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Michael K. Schwartz[1], Kristine L. Pilgrim, Kevin S. McKelvey, USDA Forest Service, Rocky Mountain Research Station, 800 E. Beckwith, Missoula, Montana 59801

Pilar T. Rivera, Wallowa-Whitman National Forest, P.O. Box 907, 1550 Dewey Avenue, Baker City, Oregon 97814

and

Leonard F. Ruggiero, USDA Forest Service, Rocky Mountain Research Station, 800 E. Beckwith, Missoula, Montana 59801

DNA Markers for Identifying Individual Snowshoe Hares Using Field-collected Pellets

Abstract

Snowshoe hare (*Lepus americanus*) abundance has been of interest to wildlife biologists, as hares are essential prey items for many rare and endangered predators. Snowshoe hare abundance has most commonly been estimated through indices such as pellet counts. While pellet counts may be useful in the areas they are developed and when hares are dense, they notably fail when hares are at low densities. Abundance estimates using capture-mark-recapture (CMR) are often preferred over indices of animal abundance, yet using CMR to estimate snowshoe hare numbers has proven a formidable and expensive task. Sample sizes obtained using traditional CMR techniques are frequently low, resulting in either biased estimates or estimates with unacceptably high variance. Here we derive a suite of 9 microsatellite DNA markers that can provide snowshoe hare individual identification at relatively low cost. We demonstrate that these markers produce no genotyping errors in a captive situation and use the markers to produce individual identification of free-ranging snowshoe hares in test plots in Montana and Idaho.

Introduction

Snowshoe hare (*Lepus americanus*) abundance has been of prime interest to wildlife biologists and managers, as hares are critical prey items for many rare and endangered predators. For instance, snowshoe hares are an important prey item of fishers (*Martes pennanti*), wolverines (*Gulo gulo*), goshawks (*Accipiter gentiles*), and martens (*Martes americanus*). However, the greatest interest in snowshoe hare numbers relates to the management of Canada lynx (*Lynx canadensis*), listed as Threatened under the U.S. Endangered Species Act (USFWS 2000). Lynx prey almost exclusively on hares and their density, survival, reproductive rates, dispersal, and home range sizes are all correlated to hare abundance (Poole 1994, Mowat et al. 2000).

While formal abundance estimates using capture-mark-recapture (CMR) are often preferred over indices of animal abundance, using CMR to estimate snowshoe hare numbers has proven challenging (Mills et al. 2005). The sample sizes likely to be obtained using traditional CMR techniques are often too low, resulting in either a biased estimate or an estimate with unacceptably high variance (McKelvey and Pearson 2001, Mills et al. 2005). Furthermore, costs associated with capturing a sufficient number of animals for a CMR have been prohibitive. Thus, most estimates of snowshoe hare abundance are currently based on indices.

The most common index of snowshoe hare abundance has been pellet counts, where the number of pellets detected is used as a relative metric of hare abundance (Litvaitis et al. 1985, Mowat and Slough 2003). Some researchers have regressed pellet counts on abundance estimates derived from captured hares to transform pellet indices into abundance estimates (Krebs et al. 1987, Krebs et al. 2001, Mills et al. 2005). While these equations may be useful in the area they are developed and when hares are dense, they notably fail when hares are at low densities (Mills et al. 2005). For instance, at some sites with very low hare densities Mills et al. (2005) found the deviation between density estimated using locally derived equations based on pellet counts and directly estimated using CMR to be 1,000% or greater.

[1]Author to whom correspondence should be addressed.
E-mail: mkschwartz@fs.fed.us

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Unfortunately, lynx conservation needs may be greatest at the periphery of their geographic range where snowshoe hares exist at low densities (Ruggiero et al. 2000, Mowat et al. 2000). Given that the pellet-based indices can produce large variances and CMR methods likely produce biased estimates at small sample sizes, we investigated the possibility of identifying individual hares reliably based on fecal pellets.

Many wildlife projects have used molecular genetic methods for enumerating and monitoring elusive and difficult to study wildlife populations (Kohn et al. 1999, Mowat and Paetkau 2002, Schwartz et al. 2007). Non-invasive genetic samples (hair, scat, urine, etc. obtained without ever seeing or handling the animal) can be collected by a variety of means including following a snow-track to find a sample (McKelvey et al. 2006, Ulizio et al. 2006), inducing an animal to leave a sample at a specific "rub" station (Woods et al. 1999, McDaniel et al. 2000), or by opportunistic collection. Advantages of non-invasive genetic sampling rests in the ease of sample acquisition and the fact that animals already have "tags", their genetic code, that cannot be lost. What is required is the development of appropriate molecular markers allowing unique identification of individuals and removal of errors caused by low-quality DNA obtained from non-invasive samples (e.g., McKelvey and Schwartz 2004). Microsatellites, neutral, highly variable repeat regions of the genome, have been the most popular molecular tool for identifying individuals collected by non-invasive genetic sampling.

In this study we screened a panel of microsatellite markers developed for the European rabbit (*Oryctolagus cuniculus*) on snowshoe hare DNA. It had already been documented that some European rabbit markers can be used for hares (Burton 2002, Burton et al. 2002), but we were searching for a panel of microsatellites that could be used to estimate hare abundance from low quantity DNA associated with non-invasive genetic samples (in this case, pellets). Here we report on this panel, the error rate in a laboratory test, and its efficacy in the wild.

Study Area

The field component of the study occurred in two different stands on the Clearwater and Bitterroot National Forests in Idaho and Montana, respectively. The first stand in the Clearwater National Forest was primarily composed of grand fir (*Abies grandis*) and western hemlock (*Tsuga heterophylla*) (UTM Zone 11 682521E 5161479N, elevation: 1,274m), while the second stand (UTM Zone 11 691802E 5172449N, elevation: 1469m) in the Bitterroot National Forest was characterized by young mountain alder (*Alnus tenuifolia*) and Douglas-fir (*Pseudotsuga menziesii*). The general area where both these stands are located can be characterized as inland maritime ecosystem with an average of 100 cm of annual precipitation and greater than 400 cm of snow in areas above 1,800m.

Methods

The research was conducted in three separate phases. First, we tested domestic rabbit microsatellite markers on high-quality snowshoe hare tissue samples. Second we collected fresh fecal pellets from captive snowshoe hares to test whether the markers effectively identified individuals based on scat samples, and to compare genetic error rates associated with each marker. Lastly, we tested the microsatellite panel on fecal pellets collected from free-ranging hares.

Captive Colony and Laboratory Methods

We collected an ear punch and matching fresh (collected within hours) fecal sample from each of five wild snowshoe hares held in captivity (All procedures were approved by the University of Montana Animal Care and Use Committee; ACC 019-02). Hares were held in cages on the sites where they were captured, were fed exclusively native plants collected on the site, and were held in captivity for no more than 4 days. Only "hard" pellets were analyzed as "soft" pellets are re-ingested by hares (Watson and Taylor 1955) and will not be present in field-collected samples. Snowshoe hare pellets were stored in 100% ethanol at room temperature until they were brought to the lab for extraction. We extracted DNA from tissue samples with the *DNeasy Tissue Kit* (*Qiagen* Inc.) following the manufacturer's protocol and extracted genomic DNA from single pellets using the protocol of Maudet et al. (2004) with the following modifications: we used 900µl of lysis washing buffer to completely coat the pellet, and processed the sample through two rounds of the *AL* buffer and *proteinase k* steps.

To test for the presence of usable DNA we amplified cytochrome b (*cytb*) using the polymerase chain reaction (PCR) and primers (*L14724* and *H15149*; Gottelli et al. 1994). There is thought to be approximately 20 fold more mitochondrial DNA extracted than nuclear DNA, thus even if microsatellites could not be amplified from pellets we wanted to know if any target DNA was recovered. The reaction volume (50µl) contained 50-100 ng DNA, 1x reaction buffer (*Perkin-Elmer*), 2.5 mM $MgCl_2$, 200µM each dNTP, 1µM each primer, 1 U *Titanium Taq* polymerase (*BD Biosciences*). The PCR program was 94°C/5 min, [94°C/1 min, 50°C/1 min, 72°C/1 min 30s] x 34 cycles, 72°C/5 min. PCR products (442bp) were run in a 2% agarose gel containing ethidium bromide (1.5µl) and 1x TAE buffer (Ausubel et al. 1989).

We screened 15 microsatellite primers for variability and suitability for use with DNA from snowshoe hare pellets (Table 1). Seven of these have been previously used on snowshoe hare tissues (Burton 2002). DNA was amplified in a 10 µl reaction volume containing 1.0-3.0µl DNA, 1x reaction buffer (*Applied Biosystems*), 2.0 mM $MgCl_2$, 200µM of each dNTP, 1µM reverse primer, 1µM dye-labeled forward primer, 1.5 mg/ml BSA, and 1U *Taq Gold* polymerase (*Applied Biosystems*). The PCR profile was 94°C/5 min, [94°C/1 min, X°C/1 min, 72°C/30s] x 29 cycles (see Table 1 for annealing conditions) for tissue samples, and was increased to 45 cycles for use with pellet samples. PCR products were run in a 6.5% acrylamide gel for 2 hours on a *LI-COR DNA analyzer* (*LI-COR Biotechnology*).

Field Testing

We collected pellets of unknown age from the field. We collected 20 pellets on May 4, 2004 along a 100 meter transect on the Clearwater National Forest, Idaho. Collected pellets were spaced at least 5m apart. We chose this location for three reasons: first, snowshoe hare densities have been studied in this area and are known to be low (Murray et al. 2002, Wirsing et al. 2002); second, we were interested in prey densities in this region because of an ongoing fisher study; and finally because this location is an inland maritime ecosystem with approximately 100 cm of annual precipitation. If the technique was successful in a wet environment, where hydrolysis degrades DNA (Burger et al. 1999), it should be generalizable elsewhere.

We conducted a second test on October 12, 2004 in the same region, but in a different stand. Here we tried a different sampling scheme, due to the large number of pellets observed. We collected 1 pellet every 10 m along a 190 m transect

TABLE 1. Table showing microsatellite loci used with snowshoe hares and their optimal running conditions. Loci in bold make up the panel used for amplifying pellets. [a]Primers known to work on tissue samples (Burton 2002); however, *Sol 03* was monomorphic in Montana samples and *Sat 5* amplified null alleles in this study. [b]This primer has been shown to be variable in other populations (Burton et al. 2002), but was monomorphic in our study area. A = number of alleles, H_o is observed heterozygosity, H_e is expected heterozygosity, and PIC is polymorphic information content. All genetic variability measures are calculated based on 5 tissue samples.

Locus	Temp (°C)	Size Range (bp)	Amplify w/Tissue	Amplify w/pellets	A	H_o	H_e	PIC	Reference
Sat 12[a]	63	119-125	Y	Y	4	1.00	0.73	0.60	Mougel et al. 1997
Sat 13[a]	54	116-124	Y	Y	4	0.60	0.73	0.61	Mougel et al. 1997
Sat 16[a]	57	101-111	Y	Y	5	0.80	0.82	0.70	Mougel et al. 1997
Sol 08	56	119-127	Y	Y	4	0.60	0.73	0.60	Rico et al. 1994
Sol 30	55	156-184	Y	Y	6	0.60	0.89	0.77	Rico et al. 1994
Sol 33[a]	54	214-230	Y	Y	2	0.4	0.36	0.27	Surridge et al. 1997
5L1A8	60	133-139	Y	Y	2	0.40	0.53	0.37	Korstanje et al. 2003
7L1D3	55	84-92	Y	Y	3	0.20	0.38	0.31	Korstanje et al. 2003
Sat 3[a,b]	62	130	Y	Y	1	0.00	0.00	0.00	Mougel et al. 1997
Sol 28	56	171-183	Y	N	5	NA	NA	NA	Surridge et al. 1997
Sat 2[a]	56	230-244	Y	N	5	NA	NA	NA	Mougel et al. 1997
Sol 03[a]	54	238-266	Y	N	5	NA	NA	NA	Rico et al. 1994
Sat 5[a]	56	NA	N	N	NA	NA	NA	NA	Mougel et al. 1997
63LF8	60	NA	N	N	NA	NA	NA	NA	Korstanje et al. 2003
6L3B8	50	NA	N	N	NA	NA	NA	NA	Korstanje et al. 2003

for a total of 20 pellets. Samples were brought to the laboratory and tested with mtDNA and the microsatellite panel we developed.

To test for genotyping errors (scoring errors, false alleles, or allelic dropout) we compared pellet samples to tissue samples from known individuals in our captive colony. To eliminate any bias in the scoring, pellet samples were scored separate from the tissue samples (i.e., pellet samples were run on different gels and entered into a database without knowledge of the tissue samples) by two independent observers. Additionally we tested our field samples for errors with the EB and DCH tests described in McKelvey and Schwartz (2004).

Results

We found 12 microsatellite primers that produced scorable products (i.e., with low stutter and high consistency) on snowshoe hare tissue (Table 1). Three other loci tested, *Sat5*, *63LF8*, and *6L3B8* did not amplify snowshoe hare tissue reliably (Table 1).

We examined the matching pellet samples from captive hares by testing the 12 microsatellites that were successful on tissues. We found 9 primers amplified and produced scorable, variable products with DNA from pellets (Table 1). Marker *Sol 28* produced too many stutter bands under all PCR conditions, and markers *Sat 2* and *Sol 03* did not amplify with any of the pellets; thus were discarded. There was no genotyping error found for any of the 9 microsatellites that amplified successfully on fresh pellets when compared to the matching tissue samples. With a 9-locus panel (including monomorphic *Sat 3*) we have ample power to detect unique individuals, as the probability of identity (PI; Paetkau and Strobeck 1994) was 3.55×10^{-6} and $PI_{(sib)}$ was 4.51×10^{-3} (Evett and Weir 1998, Waits et al. 2001).

DNA from pellets collected in the field during the spring (n=20) and fall (n=20) had a 65% amplification success rate at *cytb* per season. We ran those samples that amplified at *cytb* with our 9-locus panel of microsatellites, running each sample 3 times to ensure consistency (Taberlet et al. 1996, Schwartz et al. 2004). The error checking algorithms of McKelvey and Schwartz (2004) suggest that the resulting identifications were largely error free. Fifty-four percent of the pellets collected in the spring and 69% of the pellets collected in the fall that amplified using *cytb* produced reliable (e.g., produced the same genotype across 3 replicates, scored independently) genotypes. We identified 5 individual hares in the spring pellet sample, and 3 individuals in the fall sample (Figure 1).

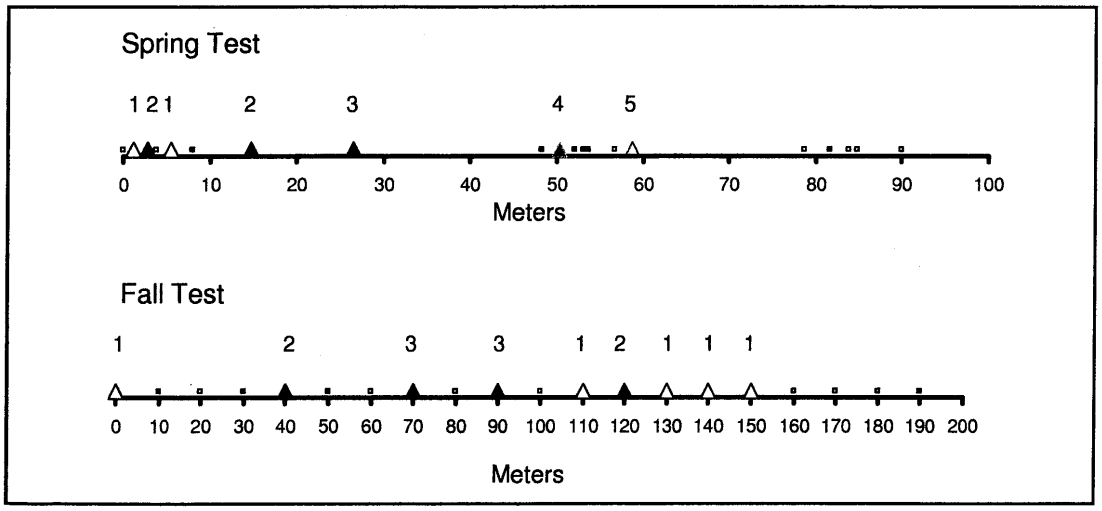

Figure 1. The top line represents the test of the methods during the spring 2004, and the bottom line represents the test of the methods during the fall 2004. In the spring a 100 meter transect was established and all pellets along that transect, regardless of age, were collected. In the fall, 20 stations placed at 10 meter intervals were established and pellets within 1 meter of the location were collected. The white squares represent samples where no DNA was obtained from the pellet, and black squares are where DNA was extracted, but only mtDNA analysis was successful. Each color triangle is a unique individual in a field test. In the spring we identified 5 individuals and the fall we identified 3 new individuals. Numbers above the triangles are individual hare identifications.

Discussion

Pellets have been used to estimate hare abundance because they are easy to collect. However, when hares are present at low densities, pellet counts exhibit high plot-to-plot variance and become poor predictors of numbers of hares trapped (Mills et al. 2005). Hares, however, produce an abundance of pellets—over 500/hare/day (Hodges 1999) providing ample sign of use by individual hares. This panel of microsatellite DNA markers for obtaining individual identification from pellets, therefore has much promise for indexing or estimating hare numbers, particularly when hares are scarce.

Before these methods are used for CMR, several sampling issues need to be resolved. For instance, if multiple sampling occasions are used, both temporal and spatial closure assumptions need to be considered. Ideally, all pellets from the area of interest should be removed prior to the time frame of interest, to prevent identifying an individual that is no longer in the area due to death or emigration. DNA may prove ideal for this situation as DNA degrades over time, and pellets that successfully amplified DNA were more likely recently deposited than those that failed. Further research on the degradation rates of pellets should be undertaken before conducting a formal study to estimate hare abundance. The number of pellets produced by hares in a stand can be substantial (Hodges 1999), thus a sampling strategy to minimize redundant sampling will also need to be developed.

DNA data are not without potential problems. When DNA is collected from non-invasive samples, genotyping errors can lead to an overestimation of abundance (Waits and Leberg 2000, Creel et al. 2003, McKelvey and Schwartz 2004, Pompanon et al. 2005). However, there are both mechanical (Taberlet et al. 1996) and analytical approaches (Paetkau 2003, McKelvey and Schwartz 2004) to detect errors in genetic datasets, as well as ways to model error rates using formal mark-recapture methods (Lukacs and Burnham 2005).

In this study, we specifically identified primers that produced reliable genotypes from pellet samples—amplification rates for cyt-b screened pellets were over 50% on samples of unknown age. An important goal of future research will therefore be to better identify those pellets likely to produce quality DNA (e.g., through the use of quantitative PCR, Morin et al. 2001). As an example, during the process of extracting DNA from scat we anecdotally noticed that when the wash buffer product was light colored or clear, DNA was absent or in low quantity. This property and other physical characteristics associated with specific pellets may allow for much more efficient screening for sample quality.

In summary, we now have a panel of 9 microsatellites that can provide snowshoe hare individual identification at a relatively low cost providing the basis for accurate density estimation. Future research should concentrate on DNA degradation rates, identifying pellets likely to produce DNA, and evaluating sampling designs. The promise of molecular tagging has shown useful for a variety species (Taberlet et al. 1997, Paetkau 2003, Schwartz et al. 2004), and should be considered for snowshoe hares as a complement to pellet counts and trapping, especially when hare densities are low.

Acknowledgements

We thank Cory Engkjer for his work in the laboratory. This research was funded by the Rocky Mountain Research Station and the Northern Region of the U.S. Forest Service. The Lubrecht Experimental Forest provided logistic support. Field support was provided by the Lolo Pass Redevelopment Project, Idaho Transportation Department, and the Northern Region of the USFS.

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Received 14 February 2007
Accepted for publication 15 August 2007