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Original Investigation

Hair snaring and molecular genetic identification for reconstructing the spatial structure of Eurasian lynx populations

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ABSTRACT

Non-invasive genetic sampling (NGS) is being increasingly applied in wildlife monitoring and population genetic research. This study was designed to evaluate the use of NGS for reconstructing the spatial structure of populations of large felids. We developed a procedure for reliably genotyping individuals of Eurasian lynx (*Lynx lynx*) from samples obtained through a hair-trapping scheme based on a network of lynx scent-marking sites. The spatial locations of the identified genotypes were matched with the home ranges distribution of radio-tracked individuals, thus cross-checking the accuracy of the two methods. We analyzed DNA extracted from 170 hair samples and 11 blood samples from live-trapped lynx collected in 2004–2009 in the Białowieża Primeval Forest, Poland. We obtained PCR products in 96 (67%) hair samples; 82 (85%) of them were reliably genotyped at 12 autosomal microsatellite loci following a multiple-tubes protocol and stringent quality-controls of the data set. The sample included 29 distinct genotypes; 18 were found only in hair samples, five were determined only in live-trapped animals, and six in both hair and blood samples. Based on linkage disequilibrium we estimated an effective population size $N_e = 20.3$ (90% CI = 15–28). The total population size estimated with CAPWIRE was $N_c = 32$ (95% CI = 25–37) in close agreement with the observed number of genotypes. The genotypes obtained from hair samples were re-sampled on average 3.9 times and 50% of them were recorded for more than one year. The spatial distribution of six hair-genotypes was consistent with their home ranges obtained by radio-tracking in the same period. The distribution ranges of hair-trapped genotypes overlapped on average in 86.4% (mode 100%) with home ranges of the corresponding individuals. Hair-trapping and molecular identification is a reliable method for reconstructing the spatial organization of lynx population. It is likely to be also efficiently used in other rare and endangered species of felids in combination with data from other monitoring techniques, such as radio- and snow-tracking and photo-trapping.

Introduction

Although non-invasive samples, such as scats or hair, are considered valuable sources of DNA (Waits and Paetkau 2005), most of research concerning wildlife population genetics is still based on higher quality tissues or blood samples (e.g. Eizirik et al. 2001; Pilot et al. 2006; Nikolov et al. 2009). As long as tissues can be collected without threatening the conservation status of the species and without interfering with the dynamics of the studied populations, bioptic samples warrant providing sufficient material, and are used to generate genotypes that are mostly error-free (Paetkau 2003). On the other hand, elusive, rare and endangered species are often protected by strict conservation rules that preclude obtaining good quality samples, while demographic and genetic monitoring of their populations is recommended to improve the design and efficiency of conservation measures (Breitenmoser et al. 2000; Delibes et al. 2000; Nichols and Williams 2006). In these cases, non-invasive genetic sampling (NGS) might be the only practical way to generate the needed information (Schwartz et al. 2007).

NGS, eventually integrated with other monitoring tools like camera-trapping (Woods et al. 1999; Davison et al. 2002; Rowcliffe and Carbone 2008), is particularly useful in long-term monitoring projects, because demographic and genetic information can be obtained through sampling schemes replicated in time, without the need to capture or even to observe the animals (Taberlet et al. 1999). Hair can be trapped and scat can be collected within well planned randomized sampling schemes (Boulanger et al. 2006), individual capture-recapture histories can be reconstructed and analyzed using a variety of population genetic and demographic approaches (De Barba et al. 2010; Gervasi et al. 2010). Sampling schemes should

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be integrated with reliable laboratory protocols, which guarantee correct individual genetic identifications, avoiding the risks of generating false genotypes in consequence of human errors, false alleles (FA) and allelic drop-out (ADO, Pompanon et al. 2005). Errors in NGS can be identified through complex multiple-tubes and quality-control procedures. For these reasons, NGS projects should be carefully planned (Taberlet et al. 1999), and the results of only a few long-term NGS monitoring projects have been reported, so far (Boulanger et al. 2004; Fabbri et al. 2007; De Barba et al. 2010).

NGS has been successfully used in various carnivore species, including felids, with an array of hair-trapping methods (Woods et al. 1999; Mowat and Paetkau 2002; Palomares et al. 2002; Weaver et al. 2005; McKelvey et al. 2006; Schmidt and Kowalczyk 2006; Sawaya et al. 2011). Those sampling protocols were aimed at detecting presence of the species, estimating the population size and analyzing the population genetic diversity. However, no study has been designed for more detailed research on the spatio-temporal distribution of individuals. The hair-snaring methods proved to work well in a number of felids, including the North American (*Lynx canadensis*: McDaniel et al., 2000) and Eurasian (*Lynx lynx*: Schmidt and Kowalczyk, 2006) lynx species, but the potential of this method for solving ecological questions has not yet been addressed. Although some interesting insights into the lynx's population genetic structure and ecological processes were recently achieved using invasively (but opportunistically) obtained samples (e.g. Rueness et al. 2003; Schwartz et al. 2003; Janečka et al. 2006; Schmidt et al. 2009), hair-sampling procedures may open up new research possibilities, especially in studies of protected populations.

In this paper we are going a step farther compared to previous research as we aimed at providing a detailed field and laboratory protocol for studying population spatial structure with NGS data in felids. In particular we aimed at testing: (1) the reliability of the hair-sampling protocol developed by Schmidt and Kowalczyk (2006) for the Eurasian lynx for obtaining good quality DNA; (2) the feasibility of individual lynx identification by microsatellite genotyping of hair samples and following their relocations over time and space; and (3) the consistency of spatial distribution of individuals genotyped from hairs with those monitored via radio-tracking.

Material and methods

Study area

The study was conducted in 2004–2009 in the Białowieża Primeval Forest (BPF, 52°30′–53°00′N, 23°30′–24°15′E), Poland, located on the Polish-Belarussian border. The BPF is a temperate mixed lowland forest, characterized by a high percentage of natural stands (Faliński 1986). The entire forest area is 1500 km^2 and its Polish side is 600 km^2. Most of the Polish side of the BPF (500 km^2) is managed by state forestry, while the rest is protected as the Białowieża National Park (BNP, 100 km^2) with a 50 km^2 zone under strict protection. The area is flat and easily accessible for vehicles by a dense network of dirt roads that usually follow a regular grid of square forest compartments (1 km × 1 km) (Fig. 1). The average temperature during the study was −3.9 °C in the winter (December–March) and 19.1 °C in the summer (June–September). Snow cover persisted for an average of 96 days per year from November to March. The lynx population in the Polish part of BPF has been estimated by snow- and radio-tracking at 29 individuals in 1994 (Jędrzejewski et al. 1996). However, the population apparently decreased by about 30–35% in 2003–2006 (Schmidt 2008).

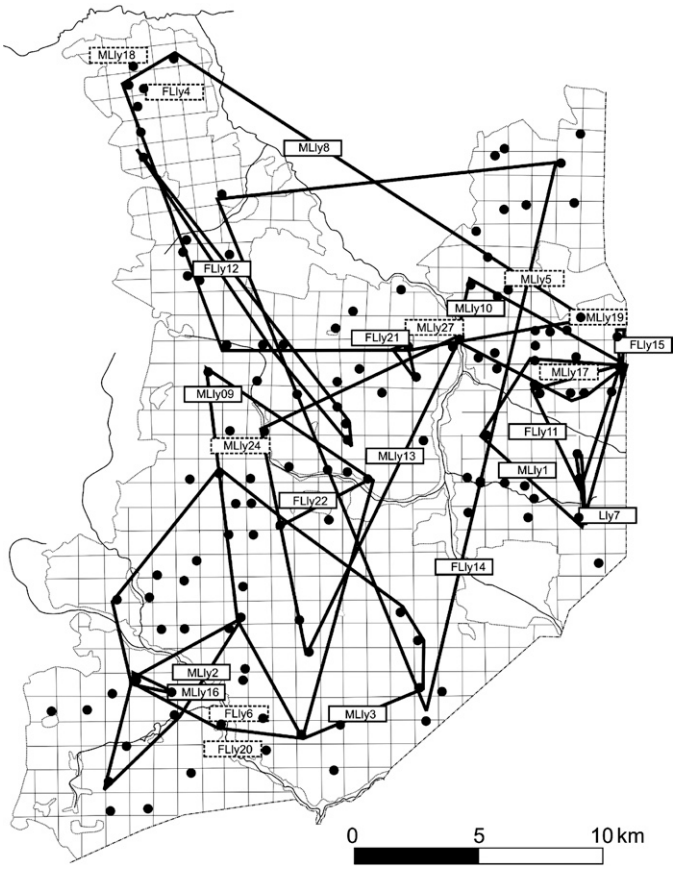

Fig. 1. Distribution of lynx individuals genotyped based on non-invasive hair sampling in the Białowieża Primeval Forest, Poland. Polygons denote ranges of particular individuals (M, males; F, females), solid rectangles refer to genotypes recorded more than two times and dashed rectangles are those recorded only once; points denote the hair-trapping sites. The square grid represents the forest area with a network of compartments (partly corresponding with road system) used to design the hair-trapping composition.

Sample collection

The hair-trapping system was based on a network of 153 lynx scent-marking sites identified by snow-tracking (Schmidt and Kowalczyk 2006). We placed hair-traps (8 cm × 8 cm pieces of carpet supplied with 10 nails) directly on the scent-marked objects spaced at 1–2 km intervals and distributed along forest roads in the entire Polish part of BPF (Fig. 1). To evoke cheek-rubbing in lynx we applied a mixture of beaver castoreum and catnip oil to each trap (see Schmidt and Kowalczyk 2006 for details on the sampling and baiting protocols applied for the Eurasian lynx in BPF and efficiency of hair collection). We conducted the surveys in 2004–2006, in different periods of the year in sessions lasting for 7–13 weeks with 36–113 (mean = 85.2 ± 29.0) scent-stations per session. Majority of samples were collected in winter months (December–March). As the lynx home ranges in BPF were 133 and 250 km^2 for females and males, respectively (Schmidt et al. 1997), there were approximately 30 traps within an average lynx home range. We attempted to check and re-bait hair-traps every 10–14 days though the actual range of intervals was 1–23 days. Hairs were dried at temperature 30–40 °C and stored in paper envelopes at room temperature in plastic boxes with silica gel for 2–5 years before analysis (following Wasser et al., 1997; Roon et al. 2005).

Additionally, blood samples were collected from 11 (5 males, 6 females) live-trapped lynx. The lynx were captured during winter using foot-snare traps (Breitenmoser 1989) set at fresh ungulate kills and with wooden box-traps (Kolbe et al. 2003). Snare

traps were equipped with radio-alarm systems (Wagener Telemetrieanlagen HF-NF Technik, Köln, Germany) and the box-traps with commercial alarm systems based on GSM (Abratronik, Wrocław, Poland) that allowed us to release the captured animals from the traps within 15 min to 2 h. Lynx were immobilized with a mixture of ketamine hydrochloride (5 mg/kg of body weight) and xylazine hydrochloride (6 mg/kg of body weight) and equipped with radio-collars (Wagener Telemetrieanlagen) that weighed approximately 230 g (that is 1–2% of the lynx body mass). The effect of xylazine was reversed with atipamezole hydrochloride (0.5 mg/kg). Blood samples (1 ml) were drawn from a radial vein with an EDTA vacutainer, and stored frozen at −20 °C. Trapping, handling and radio-tracking procedures were approved by Polish Ethical Commission for Research on Animals.

DNA extraction and amplification

DNA samples were extracted using a guanidine thiocyanate/silica protocol (Gerloff et al. 1995). Genotypes were obtained by typing twelve microsatellite loci, which were originally described in the domestic cat (Menotti-Raymond et al. 1999) and that amplified successfully in the lynx (Table 1). These loci were amplified by multiplexed PCR reactions in 8 μL total volume containing 2 μL of template solution (c. 20 DNA nanograms), 3.5 μL of 2× QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$), 0.7 μL of 5× Q-solution (QIAGEN Multiplex PCR Kit), 0.175 pmol of each primer, and the following thermal profiles: (1) 95 °C for 15 min; 12 cycles at 94 °C for 30 s, 62.2 °C for 1 min and 30 s, and 72 °C for 30 s; 27 cycles at 94 °C for 30 s, 60 °C for 1 min and 30 s, and 72 °C for 30 s; 60 °C for 10 min. PCRs were performed in a dedicated room, under a sterile air-flow hood cleaned with UV light. Negative and positive controls were added. Fragments were analysed using an ABI 3130 XL automated sequencer and software GeneMapper 3.0 (Applied Biosystems). Individual genotypes were sexed using a gender-specific marker, the zinc-finger XY gene (ZFXY; Pilgrim et al. 2005), which produces a 163 bp Y-linked allele and a 166 bp X-linked allele, generated by a 3 bp deletion in Zfy as compared to Zfx. The males were identified by heterozygote 163–166 bp genotypes, while the females produced homozygote 166–166 bp genotypes. All these loci were re-amplified to test for amplification errors, using the following multiple-tubes protocol.

The multiple-tubes genotyping protocol

Hair samples were genotyped using the following multiple-tubes procedure. (1) A screening step was designed to identify and remove low-quality DNA samples. All samples were amplified four times at two loci (Fca008 and Fca149) selected for their good amplification rates and reliability; samples showing ≤50% positive PCR (PCR+) were removed. (2) All samples which passed the screening were further processed to obtain four additional PCR replicates per locus. Multilocus genotypes were analysed with software RelioType (Miller et al. 2002), which computes the maximum-likelihood of each genotype in the sampled population (reliability score *R*), and estimates the number of additional replicates needed for each locus to obtain a reliable genotype (see step 3). Maximum-likelihood estimates are obtained assuming that allele frequencies of the sampled population are known. Reference allele frequencies were estimated from the genotypes of 11 individual blood samples collected from captured lynx. We used RelioType with the default settings. A sample was accepted as reliable if $R > 0.95$. Each allele was accepted as "true" if it has been seen at least twice in PCR replicates at putative heterozygous loci, or at least three times at putative homozygous loci. (3) Samples with low amplification success and $R < 0.95$ were further replicated four times. A final reliability analysis led to definitive genotype acceptance (if $R > 0.95$) or rejection. Reliable genotypes were used to identify the consensus genotypes, using Gimlet 1.3.3 (Valière 2002) with the threshold method: an allele was retained in the consensus genotype if it was scored at least two times. The consensus genotypes were used as "true" reference genotypes to compute the frequency of PCR errors. The blood samples were replicated twice using the same multiple-tubes protocol.

PCR errors are expressed per-genotype as the observed number of erroneous genotypes divided by the number of genotypes in which an error could have been observed (Broquet and Petit 2004). Allelic dropout (ADO) was counted as the proportion of alleles which did not amplify per each heterozygous locus. False alleles (FA) were identified as follows: (1) alleles originating from non-specific amplifications were identified by their molecular weight which was outside the range detected in the reference genotypes; all those FA were easily identified and not recorded; (2) alleles originating from cross-sample laboratory contaminations during PCR processing: alleles showing the molecular weight of true alleles were defined as FA if they were detected only once at least in seven replicated PCRs; accepting these alleles could have led to define false heterozygotes; (3) alleles originating from mixed samples, that is from DNA extracted from samples containing hair from more than one individual (Roon et al. 2005). In these cases, three or four distinct alleles could be detected at some polymorphic loci. Only the last two kinds of FA were recorded.

Genetic variability analysis and estimates of population size

Individual genotypes were recorded in a database, and the software GenAlEx 6.1 (Peakall and Smouse 2006; http://www.anu.edu.au/BoZo/GenAlEx) was used to estimate: allele frequency by locus and population, observed (H_O) and expected unbiased (H_E) heterozygosity, mean number of alleles per locus (N_A), departures from the Hardy–Weinberg equilibrium (HWE), the unbiased (PID_{unb}) and the expected (PID_{sibs}) probability-of-identity among full sib dyads (Mills et al. 2000). Wright's inbreeding estimator F_{IS} (Weir and Cockerham 1984), pairwise linkage equilibrium (LD) and departures from HWE were computed also using Genetix 4.05 (Belkhir et al. 1996–2004; http://www.genetix.univ-montp2.fr/genetix/genetix.htm). Single locus Hardy-Weinberg tests for null alleles and heterozygote deficiency were obtained using Kalinowski's ML-Relate software (Kalinowski et al. 2006a, http://www.montana.edu/kalinowski/Software/MLRelate.htm) with 50,000 randomizations. Expected and observed mismatch (MM) values were computed using MM-dist (Kalinowski et al. 2006b). In a post-process control of the genetic database, we identified and screened the laboratory record of eventual genotype pairs differing at only 1-MM or 2-MM. Any dubious genotype was further PCR replicated four times and definitely accepted or rejected.

We used software Capwire (Miller et al. 2005) for estimation of total population size (N_c) based on the genotypes identified from hair-samples. The estimation is based on the number of resampling the genetically distinct individuals at multiple sampling events (in our case different years). We used a likelihood ratio test (LRT) to choose between two available models: the even capturability model (ECM) and the two innate rates model (TIRM). Due to unequal capture probability of different individuals determined by the test in our sample ($\alpha = 6.3$) the TIRM was selected. Calculation of N_c has been done for three years pooled (2004–2006) due to low sample size in particular years and resulting wide range of confidence intervals and inconsistency of estimates between years.

Pair-wise values of linkage disequilibrium across the loci were computed using the routine LINKDOS in Genetix, and the 11

Table 1
Sequences of the PCR primers (from Menotti-Raymond et al. 1999) labelled with Hex or Fam ABI dyes, used to amplify the microsatellite loci in the studied Eurasian lynx samples. The molecular weight and frequency of each allele, per locus values of observed (H_o) and expected (H_e) heterozygosity, result of the chi-square test (χ^2) for Hardy–Weinberg Equilibrium (HWE, ns = not significant), cumulative values of unbiased (PID_{unb}) and sibling (PID_{sib}) probability-of-identity are reported.

Locus	PCR primers	Alleles	Frequency	H_o	H_e	HWE	PID_{unb}	PID_{sib}
Fca088	AGGAAAATGAAGTCAAGAAAATGG	100	0.457	0.889	0.709	ns	1.171×10^{-01}	4.220×10^{-01}
	Fam-	104	0.022					
	TTTTTCTTTTTCCCGTAATACACA	108	0.043					
		110	0.065					
		112	0.043					
		114	0.174					
		116	0.196					
Fca126	Hex-	120	0.023	0.815	0.700	ns	1.418×10^{-02}	1.791×10^{-01}
	GCCCCTGATACCCTGAATG	122	0.045					
	CTATCCTTGCTGGCTGAAGG	124	0.455					
		126	0.114					
		128	0.182					
		132	0.182					
Fca026	GGAGCCCTTAGAGTCATGCA	131	0.064	0.593	0.673	ns	2.431×10^{-03}	8.258×10^{-02}
	Fam-	133	0.128					
	TGTACACGCACCAAAAACAA	135	0.021					
		137	0.447					
		139	0.319					
		141	0.021					
Fca077	Hex-	130	0.283	0.893	0.670	ns	4.197×10^{-04}	3.756×10^{-02}
	GGCACCTATAACTACCAGTGTGA	136	0.326					
	ATCTCTGGGGAAATAAATTTTGG	138	0.370					
		140	0.022					
Fca132	Fam-	166	0.217	0.679	0.628	ns	8.506×10^{-05}	1.800×10^{-02}
	ATCAAGGCCAACTGTCCG	170	0.348					
	GATGCCTCATTAGAAAAATGGC	174	0.435					
Fca008	Hex-	126	0.500	0.500	0.600	ns	2.339×10^{-05}	9.591×10^{-03}
	ACTGTAAATTTCTGAGCTGGCC	132	0.413					
	TGACAGACTGTTCTGGGTATGG	134	0.087					
Fca096	Fam-	196	0.159	0.500	0.457	ns	7.309×10^{-06}	5.684×10^{-03}
	CACGCCAAACTCTATGCTGA	206	0.023					
	CAATGTGCCGTCCAAGAAC	208	0.705					
		214	0.045					
		222	0.068					
Fca149	CCTATCAAAGTTCTCACCAAATCA	121	0.217	0.429	0.482	ns	2.326×10^{-06}	3.331×10^{-03}
	Fam-	123	0.674					
	GTCTCACCATGTGTGGGATG	125	0.109					
Fca043	Tet-	106	0.739	0.500	0.406	ns	8.753×10^{-07}	2.115×10^{-03}
	GAGCCACCCTAGCACATATACC	108	0.022					
	AGACGGGATTGCATGAAAAG	110	0.065					
		118	0.174					
Fca058	CATCCCTGACTAGCCTGAGC	203	0.717	0.500	0.375	ns	3.813×10^{-07}	1.388×10^{-03}
	Tet-	205	0.283					
	GTGAAGAAAGCTGGTGTGCA							
Fca045	Hex-	138	0.804	0.321	0.331	ns	1.808×10^{-07}	9.750×10^{-04}
	TGAAGAAAAGAATCAGGCTGTG	140	0.130					
	GTATGAGCATCTCTGTGTTCGTG	146	0.065					
Fca023	Hex-	126	1.000	0.000	0.000	Monomorphic	1.808×10^{-07}	9.750×10^{-04}
	CAGTTCCTTTTTCTCAAGATTGC							
	GCAACTCTTAATCAAGATTCCATT							

polymorphic loci. An estimate of the effective population size (N_e) was obtained by software LDNE (Waples 2006), through the amount of linkage disequilibrium (LD) that might be generated by genetic drift in small populations due to stochastic fluctuations in allele frequency occurring from generation to generation.

Comparing the radio-tracking and hair-trapping data

We were not able to estimate reliably the total lynx numbers from radio-tracking data as we have never attempted to capture the whole population. Only one to three individuals were radio-tracked simultaneously in particular years. Therefore, we had no possibility to use these data for estimation of lynx density. The radio-tracking data, however facilitated interpretation of snow-tracking estimates, resulting in the estimation of the entire population at 20.8 ± 0.85 (average $\pm$ SE, $n = 4$ sampling years) individuals in 2003–2006 (K. Schmidt and R. Kowalczyk, unpublished data). Thus, in our study we have not aimed at comparing population density estimates between hair- and radio-tracking data. On the other hand, we used information from radio-tracking to confirm the reliability of the genotypes obtained from hair-samples by comparing the spatial distribution of individuals genetically identified from hairs with location of home ranges determined by radio-tracking. The radio-tracking was conducted with standard methods described in details in Schmidt (2008). Data on six lynx (4 males, 2 females, with an average of 172 radio-locations per individual) were used in this study for estimation of home range. The home range sizes, the areas outlined by hair-trapping points of particular individuals and the spatial overlap between these areas were estimated with the Minimum Convex Polygon method in the Biotas™ software.

Table 2
Eurasian lynx (*Lynx lynx*) samples collected from hair-traps, or from blood samples in Białowieża Forest in years 2004–2009. The total number of distinct individual genotypes identified per year in hair or blood samples are indicated. The numbers in brackets indicate the number of genotypes already identified within the whole set of samples including the hair/the number of new genotypes recorded per year.

Samples	2004	2005	2006	2008	2009	Total
Hair-traps	83	59	28	0	0	170
Genotypes	16 [0/16]	16 [9/7]	9 [8/1]	0	0	24
Blood	1	1	3	3	3	11
Genotypes	1 [0/1]	1 [0/1]	3 [2/1]	3 [2/1]	3 [0/3]	11 [6/5]
Total genotypes	16	17	10	3	3	29

Results

Sampling and minimum number of genotypes in the BPF lynx population

In total 349 hair and 11 blood samples of Eurasian lynx were collected during the study. However, only those 170 hair samples, which contained reasonable amounts of hair roots (>10) were chosen for genetic analyses. The multiple-tubes procedure led to identify in total 31 distinct genotypes: 26 genotypes from hair (Fig. 1), and five (Lly23, Lly28, Lly29 Lly30 and Lly31) only from blood. Nine to 16 genotypes were identified in particular years (Table 2). All genotypes were completely defined at all 12 microsatellite loci and sexed, with the exception of Lly07 (that was not sexed because it did not amplify at the sexing marker), and Lly17, Lly24 and Lly27 which had one missing locus each. Two hair samples performed badly producing two distinct genotypes which had four missing loci each, that is: Lly26, showing an unusual number of PCR failures, and Lly25, showing an unusual number of FA, suggesting that it might originate from contaminated or mixed hair samples. Therefore, we decided to exclude these two, likely unreliable, genotypes.

The remaining 29 genotypes, that were considered reliable (average $R = 0.992$; min 0.975–max 0.999), included 16 males, 12 females and the unsexed Lly07 individual. Hence, the sex ratio was = 1.33 M:1.00F, not significantly different from 1:1 ($\chi^2 = 0.57$, $p > 0.3$). These 29 genotypes indicate the minimum number of individual lynx which were present in the BPF study area during years 2004–2009. Eighteen genotypes were identified in hair samples only. Six genotypes were detected both in hair and blood samples, thus strengthening the reliability of the genotypes obtained from hair samples (Table 3). Five other genotypes were detected only in blood samples, thus suggesting that hair trapping could still have missed some individuals of the investigated population. However, one of them (Lly23) was a subadult female that died one month after trapping and the remaining lynx were trapped and radio-tracked after the period of hair sampling (Tables 2 and 3).

Table 3
List of the reliable lynx genotypes sampled in Białowieża Primeval Forest, Poland, in years 2004–2009. The table indicates, for each genotype: the sex (genotype Lly7 was not sexed, NI), the reliability score (*R*), the minimum number of mismatches with the most similar genotype (MM), the number of re-sampling the particular genotypes per year (*N*). Genotypes with asterisk were sampled in both hair and blood samples, genotypes labelled with b were sampled only in blood. Re-sampling was calculated for the hair-sampling period 2004–2006.

Genotype	Sex	*R*	MM	Number of samples (*N*)					Total *N* 2004–2006
				2004	2005	2006	2008	2009	
Lly1*	M	1.00	>3	5	4		1		9
Lly2*	M	1.00	>3		4	3	1		7
Lly3*	M	0.99	2	9	2	3			14
Lly4	F	1.00	>3	1					1
Lly5	M	1.00	>3		1				1
Lly6	F	1.00	>3		1				1
Lly7	NI	0.99	>3	1	2				3
Lly8	M	1.00	>3	7					7
Lly9*	M	0.98	>3	3	3	2			8
Lly10	M	1.00	>3	2	1	5			8
Lly11	F	1.00	>3	2	3				5
Lly12*	F	0.99	3	4	1				5
Lly13	M	0.98	>3	4	1	1			6
Lly14	F	0.98	>3	2	1	1			4
Lly15	F	1.00	>3	1		2			3
Lly16	M	1.00	>3		1				1
Lly17	M	0.90	>3	1					1
Lly18	M	1.00	>3			1			1
Lly19	M	1.00	>3		1				1
Lly20	F	1.00	>3		1				1
Lly21*	F	1.00	2	1	1	2			4
Lly22	F	1.00	>3	3					3
Lly23b	F	0.98	>3			1			1
Lly24	M	1.00	2		1				1
Lly27	M	1.00	>3	1					1
Lly28b	M	1.00	>3				1		–
Lly29b	F	0.98	>3					1	–
Lly30b	F	1.00	>3					1	–
Lly31b	F	1.00	2					1	–

Table 4
Average proportion of PCR success (PCR+), allelic dropout (ADO) and false alleles (FA), obtained in lynx hair or blood samples.

Samples	PCR performance	Values
Hair	PCR+	0.785
	ADO	0.207
	FA	0.003
Blood	PCR+	0.834
	ADO	0.034
	FA	0.000

Genotyping errors

The average PCR success rate at the screening was 67% (96/170) of all hair samples. The number of hair samples that passed the screening step (PCR+ >50%) were 56/83 (67%), 34/59 (58%), and 21/28 (75%) in 2004, 2005 and 2006, respectively. After the screening step, the genotyping success rates were: 48/56 (86%), 28/34 (82%), and 18/21 (86%) in 2004, 2005 and 2006, respectively, corresponding to a very satisfying 85% success rate, on average. All the 31 genotypes were identified through 8 replicated PCR per locus per sample, on average (min 4, max 12). The average error rates in the non-invasive hair samples were: ADO = 0.21 and FA = 0.003, which was, not surprisingly, higher than error rates in the blood samples (ADO = 0.03 and FA = 0.000; Table 4).

Genetic diversity

All but one (Fca023) microsatellite loci were polymorphic in the BPF lynx sample (Table 1). The number of alleles ranged from 2 (in locus Fca058) to 7 (in locus Fca088); the observed heterozygosity was from $H_o = 0.33$ (in locus Fca045) to 0.89 (in locus Fca077). All loci were in HWE and, on average, the observed ($H_o = 0.55$; standard error SE = 0.07), expected ($H_e = 0.50$; SE = 0.06) and unbiased expected ($UH_e = 0.51$; SE = 0.06) values of heterozygosity were not significantly different (at threshold $P = 0.05$). Consequently, the slightly negative F value = −0.099 indicates a non-significant excess of observed heterozygotes ($P = 0.03$), which could be due either to spatial population structuring and non-random mating, but that should exclude undetected null alleles or ADO in the non-invasive genotypes. Results of Kalinowski's ML-Relate test for null alleles showed that only locus Fca026 had significant ($P = 0.0184$; no Bonferroni correction) departure from HWE, indicating heterozygote deficiency. However, after Bonferroni correction, also this locus did not show any significant departure from HWE ($P = 0.01/12 = 0.0008$). It should be noted that locus Fca026 has 6 alleles in the BPF lynx population, and some low frequency heterozygotes were not observed likely due to stochastic effects in this small sample size of 29 genotypes.

The cumulative probability-of-identity with 11 loci (the monomorphic locus Fca023 was excluded) were: $PID_{unb} = 2.3 \times 10^{-7}$, and $PID_{sibs} = 1.1 \times 10^{-3}$, sufficiently small to exclude any "shadow effect" in a population which should not be larger than 20–29 individuals. In fact, the estimated expected number of individuals with the same genotype (calculated as the PID × population size) was: 6.1×10^{-6} (computed using PID_{unb}) and 2.0×10^{-2} (computed using PID_{sibs}), which means that if the sampled population was composed only by full-sibs, the probability that two individuals have the same genotype is of 2 over 100 siblings.

The mismatch distribution showed that majority of genotypes differed at more than three loci (Table 3). Only two genotype pairs (Lly03–Lly24 and Lly21–Lly31) differed at two loci. The mismatch distribution of the empirical and expected MM-distributions were closely concordant: we did not observe any excess of 1-, 2- or 3-MM suggesting that the genotypes should not contain errors (Fig. 2). The MM-distribution also suggests that the population should be composed mainly of unrelated and parent-offspring (PO) individuals, because the observed and the expected unrelated distributions are closely overlapping. This panel of markers can be used also for reconstructing the genealogies in the studied population. In fact, the probability of exclusion when the genotypes of both parents are known is 0.99 with the 11 loci; and the probability of excluding two putative parents is 1.0 using only seven loci.

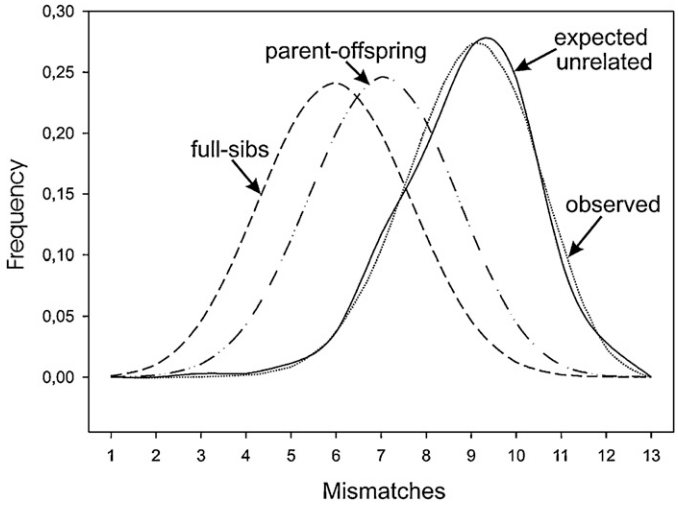

Fig. 2. Distributions of mismatches among genotypes of Eurasian lynx in Białowieża Forest, Poland. Plotting of the proportions of expected and observed mismatch values in lynx genotypes computed using the software MM-dist (Kalinowski et al. 2006b).

Linkage disequilibrium and estimates of the population size

Among pair-wise values of linkage disequilibrium across the loci only one comparison was significant at the threshold $P = 0.01$ (corresponding to $P = 0.0002$ after Bonferroni correction). The average value of linkage disequilibrium across loci was LD = 0.054. We used the slight observed LD, which could have been generated by stochasticity in the small isolated lynx population, to estimate an effective population size $N_e = 20.3$ (90% CI = 15.4–28.1). The total population size (N_c) estimated with Capwire was found to be 32 individuals (95% CI = 25–37), which is in close agreement with the observed minimum number of 29 genotypes.

Spatial distribution of the sampled genotypes

The 24 reliable hair-genotypes were resampled on average 3.88 times (range: 1–14) (Table 3, Fig. 1). More than half of the genotypes (14/24) were re-sampled 3–14 times, the others were sampled only once. The resampling frequency was insignificantly (Mann–Whitney U-test, $U = 52.5.0$, $P = 0.5$) male biased: each individual male was resampled 4.7 times, on average, while the females were resampled 3.0 times (Table 3). Male genotypes ($n = 14$) were recorded in sites distributed within larger (but not significantly, Mann–Whitney U-test, $U = 58.0$, $P = 0.8$) areas than those of females ($n = 9$; 35 and 28 km^2, respectively; Figs. 1 and 3). Twelve of the resampled hair-genotypes (50%) were identified in more than one year; a further evidence that these genotypes are reliable (Table 3).

Six genotypes obtained from hair-trapping (males: Lly1, Lly2, Lly3, Lly9 and females: Lly12 and Lly21) have been confirmed by genetic identification of the radio-tagged lynx individuals studied in 2004–2008. Spatial distribution of the hair-trapping sites

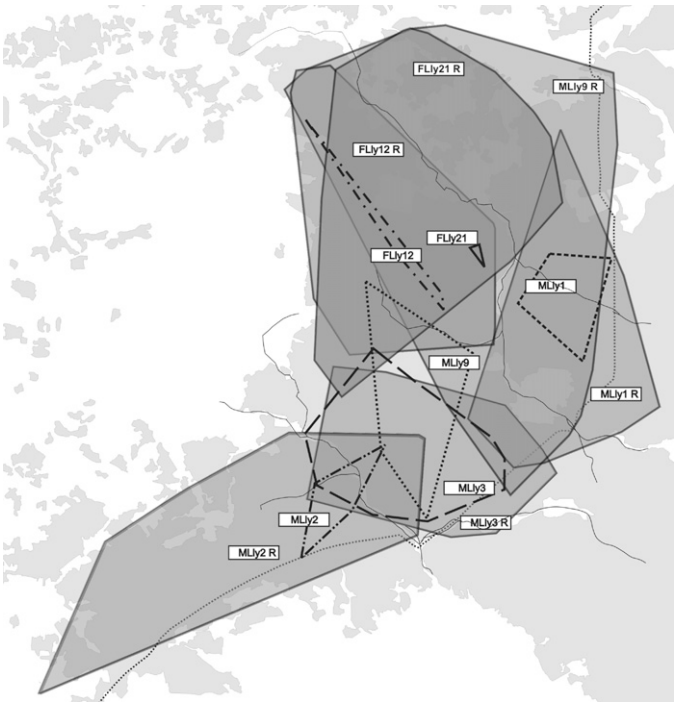

Fig. 3. Distribution of lynx home ranges based on radio-tracking (solid shaded polygons) and the ranges of the same individuals identified by genotyping hair samples (polygons with broken lines) in the Białowieża Primeval Forest. M, male; F, female; *R*, radio-tracked individual. Forest area is grey-shaded. Thin dotted line is the state border between Poland and Belarus.

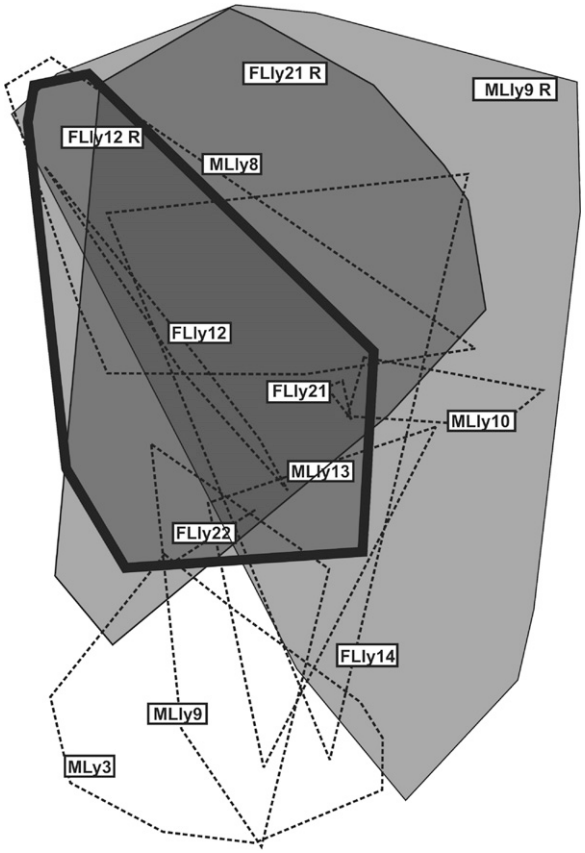

Fig. 4. An example of a lynx home range determined by radio-tracking (FLly12 R – polygon demarked with thick solid line) overlaid with overlapping home ranges of other radio-tracked lynx (MLy9 R and FLly21 R – polygons demarked with thin solid lines) and all individuals revealed by genotyping hair samples within its boundary (dashed-line polygons).

from where the samples were genetically identified mostly concurred with the home ranges of respective individuals (Fig. 3). The areas encompassed by scent-marking sites, where particular hair-trapped genotypes were recorded overlapped with home ranges of respective individuals on average in 86.4% (mode 100%). Only location of the genotype Lly9 determined from hair samples was poorly corresponding to the location of its radio-tracked home range (22.4%, Fig. 3). However, the movements of this individual were wide and aberrant anyway, with lack of contact for long periods giving possibility for missing occasional wandering outside the known range by radio-tracking.

The lynx home ranges determined by radio-tracking overlapped on average with three (from 1 to 4) ranges of other radio-collared individuals. In contrast, there were 7.3 (from 3 to 13) individuals recorded by genotyping the hair samples within the same home ranges (Fig. 4). The sex ratio among the radio-tracked lynx found within the boundaries of particular home ranges was on average 1.8:1 (M:F), while among the hair-genotyped individuals was 1.3:1. The size of lynx home ranges determined with radio-telemetry (mean for males and females pooled, $n = 6$: 272.5 ± 120.1 km^2) was on average 31 times (from 1.3 to 148.5) larger than the area determined with hair-trapped points of respective individuals.

Discussion

The main achievement of this study was to show the potential of using hair-trapping for describing the spatial structure in an Eurasian lynx population and its validation with data obtained from radio-tracking of known, genetically identified individuals. The protocol of hair sampling and storage presented in this paper proved to be an efficient method for obtaining DNA and reliably genotyping individuals. We have shown that it is possible not only to identify individuals, but also monitor their spatio-temporal relocations via subsequent repeated sampling and genotyping of their hairs collected within a specifically designed network of hair-snare system during a long-term study.

The rate of PCR success (67%) obtained in our study was satisfactory. Other studies based on non-invasively collected DNA samples (hairs and scats) provided similar or sometimes better results (Woods et al. 1999: 90%; Davison et al. 2002: 53%; Castro-Arellano et al. 2008: 60%; Haag et al. 2009: 50–78%). The amplification success rate may depend on weather conditions, sample age and storage time (Haag et al. 2009), and in case of the hairs also on size of the root (Ruell and Crooks 2007), which in lynx is very tiny. The good results obtained in our study using lynx hair collected at relatively long time intervals and stored for 5 years, suggest that our field and laboratory protocols can be successfully applied for NGS population genetic studies in felids.

As the non-invasively collected samples can be a source of genotyping errors (due to ADO and FA effects) we followed a very rigorous procedure to obtain high confidence in the reliability of genotyping the lynx individuals (Taberlet et al. 1996). By calculating the maximum likelihood of each genotype we were able to exclude non-reliable genotypes with high accuracy (Miller et al. 2002). Thus, we are certain enough that our procedures correctly identified true individuals present in the population. The number of unique lynx genotypes obtained during our research was higher than the population size estimated by simultaneous radio- and snow-tracking surveys (K. Schmidt and R. Kowalczyk, unpublished data). This finding could be the result of the protracted hair survey that allowed for recording new individuals recruited or immigrated into the population in different years. In fact, our study area encompassed only a part of the population, the majority of which inhabits

the Belarussian side of the forest (Jędrzejewski et al. 1996). The effective population size N_e estimated from linkage disequilibrium fell below the range of the total lynx population size N_c determined from hair-trapped genotypes for our study area in the Białowieża Forest, which is concordant with theoretical population genetics assumptions (Crow and Kimura 1970).

A substantial (50%) part of the hair-trapped genotypes were recorded for more than one year. The spatial locations of individual home ranges of radio-tracked lynx was highly concordant with the distribution of the hair-trapping sites from where the genetically identified hair samples were obtained. These results support the reliability of our methods on the one hand and emphasize their utility for tracking the spatial structure and long-term dynamics of the population. Moreover, hair-sampling revealed the presence of twice more individuals within the boundaries of radio-tracking home ranges than determined by live-trapping and radio-collaring. Genotypes' sex ratio was also closer to the expected 1:1 ratio (Breitenmoser-Würsten et al. 2007) than in case of radio-tracked individuals.

Low genetic variability in the sampled population, or small number of markers used in genotyping, might cause different individuals to show the same multilocus genotype. This "shadow effect" (Mills et al. 2000) can be minimized by increasing the number of loci. However, the genetic variability of the lynx population in the Białowieża Forest, was high enough to exclude any "shadow effect". The values of PID_{unb} and PID_{sibs} obtained with 11 unlinked microsatellites allowed excluding risks of sampling 2 genotypes identical by chance in a population of 100 individuals. As our study area was most likely occupied by 32 animals, we consider that the "shadow effect" has not affected the accuracy of genotyping in our study.

Despite high efficiency in obtaining hair-samples and genotyping the lynx individuals in presented protocol it has also some disadvantages that should be mentioned. There's a risk to collect mixed hair samples of different individuals at one trap (Roon et al. 2005), which is particularly likely if females with kittens were rubbing the same scent-stations. This may have produced false alleles in the genotypes. However, the FA rate among our hair-samples was negligible and the mixed samples were easily recognized and rejected. Another shortcoming arises if the study is aiming at reconstructing home ranges and spatial relationship among individuals. In this case it might be difficult to obtain replicated individual samples large enough for generating statistically acceptable results. For instance, the hair-trapping failed to provide reliable information on the size of home range in our study. Therefore, the research questions addressed with hair-sampling should have in mind these potential limitations. The design of the study should rather concentrate on an approximate estimation of the population composition and spatial distribution of individuals than an absolute calculation of home ranges and their reciprocal overlapping areas. However, the problem of low sample size for individuals might be partly compensated if this method is used as a supplementary to radio- and GPS tracking or photo-trapping.

If correctly designed, the genetic information achieved based on the hair-trap system may replace or even eliminate the necessity of live-trapping the animals in some instances. Thus, it may decrease the high costs of research related to telemetry equipment and logistics during fieldwork as well as the costs and intensity of labor. Moreover, monitoring the spatial organization of the population by telemetry might appear unfeasible in case of endangered or protected species. Among non-invasive methods of population genetic monitoring (feces, urine), the hair-trapping protocol designed at fixed stations as suggested in this paper, gives an advantage of more standardized, regular method. Scat or urine sampling can be less predictable with regard to the distribution and repeatability of collection sites, which is crucial for reliable estimation of temporal trends in population size (Sutherland 1996).

Although collection of hair from wild felids has occasionally been found discouraging (Downey et al. 2007, but cf. Castro-Arellano et al. 2008), our results suggest that it may be feasible not only for species detection and estimation of population size (as reported in many previous studies), but also for more detailed studies of the population spatial structure. Considering cheek rubbing as a common behavior for various felids (Mellen 1993), under certain scrutiny it may provide a reliable source of data for answering questions on the population spatial and social organization, density or habitat use in different cat species. We recommend it as a supplementary method to other monitoring techniques, such as radio-tracking, photo-trapping, snow-tracking, especially in case of limited possibilities of live-trapping (e.g. endangered species).

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