellites, we established allele standards using representative samples from our first gel and then standardized all other gels using those same samples. Gels were scored visually, and allele standards were run on both sides of a gel to account for gel ambiguities that cause slight variations in migration distances. Further, a subset of individuals ($N = 23$) was genotyped a second time to validate scores for microsatellite markers. For the CHD marker, we ran all known females together ($N = 40$) and all known males ($N = 29$) together to familiarize ourselves with allele morphology. Although not sequenced, the fragments were ca. 240–260 base pairs, and Z and W alleles were similar in size, but were differentiated by the SSCP analysis (Hiss et al. 1994).

Population Genetic Analysis. Population substructure, inbreeding, and genetic drift can reduce heterozygosity in populations. However, low yield and degraded DNA

sampled from sources such as molted feathers can artificially reduce population heterozygosity if allelic dropout (ADO; one of two alleles in a heterozygous individual fails to amplify) at one or more markers occurs. It is, therefore, important to use high yield sources of DNA (typically blood) from a known reference population to determine frequency of ADO (or null alleles) and true levels of heterozygosity (Taberlet et al. 1999).

We used CERVUS 2.0 (Marshall et al. 1998) to estimate observed (H_O) and expected (H_E) population heterozygosity and null allele frequencies. CERVUS provides estimates of null allele frequencies with an iterative algorithm based on differences between observed and expected homozygote frequencies. We used GENEPOP 3.4 (Raymond and Rousset 1995) to test for departures from Hardy-Weinberg equilibrium (random mating) and GDA 1.0 (Lewis and Zaykin 2001) to test for linkage-disequilibrium (genotypes at one marker are independent from genotypes at other markers) and to estimate F_{IS} , an indicator of population substructure and inbreeding. For a review of F -statistics and microsatellite genetic markers, see Balloux and Lugon-Moulin (2002).

To test our assumption that our sample of adult goshawks was not comprised of closely-related individuals, we used IDENTIX 1.1 (Belkhir et al. 2002) to estimate mean pairwise relatedness. We used Queller and Goodnight's (1989) estimator option, and tested the null hypothesis of no relatedness by comparing our estimate to a distribution of coefficients derived through conventional Monte Carlo resampling procedures (1000 permutations).

Probability of Identity. The uniqueness of an individual's genotype depends on the number and polymorphism (heterozygosity) of the markers. Multi-locus genotypes based on few highly-variable markers can be as powerful as those based on many less variable markers (Waits et al. 2001). Mills et al. (2000) suggested for studies of genetic demography that profiles should be based on multi-locus genotypes capable of discriminating individuals with 99% certainty. Estimating probability of identity ($P_{(ID)}$) is one way to establish this certainty when it is expressed as $1 - P_{(ID)}$. $P_{(ID)}$ is similar to the match probability used in human forensics (Evetts and Weir 1998, Avise 2004, Hedrick 2005), but is less susceptible to violations of linkage-disequilibrium and Hardy-Weinberg equilibrium, both of which can be prevalent in small, isolated, or substructured populations (Waits et al. 2001).

$P_{(ID)}$ analysis includes two steps. First, two theoretical $P_{(ID)}$'s, one for unrelated individuals ($P_{(ID)unbiased}$) and one for siblings ($P_{(ID)sibs}$), are estimated (for equations see Waits et al. 2001). Both estimators use population allele frequency data, and $P_{(ID)unbiased}$ is corrected for bias in small samples. The two estimators provide lower and upper confidence bounds on the number of markers needed to discriminate individuals accurately. If the study population is composed of many related individuals, then resolving those individuals requires more markers. Step two involves calculating an observed $P_{(ID)obs}$ based on actual multi-locus genotypes from a known population sample and is simply the proportion of all possible pairs of individuals with identical multi-locus genotypes (Waits et al. 2001).

To estimate both theoretical $P_{(ID)}$'s and to quantify

$P_{(ID)obs}$ (the proportion of individuals that share genotypes), we used PROB-ID5 (Waits et al. 2001). We used multi-locus genotypes derived from 69 adult goshawks, which we assume to be unrelated (see below), and 44 of their nestlings (sibling groups of 2–4 nestlings). We first analyzed the adults and then added the offspring/sibling groups. We used all five microsatellite markers and the CHD sex-linked gene and added markers sequentially starting with those having the highest number of alleles.

Effective Population Size. To evaluate whether immigration and gene flow influenced genetic structure of the Kaibab population we estimated its effective size (N_e). Effective population size is the idealized number of individuals in a population measured either demographically, in terms how many individuals actually contribute to breeding (i.e., variance in productivity), or genetically, using F -statistics and measures of inbreeding where the assumption of non-overlapping generations exists (Barton and Whitlock 1997, Hedrick 2005). Goshawks sampled on the Kaibab Plateau during the study period likely represent at least three overlapping generations; thus, we relied on reproductive data to estimate N_e .

Effective population size is generally smaller than the censused population (N). Counts of breeding pairs of goshawks can be used to index N_e , but not all goshawk pairs on the Kaibab produced an equal number of offspring during the study (Wiens and Reynolds 2005). We therefore estimated annual N_e 's (equation 6.8a in Hedrick 2005) as:

$$N_e = \frac{N\bar{k} - 1}{\bar{k} - 1 + \frac{V_k}{\bar{k}}}$$

where \bar{k} is mean productivity measured as the number of young fledged per used nest, eggs laid, subsequent young fledged, or the nest failed (1991–2003; Reynolds et al. 2005), V_k is the variance in annual mean productivity, and N is the annual count of breeding pairs for the year. We then calculated a 13-yr harmonic mean of annual N_e 's (equation 6.12b in Hedrick 2005) for our final size estimate.

RESULTS

Genetic Markers. Of nine cross-specific and sub-specific markers tested, two did not resolve alleles successfully, six amplified successfully but lacked variability, and one both amplified and was polymorphic (AGE 1a, Table 1). All four microsatellites originally isolated from Northern Goshawks amplified and were polymorphic (Table 1). The CHD sex-linked gene amplified and SSCP genotypes were consistent within the sexes (females, $N = 40$; males $N = 29$), making it useful for distinguishing between male and female goshawks. We validated our amplification and scoring of microsatellite markers after all individuals were genotyped and scored the first time. We genotyped the 23 individuals used as standards a second time using DNA that was archived and remained untouched in our

Table 1. Statistics for microsatellites tested on blood-derived DNA sampled from female ($N = 40$) and male ($N = 29$) Northern Goshawks (*Accipiter gentilis*), Kaibab Plateau, AZ (1991–1993, 2000–2002).

MARKER	SPECIES	ALLELES	NA ^a	H _O	H _E	H-W ^b P (SE)	F _{IS} (95% CI) ^c
AGE 1	<i>A. g. atricapillus</i>	15	0.0425	0.77	0.84	0.21 (0.020)	0.0825
AGE 2	<i>A. g. atricapillus</i>	11	0.0006	0.83	0.83	0.08 (0.009)	0.005
AGE 6 ^d	<i>A. g. atricapillus</i>	11	0.3805	0.38	0.85	0.00 (0.000)	0.556
AGE 4	<i>A. g. atricapillus</i>	10	-0.0062	0.86	0.85	0.06 (0.005)	-0.007
AGE 1a	<i>A. g. gentilis</i>	5	-0.0506	0.77	0.70	0.09 (0.005)	-0.100
All markers		52		0.72 ^e	0.81	See text	0.115 (-0.039, 0.346) -0.001 ^f (-0.070, 0.063)

^a Frequency of null alleles estimated with CERVUS 2.0.

^b Departures from Hardy-Weinberg expectations. Exact *P*-values and SE derived using Markov-Chain methods (1000 dememorizations, 200 batches and 1000 iterations) in GENEPOP 3.4.

^c 95% Confidence Interval (CI) calculated using bootstrapping over markers (10,000 replicates) in GDA 1.0.

^d Suspected sex-linked marker.

^e Simple average.

^f AGE 6 omitted.

freezers from the time of original DNA extraction. We found only a single scoring error out of 230 opportunities (23 samples genotyped twice for five markers). This was a recording error; the sample had actually genotyped correctly.

In comparisons of expected (H_E) and observed (H_O) heterozygosity at each marker, four of the five microsatellites were similar. However, one marker (AGE 6) significantly departed from Hardy-Weinberg expectations ($P < 0.01$) due to a deficiency of heterozygote genotypes (Table 1). Based on our data, we suspected this marker was sex-linked, as we found strong linkage-disequilibrium between AGE 6 and the sex-linked CHD marker ($P < 0.001$, Fisher's method, 3200 runs) indicating that the two markers segregate together. It appeared that the marker was located on the Z sex-chromosome because all females (ZW) had only a single allele (homozygous), while most males (ZZ) were heterozygous. We hypothesize that alleles on the female's W-chromosome are non-amplifiable (null) because of mutations in the priming sequences flanking the marker (Scribner and Pearce 2000) or because the marker on the Z-chromosome simply has no homologous region on the W-chromosome. We found no evidence for linkage-disequilibrium between the other four microsatellite markers (Table 1) when AGE 6 and CHD were excluded from the analysis. We found no evidence of null alleles, which is important for future assessments of genotyping error when using feathers as a source of DNA.

Population Genetics. F_{IS} measures departures of observed and expected heterozygosity under assumptions of random mating and indicates either inbreeding ($F_{IS} > 0$) or inbreeding avoidance ($F_{IS} < 0$). Thus, highly structured or isolated populations that experience genetic drift generally exhibit positive F_{IS} values. Alternatively, large populations or those experiencing high gene flow generally exhibit nonsignificant or negative F_{IS} values.

We found no evidence of inbreeding or inbreeding avoidance ($F_{IS} = -0.001$; 95% CI = -0.070–0.063; AGE 6 excluded; Table 1), suggesting that Kaibab goshawks mate randomly. Lack of inbreeding could result from large population size, gene flow by immigrants, or both. However, our demographically derived estimate of effective population size ($N_e = 37$ individuals; range = 10–86) indicated that the population was demographically small, thus making gene flow a more likely source of genetic variability. This is consistent with our estimate

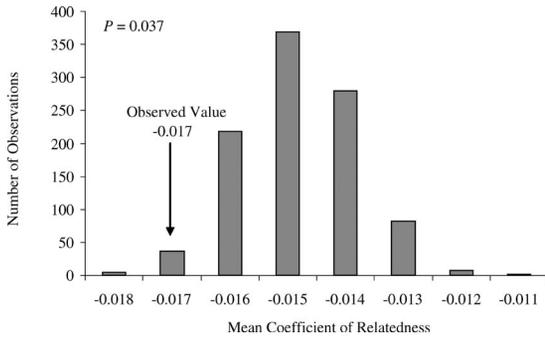


Figure 1. Observed mean relatedness relative to 1000 randomized populations assumed to lack relatedness. The observed mean falls below that which is expected at random, occurring with a probability of 3.7%, indicating that Northern Goshawks (*Accipiter gentilis*) on the Kaibab Plateau, Arizona, are less related than expected at random.

of relatedness among adults goshawks, where mean relatedness ($r_{xy} = -0.017$) was less than expected by random (Fig. 1).

Probability of Identity. Certainty of individual identification is equal to $1 - P_{(ID)}$, and therefore the goal in estimating probability of identity is to obtain small values of $P_{(ID)}$. Waits et al. (2001) suggested a value ≤ 0.0001 for forensic investigations where estimates of demographic parameters are needed. This threshold is interpreted as a 1:10 000 chance that two individuals sampled from the same population will have the same multi-locus genotype.

We found that all 69 unrelated adults had unique multi-locus genotypes with the inclusion of the first three markers ($P_{(ID)obs} < 0.0001$), and likewise the estimated $P_{(ID)}$ met the 0.0001 threshold ($P_{(ID)unbiased} = 1.13 \times 10^{-4}$; Fig. 2a). With five markers, the same sample had a $P_{(ID)unbiased} = 7.03 \times 10^{-7}$. However, based on demographic data, we know that siblings and parent-offspring nested simultaneously in the Kaibab population (R. Reynolds unpubl. data). To model this effect we added 44 nestlings-siblings to the sample. While the two theoretical $P_{(ID)}$'s did not change, all five markers were required to differentiate individuals ($P_{(ID)obs} < 0.0001$, Fig. 2b).

In both cases (adult only and adults with offspring-sibling groups), our sample of markers was insufficiently large to bring the $P_{(ID)sibs}$ to the 0.0001 threshold. Thus, we were not able to estimate an upper number of markers needed for this

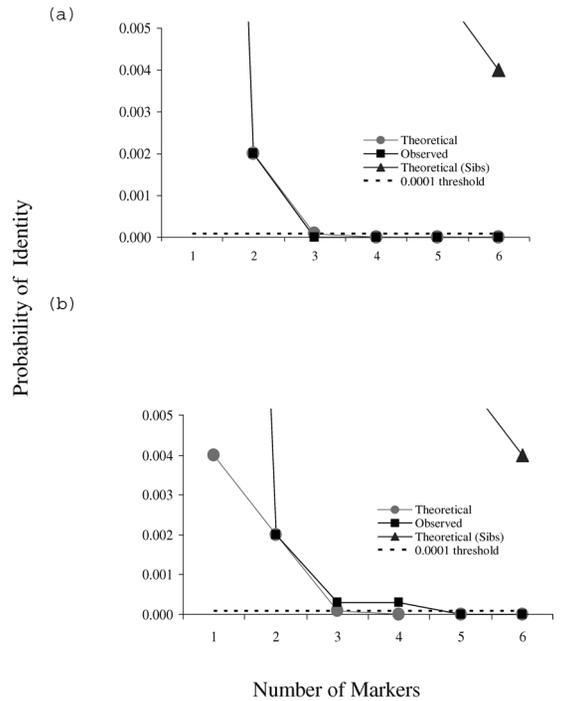


Figure 2. Relationship between theoretical, observed, and sib probability of identity ($P_{(ID)}$) for Northern Goshawks (*Accipiter gentilis*) on the Kaibab Plateau, Arizona. The first five markers are microsatellites and the sixth is the CHD sex-linked gene. Observed data closely tracked that of the theoretical estimator; however, while (a) all 69 unrelated adult goshawks were resolved after the first three markers (observed $P_{(ID)} < 0.0001$), (b) it took an additional two markers to resolve sibling and parent-offspring goshawks when 44 nestlings were added to the sample. In both cases, the theoretical $P_{(ID)}$ met our 0.0001 threshold (a 1:10 000 chance that two individuals sampled from the same population have identical multi-locus genotypes). However, we did not analyze enough markers to bring the sibling $P_{(ID)}$ to the threshold level.

resolution. Nonetheless, with five markers $P_{(ID)sibs} = 6.17 \times 10^{-3}$, which translates into a six in a 1000 chance of drawing two identical genotypes. Because we sampled many parent-offspring pairs that we could nevertheless distinguish, we are confident the five combined markers provide unique genetic marks.

DISCUSSION

Our intent in this study was to develop a set of genetic markers that uniquely identified individual goshawks. We desired to establish this marker set using high-yield DNA (blood) sampled from a

known reference population. While most microsatellites tested did not amplify or were monomorphic (most *A. g. gentilis* markers), we did find a set of highly variable markers that consistently amplified DNA from blood. Elsewhere (S. Bayard de Volo unpubl. data), we found that the same genetic markers consistently and reliably amplified DNA from molted feathers. We note that because AGE 6 is probably on the Z sex chromosome, its utility for estimating within population relatedness and levels of inbreeding is limited to samples from males. However, differences in allele frequencies between populations will still be useful for larger-scale studies comparing populations. In a study of goshawks in Utah, Sonsthagen (2002) used a different set of microsatellite markers than ours. Of eight markers, only one of theirs exhibited the same number of alleles (11 alleles, $H_O = 0.73$, $H_E = 0.74$) as AGE 6 did in our study. This alternative marker (BV 20; Gautschi et al. 2000) would be useful if it exhibited similar levels of heterozygosity and allelic diversity in the Kaibab population. Replacing AGE 6 with a less variable marker would result in having to add more markers to the entire genetic profile, which would introduce more opportunities for genotyping error. We are currently testing BV 20 to see if it is an effective replacement for AGE 6.

With the five microsatellite markers tested (Table 1), all 113 goshawks sampled had unique multilocus genotypes resulting in a $P_{(ID)obs} < 0.0001$ (Fig. 2b) and a $P_{(ID)unbiased} = 7.03 \times 10^{-7}$. This was a powerful result considering that our sample included many parent-offspring and sibling pairs from the same nest. Likewise, the five microsatellites showed a high level of expected heterozygosity ($H_E = 0.81$). Others have shown that marker sets composed of five markers that result in $H_E \geq 0.80$ will have a theoretical $P_{(ID)} \leq 0.0001$ (Waits et al. 2001). In Paetkau's (2003) retrospective analysis of 21 non-invasive genetic studies in bears (*Ursus americanus*, *U. arctos*), the number of markers used was determined by whether the first five most-variable microsatellite markers together had $H_E > 0.80$. He found that for some black bear populations H_E was >0.80 for five markers; however, for others, and for all grizzly bear populations, that H_E was <0.80 , requiring the marker set to be increased to six or seven loci in order to discriminate among individuals.

We note that our estimates of $P_{(ID)}$ are specific to the Kaibab goshawk population; we cannot pre-

dict with complete certainty that these same markers will uniquely mark goshawks from other populations. Power of discrimination depends on population-specific levels of genetic variability (heterozygosity); goshawk populations that are less variable because of geographic isolation or habitat fragmentation may require more markers to uniquely genotype individuals (Paetkau and Strobeck 1994). However, goshawks are highly vagile, and we suspect gene flow is high among populations. These goshawk populations will probably exhibit similar heterozygosity, and the marker set tested here should prove useful for other studies.

The Kaibab goshawk population exhibits high genetic variability (Table 1), despite its geographic isolation and small effective population size (based on demographic data; 13-yr $\bar{x} = 37$ individuals). Several explanations may account for this. First, it is possible that the markers used in this study are under selective sweeps with genes that are affected by balancing selection for heterozygous genotypes. Such selection has been found for the genes of the MHC (major histocompatibility complex) in mammals (Awise 2004), in which heterozygous individuals experience a fitness advantage. However, we suggest it is unlikely that all four nonsex-linked markers would be under the same selective pressures, given that they exhibit independent segregation (no evidence of linkage disequilibrium; see Black et al. 2001).

A second and more likely explanation is that actual N_e for this goshawk population is much larger because geographically distant populations in the region are connected by migration and gene flow. While adult goshawks are mostly sedentary on breeding territories (Detrich and Woodbridge 1994, Squires and Ruggiero 1995, Reynolds and Joy 2005), band recoveries of first-year goshawks from the Kaibab Plateau indicate dispersal distances of up to 440 km (Wiens 2004). In addition, telemetry data show that juvenile goshawks disperse from the Kaibab Plateau in their first year, with the majority moving beyond the 80 km detection distance (Wiens 2004). Further, Wiens (2004) showed that only 11% of 614 banded nestlings returned to be recruited into the Kaibab breeding population, indicating high first-year mortality or low natal-site fidelity. Evidence for the latter is indicated by the lack of population genetic structure for goshawks in Utah (Sonsthagen et al. 2004). To better assess actual effective population size for goshawks in western North America, we are expanding our

studies to include populations in the western portion of the species range. Data from these studies should allow a more comprehensive evaluation of the genetic structure and effective population size for goshawks in the West.

CONCLUSIONS

Genetic marking of Northern Goshawks on the Kaibab Plateau is both feasible and reliable. Likewise, non-invasive genetic sampling will provide an alternative method for demographic and genetic data collection, as we have found that molted feathers are as reliable a source of DNA as blood (S. Bayard de Volo unpubl. data). Because goshawks show high territory fidelity (e.g., Detrich and Woodbridge 1994, Reynolds and Joy 2005), they are particularly well suited for non-invasive genetic sampling. We recommend that monitoring programs implement rigorous field collection of molted feathers. As with any demographic study, valid inferences to the population depend on appropriate spatial and temporal sampling from that population. Researchers and managers interested in implementing non-invasive genetic mark-recapture to study goshawks should contact the corresponding author or refer to Bayard de Volo (2005).

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LITERATURE CITED

- AVISE, J.C. 2004. Molecular markers, natural history and evolution. Sinauer Associates, Inc., Sunderland, MA U.S.A.
- BALLOUX, F. AND N. LUGON-MOULIN. 2002. The estimation of population differentiation with microsatellite markers. *Mol. Ecol.* 11:155–165.
- BARTON, N.H. AND M.C. WHITLOCK. 1997. The evolution of metapopulations. Pages 183–209 in I. Hanski and M.E. Gilpin [Eds.], *Metapopulation biology: ecology, genetics and evolution*. Academic Press, San Diego, CA U.S.A.
- BAYARD DE VOLO, S. 2005. Molt collection protocol. <http://lamar.colostate.edu/~sbdv/protocols-page/protocols.htm>.
- BELKHIR, K., V. CASTRIC, AND F. BONHOMME. 2002. IDENTIX, a software to test for relatedness in a population using permutation methods. *Mol. Ecol. Notes* 2:611–614.
- BLACK, W.C., IV, C.F. BAER, M.F. ANTOLIN, AND N.M. DU TEAU. 2001. Population genomics: genome-wide sampling of insect populations. *Ann. Rev. of Entomology* 46:441–469.
- BLOCK, W.M., M.L. MORRISON, AND M.H. REISER. 1994. Symposium overview: introduction. *Stud. Avian Biol.* 16:1–2.
- DETRICH, P. J. AND B. WOODBRIDGE. 1994. Territory fidelity, mate fidelity, and movements of color-marked Northern Goshawks in the southern Cascades of California. *Stud. Avian Biol.* 16:130–132.
- ELLEGREN, H. 1992. Polymerase-chain-reaction (PCR) analysis of microsatellites—a new approach to studies of genetic relationships in birds. *Auk* 109:886–895.
- ESTOUP, A., P. JARNE, AND J.-M. CORNUET. 2002. Homoplasy and mutation model at microsatellite loci and their consequences for populations genetics analysis. *Mol. Ecol.* 11:1591–1604.
- EVETT, I.W. AND B.S. WEIR. 1998. Interpreting DNA evidence: statistical genetics for forensic scientists. Sinauer Associates, Inc. Sunderland, MA U.S.A.
- GALBUSERA, P., S. VAN DONGEN, AND E. MATTHYSEN. 2000. Cross-species amplification of microsatellite primers in passerine birds. *Conserv. Genetics* 1:163–168.
- GAUTSCHI, B., I. TENZER, J.P. MULLER, AND B. SCHMID. 2000. Isolation and characterization of microsatellite loci in the bearded vulture (*Gypaetus barbatus*) and cross-amplification in three old world vultures. *Mol. Ecol.* 9:2193–2195.
- GOLDSTEIN, D.B. AND D.D. POLLOCK. 1997. Launching microsatellites: a review of mutation processes and methods of phylogenetic inference. *J. Heredity* 88:335–342.
- HAG, S.M. 1998. Molecular contributions to conservation. *Ecol.* 79:413–425.
- HEDRICK, P.W. 2005. Genetics of populations. Jones and Bartlett Publishers, Inc., Sudbury, MA U.S.A.
- HISS, R.H., D.E. NORRIS, C.H. DIETRICH, R.F. WHITCOMB, D.F. WEST, C.F. BOSTO, S. KAMBHAMPATI, J. PIESMAN, M.F. ANTOLIN, AND W.C. BLACK, IV. 1994. Molecular taxonomy using single-strand conformation polymorphism (SSCP) analysis of mitochondrial ribosomal genes. *Insect Mol. Biol.* 3:171–182.
- JARNE, P. AND P.J.L. LAGODA. 1996. Microsatellites, from molecules to populations and back. *Tree* 11:424–429.
- KAHN, N.W., J. ST. JOHN, AND T.W. QUINN. 1998. Chromosome-specific intron size differences in the avian

- CHD gene provide an efficient method for sex identification in birds. *Auk* 115:1074–1078.
- LEWIS, P.O. AND D. ZAYKIN. 2001. Genetic data analysis: computer program for the analysis of allelic data. Version 1.0 (d16c).
- LUKACS, P.M. AND K.P. BURNHAM. 2005. Estimating population size from DNA-based capture-recapture data incorporating genotyping error. *J. Wildl. Manag.* 69: 396–403.
- LUIKART, G. AND P.R. ENGLAND. 1999. Statistical analysis of microsatellite DNA data. *Tree* 14:253–256.
- MARSHALL, T.C., J. SLATE, L.E.B. KRUK, AND J.M. PEMBERTON. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.* 7:639–655.
- MARTINEZ-CRUZ, B., V.A. DAVID, J.A. GODOY, J.J. NEGRO, S.J. O'BRIAN, AND W.E. JOHNSON. 2002. Eighteen polymorphic microsatellite markers for the highly endangered Spanish Imperial Eagle (*Aquila adalberti*) and related species. *Mol. Ecol. Notes* 2:323–326.
- MILLS, L.S., J.J. CITTA, K.P. LAIR, M.K. SCHWARTZ, AND D.A. TALLMON. 2000. Estimating animal abundance using non-invasive DNA sampling: promise and pitfalls. *Ecol. Appl.* 10:283–294.
- NEŠJE, M. AND K.H. RØED. 2000. Microsatellite DNA markers from the Gyrfalcon (*Falco rusticolus*) and their use in other raptor species. *Mol. Ecol.* 9:1433–1449.
- PAETKAU, D. 2003. An empirical exploration of data quality in DNA-based population inventories. *Mol. Ecol.* 12: 1375–1387.
- AND C. STROBECK. 1994. Microsatellite analysis of genetic variation in black bear populations. *Mol. Ecol.* 3:489–495.
- PARKER, P.G., A.A. SNOW, M.D. SCHUG, G.C. BOOTON, AND O.A. FUERST. 1998. What molecules can tell us about populations: choosing and using a molecular marker. *Ecology* 79:361–382.
- PECK, N. 2000. DNA forensics of raptors and the isolation and characterization of microsatellite markers in Accipitridae. Ph.D. dissertation, Univ. of Nottingham, Nottingham, U.K.
- PRIMMER, C.R., A.P. MØLLER, AND H. ELLEGREN. 1996. A wide-range survey of cross-species microsatellite amplification in birds. *Mol. Ecol.* 5:365–378.
- QUELLER, D. AND K. GOODNIGHT. 1989. Estimating relatedness using genetic markers. *Evolution* 43:258–275.
- RAYMOND, M. AND F. ROUSSET. 1995. GENEPOP Version 3.1d: population genetics software for exact tests and ecumenicism. *J. Heredity* 86:248–249.
- REICH, R.M., S.M. JOY, AND R.T. REYNOLDS. 2004. Predicting the location of Northern Goshawk nests: modeling the spatial dependency between nest locations and forest structure. *Ecol. Modeling* 176:109–133.
- REYNOLDS, R.T. AND S.M. JOY. 2005. Demography of Northern Goshawks in northern Arizona, 1991–1996. *Stud. Avian Biol.* In press.
- , ———, AND D.G. LESLIE. 1994. Nest productivity, fidelity, and spacing of Northern Goshawks in northern Arizona. *Stud. Avian Biol.* 16:106–113.
- , J.D. WIENS, S.M. JOY, AND S.R. SALAFSKY. 2005. Sampling considerations for demographic and habitat studies of goshawks. *J. Raptor Res.* 39:274–285.
- SCRIBNER, K.T. AND J.M. PEARCE. 2000. Microsatellites: evolutionary and methodological background and empirical applications at individual, population and phylogenetic levels. Pages 235–273 in A.J. Baker [ED.], *Molecular methods in ecology*. Blackwell Science Ltd., Oxford, U.K.
- SONSTHAGEN, S.A. 2002. Gene flow and genetic characterization of Northern Goshawks (*Accipiter gentilis*) breeding in Utah, USA. M.S. thesis, Brigham Young Univ., Provo, UT U.S.A.
- , S.L. TALBOT, AND C.M. WHITE. 2004. Gene flow and genetic characterization of Northern Goshawks (*Accipiter gentilis*) breeding in Utah. *Condor* 106:826–836.
- SQUIRES, J.R. AND R.T. REYNOLDS. 1997. Northern Goshawk (*Accipiter gentilis*). In A. Poole and F. Gill [EDS.], *The birds of North America*, No. 298. The Birds of North America, Inc., Philadelphia, PA U.S.A.
- AND L.F. RUGGIERO. 1995. Winter movements of adult Northern Goshawks that nested in south-central Wyoming. *J. Raptor Res.* 29:5–9.
- TABERLET, P. AND G. LUIKART. 1999. Non-invasive genetic sampling and individual identification. *Biol. J. Linn. Soc.* 68:41–55.
- , L.P. WAITS, AND G. LUIKART. 1999. Noninvasive genetic sampling: look before you leap. *TREE* 14:323–327.
- TOPINKA, J.R. AND B. MAY. 2004. Development of polymorphic loci in the Northern Goshawk (*Accipiter gentilis*) and cross-amplification in other raptor species. *Conserv. Genetics* 5:861–864.
- WAITS, L.P., G. LUIKART, AND P. TABERLET. 2001. Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Mol. Ecol.* 10:249–256.
- WIENS, J.D. 2004. Post-fledging survival and natal dispersal of juvenile Northern Goshawks in Arizona. M.S. thesis, Colorado State University, Fort Collins, CO U.S.A.
- AND R.T. REYNOLDS. 2005. Is fledging success a reliable index of recruitment in Northern Goshawks? *J. Raptor Res.* 39:210–221.

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Appendix. Source, repeat qualities and accession numbers or primer sequences for microsatellites found to be useful for genotyping Northern Goshawks (*Accipiter gentilis*) on the Kaibab Plateau, Arizona.

MARKER	REPEAT MOTIF ^a	ALLELE SIZE IN BASE PAIRS	ACCESSION NO. OR PRIMER SEQUENCES 5' TO 3'	AUTHOR
AGE 1	(gggaa) ₈ ..(gaga) ₉ ..(gagaa) ₃	216	AY312451	
AGE 2	(gagaa) ₁₀ (ga) ₄	170	AY312452	Topinka and May 2004
AGE 6	(gagaa) ₄ ..(gagaa) ₂ ..(gagaa) ₅	259	AY312456	
AGE 4	(gagaa) ₁₉	275	AY312454	
AGE 1a	(ggat) ₅	208 ^b	f acaactgggctgtgctttgc r cttcccggtggctgaggctt	Peck 2000

^a Sequenced by authors.

^b Mean allele size in European goshawk (*A. g. gentilis*).