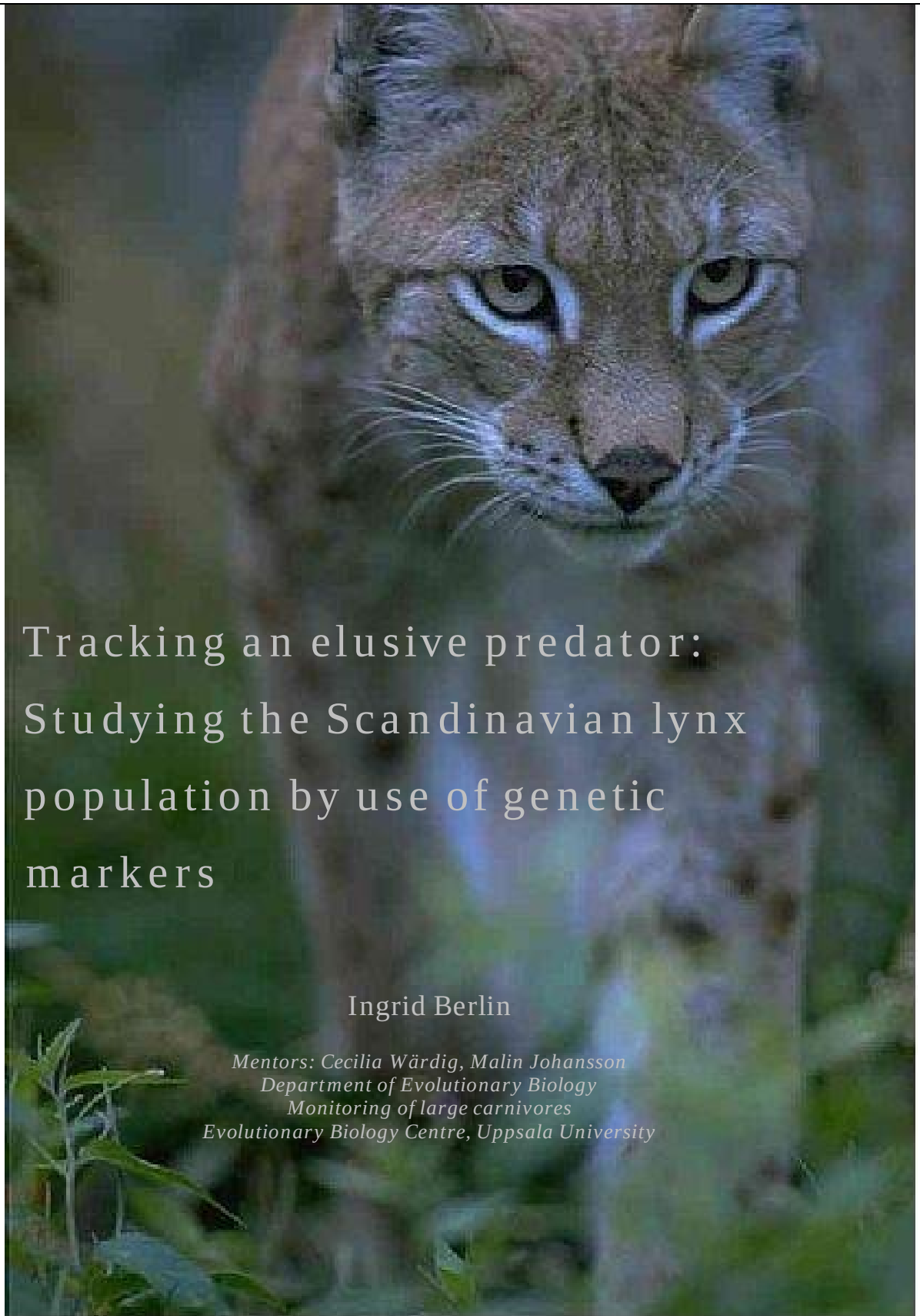
UPPSALA UNIVERSITET

Degree project 10 p C-level Spring 2007

Department of Medical Biochemistry and Microbiology

Biomedical sciences programme



Tracking an elusive predator: Studying the Scandinavian lynx population by use of genetic markers

Ingrid Berlin

Mentors: Cecilia Wärdig, Malin Johansson
Department of Evolutionary Biology
Monitoring of large carnivores
Evolutionary Biology Centre, Uppsala University

ABSTRACT

Gaining accurate population information is crucial for the conservation and management of species. The National Monitoring Program for Large Carnivores monitors the Swedish lynx population (species *Lynx lynx*) by surveying family groups, non-invasive sampling and genetic analysis. Ten microsatellite regions were used as genetic markers to retrieve unique individual genotypes, through polymerase chain reactions (PCR) with specific primer-pairs and capillary-electrophoresis. Complete genotypes were matched using an internal database. The aim of this degree project was to show how monitoring of lynx through genetic analysis is carried out at the Department of Evolutionary Biology at Uppsala University, and to evaluate how effective these methods are and how they might be improved.

Even though most of the methods used were fairly robust and reproducible, non-invasive sampling and microsatellite analysis posed some problems regarding DNA quality and quantity, and increased the risks of certain genotyping errors. These risks might be worth taking though, as genetic analysis, in combination with field observations, gives a more comprehensive picture of the Swedish lynx population.

KEYWORDS

Lynx, Genetic marker, Microsatellite, Non-invasive sampling, Faecal DNA

INTRODUCTION

Lynx of the species *Lynx lynx* (Eurasian lynx) are only found in central Europe and certain parts of northern Europe and Asia. After a strong decrease of animals being observed up until the 1960s, attempts to reintegrate have been made in several central European countries with varying results. Attempts of reintegrating animals in the Slovenian Alps and Switzerland were, for instance, very successful with lynx gradually spreading to Italy, Austria and north eastern France. Lynx can also be found throughout the Russian Taiga forest into Siberia, as well as in the mountains towards Himalaya, Mongolia, northern China and Pakistan.

The distribution and numbers of lynx in Sweden have varied greatly during the 19^{th} and 20^{th} centuries and still does to present date (*Action program for the conservation of lynx*, 2000). During the first half of the 1800s lynxes were fairly abundant in Scandinavia and in 1928 the lynx was declared a protected species which resulted in a strong increase in population. This led to public hunting being accepted in 1943, which meant that an unlimited number of animals were allowed to be killed in hunting areas. Until the year of 1986, public hunting was allowed in the entire country for at least one month every year. When the *Swedish Environmental Protection Agency* announced lynx as protected in 1991, only a couple of hundred animals were estimated to inhabit Sweden. Since then the lynx has made a big recovery and has repopulated mainly middle and northern regions of Sweden. In the winter of 2003, between 1200 and 1400 animals were estimated to live in Sweden. The conflict between carnivore and humans has gradually escalated as the population of lynx has grown. Domestic and farm animals are known to be subject to some damage by lynx, but the biggest problem is its preying on tame reindeer. The economic compensation to reindeer keepers correlates to the estimated amount of newborn lynxes in any given year.

Improved methods for mapping newborns are continuously being investigated and developed. Research has shown the possibility to identify individuals through DNA analysis with so called non-invasive sampling. Microsatellites or Simple sequence repeats (SSRs) are polymorphic regions found in nuclear DNA consisting of repeated sequences of one to four base pairs. These regions are, in most cases, located in non-coding DNA. The repeated sequence in microsatellites usually consists of two, three or four nucleotides. An example of a fairly common microsatellite, in human and other genomes, is the $(CA)_n$ repeat – repeats of the two nucleobases Cytosine and Adenine - where n is variable between alleles.

In diploid organisms such as humans or lynxes, microsatellites are biparentally inherited, which makes genotyping individuals within a population informative. Over time individuals in a population will recombine their microsatellites through sexual reproduction, which means that the population will maintain a variety of microsatellites characteristic for that population and distinct from other populations. These markers can provide individuals with unique DNA profiles when several microsatellite markers are used in combination. Genetic investigations in which microsatellites can be used as molecular markers are numerous and can include kinship, population studies and gene mapping (Griffiths, 1996).

Mutations resulting in new length alleles are most commonly thought to originate from so-called slip-strand mispairing (SSM) replication errors, making microsatellite repeat numbers highly polymorphic. The process of slip-strand mispairing begins with the DNA polymerase loosing grip or 'slipping' during replication (Eisen, 1999). This is believed to cause temporary misalignment between the template and newly replicated strand. If the strands are imperfectly realigned, mutations will be generated. The reason why SSM seems to occur more frequently in microsatellites is that rising numbers of repeats increase the likelihood of misalignment after slippage. Aside from SSM, mutations may also occur during recombination in meiosis.

The Swedish parliament has decided to try to preserve the large carnivores – lynx, wolverine, wolf and bear – in Sweden by minimizing the conflict between these carnivores and humans (www.naturvardsverket.se). Different actions have been taken to try to prevent and replace damage made by these animals, to prevent illegal hunting and to inventory the animal population. In 2001, the 'Merged politics of carnivores' (Proposition 2000/01:57) was produced. *The Swedish Environmental Protection Agency* and the *County Administrative Boards* are responsible for putting these policies into action. Carnivores are to be allowed to spread within their natural habitats, but the parliament has set goals for the size of the different animal populations. Lynx are to be maintained at an approximate number of 1500 individuals, which would mean 300 female lynx having kittens in Sweden every year.

Gaining accurate information about the population – such as survival, recruitment and population size - is crucial for the conservation and management of the species. Lynx are difficult to capture, which makes collecting and genotyping shed DNA, so called non-invasive sampling, very useful. The project for monitoring large carnivores at Uppsala University, monitor the large carnivore's population sizes through genetic analysis. In this project, ten microsatellite regions originally mapped in the domestic cat, were used as genetic markers to retrieve unique individual genotypes for Swedish lynxes. Primers developed to bind flanking regions of these microsatellites were used to produce different size fragments in a polymerase chain reaction (PCR). Alleles for the loci were analysed for fragment size using capillary-electrophoresis, and complete genotypes for all ten loci were used in individual matching with an internal database of genotypes of 'known' individuals.

The project for monitoring large carnivores at Uppsala University is funded by *The Swedish Environmental Protection Agency*. This project makes it possible to measure the number of newborns and the geographic location of lynxes in Sweden. Annual reports by a researcher at the *Norwegian Institute for Nature Research*, based on this data, estimate the total lynx population size in Sweden. It is then used by the parliament to develop plans for the conservation and management of lynx.

The aim of this report was to show how non-invasive sampling and the use of genetic markers enabled the identification of lynx individuals, and to some extent to evaluate the methods used for monitoring of Swedish lynx.

MATERIALS AND METHODS

Materials

Samples

Samples in the form of faeces, fur, hairs and saliva from wild lynx were collected by trackers employed by *County Administrative Boards* throughout Sweden. Specimens of animal tissue were acquired from the *National Veterinary Institute of Sweden.* Samples were sent to the laboratory for monitoring of carnivores in the Evolutionary Biology Centre in Uppsala.

Faeces samples were stored in -80ºC for a week upon arrival, before sample registration and additional storage at -20ºC until DNA isolation. Hairs were registered and stored in paper envelopes at room temperature until extraction. Saliva was kept in original containers at room temperature. Tissue samples obtained from the *National Veterinary Institute* arrived in tubes filled with alcohol, and were kept in -20ºC in original containers until extraction.

DNA isolation and amplification were conducted in assigned areas of the laboratory to minimize risk of contamination. Furthermore, extractions of samples with low-quality DNA were carried out in a separate laboratory from the high-quality DNA[1] to avoid contamination by the latter. All samples were, upon arrival, assigned a sample-number (numbered consecutively from 1 upwards) for that year. For example LS07-001 would be lynx from Sweden (LS), received in the year 2007 (07) and the first sample of the year (-001).

A total of 146 samples were selected for this study; collected from all over Sweden in 2006 and 2007. Table 1 shows geographic origin and type of samples selected for this study.

[1] Low quality DNA meaning DNA isolated from non-invasive samples i.e. faeces and hairs, whilst high quality DNA would be DNA isolated from blood or tissue, samples that presumably would give DNA of higher quality and of larger quantity.

Table 1. Type and geographic origin of samples

Faecal samples		**Other type of samples**
County of Origin	***Number***	***Number****
Blekinge län	**12**	**10**[1], **5**[4], **2**[3]
Dalarnas län	**3**	-
Göteborg and Bohus län	**8**	-
Hallands län	**-**	**1**[3], **1**[1]
Jämtlands län	**29**	**6**[2], **2**[1]
Jönköpings län	**3**	-
Kalmar län	**11**	-
Kronobergs län	**-**	**1**[3]
Norrbottens län	**24**	**9**[1]
Skaraborgs län	**-**	**1**[3]
Skåne län	**1**	-
Södermanlands län	**1**	-
Unknown location	**2**	-
Uppsala län	**4**	-
Västra Götalands län	**-**	**3**[3]
Örebro län	**1**	-
Östergötlands län	**3**	**3**[3]

** Superscript numbers refer to sample type: 1; hair, 2; fur, 3; tissue, 4; saliva*

Methods

DNA extraction – faeces, fur/hairs, saliva and tissue

Faeces were cut to pieces of approximately 180-220 mg weight and DNA was extracted using the 'QIAamp DNA stool mini kit' from Qiagen (http://www.qiagen.com). In this extraction method, epithelial cells shed from the intestinal lining and deposited in and on the surface of faeces, were lysed in a buffer. Substances harmful to DNA and PCR inhibitors in the sample were adsorbed by Inhibitex, applied in tablet form. Inhibitex reagent was pelleted by centrifugation and the DNA in the supernatant was purified on a spin column. Several buffers were used for washing , and a final buffer was used to elute DNA from the column. Protocol developed by Qiagen was used with some modifications. Stool samples were left overnight in lysis buffer to homogenize. Centrifugations with the Inhibitex tablet were done for 6 minutes instead of 3 on recommendation by Qiagen. 95% ethanol was used consistently, also the first and second centrifugation steps of spin columns were done at 9740 *x g* instead of 13604 *x g*. The optional step, where spin columns were centrifuged in new microcentrifuge tubes to remove residues of washing

buffer, was made obligatory. The final elution was divided into two steps of adding 100µl buffer each time, with incubation at room temperature for 3 minutes and centrifugation at 9740 *x g* after each step.

DNA was also extracted from the roots of hairs. By treating the hair with dithiothreitol (DTT), disulphide-bridges in the hair were removed. Proteinase K was used to break down proteins in the sample. Metal ions in the hair that might be harmful to DNA were bound to Chelex 100 Resin from Bio-Rad. Chelex 100 is styrene divinylbenzene copolymers containing ions which bind polyvalent metal ions, for example copper, iron and other heavy metals. A buffer was prepared as follows; 5 g of Chelex were added to 1ml of 1M Tris-HCl, purified water was then added to a final volume of 100ml. A magnet was added . Hair roots were placed in microcentrifuge tubes containing 200µl hair buffer. 1µl Proteinase K (20mg/ml) and 7µl DTT (1M) was added, and the solution was then stirred using a vortex MS1 minishaker (IKA). Hair roots were then incubated in the solution for 3-6 hours at 56ºC using the DB-2D Dri-block (Techne), or until the hair was completely dissolved. The sample was vortexed and incubated at 100ºC for 8 minutes. After incubation, the sample was vortexed again and centrifuged for 1 minute at 8050 *x g*. The supernatant was transferred to a new microcentrifuge tube, removing chelex resin, and stored in a refrigerator at 4^{o}C until amplification, or at -20ºC for long term storage.

Animal tissue was cut into approximately 25 mg pieces before DNA isolation was performed either with 'DNeasy Tissue Kit' (Qiagen) or using a proteinase K digestion followed by a Phenol-Chloroform extraction (Sambrook et al. 1989). Cut tissue was placed in a microcentrifuge tube with 500µl Laird's buffer consisting of; 1,21g Tris, 0,19g EDTA disodium salt, 1,17g sodium chloride, 1ml 20 % sodium dodecyl sulfate (SDS) and 99ml double distilled water (ddH_2O). 15µl Proteinase K enzyme (20mg/ml) was added to the tubes that were incubated at 37ºC overnight, or until the tissue was dissolved, in a rotator incubator Hir10M (Boekel Scientific). Extraction was carried out with the Phenol-Chloroform method.

Saliva was collected from bite marks on dead prey. Saliva was at sampling smeared on a 'cotton bud' and placed in a tube designed for this purpose. Upon arrival at the laboratory, samples were extracted by the standard Phenol-Chloroform extraction method with some additional preparations. The cotton buds with samples were usually dried at the time of extraction, so the tip of the cotton bud was broken off and placed in a tube with 1ml of Laird's buffer and were incubated for approximately 4 hours at room temperature. 400µl were then transferred into a second tube containing 15µl proteinase K enzyme (20mg/ml) and incubated at 37ºC overnight in a rotator incubator Hir10M (Boekel Scientific). Extraction was carried out according to the Phenol-Chloroform method described in Sambrook et al. 1989. The dried pellet acquired from the DNA-isolation was dissolved in 100µl purified water and cooled overnight at 4^{o}C until absorption measurement of DNA-concentration with a ND-1000 spectrophotometer (NanoDrop Technologies).

DNA quality testing

Extracted samples of droppings, hairs and saliva were quality tested to see in which samples DNA isolation had been successful, and to ensure that the samples came from lynx. A microsatellite marker called Fca001 (Fca from *felis catus* meaning domestic cat) (Menotti-Raymond, 1999), only found in felines, was amplified using specific primers in a PCR reaction. DNA amplification was carried out in 10µl reactions containing 1x PCR buffer, 3 mM $MgCl_2$ (Naxo), 0.2 mM dNTPs (GE Healthcare), 0.32µM of each primer (Invitrogen) and 0.45 units (U) Smart Taq Hot Thermostable DNA polymerase (Naxo). 0.1mg/ml bovine serum albumin (BSA) was also added to the reaction (GE Healthcare). Amplification reactions were performed in 'Geneamp PCR System 9700' from Applied Biosystems. Initial denaturation was carried out at 95ºC for 15 minutes, followed by 45 cycles at 94ºC for 30 seconds, 55ºC for 40 seconds, 72ºC for 45 seconds. Final extension was made at 72ºC for 10 minutes. All samples were run in duplicates, and with every run negative controls from extraction, a PCR blank and a positive control (sample positive for marker FCA001) were included. The PCR-product was then separated in an agarose gel electrophoresis using a 2 % mini-gel 'E-Gel 96', containing ethidium bromide and 'E-Base system' (Invitrogen). Amplification products were visualized with UV-light and photographed.

Table 2. Feline microsatellite markers and their known allele size range in Swedish lynx

Micro-satellite	**Forward primer**	**Reverse primer**	**PCR product range (base pairs)**
FCA001	TGCTTGTCCTCTCCCTCG	TGACTGCGCCATAGCTTTC	172-190
FCA008	ACTGTAAATTTCTGAGCTGGCC	TGACAGACTGTTCTGGGTATGG	131-139
FCA043	GAGCCACCCTAGCACATATACC	AGACGGGATTGCATGAAAAG	111-115
FCA045	TGAAGAAAAGAATCAGGCTGTG	GTATGAGCATCTCTGTGTTCGTG	142-146
FCA090	ATCAAAAGTCTTGAAGAGCATGG	TGTTAGCTCATGTTCATGTGTCC	99-113
FCA149	CCTATCAAAGTTCTCACCAAATCA	GTCTCACCATGTGTGGGATG	126-131
FCA391	GCCTTCTAACTTCCTTGCAGA	TTTAGGTAGCCCATTTTCATCA	222-238
FCA506	AATGACACCAAGCTGTTGTCC	AGAATGTTCTCTCCGCGTGT	179-203
FCA559	GCCAAAATGTTCAAGAGTGG	TTTTGGCTTGATGAGCATCA	105-125
F115	CTCACACAAGTAACTCTTTG	CCTTCCAGATTAAGATGAGA	223-248

Microsatellite analysis

Samples positive for the feline species microsatellite marker (Fca001), were analyzed further with multiplex PCR for 10 different microsatellite markers followed by capillary-electrophoresis. The markers were Fca001, Fca043, Fca008, Fca045, Fca090, F115, Fca391, Fca149, Fca506 and Fca559 (Menotti-Raymond, 1999) as seen in table 2. And they were pooled in the following fashion for PCR reactions;

- Fca001 + Fca043
- Fca008 + Fca045
- Fca090 + F115 + Fca391
- Fca149 + Fca506 + Fca559

DNA amplification was carried out in 10µl reactions containing 1x PCR buffer, 3 mM $MgCl_2$ (Naxo), 0.2 mM dNTPs (GE Healthcare), 0.32µM of each primer (Invitrogen) and 0.25 U Smart Taq Hot Thermostable DNA polymerase (Naxo). 0.1mg/ml BSA was also added to the reaction (GE Healthcare). Amplification reactions were performed in 'PTC-225^{TM} - DNA Engine Tetrad' (MJ Research) and initial denaturation was carried out at 95ºC for 15 minutes. This was followed by 39 cycles at 94ºC for 30 seconds, 55ºC for 40 seconds and 72ºC for 45 seconds. Final extension was made at 72ºC for 10 minutes. All samples were analyzed in three replicates, as well as a PCR blank. The PCR-products were then pooled and diluted for fragment size analysis. Two pools were created by dividing PCR-products for markers accordingly:

- Fca001 + Fca043, Fca149 + Fca506 + Fca559
- Fca008 + Fca045, Fca090 + F115 + Fca391

Diluted and pooled PCR-products were analyzed together with size standard ET-ROX 400 for fragment sizes in the capillary-electrophoresis instrument MegaBace 1000^{TM} (Amersham) following the manufacturers protocols. In cases when multiplex-PCR proved unsuccessful, samples were tested in single PCR-reactions for separate markers. PCR-reactions were then carried out in the same way as for the DNA quality test. Analysis of data for allele sizes was done using the software program 'Genetic Profiler v2.2' (Amersham). Figure 1 represents an example of how alleles could look when scoring them for sizes for a specific marker.

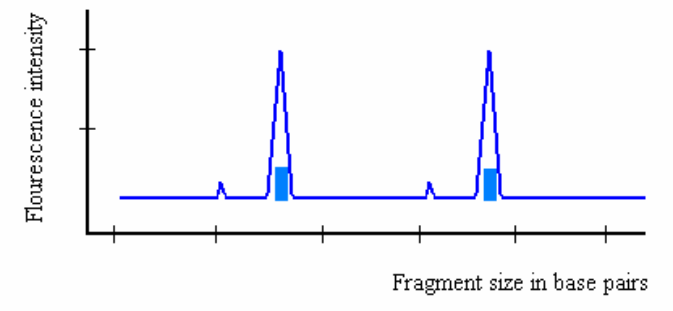


Figure 1. Basic representation of alleles when scoring allele sizes for a specific marker

Criteria for heterozygotes and homozygotes

For an individual to be accepted as heterozygous for a specific locus, the two alleles had to be confirmed by two unanimous replicates. Homozygosity had to be confirmed by three unanimous test results for the locus.

Table 3. Example of compiled genotype for a sample

Sample-number	Fca001		Fca008		Fca043		Fca045		Fca090		Fca115		Fca149		Fca391		Fca506		Fca559	
LS07-011	176	180	137	137	115	115	144	144	111	111	231	244	129	129	226	238	201	201	113	113

Genotype matching

Genotypes retrieved from samples were matched to each other and genotypes of already identified individuals (assigned separate identification numbers) in an internal database, using a Microsoft Excel macro called 'Microsatellites'. Table 3 is an example of how the compiled genotype of a sample could look.

RESULTS

DNA quality testing

DNA quality tests were carried out on 128 of the samples, 18 samples were accepted without DNA quality testing to fill out spare room on PCR master plates. Out of 128 samples tested, 75 were positive for the Fca001 marker specific for felines.

Microsatellite analysis

48 of the 146 samples could be genotyped for all ten markers during the ten weeks of this study. Another 40 samples had an incomplete set of markers identified, which with additional analysis could have been genotyped for all ten microsatellites. Of the samples with all ten markers, 12 were matched to individuals already tested in the internal lynx database (as seen in table 4). The remaining 36 samples were registered as 'new' individuals, and of these, three 'new' individuals had been sampled twice.

Table 4. Samples matched to each other or to identified individuals in internal lynx database

Unique Identification number	Matched sample(s)	Sampled first time in	Sample-number(s)	Sex	County of origin
L29	LS06-160	January 2003	LS03-40	Female	Jämtlands län
			LS03-63		
L113	LS06-169	January 2006	LS06-103	Male	Kalmar län
	LS06-172		LS06-104		
L83	LS07-037	~ April 2005	LS05-067	Male	Blekinge län
-	LS07-053	January 2007	-	Male	Blekinge län
	LS07-039				
L41	LS07-063	February 2003	LS04-005	Male	Kalmar län
			LS04-006		
			LS05-063		
			LS05-068		
			LS06-084		
			LS06-098		
			LS06-099		
			LS06-100		
-	LS07-045	March 2001	LS04-013	Male	Östergötlands län
	LS07-066				
L98	LS07-078	February 2005	LS05-106	Female	Jämtlands län
-	LS07-106	January 2007	-	-	Norrbottens län
	LS07-136				
-	LS07-114	March 2007	-	-	Kalmar län
	LS07-115				
-	LS07-128	January 2007	-	-	Norrbottens län
	LS07-132				
L128	LS07-146	February 2006	LS06-047	Female	Norrbottens län
	LS07-147				

The number of samples that failed in the DNA quality test, or that were discarded because of inconsistent PCR amplification across all loci, came to a total of 58. This would suggest a total success-rate of ~60 %, when the 40 samples with an incomplete set of genotyped microsatellite markers were added to the equation.

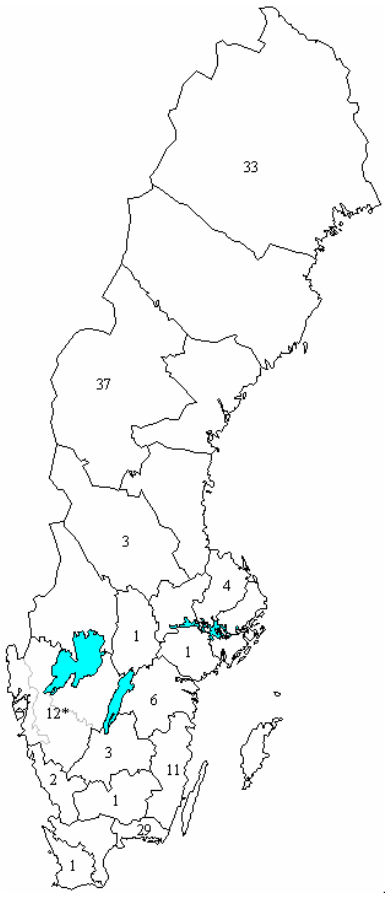


Figure 2. Map showing number of samples collected by county. Two samples were of unknown origin.

** Samples from Skaraborg län, Göteborg and Bohus län and Älvsborg län were grouped into one (Västra Götaland), according to the joining of counties in 1999.*

Figure 2. shows counties where the samples used in this study were collected. The number of successful samples compared between northern and central/southern Sweden were similar (61,4 % and 60,8 % respectively).

Out of 102 stools sampled, 59 were successfully genotyped (including samples that with additional analysing could have been genotyped for all ten microsatellites), and from six fur samples, four could be analysed. 22 hair samples were included in the study, and a total of ten samples were successfully analysed. Five out of five specimens of saliva could be genotyped, though no success-rate was calculated for saliva since, based on this data, it would be misrepresentative (see Discussion). Out of eleven tissue samples ten could be analysed. Success-rates for the different types of samples are illustrated in figure 3. Table 5, on the other hand, shows the genetic variability of the ten microsatellite markers used in this project.

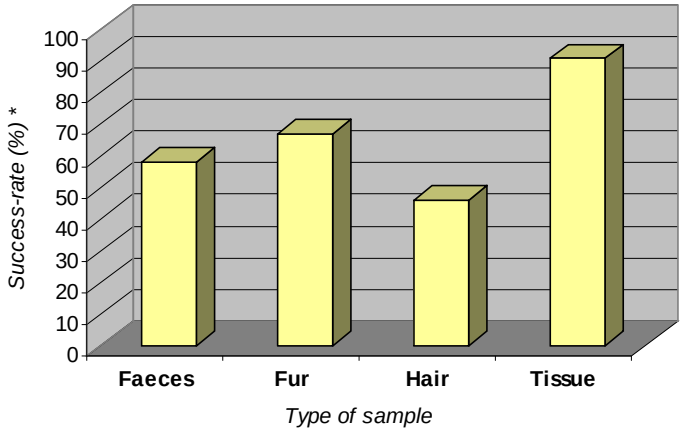


Figure 3. Success-rate by sample type

** Samples, that with additional analysing could have been genotyped for all ten microsatellites, were treated as successful.*

Table 5. Genetic variability of ten feline microsatellite markers in Swedish lynx

Locus	Number of alleles
Fca001	4
Fca008	4
Fca043	3
Fca045	2
Fca090	3
F115	7
Fca149	3
Fca391	4
Fca506	6
Fca559	4

DISCUSSION

The aim of this report was to show how non-invasive sampling and the use of genetic markers enables the identification of lynx individuals and also, to some extent, to evaluate the methods used for this purpose.

Recent developments in molecular technology and non-invasive sampling provide opportunities to improve the accuracy of population estimates, information of home range-sizes, habitats and more. Non-invasive sampling is a useful alternative to the

trapping of animals to retrieve tissue or blood samples, especially for elusive or rare species such as lynx. It does have its limitations though; the low quantity and quality of DNA obtained from such samples results in an increased risk of genotyping errors.

Only 48 of the 146 samples were genotyped for all ten markers and another 40 samples had incomplete genotypes. This shows that the time span of ten weeks was not long enough for acquiring sufficient data for this study. With more time I am confident that additional analysis could have produced complete genotypes for these individuals. Some samples failed the DNA quality test or were discarded after genotyping because of poor amplification in the PCR (*n: 58*). The most common reason why some samples could not be analysed was because of low quality of the sample. This could be due to low DNA-concentration because of degradation of the DNA by micro-organisms if samples were not kept frozen after sampling. A low DNA-concentration can also occur because of PCR inhibiting substances (such as polysaccharides from food residue) in the droppings that prevents laboratory analysis (Prugh et al. 2005). A possible connection has been suggested between supernatant colour and the level of inhibitors in the sample. There was also the possibility that some droppings were wrongly identified as lynx droppings, and actually came from a different species.

It can be argued that different storing routines for faeces might improve amplification success-rates. It has been shown that storing samples at -80ºC in a buffer consisting of dimethyl sulfoxide, EDTA, Tris and sodium chloride gives higher success-rate in amplification, compared to samples stored in -80ºC without buffer. The same study shows that samples stored for a shorter period of time before amplification result in higher amplification success (33 months vs. 45 months gave 91% and 79% respectively). Also, the probability of successful amplification appeared to decrease as the age of the stool-sample increased.

The DNA extraction by the 'QIAamp stool mini kit' can be considered to be fairly robust. Although the colour of the supernatant of faeces samples extracted by the kit might play a role in amplification success-rates, samples with lighter supernatant colour have higher amplification success than darker samples (Prugh et al. 2005). Addition of a preparing step of stool samples – with gentle incubation in phosphate-buffered saline (PBS) once to isolate exfoliated epithelial cells – could give a significantly higher success in amplification through PCR, than from homogenate of faeces (Palomares et al. 2002). Phenol-chlorophorm extraction performed on tissue and saliva samples is a well-known and oft used method for DNA isolation; the only drawbacks being the hazardous qualities of the chemicals used and the risk of ethanol remains in the final DNA solution if executed poorly. The Proteinase K/Chelex resin method used for DNA isolation from hair in this project was the least successful method (success rate 46 %).

An approximated success-rate of 58 % for faeces samples was somewhat lower than the success-rate for faeces reported in 2006 (62 %) (Ø. Flagstad et al. 2006). The success-rates of hairs and fur (46 % and 67 % respectively) were lower than that of tissue (91 %). The success-rate of tissue samples was somewhat lower than results obtained in previous years. A problem regarding the samples of saliva also appeared during the scoring of alleles after genotyping. Some saliva-samples showed >2 alleles for some markers, which could suggest saliva from more than one animal. That could, most likely, be traced back to the actual sampling; since saliva is swabbed from the wounds of killed

animals, there is no way to know that only the preying lynx has eaten from the cadaver. Also, the success-rate for saliva-samples could not be calculated, since the results would be misrepresentative. Samples of saliva included in this study, were samples that showed good spectrophotometrically determined values after DNA-isolation. We failed to extract DNA from most of the saliva-samples from each batch. Unsuccessful saliva-samples were not included in this study, hence the excluding of the success-rate of saliva-samples in figure 1.

12 samples were matched to individuals sampled in previous years, and 36 samples were categorised as 'new' individuals. It must be noted that matching for individuals, based on genotypes acquired through PCR and autosomal microsatellite analysis, is rarely 100 % accurate. The enzymatic amplification of microsatellite regions often produces artefact fragments that differ in size by multiples of the number of nucleotides in the repeated unit (Ellegren, 1993). This is thought to occur due to polymerase slippage during the amplification process (Luty et al. 1990). One theory suggests that if a single DNA sequence is used as template, and polymerase slippage occurs during the first cycle of the PCR, the artefact could be amplified in the same proportion as the template DNA (Taberlet et al. 1996). These artifacts can – if especially intense – produce incorrect scoring of alleles that are similar in size.

A well known problem when working with autosomal microsatellite genotyping is a phenomenon called 'null alleles' (Björklund, 2005). This means that there are alleles in the population that do not amplify, or if they do, then they amplify poorly. The consequence of this occurring for a locus would be that one allele would amplify whilst the other would not, resulting in the sample being falsely scored as homozygous. Also, shorter fragments (100-200 bp) amplify more frequently than longer fragments (300-700 bp) in the PCR amplification process (Frantzen et al. 1998), and maybe even more so in these non-invasive samples.

Another problem, which I myself encountered whilst matching sampled genotypes with genotypes in the database, is so called allele drop-outs. Similar to the phenomenon 'null alleles', this means that the amplification of one allele disturbs the amplification of another (Björklund, 2005). It occurs more frequently in loci with large differences in allele sizes and it is thought to occur as a result of one allele (often the smallest) being amplified earlier in the PCR than the larger allele (Gagneux et al. 1997). On the electropherogram this can be seen as a shorter fragment giving off a much stronger signal than a longer fragment. If the total amount of DNA analysed is small, which often is the case when working with low quality DNA, the larger allele might fail to give off a visible signal and 'become' a drop-out. This effect can result in false homozygosity for certain loci. After matching, some of the genotyped samples in this study were, for certain loci 'questionable' homozygous, when considering sampling location compared to old sample locations for the matched individual and the possibility of allele drop-out. Such samples were placed in the category of 'new' individuals.

Strict rules regarding scoring of heterozygous and homozygous samples were employed, based on the combination of alleles for a specific locus. To obtain an even higher confidence level, Taberlet et al. (1996) suggest an initial procedure of acquiring three positive PCR results for a locus. An allele should then only be recorded if observed at least twice, and homozygous samples should only be scored as such after seven

experiments detecting the same allele. Their study claims that this procedure would ensure a confidence level of 99 %.

Regarding the genetic variability of the microsatellite markers employed in this project, the low variability of some of the markers brings forth the question how informative these markers are for individual matching? For example Fca-markers 043, 045, 090 and 149 presents only 2-3 different alleles for the samples used in this study. Genetic variability for two of these markers is presented in figure 4, below.

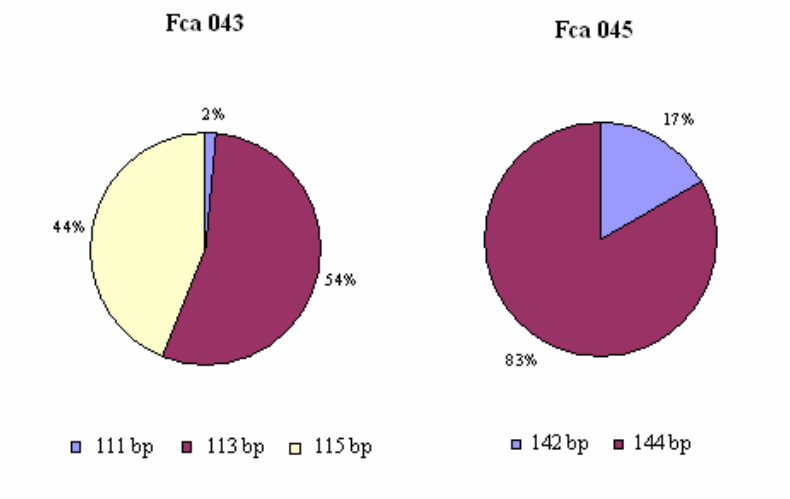


Figure 4. Genetic variability for loci Fca043 and Fca045 in Swedish lynx

Hellborg et al. (2002) estimated the mean number of microsatellite alleles as being significantly lower in Scandinavia compared to Finnish and Baltic lynx populations, with heterozygosity in Scandinavian lynx being consistent with a recent population bottleneck. This can most likely be traced back to the periods of intense hunting of lynx in Sweden.

Alternatives to this kind of microsatellite genotyping could be the analysing of samples for single nucleotide polymorphisms (SNPs) or Insertions/Deletions. Higher numbers of genetic markers would then have to be used for SNP analysis, since only two possible alleles for every marker would be available. Intron sequences with SNPs would then need to be mapped, and specific primers produced for PCR of fragments, and genotype information retrieved from pyrosequencing, for example. However, sequencing all samples would not only be very time consuming, but also presumably much more costly. Insertion/deletion analysis would also only produce two alternative genotypes for each loci; either the fragment is subject to an insertion/deletion or not, hence a higher number of markers would have to be used. Analysing for fragment size could be carried out with the capillary-electrophoresis instrument presently used, but several other alternatives would be available; determining the fragments molecular weight using a microchip-technique, for example. However, compared to microsatellites, both SNP and insertion/deletion would, as genetic markers, contain much less information useful for population studies.

In conclusion the risks associated with genotyping non-invasive samples by microsatellite markers, such as inaccurate homozygous scoring of alleles, might be worth taking, as the information acquired through genetic analysis in combination with field observations gives a more comprehensive picture of the Swedish lynx population. Further development of current methods for DNA isolation and genotyping, combined with non-invasive sampling, could perhaps give a greater accuracy to population estimates.

ACKNOWLEDGEMENTS

Firstly I would like to thank Professor Hans Ellegren for giving me the opportunity to gain insight into this unique project. Many thanks to Malin Johansson and Cecilia Wärdig for mentoring me and generously sharing in discussion, advice and knowledge. Thank you Cecilia Lepp, Patrik Rumin and Axel Robertsson for your support and feedback. And last but not least my thanks to Michael Jones who helped me linguistically on revision of this report.

REFERENCES

M. Björklund (2005) *Molecular Ecology Notes*. A method for adjusting allele frequencies in the case of microsatellite allele drop-out **5**, 676-679

J.A. Eisen (edited by David B Goldstein and Christian Schlötterer) 'Microsatellites – evolution and applications' Oxford university press. 1999.

H. Ellegren (1993) Genome analysis with microsatellite markers, Swedish University of Agricultural Sciences.

Ø. Flagstad, C. Wärdig, M. Johansson et al. (2006) Foryngringer i den svenske gaupebestanden i 2006 belyst fra genetisk analyse av spillning (Restricted availability from the Evolutionary biology centre, Uppsala University)

M.A.J. Frantzen, J.B. Silk, J.W.H. Ferguson et al. (1998) *Molecular Ecology.* Empirical evaluation of preservation methods for faecal DNA **7**, 1423-1428

P. Gagneux, C. Boesch, D.S. Woodruff (1997) *Molecular Ecology*. Microsatellite scoring errors associated with non-invasive genotyping based on nuclear DNA amplified from shed hair **6**, 861-868

A.J.F. Griffiths, J.H. Miller, D.T. Suzuki et al. 'An introduction to genetic analysis', 6th edition. Freeman. 1996.

L. Hellborg, C.W. Walker, E. Knispel Rueness et al. (2002) *Conservation genetics.* Differentiation and levels of genetic variation in northern European lynx (*Lynx lynx*) populations revealed by microsatellites and mitochondrial DNA analysis **3**, 97-111

J.A. Luty, Z. Guo, H.F. Willard et al. (1990) *American Journal of Human Genetics*. Five polymorphic microsatellite VNTRs on the human X chromosome **46**, 776-783

M. Menotti-Raymond, V.A. David, L.A. Lyons et al. (1999) *Genomics*. A genetic linkage map of microsatellites in the domestic cat (*felis catus*) **57**, 9-23

F. Palomares, A. Godoy, A. Piriz et al. (2002) *Molecular Ecology*. Faecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx **11**, 2171-2182

L.R. Prugh, C.E. Ritland, S.M. Arthur et al. (2005) *Molecular Ecology*. Monitoring coyote population dynamics by genotyping faeces **14**, 1585-1596

J. Sambrook 'Molecular cloning: a laboratory manual', 3rd part title. Cold Spring Harbor Laboratory Press. 1989.

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

The Swedish Environmental Protection Agency 'Action program for the conservation of lynx' number 22, produced 28th June 2000, revised 13th November 2003. [author's translation]

The Swedish Environmental Protection Agency [author's translation]

www.naturvardsverket.se/index.php3?main=dokument/natur/rovdjur/rovdok/politik.htm, accessed 070301

Picture on front cover: S. Widstrand / www.de5stora.com