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Genotyping success of historical Eurasian lynx (*Lynx lynx* L.) samples

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Abstract

Historical samples, like tanned hides and trophy skulls, can be extremely important for genetic studies of endangered or elusive species. Selection of a sampling protocol that is likely to provide sufficient amount and quality of DNA with a minimum damage to the original specimen is often critical for a success of the study. We investigated microsatellite genotyping success of DNA isolated from three different types of Eurasian lynx historical samples. We analysed a total of 20 microsatellite loci in 106 historical samples from the endangered Dinaric lynx population, established from re-introduction of three pairs of lynx in 1973 from Slovakian Carpathians. Of the three tested sample types, turbinal bone and septum from the nasal cavity of the trophy skulls had the lowest percentage of samples successfully genotyped for all 20 microsatellite loci. Footpad samples, collected using a cork drill, exhibited better results in polymerase chain reaction amplification and genotyping than samples of footpad epidermis cut with a scalpel. We report simple and efficient sampling protocols, which could be widely applied for future studies utilizing historical samples.

Keywords: Dinaric population, DNA, Eurasian lynx, genotyping, historical samples, *Lynx lynx*

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Introduction

Analysis of historical samples can reveal temporal variation in genetic diversity and structure or provide data for phylogenetic and evolutionary studies (Higuchi *et al.* 1984; Westemeier *et al.* 1998; Wandeler *et al.* 2007; Casas-Marce *et al.* 2010). Understanding of the temporal variation patterns is important in several ecological and population genetic contexts, as well as in conservation (Hedmark & Ellegren 2005). For many endangered or extinct species, historical samples are the only source of DNA for genetic analysis. Additionally, elusive lifestyle makes collection of nondestructive or noninvasive DNA samples a difficult alternative for many species, making historical samples an irreplaceable source of information. The critical point is a selection of the appropriate material from historical samples, which would provide a sufficient amount of DNA for polymerase chain reaction (PCR) with minimal damage to the original specimen (Horvath *et al.* 2005; Casas-Marce *et al.* 2010).

Tanned hides and skulls can be an important source for genetic studies of endangered or elusive species, especially the ones that are or have been hunted for trophies in the past. Preparation of hides through tanning is a common preservation method for mammalian specimens, both in museum collections and trophies. It is known that the treatments involved in tanning and processing of trophies can cause DNA degradation and can inhibit enzymatic reactions used during DNA isolation and amplification (Hall *et al.* 1996; Hedmark & Ellegren 2005; Casas-Marce *et al.* 2010). PCR amplification success relies on the initial number of intact DNA templates (Casas-Marce *et al.* 2010). DNA quantity and quality in museum specimens mainly depend on the preservation treatments and the age of the samples (Wandeler *et al.* 2007).

Eurasian lynx (*Lynx lynx* L.) is one of the most endangered European mammals. At the beginning of the 19th century, this predator was still roaming over the most of its historical range. As in the majority of other European habitats, a combination of habitat loss, a depletion of its prey base and an intensive prosecution led to the extinction of this species from Dinaric Mountains at the beginning of the 20th century (Breitenmoser *et al.* 1998; Kos *et al.* 2005). The Dinaric population has been re-established in 1973 through the re-introduction of six animals from Slovakian Carpathian Mountains to Slovenia. The newly founded population rapidly expanded into neighbouring Croatia, as well as to Bosnia and Herzegovina, and the re-introduction itself became one of the most successful re-introductions of a

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large predator to date (Čop 1987; Breitenmoser *et al.* 1998; Staniša *et al.* 2001; Potočnik *et al.* 2009). Today, the Dinaric lynx population is relatively small (estimated at max. 130 individuals), isolated and endangered (Sindičić *et al.* 2010). A small population size and an elusive lifestyle of lynx make noninvasive and other nondestructive samples a limited option as a source of DNA for genetic studies. However, an extensive collection of historical samples for the Dinaric lynx population exists from trophy hunting that took place in the past. During the 1974–2007 period, around 395 individuals of the Dinaric lynx population have been registered as legally killed, poached, killed in traffic accidents or were found dead, because of other causes (Breitenmoser-Würsten & Breitenmoser 2001; Frković 2001; Jonozovič 2003; Kos *et al.* 2005; Sindičić *et al.* 2010). Tanned hides and trophy skulls of these animals are mostly kept in private collections of hunters or hunting clubs, which makes minimizing of the damage to the specimen during sample extraction an important issue.

The goal of this research was to investigate microsatellite genotyping success of DNA isolated from Eurasian lynx historical samples. We compared three different types of samples to discover the optimal source of DNA, taking into consideration the final genotype quality and specimen damage.

Materials and methods

A total of 106 samples of lynx from the Dinaric population from Slovenia, Croatia, and Bosnia and Herzegovina were used in this study. Samples taken from private and scientific collections originated from the period from 1974 until 2001. Three types of samples were collected: cut footpad ($N = 43$); drilled footpad samples ($N = 39$) and turbinal bone and septum from the nasal cavity of the trophy skulls ($N = 24$).

The cut footpad samples consisted of approximately 2-mm-deep cut (approximate mass 0.05–0.1 g) in the epidermis layer using a sterilized scalpel. The drilled footpad samples were collected with a sterilized cork drill (3 mm in diameter). Approximate mass of drilled samples was 0.03–0.06 g. We were manually drilling between the claws into the footpad dermis. In this manner, we obtained a sample of the dermis layer, which should be less exposed to the tanning processes than the epidermis layer of the footpad. We were careful not to drill through the entire footpad, and we covered the drill hole with hair, so the visible damage to the paw was minimal. The turbinal bone and septum samples from the nasal cavity of the trophy skulls were collected using sterilized tweezers and a scalpel.

As a precaution against contamination, DNA extractions and PCR set-up were prepared in a laboratory dedicated only to processing of low copy number DNA samples, with a strict separation from downstream laboratories where PCR products were handled. Each step of the process included negative controls and followed a strict contamination prevention and quality control protocol.

The DNA from the footpads was extracted using GeneElute™ Mammalian Genomic DNA Miniprep Kit (Sigma), following the manufacturer recommended protocol, but with an extended proteinase K digestion step (12 h). Bone samples were digested for 48 h with buffer containing 100 mM Tris–HCl (pH 8.0), 1 M NaCl, 100 mM EDTA (pH 8.0), 10% SDS and proteinase K, and extracted using the standard phenol–chloroform protocol. All extracts were stored at −20 °C until used.

We amplified a total of 22 microsatellite loci, 20 of which were designed for the domestic cat (*Felis catus*): FCA001, FCA82, F85, FCA123, FCA132, FCA161, FCA201, FCA247, FCA293, FCA369, FCA391, FCA424, FCA506, FCA559, FCA567, FCA650, F53, F115, FCA723, FCA742 (Menotti-Raymond *et al.* 1999; Butler *et al.* 2002); one for Sumatran tiger (*Panthera tigris sumatrae*): HDZ700 (Williamson *et al.* 2002) and one for Canadian lynx (*Lynx canadensis*): Lc106 (Carmicheal *et al.* 2000) (Table 1).

The DNA was amplified in three multiplex PCR, with primer concentrations and PCR protocols as shown in Table 2. Loci FCA506 and F85 were excluded from further analysis, because of their poor amplification success during the optimization of multiplex PCRs. We prepared 10 μL of reactions, which included 1 μL of template DNA, 5 μL of Multiplex PCR kit Mastermix (Qiagen), 1 μL of Q solution and 3 μL of the primer mix (different for each of the multiplexes) and Ultra High Quality water.

We used a multitube-based genotyping procedure (Taberlet *et al.* 1996), modified from (Adams & Waits 2007). A fragment analysis was carried out on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Inc.) and interpreted using GeneMapper 4.0 software (Applied Biosystems, Inc). For the genotype at each locus to be accepted, at least two matching replicates had to be confirmed by two different people. All samples were amplified at least twice. Up to eight amplifications were performed on samples that successfully produced PCR products. We have conducted a two-phase screening of PCR products. In the first phase, samples were discarded if they did not produce any PCR products after two replications, and at the second phase, samples were discarded if after four replications they gave PCR products usable for genotyping at less than seven loci. The samples that had the quality index (QI) below 0.4 (Miquel *et al.* 2006) and reliability estimated using program Reliotype (Miller *et al.* 2002) below 0.95 even after eight replicates were discarded (Skrbinšek *et al.* 2010). We used the methods

Table 1 List of genotyped microsatellite loci

Locus	Repeat structure	Primer sequences (5′–3′)	Size range (bp)	Dye label	References	GenBank Accession no.
Lc106	GT	(F)TCTCCACAATAAGGTTAGC (R)GGGATCTTAAATGTTCTCA	96–108	6-FAM	Carmicheal *et al.* (2000)	AF288054
HDZ700	GT	(F)CCTCCTTCCAGGATGCCA (R)GGATGGGGGAAAATCTCTC	133–149	6-FAM	Williamson *et al.* (2002)	AF296747
FCA201	AC	(F)TCTGCAGGACCAGTCAGATG (R)AGCATACACAAATTGATGCTGG	90–169	6-FAM	Menotti-Raymond *et al.* (1999)	AF130563
FCA369	AC	(F)TGAAGAGCAGAAATAGCTTCA (R)TTTTTTGAGGTTTTTTGCAGC	156–184	6-FAM	Menotti-Raymond *et al.* (1999)	AF130623
FCA293	AC	(F)GATGGCCCAAAAGCACAC (R)CCCACATCTTGTCAACAACG	176–204	6-FAM	Menotti-Raymond *et al.* (1999)	AF130598
FCA506	GT	(F)AATGACACCAAGCTGTTGTCC (R)AGAATGTTCTCTCCGCGTGT	182–258	6-FAM	Menotti-Raymond *et al.* (1999)	AF130639
FCA650	AC	(F)GCACATTGTGACAATACACAG (R)AGGTTTCTAGAAGGTTCCCAGG	116–138	VIC	Menotti-Raymond *et al.* (1999)	AF130683
FCA247	GT	(F)GGAAATTAGGAGCTCTGCCA (R)AAGATTTACCCAGTTGCCCC	149–151	VIC	Menotti-Raymond *et al.* 1999	AF130583
FCA132	GT	(F)ATCAAGGCCAACTGTCCG (R)GATGCCTCATTAGAAAAATGGC	137–152	VIC	Menotti-Raymond *et al.* 1999	AF130535
F85	TTTC	(F)TCTGGTCCTCACGTTTTCCT (R) ATGTCTGTATGAGATGCGGT	175–311	VIC	Butler *et al.* 2002	AY988120
FCA391	GATA	(F)GCCTTCTAACTTCCTTGCAGA (R)TTTAGGTAGCCCATTTTCATCA	129–273	VIC	Menotti-Raymond *et al.* (1999)	AF130624
F53	AAGA	(F)GTTGGGAGTAGAGATCACCT (R)GAAAAAGACTCCTGCTTGCA	288–344	NED	Butler *et al.* (2002)	AY988119
FCA742	CTTT	(F)TCAATGTCTTGACAACGCATA (R)AGGATTGCATGACCAGGAAC	122–175	NED	Butler *et al.* (2002)	AY988143
FCA001	AC	(F)TGCTTGTCCTCTCCCTCG (R)TGACTGCGCCATAGCTTTC	190–212	NED	Menotti-Raymond *et al.* (1999)	AF130472
FCA82	GT	(F)TCCCTTGGGACTAACCTGTG (R)AAGGTGTGAAGCTTCCGAAA	239–246	NED	Menotti-Raymond *et al.* (1999)	AF339955
FCA123	AC	(F)ACTGCGAGAGGACTTTCGAA (R)CTTCTGACAGGCTCCAGGTT	137–149	NED	Menotti-Raymond *et al.* (1999)	AF130530
FCA424	GATA	(F)TGGAAAAATGTGGAATACTGA (R)CCAATTTGTAGTGACATCCCC	159–187	NED	Menotti-Raymond *et al.* 1999)	AF130625
FCA567	GT	(F)TCAGGGTTTTCCAGAGAAACA (R)TAGACACATACAGATGGGGTGC	92–106	PET	Menotti-Raymond *et al.* (1999)	AF130661
FCA559	AGAA	(F)GCCAAAATGTTCAAGAGTGG (R)TTTTGGCTTGATGAGCATCA	113–195	PET	Menotti-Raymond *et al.* (1999)	AF130659
FCA161	GT	(F)TTACCGATACACACCTGCCA (R)CACAGACGTGCTCTAGCCAA	179–187	PET	Menotti-Raymond *et al.* (1999)	AF130549
F115	GAA	(F)CTCACACAAGTAACTCTTTG (R)CCTTCCAGATTAAGATGAGA	193–217	PET	Menotti-Raymond *et al.* (1999)	AY988109
FCA723	AAAG	(F)TGAAGGCTAAGGCACGATAGA (R)CGGAAAGATACAGGAAGGGTA	243–317	PET	Butler *et al.* (2002)	AY988124

recommended by Broquet & Petit (2004), Pompanon *et al.* (2005) and Luikart *et al.* (2008) to estimate the frequency of allelic dropouts (ADO), false alleles (FA) and genotyping error rate per locus. The consensus genotypes from multiple replicates and error estimates were carried out in a database application programmed in Microsoft Access (T. Skrbinšek, in preparation).

Amplification success rates for different sample types were calculated based on the ratio of the number of successful amplifications per locus to the total number of amplifications per locus, while per locus genotyping success rates were calculated based on the ratio of the number of successfully genotyped samples per loci to the total number of analysed samples per loci. We used the Wilcoxon nonparametric test to compare QIs of different sample types. Tests were carried out in R statistical environment (R Development Core Team, 2011). The probability of identity (PI), the probability of identity

Table 2 Multiplex primer concentration and PCR protocol*

Multi A		Multi B		Multi C	
Primer mix	Concentration (μM)	Primer mix	Concentration (μM)	Primer mix	Concentration (μM)
F53 F & F53 R	0.28	Fca567 F & Fca567 R	0.12	Fca 559 F & Fca 559 R	0.20
Fca161 F & Fca161 R	0.30	Fca247 F & Fca247 R	0.18	Fca123 F & Fca123 R	0.40
Lc106 F & Lc106 R	0.40	Fca723 F & Fca723 R	0.22	F115 F & F115 R	0.20
Fca369 F & Fca369 R	0.28	Fca650 F & Fca650 R	0.10	Fca001 F & Fca001 R	0.40
HDZ700 F & HDZ700 R	0.20	Fca132 F & Fca132 R	0.10	Lc106 F & Lc106 R	0.40
		Fca391 F & Fca391 R	0.40		
		Fca201 F & Fca201 R	0.10		
		Fca293 F & Fca293 R	0.10		
		Fca82 F & Fca82 R	0.15		
		Fca424 F &Fca424 R	0.40		
PCR protocol		PCR protocol		PCR protocol	
95 °C	15 min	95 °C	15 min	95 °C	15 min
52 cycles		52 cycles		52 cycles	
94 °C	30 s	94 °C	30 s	94 °C	30 s
57 °C	90 s	60 °C	90 s	53.8 °C	90 s
72 °C	60 s	72 °C	60 s	72 °C	60 s
60 °C	30 min	60 °C	30 min	60 °C	30 min

*Loci FCA506 and F85 were excluded because of poor amplification success in multiplex PCR.

among sibs (PIsib) and cumulative PIsib per locus were calculated according to Paetkau & Strobeck 1994 and Waits *et al.* 2001, using access application programmed in Microsoft Access (T. Skrbinšek, in preparation).

Results

A total of 106 historical samples were analysed at 20 microsatellite loci. In total, 43 samples (41%) were successfully amplified across all 20 loci. Of 43 cut footpad samples, we managed to obtain the full genotype for 15 samples (35%), while six samples (14%) had no successful amplifications at any locus. The amplification success rate for cut footpad samples was 58% and mean per locus genotyping success was 39%. In the drilled footpad samples, we managed to obtain the full genotype for 69% of the samples (27 of 39) and one sample (3%) had no successful amplifications at any locus. The drilled footpad samples also demonstrated the highest amplification success rate (74%) and the highest mean per locus genotyping success (72%). Of 24 skull samples, we obtained the full genotype only for one sample (4%) and three samples (13%) had no successful amplifications at any locus. An amplification success rate was 44%, while the mean per locus genotyping success was 4% (Table 3).

The samples with the lowest ADO were cut footpad samples (4.34%), while the highest rate of ADO was detected at drilled footpad samples (5.58%). The drilled footpad samples had the lowest rate of false alleles (0.29%), as well as the lowest genotyping error rate per locus (2.83%). The highest rate of false alleles (2.68%) and the highest genotyping error rate per locus (5.67%) were detected in skull samples (Table 3).The locus Fca742 is the most informative locus, with 0.12 PI) and PIsib of 0.42, while the overall across loci PI was 2.1E-05 (Table 4).

Table 3 Genotyping success and mean error rates per sample type

Sample type	No. of analysed samples	Distinct PCR product (%)	No. of samples with full genotype (%)	Amplification success rate (%)	Median quality index (%)	Mean per locus genotyping success (%)	Mean per locus ADO (%)	Mean per locus FA (%)	Mean per locus error rate (%)
Skull	24	51.9	1 (4.2)	43.9	0.26	4.35	5.22	2.68	5.67
Cut footpad	43	70.1	15 (34.9)	58.4	0.64	39.30	4.34	0.82	2.91
Drilled footpad	39	90.5	27 (69.2)	74.1	0.77	71.67	5.58	0.29	2.83

Table 4 Probability of identity (PI), probability of identity among sibs (PIsib) and cumulative PIsib

Locus	PI	PIsib	Cumulative (PIsib)
Fca742	0.12	0.42	0.42
Fca201	0.18	0.47	0.20
HDZ700	0.20	0.48	0.10
Fca247	0.20	0.48	0.05
Fca123	0.21	0.49	0.02
Fca82	0.24	0.52	0.01
Lc106	0.26	0.54	6.3E-03
Fca559	0.28	0.57	3.6E-03
Fca161	0.30	0.56	2.0E-03
F115	0.33	0.60	1.2E-03
Fca369	0.34	0.59	7.2E-04
Fca424	0.37	0.60	4.3E-04
Fca293	0.38	0.60	2.6E-04
Fca567	0.39	0.61	1.6E-04
Fca132	0.40	0.62	9.9E-05
Fca391	0.40	0.63	6.2E-05
F53	0.43	0.67	4.2E-05
Fca650	0.46	0.68	2.8E-05
Fca001	0.67	0.83	2.4E-05
Fca723	0.81	0.90	2.1E-05

The QIs of the footpad samples (cut and drilled) that provided PCR products showed bimodal distribution, with a group of samples with high QI and another group with very low QI. The drilled skins showed a higher QI (median = 0.77) than the cut skins (median = 0.64, $P = 0.002$). The skull samples performed considerably worse than the skin samples collected by drilling (median QI = 0.26, $P < 0.001$), but this difference was less convincing for samples collected by the cut method ($P = 0.096$).

Discussion

We have analysed the genotyping success in 106 Eurasian lynx historical samples genotyped using 20 microsatellite loci. Our results show that the drilled footpad samples are a useful source of good quality DNA for genetic studies. The drilled footpad samples demonstrated the highest success in obtaining the complete genotype (69.2%), the highest amplification success rate (74.1%), the highest mean per locus genotyping success (71.7%), as well as the highest QI (0.77) and the lowest genotyping error rate per locus (2.83%). Of the three tested types of samples, the skull samples had the lowest genotyping success and the highest genotyping error rate per locus (5.67%). Interestingly, we observed a bimodal distribution of QIs for both footpad samples types (but not for skulls). This may be the result of different tanning processes having different effects on DNA quality; however, we had no data on specific tanning process used on each hide to explore this further.

A successful PCR amplification of tanned hides and skull samples depends on several factors, such as the amount of recoverable DNA, the level of DNA damage and the inhibition agents present. Tanning processes typically involve treatments with salt, aluminium, soda, formic acid and tannic acid (Hedmark & Ellegren 2005), which can degrade DNA and inhibit digestion and PCR enzymatic reactions. Bones are subjected to different conservation treatments than tanned hides. After boiling in water, trophy skulls are treated with hydrogen peroxide. Sometimes, they are also bleached with a mixture of ethanol and calcium carbonate. Bone structure presents a safe environment for the DNA conservation, as light, oxygen and other damaging factors may not reach the inner tissue (Cooper 1993). Casas-Marce *et al.* (2010) reported that claws are the best source of DNA in tanned hides, as the entrance of damaging and inhibiting agents to the inner tissue is obstructed by keratin. Unfortunately, sampling of a claw also visibly damages the trophy, so our goal was to test an alternative sampling method. Casas-Marce *et al.* (2010) used a Dremel bit tool to drill into a footpad and collect the footpad powder sample. We did not use any automated drilling machines that could additionally damage DNA by overheating. Instead, we applied a cork drill as a simple tool to obtain the inner footpad tissue, which was, similar as in the bones and claws, less exposed to the tanning processes, air, humidity and UV light than the epithelium layer of the footpad.

The analysed tanned hides and skulls mostly came from the private hunting trophy collections. We had to be very cautious not to cause any visible damage and were constantly monitored by the owners of the trophies during the sample collection process. With this in mind, we decided not to take the more commonly used samples of the mastoid processes, where we could have damaged the trophy, and rather took the samples of turbinal bones and septum from the nasal cavity of trophy skulls. The turbinal bone and septum samples in our study displayed a 43.9% amplification success rate, which is considerably lower than the maxilloturbinal bone amplification rate described by Wisely *et al.* (2004) and Casas-Marce *et al.* (2010). Casas-Marce *et al.* (2010) observed that the drilled footpad samples have similar amplification response rates as bones (mastoid processes and maxilloturbinal bone). As our sample was a mix of bone and cartilage tissue (septum), it seems that cartilage is less resilient than bone.

In this study we are reporting a useful method for the collection and analysis of genetic samples extracted from the Eurasian lynx tanned hides, which could be of considerable importance for further genetic studies of this species. We demonstrated a higher success in PCR amplification and genotyping of the footpad samples collected with a cork drill compared with the samples that were

simply cut from the footpad surface. We believe that this simple and efficient sampling technique can provide good quality DNA with minimal or no visible damage to the specimen and has potential utility also in studies of other mammal species where such material is available. The described new sampling technique has the possibility to be widely applied in the future.

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Data Accessibility

For detailed information regarding genotyped microsatellite loci, please see Table 1.