Conservation Genetics **5:** 405–410, 2004.

Short communication

DNA-based individual and sex identification from wolverine (*Gulo gulo*) faeces and urine

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Received 27 January 2003; accepted 9 June 2003

Key words: allelic dropout, management, non-invasive molecular techniques, reliable genotypes, Y-linked markers

Abstract

Non-invasive genetic analyses are important for studies of species that are rare, sensitive or at risk of extinction. This study investigates the possibility of using faeces and urine to obtain microsatellite genotypes for individual identification of wolverines (*Gulo gulo*). The reliability of the employed method was assessed by analysing independent amplifications of non-invasive samples (a multiple-tube approach) and by comparing genotypes obtained from faeces to genotypes obtained from blood or tissue of the same individual. Ten microsatellite markers were successfully amplified in 65% of the faecal samples (n = 32) and 40% of the urine samples (n = 22). Allelic dropout was found in 12 and 14% of the amplifications from extracts of faeces and urine, respectively. Nevertheless, all multi-locus genotypes were correct, as judged from comparison to data from tissue or blood samples, after three replicates. These results suggest that a non-invasive approach based on DNA-analysis of faeces can be a powerful tool in population monitoring of wolverines, potentially providing reliable estimates of population size and immigration rate. A second objective of the study was to develop markers for DNA-based sex identification in wolverines using non-invasive samples. We developed two Y-linked markers, one that was specific to wolverine and one that also successfully identified sex in another mustelid. Importantly, none of the markers amplified potential prey species such as reindeer or rodents.

Introduction

Appropriate management of small, vulnerable or endangered populations requires reliable estimates of the population size. Traditional census methods such as direct counts and radio tracking can be inconvenient and inefficient when applied to rare animals living in remote areas (Greenwood 1996, Mills et al. 2000). During the last decade, several studies have demonstrated the possibility of obtaining amplifiable DNA from non-invasive samples such as faeces, urine, or shed hair (e.g., Höss et al. 1992; Flagstad et al. 1999; Woods et al. 1999; Valiere and Taberlet 2000). The implementation of non-invasive DNA techniques as tools for individual identification (e.g., Taberlet et al. 1997) has opened up an alternative approach to census a population, either by direct count estimates of minimum population size (e.g., Ernest et al. 2000, Lucchini et al. 2002) or by using capture–recapture methodology (Kohn et al. 1999, Levy 1999, Mills et al. 2000).

The wolverine is a rare and elusive large carnivore of the northern hemisphere. Due to habitat fragmentation and human persecution over the last two centuries, population sizes have declined dramatically throughout the circumpolar range of the species. In Scandinavia, the current population is composed of approximately 600 individuals (Landa et al. 2001) and is considered highly vulnerable. Since the wolverine is a shy and rare animal, proper monitoring is problematic, time-consuming and expensive. A non-invasive molecular approach may therefore provide an alternative way to assess the demography of the species.

Here, we report a study where we examined whether a non-invasive approach based on faecal and urine samples could be applied to the genetic monitoring of wolverines. In particular, we assess the number of replicates needed for reliable genotyping of wolverine faeces. We also report the development of two Y chromosome markers and their ability to identify the sex of wolverine faecal samples.

Methods

Sampling and DNA extraction

Thirty-two faecal samples and 139 blood or tissue samples were collected in northern Sweden. All the faecal samples originated from animals that were also sampled for blood or tissue. Faecal samples were collected either directly from animals that were caught for marking or from snow in connection with tracking of known individuals. In this way the field collectors knew the individual origin of each sample. In addition, 22 urine samples were collected on snow in northern Norway. As far as possible excrement samples were kept cold or frozen until used for DNA extraction. In the field, collected samples were stored buried in snow and during transportation to the laboratory they were packed with ice.

At the laboratory, DNA from frozen excrements was isolated using the QIAamp DNA stool mini kit (GmbH, Hilden, Germany), following the protocol provided by the manufacturer. A slight modification was done for the urine samples: a small amount (≈300 μl) of snow where urine appeared particularly concentrated was added to 1.6 ml of the lysis buffer provided in the kit. To avoid precipitation during lysis the tube was incubated at 70 °C for 90 s before vortexing the sample. Each round of extraction included 8–12 samples and a negative control. A special room dedicated to low-copy number DNA research was used during extractions and PCR set-up. DNA from blood and tissue was extracted in other localities using a standard phenol: chloroform protocol (Sambrook et al. 1989).

Microsatellite genotyping

As an initial test of sample performance, amplification of nuclear DNA was carried out using one marker (*Ggu14*; Walker et al. 2001) that gives strong amplification products. Amplifications were performed in 10 μl reactions containing 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 3.2 pmol of each primer, 0.5 μg of bovine serum albumine (BSA), 0.45 U of HotStar DNA polymerase (Qiagen) and 2 μl of template. A 15-min pre-denaturation step at 95 °C was followed by 45 cycles of amplification with 30 s at 94 °C, 30 s at 52 °C and 1-min at 72 °C. A final 10-min extension step was added. In this initial test, all samples were run in two replicates and amplification products were visualized on a 2% agarose gel. Samples that gave visible products were subsequently amplified with fluorescently labeled primers across 10 microsatellite loci (Table 1). Amplifications were performed using the same PCR profile as described for *Ggu14*, except for the number of cycles, which now were 37. Amplification from tissue extracts was identical to the protocols described above for all 10 loci, but with a reduced number of cycles (32 or 34). PCR products were run on an ABI 377 instrument and the subsequent determination of allele lengths was performed with GENESCAN and GENOTYPER software (Applied Biosystems, Foster City, CA, USA).

Assessment of genotyping errors

For excrement samples, a single-locus genotype was not accepted before it had shown a minimum of four identical homozygous profiles or a minimum of two identical heterozygous profiles. Allelic dropout was interpreted in cases where one or more replicates were homozygous while others were heterozygous. A false allele was interpreted when a

Table 1. Microsatellite markers used in this study

Marker	Annealing temperature	$MgCl_2$ concentration	Number of alleles	PI[a]	PI_{sibs}	References
Ggu7	55 °C	3.0 mM	2	0.377	0.597	Davis and Strobeck (1998)
Ggu10	52 °C	4.0 mM	5	0.268	0.550	Walker et al. (2001)
Ggu14	52 °C	1.5 mM	5	0.278	0.535	Walker et al. (2001)
Ggu25	58 °C	3.0 mM	4	0.310	0.555	Walker et al. (2001)
Ggu42	52 °C	4.0 mM	3	0.457	0.692	Walker et al. (2001)
Gg216	52 °C	3.0 mM	5	0.175	0.464	Duffy et al. (1998)
Gg443	58 °C	4.0 mM	4	0.470	0.690	Walker et al. (2001)
Gg452	58 °C	1.5 mM	4	0.237	0.532	Walker et al. (2001)
Gg454	55 °C	3.0 mM	5	0.189	0.476	Walker et al. (2001)
Gg465	58 °C	3.0 mM	4	0.321	0.578	Walker et al. (2001)

[a] PI is the probability of identity based on the observed allele frequencies of the 139 tissue and blood samples. All 10 markers combined give an average probability of identity in two unrelated individuals of 4.7×10^{-5}, for siblings 3.1×10^{-3}.

spurious allele occurred in a single replicate but was not possible to reproduce in five other replicates. In order to assess the genotyping error rate and to test whether individuals could be correctly identified through faecal genotyping, each multilocus genotype obtained from faeces were compared and matched against genotypes obtained from tissue or blood. This part of the analysis was performed as a blind test, where it was not known at the laboratory which blood/tissue samples were represented among the faecal samples.

Sex identification

In a first step, we used conserved exon primers (*DBY3* and *DBY7*; Hellborg and Ellegren 2003) flanking two different introns of the Y-linked *DBY* gene to amplify and subsequently sequence these introns (sequences have been submitted to GeneBank; accession numbers AY333757 and AY333758). From the obtained intron sequences, we designed two internal pairs of primers, DBY3Ggu-F (5′-GGTAATATCAACTTTTCC TG-3′) + DBY3Ggu-R (5′-CACCTA TCAAGA-AATGTTTG-3′), and DBY7Ggu-F (5′-GACAA-ATATTAGTTGGGACC-3′) + DBY7Ggu-R (5′-CTGCTCATGCTCTCTTAAAT-3′). The new markers were initially tested for amplification using 20 tissue samples (10 of each sex). Wolverine specificity was tested against male samples from a range of other mammalian species. For application to excrement samples, only samples that amplified well for microsatellites were analysed and both Y-markers were always amplified in two replicates. PCR amplifications were run through 37 or 42 cycles (for tissue and excrement, respectively) with profiles and conditions identical to those described above for the microsatellite markers (annealing temperature: 55 °C, $MgCl_2$ concentration: 3.0 mM). Samples that could be successfully amplified were interpreted as representing males.

Results and discussion

Genotyping from faeces

In the initial test, the microsatellite *Gg14* could be amplified from 23 (72%) of the 32 wolverine faecal samples. Twenty–one of these could subsequently be amplified and scored for all 10 microsatellites. This corresponds to an overall amplification success of 65%, which is in the upper range compared to previous studies using faeces as the source of DNA. Allelic dropout was detected 41 times (12% of amplifications at heterozygous loci; Table 2). The presence of dropout appeared to vary both among markers and samples. In fact, the three samples showing a dropout rate of >20% (Table 2) account for more than half of all observed allelic dropouts. Finally, it appeared that longer alleles drop out at a significantly higher rate than shorter alleles (11 dropouts occurred in the shorter allele, 30 in the longer; $\chi^2 = 8.80$, $P < 0.01$). False alleles were observed only five times (<1%; Table 2), suggesting that this type of error should

Table 2. Simplified genotypes from positive amplifications of analysed faecal samples

Sample	*Gg7*	*Gg10*	*Gg14*	*Gg25*	*Gg42*	*Gg216*	*Gg443*	*Gg452*	*Gg454*	*Gg465*	Average dropout (%)
S1	22	1111	22	22	1111	1111	1111	1111	22	1111	0
S2	22	1111	22	22	1111	22	1111	1111	22	22	0
S3	22	1111	22	22	1111	22	1111	1111	22	22	0
S4	22	**1**222	1111	22	**1**2**1**2	**11**2122	22	22	2**11**2	**1**222	32
S6	22	1111	22	111**2**11	22	22	22	22	**1**22	22	6
S7	22	22	1111	22	22	22	22	22	222	**1211**22	13
S8	22	1111	22	22	1111	22	**11**22	22	22	22	10
S9	2**1**22	**1**2**1**2	22	22	1111	22	22	1111	22	1111	17
S10	22	**1**22	22	1111	1111	22	22	22	22	1111	7
S11	22**1**	1111	22	1111	1111	22	22	22	22	22	7
S12	22	1111	22	1111	22	22	22	22	22	22	0
S13	22	22	22	1111	1111	22**1**	**1**22	22	22	1111	13
S16	11111	1111	22	22	22	22	222	22	22	**1**222	5
S17	22	1111	1111	1111	1111	**2**11111	1111	1111	22	22	0
A2	1111	2**1**2	11111	1111	**11**2**1**2	**1**22**1**	1111	**11**22	1111	1111	47
A3	22	11**2**111	22	22	1111	22	**1**22	22	22	22	6
A7	22	2222	1111	22	1111	22	1111	1111	1111	22	0
A9	22	**32**1222	1111	22	1111	2**111**2	111111	11111111	22	1111	24
A11	1111	1111	22	22	1111	22	1111	1111	1111	22	0
A12	22	1**2**1111	2222	22	22	2**1**2	**1**22	22	22	22	9
A15	2222	1111	2**1**22**1**	22**1**2	22	22	2222	222222	22	22	10
Average dropout (%)	5	21	6	3	25	20	15	6	8	13	12

The outcome of each replicate is indicated by the number of amplified alleles (1 = homozygote, 2 = heterozygote, 3 = three alleles). Replicates showing an incorrect genotype (as initially identified using our pre-defined scoring criteria and later verified from comparison to genotype data obtained from blood or tissue of the same individual) are indicated in bold. Hence, bold **1** indicates an allelic dropout, and bold **2**/**3** indicates that a false allele was present.

cause only minor problems to the interpretation of consensus genotypes (see also Taberlet et al. 1996, Sloane et al. 2000).

The 21 faecal samples that were successfully amplified at all 10 loci were found to represent 16 different individuals. Importantly, all 16 multi-locus consensus genotypes could be perfectly matched to a blood or tissue sample. These genetically defined pairs of samples were in full agreement with the information on individual origins obtained during sampling of faeces. The perfect match between corresponding samples demonstrated that no allelic dropouts or other errors had passed undetected when scoring the faecal samples according to the multiple-tube criteria. Correct consensus genotypes were obtained at all loci and for all faecal samples already after the first three replicates. This was true even for three samples that showed high dropout rates (S4, A2, A9), suggesting that three replicates should be sufficient for reliable genotyping of wolverine faeces using the described methodological approach.

Considering amplification success, allelic dropout rate, as well as the perfect matches between faecal and tissue samples, we hypothesize that the relatively good performance may largely be due to three critical factors. (1) Samples were either very fresh or collected on snow. This would be consistent with observations of Lucchini et al. (2002), indicating that fresh samples and samples collected on snow in general amplified better than old samples and samples collected on bare ground. (2) The use of inhibitor-binding substances during DNA extraction (the anti-inhibitor provided in the applied kit) as well as during PCR amplification (BSA). (3) The use of a particularly efficient DNA

polymerase (HotStar DNA polymerase). It should also be noted that species-specific factors may play a role. For example, effects of diet have been demonstrated for brown bears (Murphy et al. 2003) and for herbivore species (Huber et al. 2002).

Sex identification

The initial test of the Y-markers, using tissue samples from 10 males and 10 females, consistently gave the anticipated result; i.e., a single band of expected size for males and no band for females. Unfortunately, the possibility to test the markers on faecal samples of known sex was limited as only one of the 16 individuals amplifying well was a male, a sampling bias explained by the fact that radio-tracking is directed towards females in our study area. Nevertheless, none of the 15 known females showed positive PCR products, while both markers were successfully amplified for the single male.

To ensure wolverine specificity of the developed markers, amplification was also attempted for males of several other mammalian species. We included one mustelid, the otter, and several other carnivores like brown bear, lynx, grey wolf, and dog. In addition, the primers were tested for human samples and several potential prey species like reindeer, roe deer, hare, shrew and vole. None of the other mammalian species amplified for *DBY3Ggu*. For *DBY7Ggu* a positive amplification product was obtained in male otters. Thus, it appears that *DBY3Ggu* is wolverine-specific while *DBY7Ggu* may be specific to mustelids (both being male-specific).

Microsatellite genotyping from urine samples

Nine of 22 urine extracts (40%) were successfully amplified across 10 loci. These nine samples were found to represent eight different individuals. Using the procedure described above, two were identified as males and six as females. An allelic dropout rate of 14% was observed from the successfully amplified urine extracts (Table 3). Despite a lower amplification success than from faeces, this indicates that urine samples collected in snow may also be a valuable source of DNA as suggested by Valiere and Taberlet (2000). Our results suggest that the silica-based method may be applied directly to frozen urine samples without an initial melting and precipitation step, especially if the urine sample is concentrated.

Conclusions

This study demonstrates that reliable microsatellite genotyping from wolverine faeces and urine can be obtained with a reasonable number of replicates.

Table 3. Simplified genotypes (as in Table 2) of positive amplifications from urine samples

Sample	*Gg7*	*Gg10*	*Gg14*	*Gg25*	*Gg42*	*Gg216*	*Gg443*	*Gg452*	*Gg454*	*Gg465*	Average dropout (%)
D0	2222	2222	111111	1111	1111	222	222	222	222	22	0
D4	222	1111	2222	**1**22	1111	222	222	222	222	222	4
C3	**1**2**1**2	1111	**1**22	2222	1111	222	2**11**222	22**1**	1111	222	23
A6	2222	2222	111111	2222	1111	11111	2**11**222	222	222	11111	8
D6	11111	2222	2**1**22	2222	**1**22**1**	222	2**1**2	2**11**2	1111	222	21
F9	11111	2**111**2**1**2	1111	1111	2222	222	22**1**	222	222	22	20
F1	222	2222	2222	1111	2222	222	222	222	222	22	0
F6	1111[a]	2**1**2**1**	1111	1111	2222	22**1**	222	**1**22**11**	2**1**2	22	33
D7	111111	1111	2222	**1**22**1**	1111	11111	111111	11111	11111	222	18
Average dropout (%)	11	22	11	16	13	5	20	23	6	0	14

Replicates showing an incorrect genotype are indicated in bold.

[a] The third replicate showed a different allele from that obtained in replicate one, two and four. For this locus the scoring criterion could not be fulfilled since we ran out of extract and sample.

Importantly though, full integration of non-invasive molecular techniques requires strict criteria for accepting a consensus genotype. To minimize the occurrence of false genotypes, we recommend that individual samples clearly performing worse than an average sample be discarded.

Non-invasive sampling and subsequent genetic analysis are currently being implemented in national plans for management of carnivores in Scandinavia. This work is designed as long-term studies to allow for observations of e.g., population trends and immigration. Indeed, such data will provide an important basis for the design of appropriate conservation plans for the small and vulnerable populations of large carnivores in Scandinavia.

Acknowledgements

This work was supported by grants from the Environmental Protection Agencies in Norway and Sweden, and Nordic Council of Ministers. H. Ellegren is a Royal Swedish Academy of Sciences Research Fellow supported by a grant from Knut and Alice Wallenberg Foundation.

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