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LABORATOIRE D'ECOLOGIE ALPINE

Results from the genetic analyses performed on feces samples from bears in Västernorrland, January-May 2005

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METHODS:

DNA extractions and typing

For every collected fecal sample, DNA extractions were performed using the Qiamp DNA Stool Kit (Qiagen Inc., Netherlands), developed especially for this type of material and following the manufacturer's instructions. All extractions occurred in a room dedicated to processing hairs and feces. Tubes containing samples and tubes without feces were treated identically to check for exogenous DNA contaminations.
Six microsatellite primers (Mu10, Mu23, Mu50, Mu51, Mu59, G10L) as well as a sex primer (SRY) were amplified using PCR (Bellemain and Taberlet, 2004). Those 6 microsatellites were chosen for their discriminatory power (loci with the lowest probability of identity) based on the Scandinavian brown bear tissue dataset (Bellemain & Taberlet 2004). The probability of identity (PI), i.e. the overall probability that two individuals drawn at random from a given population share identical genotypes at all typed loci (Paetkau & Strobeck 1994; PIsibs; Waits et al. 2001) was very low: (PI= $1.38\ 10^{-6}$; PIsibs = $4.52\ 10^{-3}$).

The amplification of microsatellites was carried out following the multiplex preamplification method (Piggott et al. 2004; Bellemain & Taberlet 2004). This method allows one to maximize the number of samples that contain the critical threshold amount of DNA for accurate genotyping. One primer of each pair was synthesized with a fluorescent dye group (6-FAM, TET or HEX) on the 5' end to allow detection and sizing of fragments on an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster city, California).

We repeated each amplification four times. Samples were typed as heterozygous at one locus if both alleles appeared at least twice among the four replicates and as homozygous if all the replicates showed identical homozygous profiles. If neither of those cases occurred, the alleles were treated as missing data. The gels were analyzed using Genemapper (version 3.0) software package (Applied Biosystems, Foster city, California).

Reliability of the DNA results

Genotypes from different samples were considered to represent an identical bear when all the alleles at all loci were identical. However, to be conservative, we assumed that two samples belonged to the same individual when there was only one mismatch for one allele at one locus. To verify the reliability of the genotypes and to calculate an error rate, about 5% of the successfully genotyped samples were randomly and blindly chosen to be amplified another four times (including the sex primer). The genotypes were then compared with the first typing.

The geographical consistency of the results was verified: for individual bears that were characterized by more than 1 fecal sample, the coordinates of the samples were plotted and the geographical grouping of those samples was visually checked.

Population size estimation

Three population size estimators were applied to obtain population size estimates:

Two rarefaction indices

Following the method described in Kohn et al. (1999), we compared the multilocus genotype of each sample with all those drawn previously and calculated the population size as the asymptote of the relationship between the cumulative number of unique genotypes and the number of samples typed. This curve is defined by the equation $y = (ax)/(b+x)$, where a is the asymptote, x the number of feces sampled, y the number of unique genotypes, and b the rate of decline in the value of slope. Eggert et al. (2003) derived another estimator with a similar equation; $y = a(1-e^{bx})$. Data was analysed with the software package GIMLET (Valière 2002), with 1000 random iterations of the genotype sampling order and rarefaction curves obtained from those iterations were plotted using R software (version 1.7.1; available at http://www.r-project.org).

MARK estimator (based on a capture mark recapture (CMR) principle):

Identical multilocus genotypes were grouped and compiled a "capture" and "recapture" history for each individual by dividing the dataset into 12 weekly sampling periods (only the weeks with an adequate sample size for estimating population size were considered). If an individual's feces were "captured" two or more times within the same capture period, only one capture was considered.

Data was analyzed with the open-capture models of MARK (White & Burnham 1999). A set of a priori models was developed, analyzed, and then ranked by AICc (Akaike's information criterion corrected for small sample size) values following analysis in the program MARK.

RESULTS:

Microsatellite typing

Extraction was performed on the totality of the 690 feces samples received at the laboratory. Among those samples, 434 (~63%) were successfully amplified for 6-7 loci (including the sex locus). From these 434 samples, 248 corresponded to male bears, 182 to female bears (and the sex could not be determined for 4 samples). **Figure 1** shows the geographical distribution of female, male and untyped samples. 140 unique genotypes were obtained, including 59 female genotypes (42.1%), 77 male genotypes (55%), and 4 "unknown sex" genotypes (2.9%) (**Table 1)**. Each multilocus genotype was found from 1 to 17 times, with a mean of 3.01±2.82 (SE) times (3.07 ± 3.11 for females and 3.22 ± 2.60 for males).

Reliability of the DNA results

About 5% of the 434 "positive" samples were blindly amplified another four times to check the reliability of the results. Totally, 280 alleles were compared (between the first and second typing): 274 were identical and 6 were different. This error rate of about 2% was due to allelic dropout, false alleles or contaminations (Taberlet et al. 1996).

Geographical consistency of the results was verified for 85 individuals that were characterized by more than one sample (totally 379 samples). Generally the samples corresponding to the same individual were geographically grouped. However, 5 samples (1.3%) were found to be geographically far away from the "same genotype samples", and could be considered as "suspicious".

Provnr	Group	Sex	X Coordinate	Y Coordinate	Comment
2871	20	M	6979854	1583535	About 100 km away from the other samples
3273	70	F	7047297	1555631	About 90 km away from the other samples
2406	84	M	7001806	1609866	All samples from this male are quite dispersed.
431	100	F	6967377	1535919	2 samples about 90 km away
1018	69	F	6963836	1561001	About 100 km away from the other samples

Although possible from a behavioral consideration, there might be a mistake in the results for those samples that could have occurred at all steps of the process (from sampling to genotyping).

Population size estimates:

Method	Estimate	Min	Max
Kohn's equation	228	183	267
Eggert's equation	152	138	176
MARK	159 (92 males 67 females)	148 (86 males 62 females)	180 (103 males 77 females)

See **Figure 2** for the rarefaction curve

Comments:

Rarefaction methods:

In the Dalarna and Gävleborg study (Bellemain et al. 2005), the rarefaction method gave results that were above (for Kohn's equation) and below (for Eggert's equation) what we thought was the best estimate.

We would also like to caution that the closure assumption, which is a requirement of the rarefaction methods, might be violated in the present study population. Therefore the validity of the results is not guaranteed with this method.

MARK method:

The best approximating model of the MARK open-capture (POPAN) model included constant parameters for "apparent survival", "recapture" and "probability of entry". The models with heterogeneity and temporal variation in capture probability received less support. We used the "POPAN" model rather than the "Jolly Seber" model because the likelihood function better converged with "POPAN".

Estimation of real function parameters:

			95% Confidence Interval	
Parameter	**Estimate**	**Standard Error**	**Lower**	**Upper**
Apparent survival	0.61	0.02	0.56	0.66
Probability of recapture	0.84	0.01	0.82	0.85
Probability of entry	1.00	0.48E-08	1.00	1.00

The high recapture probability shows that a high proportion of the population has been sampled. However, when considering the population size estimates for males and females, it should be kept in mind that individuals can move outside of the sampling area (about 39% of emigration if we consider the survival rate) and enter in the sampling area (probability of entry of 1).

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Figure 1: Geographic distribution of genotyped fecal samples (males and females) and fecal samples without amplifiable DNA. The X and Y axis represent X and Y geographical coordinates respectively.

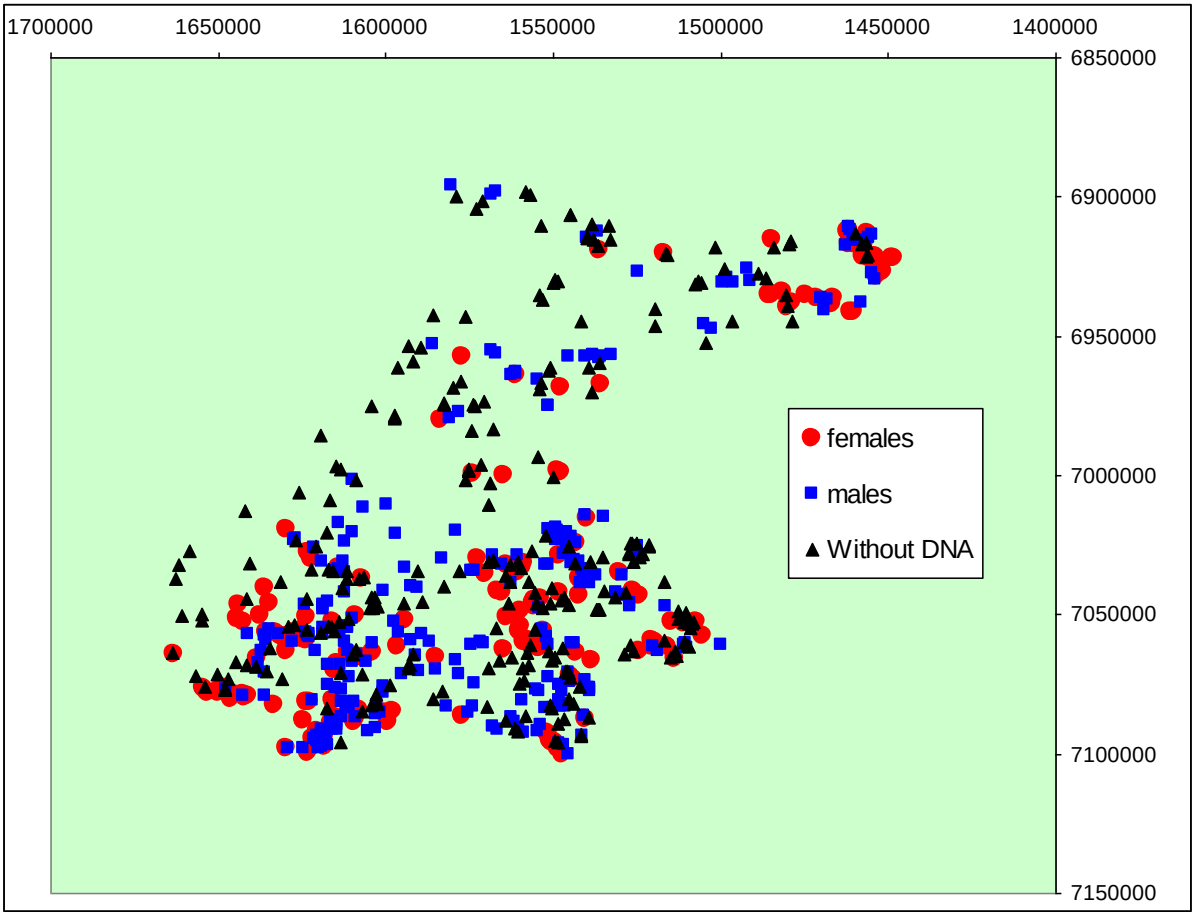

Figure 2: Rarefaction curve obtained from the observed data compared with estimates obtained from the Kohn and Eggert methods. The different curves represent the different iterations of genotype sampling order.