Laboratoire d'Ecologie Alpine
Génomique des Populations et Biodiversité
UMR CNRS 5553
Université Joseph Fourrier
BP 53
38041 GRENOBLE CEDEX 09
Tel : (0033) 4 76 63 54 78
Fax : (0033) 4 76 51 42 79

Results from the genetic analyses performed on faeces and hairs samples from Norwegian bears

Technical report 22th January- 27th March

□/ Species identification

Objective : Verify that faeces and hair are brown bear samples and that they contain enough DNA to be amplified.

Principle :

1st step : DNA extraction : DNA was extracted in a room dedicated to processing ancient samples, hairs and faeces. DNA from faeces samples was extracted as described in Waits et al.(2000), using Qiagen DNeasy kit, involving overnigh digestion with Proteinase K. DNA from hair samples was extracted using the Chelex method as described by Walsh et al.(1991).

2nd step : Amplification of the mitochondrial DNA control region.
Primers used : L 6164 : 5'-GCCCCATGCATATAAGCATG-3' (foward)
H 6299 : 5'-GGAGCGAGAAGGTACACGT-3' (reverse)

Samples used for DNA extraction and amplification are indicated in **Table 1**.

Results : The mtDNA amplification was checked by electrophoresis of the PCR products on an agarose gel. Around 87% of the faeces samples (103/118) and 38% of the hairs samples (5/13) showed positive bear DNA amplification, i.e those samples were confirmed to be brown bear samples and contained enough mtDNA to be amplified.
Nevertheless, in order to be sure not to miss any sample, we decided to amplify nuclear DNA for all the samples.

/ Individual typing

Objective: Establish a genotype for each sample and group samples with the same genotype in order to estimate the number of different bears present among the samples.

Principle : We analysed the polymorphism of microsatellite markers (hypervariable nuclear DNA).
The 5 microsatellite primers used for this analysis were selected to be the most polymorphic and informative markers among 19 microsatellite markers usually used for genetic analyses in Scandinavian brown bears.
We used a newly developed method (multiplex pre-amplification method) that significantly improves microsatellite amplification and error rates for faecal DNA in limiting conditions (Pigott et al., in prep). Each DNA extract corresponding to each sample was first pre-amplified using all the primers (that are multiplexed). Secondly, using the multi-tube approach (Taberlet et al.,1996), each locus was individually amplified from the pre-amplification PCR product. Samples were first replicated 4 times, and depending on the quality of the resulting genotype, 4 more replicates were amplified where necessary.
Moreover a "blind test" was performed, as a second step, in order to check the reliability of the obtained results.
PCR products were run on an automatic sequencer ABI Prism 3100.

Five microsatellite primers and one sex primer were used for the individual typing **:**
Mu10
Mu23
Mu50
Mu59
G10L
SRY (Y-linked primer)

Results :

1/ Individual identification.

In the following, results are described step by step.
• We were able to establish a "readable" genotype (e.g. 3 or more loci typed) for 68 out of 118 faeces samples. None of the hair samples gave positive nuclear DNA amplification although the extraction process was repeated twice. Among the 68 genotypes obtained for faeces samples, we differentiated 29 individual bears (13 females, 12 males and 4 individuals of unknown sex). Individuals were characterised by 1 to 9 faeces samples, with a mean of 2.5 scats/individual.
• However, in order to increase the reliability of the results, we recommend not to consider individuals that were typed for less than 5 loci, thus only 20 individuals (genotyped with at least 5 loci) should be considered in the whole sampled area. Details about PCRs experiments are described in **Table 2** : number of PCR replicates performed for each sample, number of markers successfully typed, number of positive PCRs per sample and percentage of false alleles and allelic dropout per sample.
• As a second step, for the "blind test", we selected one faecal sample for each of those 20 individuals, extracted and amplified the DNA from those samples a second time.
Finally, **14 individuals** (8 females and 6 males) were confirmed (see **Table 2** for more details about PCR experiments and **Table 3** for genotypes).

Here is the summary of the 14 identified individuals with their corresponding faeces samples and, in brackets, the sex and individual number with which they are identified in **Tables 2 and 3**:
•1st Individual (F) : R300959; R300963; R300969; R300971; R300973; R300975; R300981; R300994; R301052 (**Individual 1**).
•2nd Individual (M) : R300943; R300952; R300953; R300955; R301004; R300974; R300954; R300944 (**Individual 4**).
•3rd Individual (M) : R300920; R300964; R300965; R300967; R300988; R300989; R300991 (**Individual 2**).
•4th Individual (F) : R300945; R300956; R300960; R300972; R301053 (**Individual3**).
•5th Individual (F) : R300896; R300966; R301058; R301060; R301062 (**Individual 5**).
•6th Individual (M) : R300891; R300907; R300910; R300917 (**Individual 6**).
•7th Individual (F) : R300978; R301057; R301059 (**Individual 7**).
•8th Individual (M) : R300948; R300951; R300984 (**Individual 8**).
•9th Individual (M) : R301384; R301385; R301387 (**Individual 9**).
•10th Individual (F) : R300922; R300924 (**Individual 10**).
•11th Individual (F) : R300961; R300990 (**Individual 12**).
•12th Individual (M) : R301054 (**Individual 15**).
•13th Individual (F) : R300899 (**Individual 16**).
•14th Individual (F) : R301056 (**Individual 26**).

2/ Results from the parentage analysis

We used the software PARENTE (Cercueil et al., 2002; available at http://www2.ujf-grenoble.fr/leca/membres/manel.html) to run the parentage analysis. We took into account genotypes from the 14 identified individuals (**Table 3**) for this analysis and did not allow any allelic incompatibility between a parent and his/her offspring. We considered only parentage probabilities higher then 25%.
As the age of individuals can not be estimated from the faeces samples, the relationship parent-offspring can be considered in both ways.
Nevertheless, those results should be considered cautiously as the number of loci is low. For a comparison, we usually use 18 loci to run parentage analysis on Scandinavian brown bears.

A/ Results "couple of parents"

Individual	Possible Mother	Number of common alleles	Possible Father	Number of common alleles	Parentage Probability
Individual 10	Ind 3	5	Ind 4	5	0.5373
Individual 7	Ind 26	8	Ind 15	7	0.7211

B/ Results "fathers"

Relationships Father-Offspring	Number of common alleles	Paternity Probability
Individual 4 - Individual 10	5	0.6214
Individual 2 - Individual 12	6	0.7223
Individual 15 - Individual 7	7	0.7626
Individual 15 - Individual 26	8	0.9080

C/ Results “mothers”

Relationship Mother-Offspring	Number of common alleles	Maternity probability
Individual 1 - Individual 12	5	0.4335
Individual 5 - Individual 16	6	0.6704
Individual 12 - Individual 2	6	0.7223
Individual 7 - Individual 15	7	0.7626
Individual 10 - Individual 3	5	0.7902
Individual 26 - Individual 15	8	0.9080
Individual 26 – Individual 7	8	0.9562

3/ Reliability of the results :

Using these 5 polymorphic microsatellite primers and one sex primer, the Probability of Identity (Probability to get the same genotype for 2 different individuals “by chance”) is very low (calculated from the genetic dataset of Scandinavian brown bears)
For related individuals (sibs) : $PI=6.10^{-3}$
For unrelated individuals: $PI=2.10^{-5}$
However, some loci were ambiguous to type for some samples (cf **Table 2**).
We suspect that this is due to the fact that faeces samples were frozen instead of being conserved in alcohol. Moreover, the samples were probably de-frozen and then re-frozen during the transport and the stay at Geneve’s airport. We also noticed leaking between the plastic bags at the opening so that possible contamination may have occurred. Consequently, even if most of the faecal samples contained DNA (results from the mitochondrial DNA), the nuclear DNA was probably much degradated and might be contaminated by other samples. This can explain why we have had a relatively high proportion of allelic dropout and false alleles. Even when repeating the DNA amplifications 8 times, a “consensus genotype” could not be established for most of the samples (not listed in **Table 2**). Genotypes for which less than 3 loci were typed were not considered. At first, genotypes with only 3 or 4 loci were considered only if some alleles allowed a differentiation from other genotypes. However, according to the problems described above, we strongly suggest to consider with caution samples that are genotyped with less than 5 loci. We precise that contaminations during the extractions process and PCRs experiments are possible but very improbable as negative controls were included in the experiments.

We also noticed that the hairs were not preserved dry as they should be, in fact they were not separated from the faeces samples, and were thus exposed to humidity. This is probably why we couldn’t establish any genotype for hair samples.

We recommend, for future investigations, to preserve faecal samples in alcohol (2 mL of faecal sample in 20mL alcohol), and hair samples in paper envelopes and in a “hermetic” box with Silica gel. For a comparison, we usually obtain a success rate of 70% using faeces samples preserved into alcohol.

References

Cercueil, A., Bellemain, E. & Manel, S. (2002) PARENTE: A software package for parentage analysis. *Journal of Heredity.* **6**, 458-459.

Piggott, M., Bellemain, E., Taberlet P. & Taylor**,** A.C. A multiplex pre-amplification method that significantly improves microsatellite amplification and error rates for faecal DNA in limiting conditions**.** In prep.

Taberlet, P., Griffin, S., Goossens, B. *et al.* (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research,* **24**, 3189-3194.

Waits, L.P., Taberlet, P., Swenson, J.E., Sandegran, F., Franzen, R. (2000) Nuclear DNA microsatellite analysis of genetic diversity and gene flow in the Scandinavian brown bear *Ursus arctos*. *Molecular Ecology,* **9**, 421-431.

Walsh, P.S, Metzger, D.A, & Higuchi, R. (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques*, **10**, 506-513.

Table 1 : Samples used for DNA extraction and amplification

Id.number	Type
R300418	Hair
R300765	Hair
R300888	Hair
R300889	Hair
R300890	Hair
R300901	Hair
R300947	Hair
R300949	Hair
R300979	Hair
R300980	Hair
R301390	Hair
R301391	Hair
R301392	Hair
R300846	Faeces
R300891	Faeces
R300892	Faeces
R300893	Faeces
R300894	Faeces
R300895	Faeces
R300896	Faeces
R300897	Faeces
R300898	Faeces
R300899	Faeces
R300900	Faeces
R300902	Faeces
R300903	Faeces
R300904	Faeces
R300905	Faeces
R300906	Faeces
R300907	Faeces
R300908	Faeces
R300909	Faeces
R300910	Faeces
R300911	Faeces
R300912	Faeces
R300913	Faeces
R300914	Faeces
R300915	Faeces
R300916	Faeces
R300917	Faeces
R300918	Faeces
R300919	Faeces
R300920	Faeces
R300921	Faeces
R300922	Faeces
R300923	Faeces
R300924	Faeces
R300939	Faeces

Id.number	Type
R300940	Faeces
R300941	Faeces
R300942	Faeces
R300943	Faeces
R300944	Faeces
R300945	Faeces
R300946	Faeces
R300948	Faeces
R300950	Faeces
R300951	Faeces
R300952	Faeces
R300953	Faeces
R300954	Faeces
R300955	Faeces
R300956	Faeces
R300957	Faeces
R300958	Faeces
R300959	Faeces
R300960	Faeces
R300961	Faeces
R300962	Faeces
R300963	Faeces
R300964	Faeces
R300965	Faeces
R300966	Faeces
R300967	Faeces
R300968	Faeces
R300969	Faeces
R300970	Faeces
R300971	Faeces
R300972	Faeces
R300973	Faeces
R300974	Faeces
R300975	Faeces
R300978	Faeces
R300981	Faeces
R300982	Faeces
R300983	Faeces
R300984	Faeces
R300985	Faeces
R300986	Faeces
R300988	Faeces
R300989	Faeces
R300990	Faeces
R300991	Faeces
R300992	Faeces
R300993	Faeces
R300994	Faeces

Id.number	Type
R300995	Faeces
R300996	Faeces
R300997	Faeces
R300998	Faeces
R300999	Faeces
R301000	Faeces
R301001	Faeces
R301002	Faeces
R301003	Faeces
R301004	Faeces
R301005	Faeces
R301006	Faeces
R301050	Faeces
R301051	Faeces
R301052	Faeces
R301053	Faeces
R301054	Faeces
R301055	Faeces
R301056	Faeces
R301057	Faeces
R301058	Faeces
R301059	Faeces
R301060	Faeces
R301062	Faeces
R301382	Faeces
R301383	Faeces
R301384	Faeces
R301385	Faeces
R301386	Faeces
R301387	Faeces
R301388	Faeces
R301389	Faeces
R301393	Faeces
R301394	Faeces
R301395	Faeces

Table 2 : Details concerning the performed PCRS

	Sex	File name	"First experiments"					Results of the "Blind test"				Results after the "Blind test"
			Number of repeats	Number of markers typed	Number of positive PCRs	Percentage of allelic dropout	Percentage of false alleles	Number of positive PCRs	Percentage of allelic dropout	Percentage of false alleles	Number of markers typed	
Individual 1	F	R300959	4	6	24/24	_	_	23/24	_	4,34%	6	**Confirmed**
		R300963	4	6	24/24	–	–					
		R300969	8	6	39/48	3.23%	6.45%					
		R300971	4	6	24/24	–	–					
		R300973	4	6	24/24	–	–					
		R300975	4	6	24/24	–	–					
		R300981	4	6	22/24	–	–					
		R300994	8	6	44/48	0	6.94%					
		R301052	8	6	45/48	0	1.35%					
Individual 2	M	R300920	8	5	36/40	3.13%	6.25%					**Confirmed**
		R300964	4	5	20/20	–	–	23/24	_	2,17%	6	
		R300965	4	6	23/24	–	–					
		R300967	8	6	38/48	10.29%	1.47%					
		R300988	8	6	44/48	2.50%	13.75%					
		R300989	8	6	35/48	1.56%	4.69%					
		R300991	8	6	46/48	3.57%	3.57%					
Individual 3	F	R300945	4	6	24/24	_	_	24/24	_	_	6	**Confirmed**
		R300956	4	6	18/24	–	–					
		R300960	4	6	21/24	8.82%	2.94%					
		R300972	8	6	41/48	7.58%	12.12%					
		R301053	8	5	35/40	9.26%	7.41%					
Individual 4	M	R300943	4	6	23/24	2.38%	2.38%	24/24	_	_	6	**Confirmed**
		R300952	4	6	18/24	–	–					
		R300953	4	6	24/24	–	–					
		R300955	8	6	31/48	9.26%	9.26%					
		R301004	8	6	41/48	5.40%	14.86%					
Individual 5	F	R300896	4	6	24/24	_	_	24/24	_	_	6	**Confirmed**
		R300966	4	6	23/24	0	7.89%					
		R301058	8	6	43/48	0	1.47%					
		R301060	8	6	45/48	0	1.35%					
		R301062	8	6	45/48	0	1.35%					

Individual 6	M	R300891	4	6	23/24	0	7.14%	23/24	2,17%	_	6	**Confirmed**
		R300907	4	6	23/24	2.38%	4.76%					
		R300910	4	6	24/24	_	_					
		R300917	4	6	21/24	2.63%	5.26%					
Individual 7	F	R300978	4	6	23/24	0	10.52%	24/24	_	_	6	**Confirmed**
		R301057	4	6	24/24	_	_					
		R301059	4	6	24/24	_	_					
Individual 8	M	R300948	4	6	24/24	_	_	19/24	_	_	6	**Confirmed**
		R300951	8	6	34/48	13.33%	3.33%					
		R300984	8	6	44/48	0	3.75%					
Individual 9	M	R301384	4	6	17/24	0	3.13%					
		R301385	4	6	16/24	_	_					
		R301387	4	6	23/24	_	_	24/24	_	_	6	**Confirmed**
Individual 10	F	R300922	4	5	15/20	0	9.09%					
		R300924	4	5	19/20	_	_	16/20	_	_	5	**Confirmed**
Individual 11	M	R300974	8	6	42/48	14.47%	2.63%					Proved to be identical
		R300954	4	6	24/24	2.27%	6.82%	24/24	_	_	6	to individual 4
Individual 12	F	R300961	8	6	46/48	_	_	24/24	_	_	6	**Confirmed**
		R300990	8	6	43/48	0	2.85%					
Individual 13	M	R300996	8	3	11/24	31.82%	4.55%					Not enough loci typed
		R300998	8	3	11/24	27.27%	9.09%					to be considered
Individual 14	M	R301000	4	5	16/20	0	(12.5%)					Not confirmed
Individual 15	M	R301054	4	6	20/24	(2.63%)	0	24/24	_	2,08%	6	**Confirmed**
Individual 16	F	R300899	4	6	23/24	_	_	23/24		4,35%	6	**Confirmed**
Individual 17	F	R300997	8	6	25/48	(7.14%)	(14.29%)					Sample contaminated
Individual 18	F	R300916	8	6	21/48	(5.55%)	(8.33%)					Not confirmed
Individual 19	F	R300940	4	5	15/20	(22.73%)	0					Not confirmed
Individual 20	M	R300944	8	5	40/40	(13.88%)	0	23/24	_	_	6	Identical to individual 4
Individual 21	F	R300983	8	3	14/24	0	(16.66%)					Not considered
Individual 22	M	R300985	8	4	19/32	(6.67%)	(16.67%)					Not considered
Individual 23	F?	R300993	8	3	12/24	0	(12.5%)					Not considered
Individual 24	M?	R300995	8	3	14/24	(30%)	(5%)					Not considered
Individual 25	M	R301006	8	3	18/24	(19.23%)	(15.38%)					Not considered
Individual 26	F	R301056	8	6	46/48	_	_	24/24	_	_	6	**Confirmed**
Individual 27	M?	R300903	8	4	21/32	(23.53%)	0					Not considered
Individual 28	F?	R300921	8	3	18/24	(15%)	0					Not considered
Individual 29	F	R300939	8	3	15/24	0	(7.14%)					Not considered

Table 3 : Genotypes for the 14 identified individuals

Individual Number	Sex	Mu50	Mu50	Mu10	Mu10	G10L	G10L	Mu59	Mu59	Mu23	Mu23
Individual 1	F	92	100	125	129	150	150	100	118	141	149
Individual 2	M	92	92	119	129	140	150	118	120	149	153
Individual 3	F	92	100	119	125	150	152	116	118	143	149
Individual 4	M	92	92	129	129	146	150	116	118	147	147
Individual 5	F	96	96	113	129	150	150	94	114	149	149
Individual 6	M	100	102	119	127	150	150	116	118	143	151
Individual 7	F	100	100	113	129	150	150	94	108	149	149
Individual 8	M	92	100	125	129	138	138	100	120	143	147
Individual 9	M	98	100	119	125	140	152	114	118	149	153
Individual 10	F	92	92	125	129	150	152	116	116	?	?
Individual 12	F	92	96	119	125	140	150	100	120	141	153
Individual 15	M	100	100	125	129	150	150	108	120	143	149
Individual 16	F	96	100	119	129	150	152	94	116	149	149
Individual 26	F	100	100	113	125	150	150	94	108	143	149