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Genetic differentiation of the Golden Jackal (*Canis aureus*) populations in southern Hungary and southern Romania as revealed by microsatellite data analysis

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Abstract. The golden jackal (*Canis aureus*) is a widespread opportunistic mid-sized canid, distributed throughout southern Asia, the Middle East and South-eastern and Central Europe. European populations have undergone significant population changes in recent decades. During the first two decades of the 21st century the size of the jackal populations increased in their distribution and abundance across Europe. Hungary and Romania apply similar game management practices, and the golden jackal appeared approximately at the same time in both countries. With the aim to determine the genetic structure and the origin of the expanding jackal populations, we analysed samples obtained from Romania and Hungary. Genotyped at 22 canine autosomal microsatellite loci (STR), samples were analysed using multivariate, Bayesian and landscape genetic methods. In the Romanian samples all loci were polymorphic with 3–12 alleles. The overall observed (Ho) and expected (He) heterozygosities were 0.552 and 0.647, respectively. In the Hungarian samples 20 out of 22 loci were polymorphic with 2–11 alleles. The overall observed (Ho) heterozygosity (0.561) was higher and the expected (He) heterozygosity (0.564) was lower than the corresponding Romanian value. Based on our clustering results, Romanian and Hungarian samples were separated into two different genetic clusters. These results show that smaller groups of golden jackals could become established in different regions following several episodes of colonization, possibly at different times and arriving from various locations.

Key words: golden jackal, autosomal microsatellites, population structure, recolonization genetics, Hungary, Romania.

Introduction

The golden jackal (*Canis aureus* L.) is a widely distributed mesopredator across the south-eastern part of Europe with a special emphasis on the Balkans (Arnold et al. 2012, Markov et al. 2018). The reason for the paramount importance of the Balkans is its status as a core area of distribution and suspected source of expansion across Europe (Spassov 1989, Kryštufek et al. 1997, Arnold et al. 2012, Šálek et al. 2014, Markov et al. 2018, Spassov & Acosta-Pankov 2019). The golden jackal is considered a native species in the Balkan and Carpathian Basin (Demeter & Spassov 1993, Kryštufek et al. 1997, Hoffmann et al. 2018). According to hunting and zoological literature, the species has been present in the Carpathian Basin since at least the 1800s (Tóth et al. 2009). After the extirpation in the 20th century, recolonization started in the 80s and 90s (Heltai et al. 2004, Tóth et al. 2009, Banea et al. 2018). In Hungary the golden jackal was considered as an extirpated species in the period between 1920 and 1945. Few and usually uncertain records were available after 1945, but in essence it seems that it was a rare canine in Hungary with irregular occurrences prior to the 1980s (Demeter 1984, Tóth et al. 2009). The first verified data on its return come from 1981 and 1983 (Demeter 1984). From the beginning of the 1990s reproducing and expanding golden jackal populations were established in three counties in the south-western part of the country (Heltai et al. 2001), and nowadays the golden jackal has a countrywide distribution and a year-round open hunting season (Szabó et al. 2009). Evolution of the distribution area of golden jackals in Romania ran a similar course. Until the 1960s the Danube was considered as the northern border of the distribution, and only random occurrences were recorded in Romania (Cotta & Bodea 1969, Rösler 2013). By 2017 golden jackals were present in 37 counties out of 41 (Farkas et al. 2017), and currently each county in Romania has a hunting quota for the species (Decree no. 1400/2020 of Ministry of Waters and Forests).

Hungary and Romania are two Central-Eastern European countries with similar wildlife management practices. The golden jackal's recolonization started in approximately the same period within both countries, and the exponential trends of stock assessment- and hunting bag data are similar as well (Banea et al. 2018, Csányi et al. 2019). The rapid and ongoing expansion of the golden jackal's distribution raises some issues about the origin of the recently founded populations, especially regarding population management (Trouwborst et al. 2015). In the absence of individual tracking of wandering individuals, a genetic approach seems to be the most adequate for population dispersal studies (Broquet & Petit 2009). Microsatellites, also called Short Tandem Repeats (STRs) are DNA sequences made up of repeated short units no longer than six bases. Owing to their multiallelic nature, co-dominant mode of inheritance, abundance and wide genome coverage they have been extensively used as genetic markers in many research areas, such as population biology, phylogeography, forensics and conservation

biology (Schoebel et al. 2013, Szabolcsi et al. 2014). The population genetics of golden jackals has been so far poorly studied. The first Europe-wide study of the genetic structure of the species (Rutkowski et al. 2015) revealed six genetic clusters; samples from Romania and Hungary belonged to the same group. In contrast, using 15 microsatellite loci Bogdanowicz et al. (2018) found that samples from Romania and Hungary formed separate groups.

Genetic diversity is also one of the important benchmarks of golden jackal populations from different geographical areas. In the Balkans (Bulgaria, Serbia and Croatia) Fabbri et al. (2014) found intermediate values of genetic diversity, whereas Rutkowski et al. (2015) reported higher genetic diversity for other European jackal populations. Examining the jackals in Hungary, Kusza et al. (2018) found moderate genetic diversity. Although there is a considerable amount of genetic data already available to researchers, the incompatibility of the current marker panels makes it difficult to identify the long-distance dispersion of populations (De Groot et al. 2016). Previous studies have had contradictory results: jackals from Hungary and Romania were reported as either part of the same genetic cluster or formed genetically distinct groups. We assumed that genetic comparisons between a larger numbers of samples from these two countries – which means a smaller geographical scale than the continent-wide studies – could reveal the jackals' south-to-north expansion (Kusza et al. 2018). Here we report genetic analyses of samples obtained from two geographic regions (southern Hungary and southern Romania). Multivariate, Bayesian and landscape genetic methods were used to determine genetic diversity and differentiation of the populations, and to investigate the possibility that Hungarian and Romanian golden jackals arrived from the direction of the Balkans along the Danube River and its tributaries.

Materials and Methods

Sampling and DNA extraction

Individual tissue samples of a total of 68 golden jackals were collected from southern Romania (close to the Bulgarian border; n = 30) and south-western Hungary (n = 38). Samples from Hungary were collected in the south-western part of the country in Somogy and Baranya (Hu) counties on the right side of the Danube, and those from Romania in Teleorman county (Ro) on the left side of the Danube (Fig. 1). Muscle tissue was obtained from free-ranging animals legally shot between 2011 and 2017 in Hungary and between 2014 and 2016 in Romania. Tissue samples were preserved in 96% ethanol and stored at –20°C. Total genomic DNA was extracted using the Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's protocol, and stored at -20°C until processing.

Laboratory analysis

Samples were individually genotyped at 22 polymorphic canine microsatellite loci: AHT137, AHTh171, AHTh260 (Breen et al. 2001), FH2004, FH2010, FH2088, FH2107, FH2309, FH3313, FH3377, PEZ02 (Dayton et al. 2009), c2001, c2054, c2096, FH2538, PEZ3, PEZ5, PEZ6, PEZ8, PEZ11, PEZ12, PEZ19 (Vilà et al. 2003). Primers were divided into four multiplex reactions of five to nine loci, and primer concentrations were optimized for efficient amplification and fidelity. Final multiplex PCRs were composed in a total volume of 25 µl, containing 1× QIAGEN Multiplex Master Mix (QIAGEN GmbH, Germany), 60 ng of template DNA, each primer in optimum concentration (0.04–0.30 mM), and filled up with water. Amplification reactions were performed using a LifeECO Thermal Cycler (Hangzhou Bioer Technology Co. Ltd., China), with the following cycling conditions: an initial activation at 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 58°C for 60 s, and 72°C for 60 s, and with a final extension at 60°C for 30 min. PCR products were separated on an ABI Prism 3100 Genetic Analyser with LIZ500 Size Standard (Applied Biosystems, USA), and the PeakScanner ver. 1.0 (Applied Biosystems, USA) was used to score microsatellite allele sizes.

Figure 1. Geographic location points of the samples analysed in Hungary and Romania.

Genetic analysis

The presence of null alleles, scoring errors and large allele dropout was assessed using MICRO-CHECKER ver. 2.2.3 (Van Oosterhout et al. 2004). Allele frequencies, the number of alleles (Na), the number of effective alleles (Ne), observed and expected heterozygosity values (Ho and He, respectively), Shannon's Information Index (I) and the Hardy-Weinberg equilibrium (HWE) were computed with GenAlEx ver. 6.5 (Peakall & Smouse 2006). Population structure was examined using the software STRUCTURE ver. 2.3.4 (Pritchard et al. 2000) to infer the most probable number of genetic clusters without *a priori* definition of populations. The Bayesian clustering method and Markov Chain Monte Carlo (MCMC) simulation were run using an admixture model and correlated allele frequencies with a burn-in period of 250,000 iterations and 750,000 replicates for a number of genetic clusters (K) from one to ten with ten independent runs for each K. We used STRUCTURE Harvester ver. 0.6.94 (Earl & vonHoldt 2012) to determine the number of genetic clusters. The second approach was a discriminant analysis of principal components (DAPC), a multivariate method implemented in the adegenet package (Jombart 2008) with R ver. 3.4.4 (R Core Team 2018) that identifies clusters of individuals without using any population genetic model (Jombart et al. 2008). We used the "find.clusters" function for the identification of the optimal number of clusters (K) with the "choose.n.clust" option and the Bayesian Information Criterion (BIC). After that, DAPC was employed to assign individuals into populations, retaining all the principal components, as suggested in the manual. Additionally, a principal coordinate analysis (PCoA) was performed on individual multilocus genotypes with the help of GenAlEx ver.6.5 (Peakall & Smouse 2006) to visualize the genetic distance between individuals. The spatial locations of genetic clusters were demonstrated by landscape genetic analyses in Geneland ver. 4.0.8 (Guillot et al. 2005), with 100,000 MCMC iterations (thinning = 1000, running number = 10), using the number of possible populations from one to ten, with the correlated allele frequency model, and with a coordinates uncertainty of 0.01.

Results

Genetic variability

Null alleles were detected at eight loci (AHT137, PEZ5, AHTh171, AHTh260, FH2010, FH2004, FH2107 and FH2309) in the jackal samples with low frequencies. Analyses using all loci and loci with null alleles excluded gave similar re-

Table 1. Variability at 22 canine microsatellite loci in the two studied regions. *Na* number of allels, *Ho* observed heterozigosity and *He* expected heterozigosity, *I* Shannon's Information Index, *HWE* deviation from Hardy-Weinberg Equilibrium * p<0.05, ** p<0.01, *** p<0.001, ns not significant.

Locus	Hungary					Romania				
	Na	Ho	He	I	HWE	Na	Ho	He	I	HWE
c2001	6	0.737	0.675	1.314	ns	6	0.700	0.764	1.506	ns
AHT137	8	0.500	0.837	1.927	***	8	0.867	0.728	1.566	ns
c2054	7	0.658	0.666	1.353	***	4	0.467	0.575	1.002	ns
c2096	4	0.447	0.373	0.727	ns	5	0.300	0.513	1.001	***
FH2538	5	0.132	0.196	0.464	**	3	0.433	0.399	0.642	ns
PEZ3	4	0.868	0.577	1.009	***	5	0.241	0.618	1.176	***
PEZ5	4	0.237	0.236	0.495	ns	5	0.167	0.373	0.806	***
PEZ6	7	0.789	0.565	1.128	ns	10	0.367	0.380	0.977	ns
PEZ8	7	0.763	0.720	1.474	ns	6	0.733	0.770	1.572	ns
PEZ11	6	0.763	0.771	1.592	ns	11	0.667	0.719	1.701	ns
PEZ12	6	0.684	0.764	1.514	ns	7	0.700	0.743	1.555	*
PEZ19	3	0.579	0.475	0.715	ns	4	0.600	0.669	1.201	ns
AHTh171	4	0.132	0.318	0.642	***	5	0.567	0.544	1.043	ns
AHTh260	3	0.211	0.258	0.513	ns	8	0.633	0.771	1.712	**
FH2088	2	0.342	0.400	0.589	ns	4	0.533	0.568	1.015	*
PEZ2	4	0.763	0.566	0.995	*	5	0.700	0.754	1.481	*
FH3377	2	0.500	0.497	0.690	ns	3	0.600	0.504	0.788	ns
FH2010	4	0.763	0.725	1.335	ns	6	0.400	0.784	1.608	***
FH2004	6	0.684	0.735	1.523	ns	9	0.667	0.781	1.744	*
FH2107	11	0.632	0.835	2.023	*	12	0.633	0.832	2.095	*
FH2309	4	0.474	0.524	0.999	ns	7	0.467	0.697	1.482	***
FH3313	6	0.684	0.702	1.373	ns	5	0.700	0.742	1.432	ns
Average	5.136	0.561	0.564	1.109		6.273	0.552	0.647	1.323	

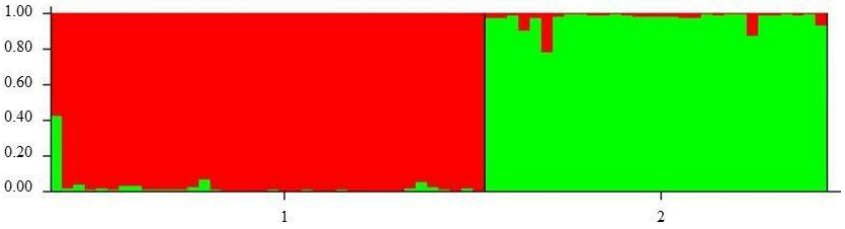

Figure 2. Bayesian clustering analysis for golden jackals from Hungary (1) and Romania (2) obtained by STRUCTURE. Each individual is represented by vertical bar fragmented in K sections of different length, according to their membership proportion in the inferred genetic cluster.

sults, all loci were therefore included in subsequent analyses. For 67 samples we could obtain a complete 22-locus genetic profile; for one sample a single locus was missing resulting in an incomplete genotype. All loci were polymorphic in the Romanian samples, and 20 out of 22 loci were polymorphic in the Hungarian samples. The average number of alleles was 5.136, with the number of alleles per locus ranging from 2 (FH2088, FH3377) to 11 (FH2107) in the Hungarian samples. The overall observed and expected heterozygosities were 0.561 and 0.564, respectively. Shannon's Information Index was 1.109, and seven of the 22 loci deviated from HWE. In the Romanian samples the average number of alleles was 6.273, with the number of alleles per locus ranging from 3 (FH2538, FH3377) to 12 (FH2107). The overall observed and expected heterozygosities were 0.552 and 0.647, respectively. Shannon's Information Index was 1.323 and 11 of the 22 loci deviated from HWE (Table 1).

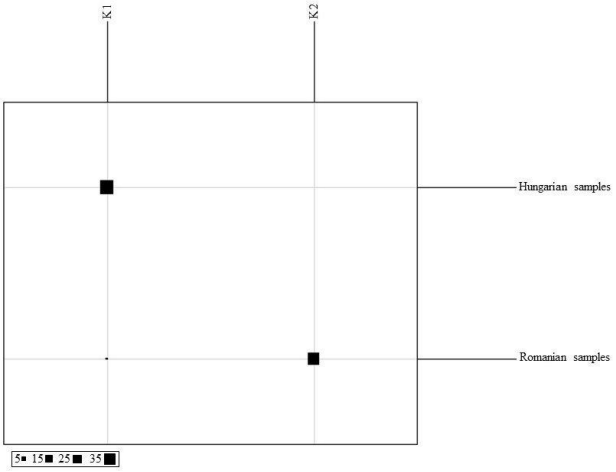

Figure 3. Clustering based on the number of individuals within the groups in the discriminant analysis of principal components (DAPC). The size of the squares is proportional to the number of individuals in the groups.

Genetic structure

Genetic structure was detected by STRUCTURE and DAPC analyses, as well as by the PCoA. Bayesian clustering analysis detected the highest average likelihood scores for the case of two genetic clusters (K = 2) in the dataset, where Hungarian and Romanian samples separated into two different clusters (Fig. 2). The DAPC also revealed the presence of genetic

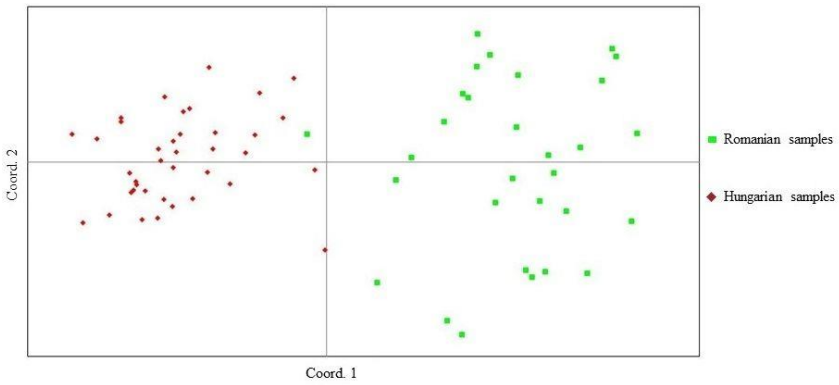

Figure 4. Principal coordinates analysis obtained by GenAlEx. Red diamonds represent the Hungarian jackal samples, green squares the Romanian samples.

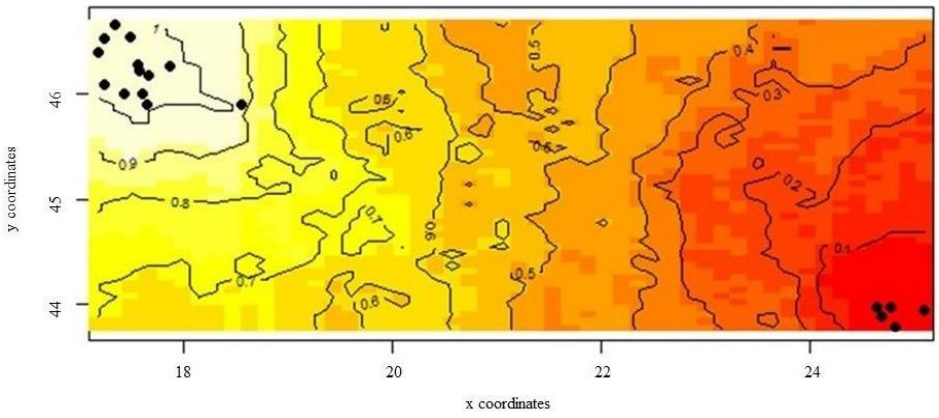

Figure 5. Results of spatial analysis of genetic structure, using Geneland.

subunits, with the lowest BIC obtained for a model comprising two clusters. The samples from Hungary were assigned into one cluster and almost all jackals from Romania were assigned into another cluster (Fig. 3), with one Romanian sample assigned to the Hungarian group. According to the PCoA, Hungarian and Romanian samples showed signs of differentiation but were not clearly separated (Fig. 4). Using the coordinates of the locations where the samples were taken, Geneland grouped samples from Romania and Hungary into two separate groups. In all 10 runs the separation into two distinct groups was found to have the highest probability (Fig. 5). Out of 13 sampling locations in Hungary, 12 were assigned to the same group with 100% probability and one with 90% probability, whereas all five sampling locations in Romania were assigned to the same group with 100% probability.

Discussion

Samples from Romania and Hungary could be clearly distinguished from each other with the software tools we used. The STR analyses we carried out showed higher heterozygosity values in both sample sets compared to previous studies in Europe. Contrary to our results, Zachos et al. (2009) found low genetic diversity (Ho = 0.28; He = 0.33) in 121 samples from Serbia, using eight STR loci. Fabbri et al. (2014) also found a more limited diversity (Ho = 0.47; He = 0.51) in the g part of the golden jackal's distribution range using 21 STR loci, similar to Rutkowski et al. (2015), who analysed samples from South-Eastern Europe (Ho = 0.55; He = 0.58) using 15 STR loci. Kusza et al. (2018) analysed 69 samples collected in Hungary using 10 STR loci and found limited genetic diversity as well (Ho = 0.35; He = 0.45). Studies of golden jackals across Europe showed a low level of genetic differentiation between various populations, except in the case of the Dalmatian population, which proved to be distinct from other populations in the Balkans as well as in the Middle East (Zachos et al. 2009, Fabbri et al. 2014). Rutkowski et al. (2015) identified two separate clusters in a study focusing on the genetic structure of golden jackal populations in South-Eastern Europe, in which samples from both Romania and Hungary were assigned to the same cluster. However, Rutkowski et al. (2015) included a total of only five samples from Romania and ten from Hungary, which could be considered as a very low sample size. Our genetic analyses, which used a higher number of samples, found that samples from Romania and from Hungary do show genetic differentiation. Both the diversity values and the parameters associated with genetic differentiation showed that the golden jackal in Europe has a lower genetic diversity (Zachos et al. 2009, Fabbri et al. 2014, Rutkowski et al. 2015) compared to other wild canines (e.g. Jansson et al. 2012). The results of Fabbri et al. (2014) suggested that the European continent had once been populated by a comparatively small population that had a more limited genetic diversity compared to the source population in the south, but more empirical data are needed to confirm this. Our results differ from these previous studies, but at the same time confirm the results of Bogdanowicz et al. (2018), who carried out the genetic analysis of close to 400 golden jackal samples collected in the Balkans, central, northern and north-eastern Europe. In their study, samples from Romania and Hungary were assigned to two separate clusters, one of which predominantly

included samples from Hungary, whereas most of the samples assigned to the other cluster were collected in Romania. However, both clusters included some individuals with discrepant geographical origin. The results of Bogdanowicz et al. (2018) imply that smaller groups of golden jackals, which later settled in different regions, were separated along the main route of the species' expansion at different times and in various locations. Our results also suggest that golden jackals could have become established in Romania and Hungary in a number of repeated waves. Genetic surveys of the golden jackal may show different results. This may also be due to the fact that researchers work with different marker panels, making it difficult to compare results and perform meta-analyses with the aim of determining the direction and rate of dispersion. In order to study the European golden jackal populations uniformly, the loci examined should be standardized (De Groot et al. 2016). Based on the results of Spassov & Acosta-Pankov (2019), we can assume that golden jackals expanded into Romania and Hungary from Bulgaria's mountainous regions along the Danube River. Since our results showed that samples from these two countries can be distinguished on the basis of genetic characteristics, we can also assume that this route was not the species' single route of expansion and other corridors can be assumed as well. The large carnivore species found in Europe are able to travel long distances, often crossing through several different countries, when they are expanding or wandering (Blanco 2012, Chapron et al. 2014). Golden jackals' capability to expand has been repeatedly proven by genetic and telemetry studies, both inside and across the borders of different countries. Members of the species have been found several hundred kilometres from the source populations including in the Baltic countries and Switzerland (Rutkowski et al. 2015). This suggests that golden jackals became established in Romania and in Hungary following several episodes of colonization, possibly arriving from different directions.

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