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POPULATION STRUCTURE AND GENETIC DIVERSITY OF OTTER POPULATIONS IN HUNGARY

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Abstract

The Eurasian otter (Lutra lutra) is a widely distributed species in Europe and in the past (in the last century) it has experienced long decline periods followed by slow recovery due to the protection (active and passive) of its habitat (wetlands) and the fact that the amount of environmental pollutants has decreased significantly in the area. Otter populations have been stable in recent decades in Hungary, but one can find only limited information on the genetic diversity and structure of the species and no information available on potential barriers that can affect gene flow. In this study, 255 tissue samples were analysed. The samples were collected since 2002 and originate mainly from road killed animals in various regions of Hungary to determine the genetic diversity and structure of the species. The Hungarian otter populations showed a relatively high level of genetic diversity (observed heterozigosities (Ho): 0.59 to 0.72; mean number of alleles per locus (A): 3.9 to 6.4). Two genetically distinct clusters were also identified at the regional level. The genetic differentiation can be caused by an extrinsic and partial habitat barrier, the Danube-Tisza Fluvial (a north-south divide of dry sand) that may limit the gene flow between the two subpopulations. Demographic history analysis does not suggest a bottleneck event in the past or that restricted dispersal has occurred in this population, which is in contrast to other reported studies on the Eurasian otter. The results provide a framework for positive implementation of otter conservation management plans in Hungary.

Keywords: Eurasian otter, microsatellite DNA markers, genetic structure

Introduction

The Eurasian otter (*Lutra lutra;* hereafter otter) has the widest distribution of all otter species as it occurs throughout Europe, Asia, and certain parts of Africa (Reuther 1993; Kruuk 2006, Ki et al. 2010). Human activities in the 1960s and 1970s resulted in population declines and fragmentation across major parts of Europe (West 1975; Macdonald and Mason 1976). This could have resulted in the loss of genetic diversity and increased genetic differentiation among populations through genetic drift (Frankham et al. 2002).

In Hungary, otter populations have been considered stable, with slight declines only reported east of the Danube due to pollution since about the 1970s (Nechay 1980; Heltai et al. 2012). However, pollution has dramatically decreased since the 1990s, including a reduction in the amounts of industrial and agricultural pollutants in the environment and the levels of heavy metals (lead, cadmium, mercury, copper, and zinc) found in the liver tissue of otters (Lanszki et al. 2009a). Although populations are considered stable, regional fluctuations and differences have been reported (Faragó 2009; Heltai et al. 2012). Numbers of otters in Hungary are estimated to be on the order of 1,000 to 10,000 animals (Lanszki et al. 2008; 2010). Lower densities of otters have been reported in mountains and in dry plain areas (in the Kiskunság region) with little wetland habitat. During this time period, the distribution range of otters in Hungary has remained wide (Heltai et al. 2012, approximately 72,000 km^2 or 77.5% of Hungary). On the basis of field surveys (Heltai et al. 2012) and local molecular genetics analyses (Lanszki et al. 2008; 2010) of Hungarian populations, it has been suggested that otter populations are currently increasing (Heltai et al. 2012).

Mucci et al. (2010) carried out the most extensive genetic analysis of Eurasian otters in Europe. Although samples from Hungary were included in that study, sample numbers were too low to adequately quantify genetic diversity and structure across the region. Thus, we report here on the genetic diversity and the structure of otter (*Lutra lutra*) populations in Hungary.

Materials and methods

Study area and sampling

The whole territory of Hungary belongs to the Middle Danube Basin catchment area and includes three Sub-River Basins (Tisza River Basin, Pannonian Central Danube Basin and Drava Basin; Fig. 1, numbered 1, 2, and 3 respectively). The 3 river basins are connected and theoretically fully accessible to otters. Otter carcasses were collected by the staff of the ten national park directorates (NPD) of Hungary (Fig. 1) opportunistically mainly from road kills (90%) and from other resources (Lanszki et al. 2009) to study the ecology of the species, with the permission of the Ministry of Environment and Waters. Tissue samples for DNA analysis (n = 255 mainly from kidney or muscle) were collected from the above-mentioned carcasses originating from the entire country (Fig. 1) and representing localities within 10 national park directorates (NPDs) of Hungary: Aggteleki (ANPD), Balaton-felvidéki (BFNPD), Bükki (BNPD), Duna-Dráva (DDNPD), Duna-Ipoly (DINPD), Fertő-Hanság (FHNPD), Hortobágyi (HNPD), Kiskunsági (KNPD), Körös-Maros (KMNPD), Őrségi (ŐNPD). The NPDs are situated in the different river basins as follows: ANPD, HNPD, and KMNDP in the Tisza River Basin; BFNPD, DINPD, ŐNPD, and FHNDP in the Pannonian Central Danube Basin; DDNDP partly in the Drava River Basin and partly in the Pannonian Central Danube Basin; the KNPD and BNPD partly in the Tisza River Basin and partly in the Pannonian Central Danube Basin. For this study, we grouped samples originating from 2 Northeastern NPDs (ANPD and BNPD), where otters are rare and habitats are poor for the species (Heltai et al. 2012).

DNA analysis

DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the extraction protocol as outlined by the manufacturer. A total of 10 cross-species autosomal microsatellites markers (Dallas and Piertney, 1998) were used to genotype all

individuals. For the amplification standard PCR protocols were followed. PCR products were pooled together and run against a Genescan 500 LIZ (Applied Biosystems, Foster City, CA.) internal size standard on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA.). Samples were visualized using GeneMapper v. 4.0 software (Applied Biosystems, Foster City, CA.) and bins were assigned to alleles and calls were then checked by eye.

Analysis of genetic diversity in the total population

MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004) was used to detect possible genotyping errors, allele dropout and nonamplified alleles (null alleles). This software package can estimate the frequency of null alleles and can adjust the data set to account for the presence of null alleles. Mean number of alleles per locus (*A*), observed heterozygosities (*Ho*), expected heterozygosities (*He*), and deviations from Hardy–Weinberg Equilibrium (HWE) proportions were calculated using GenAlEx (Peakall and Smouse 2006). Linkage disequilibrium between pairs of microsatellite loci was evaluated using GenAlEx (Peakall and Smouse 2012) according to the method of Black and Kraftsur (1985). Associated probability values were corrected for multiple comparisons using Bonferroni adjustment for a significance level of 0.05.

Population structure

To identify genetically cohesive clusters, Bayesian cluster analysis was implemented in STRUCTURE version 2.3.3 (Pritchard et al. 2000). The genetic relationship between populations and individual assignments of otters and samples of unknown location was inferred via a Bayesian clustering analysis using the statistical program STRUCTURE version 2.3.3 (Pritchard et al. 2000). The program was run without prior population information (option USEPOPINFO = 0, no LOCPRIOR). STRUCTURE was run for 10 replicates from $K = 1–11$, with a run-length of 1 million repetitions of Markov chain Monte Carlo, following the burn-in period of 100,000 iterations. For each *K*, we calculated the mean posterior probability over its runs. We then used this mean value to estimate the posterior probability of each *K* using the formula given by Pritchard and Wen (2003). We used the ΔK method of Evanno et al. (2005) using STRUCTURE HARVESTER (Earl and VonHoldt 2012) to determine the most likely value of *K*. Values of average proportion of membership (*qI*) over 10 runs were used to assign individuals to subpopulations; individuals were assigned to a single population when *qI* was greater than 0.70 or less than 0.3 (Latch et al. 2008). Alleles in Space (Miller 2005) were used to explore spatial genetic structure by the interpolation of landscape shape. This procedure first generates a Delaunay triangulation based connectivity network among the sample localities and then calculates the genetic distances between them. The results were plotted over a spatial grid of 50 × 50 that covered the sampling area, and the surface heights were calculated based on raw and residual genetic distances. Global fixation indices, standardized fixation indices, and Jost's estimate of population differentiation (Jost 2008) were calculated in GenAlEx version 6.5 (Peakall and Smouse 2012) with 10,000 bootstrap replicates and 10,000 pairwise permutations. Population genetic variance was further analyzed by analysis of molecular variance (AMOVA) performed by the method of Excoffier et al. (1992) using GenAlEx version 6.5 (Peakall and Smouse 2012). In order to identify a significant isolation by distance effect, a Mantel test was performed on average pairwise genetic distances (Nei's *D*) and average pairwise geographic distances between each NDP. In order to determine isolation by distance spatial pattern analysis

of genetic diversity, we plotted and regressed average coefficients using SpaGeDi version 1.4 (Hardy and Vekemans 2002).

Results

All loci were polymorphic with the number of alleles per locus ranging between 3 and 11 with a mean of 5 alleles per locus. Global tests of all sampled areas indicated a significant deviation from HWE ($P < 0.000$) due to a reduction in observed heterozygosity at 4 loci, namely LUT453, LUT457, LUT782, and LUT818, suggesting the existence of structure within the Hungarian otter populations as a result of the Wahlund effect (Wahlund 1928). Linkage disequilibrium was not detected for any of the markers tested. In addition, null alleles were not observed by MICRO-CHECKER. Expected heterozygosity within all populations (H_e) was 0.709 (Table 1); observed heterozygosity (H_o) was 0.669 and the number of alleles was 7.25.

Population structure and isolation by distance

Posterior probabilities (*Ln*) using Bayesian admixture analysis were calculated for K = 1-11 with K = 2 being identified as the most likely K based on Evanno's ΔK method. STRUCTURE analyses with and without USEPOPINFO and POPFLAG options provided similar results (data not shown). Clear breaks are present at q_1 or $q_2 \geq 0.7$. The majority of individuals (n = 224, 88%) were assigned to either the eastern part of Hungary (cluster 1) or the western part of Hungary (cluster 2). The 2 clusters correspond to the Tisza and Danube/Drava river basins and are hereafter referred to as "Danube" and "Tisza" (Fig. 2a and 2b). A total of 18 individuals were potentially admixed, (individuals with q_1 values between 0.3-0.7). In addition, a total of 11 individuals were identified as migrants (e.g., an individual with $q_1 > 0.7$ found in the region dominated by cluster 2). These admixed and potentially migrant individuals were more common in border areas between the Danube and Tisza river tributaries (Fig. 2a and 2b). Seven migrants (63%) occurred within 150 km of areas dominated by the other genetic cluster and 13 admixed individuals (65%) were within 110 km of the river tributaries. Thus, regions closer together seemed to share migrants more often. These results suggest that there may be geographical structure to genetic variation, specifically an isolation by distance relationship between the 2 river basins. We found evidence for isolation-by-distance with the Mantel test (R_{xy} = 0.247, $P <$ 0.001). Consistently, spatial genetic structure was found with SPAGeDI 1.4c which indicated a significant correlation between pairwise genetic distance and geographic distance (R^2 =0.8332, Fig. 3). Our genetic landscape shape interpolation analysis produced surface plots that support the STRUCTURE and isolation by distance (Fig. 4). A 'ridge,' indicating the greatest genetic distance was observed in an East versus West orientation approximately at the midpoint of the otters' range.

Genetic diversity within populations

The Danube population had 3 markers out of HWE, namely; LUT457, LUT615, and LUT782. The Tisza population exhibited a significant deviation from HWE due to disequilibrium at one locus (LUT818). In both populations, a weak deviation from HWE was observed (Danube: F_{IS} = 0.059; Tisza: F_{IS} = 0.057). The level of genetic diversity was similar in both the Tisza and Danube populations. H_o ranged from 0.643 (Danube) to 0.695 (Tisza), H_e values varied from 0.685 (Danube) to 0.734 (Tisza) and number of alleles was 6.8 in Danube and 7.7 in Tisza. Genetic differentiation of populations was also indicated through the presence of a relatively

high number of private alleles. Unique alleles were observed in the Danube population (n = 9) and Tisza population (n = 17). Most of the unique alleles were detected at low frequency (0.004 to 0.096). Based on the results of AIS and STRUCTURE, 2 populations were defined and analyzed using the spatially explicit methods described above (fixation indices, standardized fixation indices, and Jost's D). The 2 populations identified represent eastern (Tisza) and western (Danube/Drava) river basins. F_{ST}-based AMOVA showed that 7% of the variation was distributed among river basins and another 7% among individuals. Genetic differentiation was low but significant among the 2 populations (AMOVA F_{ST} = 0.069, F'_{ST} = 0.24; Jost's D_{est} = 0.19; G_{st} = 0.037, all $P < 0.001$), supporting the existence of population substructure.

Discussion

Analyses of a collection of otter samples from Hungary with a set of 10 microsatellite markers revealed moderate to high genetic variability (H_e: 0.69- 0.73; N_a: 6.8-7.7), concordant with other published studies (Dallas et al. 1999, 2002; Arrendal et al. 2004). In Britain, H_e values varied from 0.26-0.72 and the number of alleles varied from 2.1-5.3 (Dallas et al. 2002). In Europe, H_e values range from 0.37- 0.71 with the number of alleles ranging from 2.5-6.8 (Mucci et al. 2010). Levels of genetic diversity vary greatly between different regions, and the heterozygosity and number of alleles was lowest in isolated populations from Denmark and Italy and highest in northern European otter populations (i.e., Latvia, Belarus, Finland, and Sweden). Lower levels of genetic diversity in European otter populations have been attributed to recent events such as climate change or anthropogenic population declines rather than historical bottlenecks due to founder events (Beaumont 1999; Pertoldi et al. 2001; Mucci et al. 2010). As genetic diversity in Hungarian otters analyzed here is considered moderate to high, these populations may not have experienced dramatic declines, or at least declines of a magnitude that would lead to loss of diversity. The results of this study thus support the observation that Hungarian otter populations have been largely stable with large effective population sizes (Heltai et al. 2012).

Population structure and assignment

Our data indicated that the otter population in Hungary is not a single panmictic population, but is distinctly divided into 2 genetically defined groups. STRUCTURE analysis identified 2 populations that broadly correspond to the western Danube and eastern Tisza river basins. Deviation from HWE in both populations was moderate and thus is not considered to be due to inbreeding but may be due to additional cryptic subdivision (Wahlund 1928). In addition, admixed individuals were observed in regions of expected contact suggesting that there is still a small amount of migration and gene flow between the 2 populations.

In this study, consistent sampling provided a framework to identify possible barriers and their influence on genetic structure across the range of Hungarian otters. The presence of physical barriers to gene flow does not preclude an additional effect of non-physical barriers, such as female philopatry. A recent study of otters in southern Portugal found evidence that female philopatry acts as a non-physical barrier to gene flow that has resulted in fine-scale genetic structure in an interconnected river system (Quaglietta et al. 2013). Additionally, previous molecular studies have found patterns of isolation by distance (IBD) among otter populations (e.g., Cassens et al. 2000; Dallas et al. 2002; Mucci et al. 2010). The genetic structure of the Hungarian otter population is clearly dominated by 2 patterns: isolation by distance and a partial

dispersal barrier between the 2 river basins (Tisza and Danube). Gene flow between the Tisza and Danube otter populations is higher in sample sites that are geographically proximate, and it could be mediated by dispersal of males. Given that otters are so dependent upon an aquatic environment, dispersal over land is most likely avoided, resulting in genetic divergence between river basins. In addition, genetic landscape shape interpolation analysis indicated that genetic distances were shallower in the western part of the country than in the east. This result could be due to variations in otter densities throughout the region. Field surveys have indicated that the highest numbers of otter populations are found in the western part of the country (Kemenes 1991) where there are many fish ponds with dense vegetation resulting in higher food availability.

However, in addition to geographic distance, the species does seem to be spatially restricted to 1 of the 2 river basins. Hungary is rich in surface waters and wetlands which form suitable habitat for otters (Heltai et al. 2012); however, these are not uniformly distributed and available across the entire country. Specifically, the genetic delineation observed in this study between the eastern and western regions corresponds to the so-called Danube-Tisza Fluvial— a sand-based, dry, hilly area that runs in a north-south direction between the Pannonian Central Danube Basin and the Tisza Basin.

The population differentiation emphasizes the importance of conservation of the rich and connected network of water-related habitats and supports the restoration of wetland habitats. Our results emphasize that even a widely distributed top predator like the otter can face barriers to movement that limit genetic connectivity, but such barriers may not be evident from simple mapping of the species' distribution. These results have significant implications for conservation and management of the species within Hungary and can be used for management plans to retain the distinct genetic diversity present in each subpopulation.

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