GENETIC STRUCTURE OF CZECH AND SLOVAK POPULATIONS OF THE EURASIAN OTTER (*LUTRA LUTRA*): DNA TYPING OF SPRAINTS AND MICROSATELLITE VARIABILITY

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ABSTRACT: In 2003, a project aiming to assess Eurasian otter population size and structure in 2 different habitats in the Czech and Slovak Republics using genetic typing of spraints was started. Initial testing showed that analysis success rate was significantly influenced by (1) type of sample (anal jellies better than spraints); and (2) weather (negative impact of increasing temperature). Therefore, samples were collected in cold months only, and jelly samples and spraints with jelly or mucus were taken in preference. Samples need to be very fresh (≤18 hrs) and should be sub-sampled into several tubes to obtain sufficient amount of DNA. In general, around 70% of all samples were successfully genotyped. To date, 11 different genotypes, corresponding to 11 otter individuals (5 females and 6 males), have been identified in the mountainous area of the Slovenský Raj National Park, Slovakia. Tissue samples from carcasses from the Czech (n = 57) and Slovak (n = 22) Republics, and genotypes identified from spraints (n = 11), were used to study genetic diversity. Most of the Czech population formed part of a relatively strong but isolated Czech-Austrian-German population; while the Slovak population formed part of the Central and Eastern European otter distribution range. A relatively narrow (50-80 km) region with no otter presence separated the populations; however, the Czech population has very recently reconnected with the otter population in Eastern Europe through Poland. Values of allelic richness (R_s) and observed heterozygosity (H_o) were similar for both populations (R_s: Czech = 4.21, Slovak = 4.33; H_o: Czech = 0.643, Slovak = 0.565). The overall F_{st} (fixation index) value was significant (F_{st} = 0.128; $P < 0.001$), indicating the existence of a gene flow barrier between populations and confirming their moderate genetic differentiation. Both Czech and Slovak populations, however, exhibited a Hardy-Weinberg equilibrium (F_{is} = N.S.), i.e. there was no evidence for inbreeding in these populations.

KEY WORDS: Czech Republic, Eurasian otter, *Lutra lutra*, microsatellite DNA, non-invasive genetic sampling, population estimation, population genetic structure, Slovak Republic, spraints, *SRY* gene.

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Although the method of non-invasive genetic typing of otters using spraints was developed only a few years ago and is still subject to some methodological problems and high costs, it has already become a very useful and promising method for monitoring and studying otter populations. Over most of its distribution, Eurasian otters (*Lutra lutra*) are nocturnal and very elusive in their behavior, and spraints (feces; also referred to as scats) usually represent the only available biological material. Spraints contain cells shed from the gut lining, thus DNA from its owner potentially can be extracted and analyzed. Using microsatellite (highly variable simple sequence repeats of DNA) and *SRY* (a male specific gene located on the Y chromosome) markers, the method can provide identification of individuals, their sex and relatedness, estimates of population size, dispersal and range size, as well as the level of genetic polymorphism within or between populations (Kohn and Wayne 1997, Taberlet et al. 1999). All this information can be obtained without contact with the animals and, also, without disturbing them or influencing their normal behaviour. The method, however, has limitations resulting either from low DNA quantity, low DNA quality (degraded DNA), or poor extract quality (e.g., presence of PCR inhibitors) (Taberlet et al. 1999). These factors are responsible for many technical problems and the low success rate of analysis, making this method very time and cost consuming.

In 2003, we initiated a project aiming to assess otter population size and structure in 2 different habitats in the Czech and Slovak Republics using genetic typing of spraints. The project had 3 aims: 1) testing and optimization of microsatellite DNA typing of spraints; 2) identification of individuals and assessment of population size and structure (e.g., sex ratio, relatedness of individuals) at 2 study sites, which represent different habitats; and 3) analysis of genetic variability and population genetic structure of otters in the Czech and Slovak Republics. The first phase of the project included analysis of tissue samples from carcasses to assess the level of microsatellite polymorphism and probability of identity values (probability of 2 individuals having identical genotypes), and testing and optimization of a non-invasive method using spraint samples from captive otters and, subsequently, from wild (non-captive) otters. At present, the method is being used to assess otter population size and structure at 2 study sites, representing 2 different otter habitats. Data obtained through microsatellite DNA typing of both tissue and spraint samples is intended as a basis of studying genetic variability and population genetic structure of otters in the Czech and Slovak Republics.

Until recently, most of the otter population in the Czech Republic was part of a relatively healthy, but isolated Czech-Austrian-German population; whereas the Slovak population was part of an otter population with a relatively continuous distribution in Central and Eastern Europe. These 2 populations had been separated by a relatively narrow (50-80 km) region that was unoccupied by otters; however, the Czech population has recently (2000-2003) reconnected with the otter population in Eastern Europe through Poland (Roche et al. 2004).

STUDY AREA

Two different sites (each an area of 100 km^2), representing different otter habitats, were selected for the study: 1) an area of eutrophic fish ponds (Třeboňsko Biosphere Reserve & Protected Landscape Area, Czech Republic), and 2) a mountainous area with oligotrophic streams (Slovenský Raj National Park, Slovakia). The first site included a wide, slow-flowing lowland river and a high number of eutrophic fish ponds connected by a complex system of channels, brooks and marshes. This area is known for its high otter density and small and overlapping home ranges, presumably because of the high concentration of fish ponds that provide abundant food supply for most of the year (Dulfer et al. 1996, Roche and Roche 2004). Aquatic habitats at the second site were primarily riverine (“linear”), and supported a

much lower otter density, corresponding to a lower surface area and oligotrophic nature of aquatic habitats in the area (Hájková 2001).

METHODS

We used tissue samples from otter carcasses obtained in the Czech (n = 57) and Slovak (n = 22) Republics, and genotypes identified from spraints (n = 11) from the Slovenský Raj National Park (Slovakia) to analyze population genetic variability and structure. Based on results of initial testing to determine conditions best for collecting spraints, we only collected spraint and anal jelly samples in cold months (November-March, 2003-2004), and anal jelly samples and jelly or mucus part from spraints were preferred to spraints because we determined them to yield higher DNA extraction rates. Samples were collected along all suitable water habitats at the study sites. Only very fresh spraints, from previous night (≤18 hrs), were collected. Location of samples was determined using GPS units. Samples were placed into tubes with buffer (ASL, Qiagen, www.qiagen.com; SLB or Stool Stabilizer, Invitek, www.invitek.eu/) or 96% ethanol immediately in the field, except when samples were frozen in snow or ice – in this case the samples were placed into tubes upon arrival to the laboratory. To ensure sufficient amount of DNA, several sub-samples (1-4) were taken from each spraint. Samples were stored in cool boxes at the collection site for transport, and frozen at -20 °C upon arrival at the laboratory.

DNA extractions were performed using QIAamp Stool DNA Mini Kit (Qiagen,) or Invisorb Spin Stool DNA Kit (Invitek), following manufacturer's instructions with only minor modifications. Extracted DNA was used as a template in Polymerase Chain Reactions (PCR) at 5 microsatellite loci: Lut701, Lut715, Lut717, Lut832, Lut833 (Dallas and Piertney 1998), and in duplex PCR for sex identification, using part of *SRY* gene (Lut-SRY) and 1 additional microsatellite, Lut914 (Dallas et al. 2000). PCR products were analyzed through fragment analysis on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, www.appliedbiosystems.com). Appropriate precautions were taken to avoid contamination. DNA extraction from spraints was performed in a flow-box after ultraviolet sterilization in a separate pre-PCR room, and negative controls were used at each step to detect possible contamination. For PCR, HotMaster *Taq* DNA polymerase (Eppendorf, www.eppendorf.com) was used and analysis was performed using the multiple-tubes approach (3 positive PCR for heterozygotes, 6-7 for homozygotes), to reduce the possibility of incorrect genotyping from allelic dropout and false alleles (Taberlet et al. 1996).

We analyzed tissue samples from 57 otter carcasses from the Czech Republic and 22 from Slovakia. The phenol-chloroform method (Sambrook et al. 1989) or DNeasy Tissue Kit (Qiagen) was used to extract the DNA. We followed procedures identical to those used to prepare spraint samples, except the multiple-tubes approach was omitted. However, genotypes were always based on minimum of 2positive PCR amplifications. Genotypes obtained from tissue samples, together with genotypes identified from spraints were used to analyze population genetic variability and structure. The number of alleles (n_a), allelic richness (R_s), number of private alleles (n_{pa}), observed (H_o) and expected heterozygosity (H_e), and F_{is} and F_{st} indices were calculated using FSTAT software (Goudet 2001).

RESULTS

We analyzed 160 spraint samples from the area of Slovenský Raj National Park, Slovakia. Around 70% of samples were successfully genotyped (about 50% of spraints, 90% of spraints with jelly, and 96% of anal jellies). From these samples, 11 unique genotypes were identified, corresponding to 11 individual otters – 5 females and 6 males (Fig. 1). The number of positive samples for each individual varied between 1 and 23. Most individuals

were recorded several times, thereby providing information on movements and spatial distributions. A minimum of 6 otters was recorded repeatedly in different months.

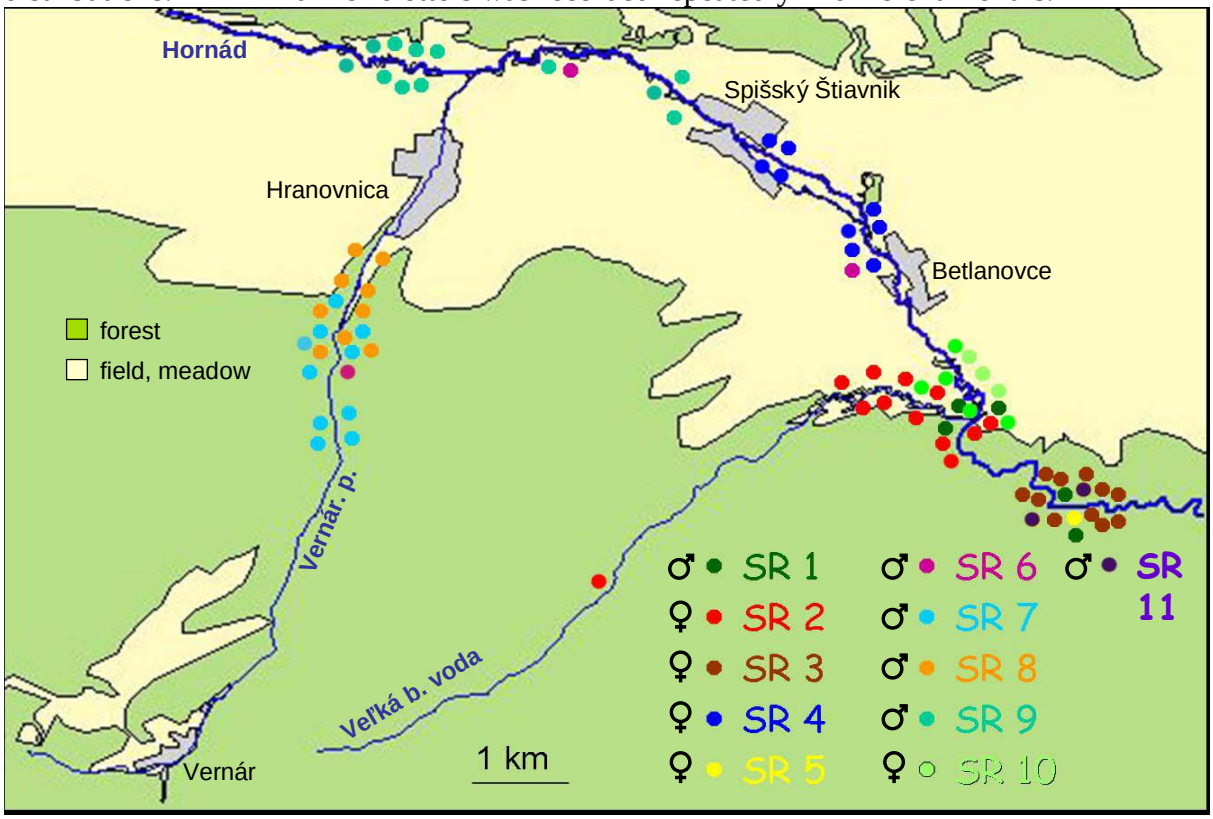

Fig. 1. Map of the study area in the Slovenský Raj National Park (Slovakia) with identified individuals. All positive samples analysed to-date are included; repeated occurrence of the individual at the same place is not marked.

Table 1. Characteristics of genetic variability (microsatellite DNA) of otter populations in the Czech and Slovak Republics based on tissue and spraint samples collected during 2002-2004.

	Czech Republic (*n* = 57)	**Slovak Republic (*n* = 33)**
No. alleles per locus	n_a = 4.50	n_a = 4.33
Allelic richness	R_s = 4.21	R_s = 4.33
No. private alleles	n_{pa} = 4	n_{pa} = 3
Observed heterozygosity	H_o = 0.643	H_o = 0.565
Expected heterozygosity	H_e = 0.634	H_e = 0.541

Table 2. Number of alleles, observed (H_o) and expected (H_e) heterozygosity for 6 microsatellite loci in otter populations in the Czech and Slovak Republics based on tissue and spraint samples collected during 2002-2004.

	Czech Republic (*n* = 57)			**Slovak Republic (*n* = 33)**		
Locus	**No. alleles**	***H*$_o$**	***H*$_e$**	**No. alleles**	***H*$_o$**	***H*$_e$**
Lut701	5	0.625	0.684	5	0.688	0.708
Lut715	3	0.614	0.604	3	0.258	0.324
Lut717	6	0.649	0.631	6	0.655	0.613
Lut832	4	0.635	0.688	4	0.800	0.623
Lut833	6	0.737	0.674	6	0.774	0.789
Lut914	3	0.595	0.524	2	0.214	0.188

The 6 loci analyzed were polymorphic in otter populations in both the Czech and Slovak Republics, with a mean number of alleles per locus of 4.50 for the Czech population, and 4.33 for the Slovak population (range = 2 to 6 alleles) (Tables 1, 2). Mean observed and expected heterozygosity was 0.643 and 0.634 for the Czech population, and 0.565 and 0.541

for the Slovak population. The highest level of polymorphism occurred at loci Lut833, Lut701 and Lut717 (Table 2).

Both Czech and Slovak populations exhibited a Hardy-Weinberg equilibrium (F_{is} = N.S.), i.e., there was no evidence for inbreeding in these populations. However, the overall F_{st} (fixation index) value was significant (F_{st} = 0.128; $P < 0.001$), indicating a moderate genetic differentiation of populations and confirming the existence of a gene flow barrier.

DISCUSSION

In this study, approximately 70% of samples were successfully genotyped, which is a relatively high proportion compared to 20-33% in previous studies on wild otter populations (Jansman et al. 2001, van Rijswijk 2001, Dallas et al. 2003). Many factors may influence the success rate of faecal DNA analysis, including a species' diet, the type of sample and its freshness, environmental conditions, sample preservation and DNA extraction method, and the type of molecular marker used (Murphy et al. 2002, 2003, Roeder et al. 2004, Maudet et al. 2004, Nsubuga et al. 2004, Piggott et al. 2004). In otters, low success rates for analysis of DNA from spraints may be connected with their diet, which predominantly consists of fish. Assessments based on captive bears fed different diets (i.e., grass, alfalfa, carrots, deer, blueberries and salmon) demonstrated that PCR amplification success rate for microsatellite DNA was only 26% when the diet was salmon, compared to 65-76% for other diet types (Murphy et al. 2003). The relatively high success rate in our study may be related to slightly improved methodology of laboratory analysis, but more likely to improvements in the sampling procedure (e.g., sampling in cold months and collection of very fresh samples). These factors can consequently save time and cost in the laboratory. Therefore, before starting a large-scale project based on a non-invasive genetic sampling, as well as when applying this method to other otter species, a preliminary study should be conducted to assess and develop best methodologies for collection, storage, and analysis of spraints.

Population genetic analysis of tissues and genotyped spraint samples indicated that values of microsatellite diversity were similar for Czech and Slovak population, and comparable with other otter populations in Europe (Dallas et al. 1999, 2002, Pertoldi et al. 2001, Randi et al. 2003). Both Czech and Slovak populations exhibited Hardy-Weinberg equilibrium (i.e., there was no evidence of inbreeding). Significant F_{st} value confirmed the existence of a gene flow barrier between Czech and Slovak population, and indicated moderate genetic differentiation. However, a reconnection of populations through Poland was found recently (2000-2003; Roche et al. 2004), and another reconnection is expected in the near future, or has already taken place, in Beskydy Mountains, north-eastern Czech Republic (K. Roche, unpublished data). Nevertheless, for more detailed studies on population genetic structure and gene flow in these populations, analysis of additional samples is needed, especially from the areas under-represented in this study. Paucity of tissue samples from some areas could potentially be supplemented by an analysis of spraint samples, which can be collected relatively easily.

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