

# Genetic Variability, Differentiation, and Founder Effect in Golden Jackals (*Canis aureus*) from Serbia as Revealed by Mitochondrial DNA and Nuclear Microsatellite Loci

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**Abstract** We analyzed 121 golden jackals (*Canis aureus*) from six sample sites in Serbia with regard to genetic variability and differentiation as revealed by mitochondrial control region sequences and eight nuclear microsatellite loci. There was no variation at all in the mtDNA sequences, and nuclear variability was very low (average observed and expected heterozygosity of 0.29 and 0.34, respectively). This is in line with the considerable recent range expansion of this species in the Balkans and indicates a strong founder effect in the recently established Serbian population. We did not find evidence of differentiation between the northeastern jackals and those from the plain of Srem or those in between. *F*-statistics and Bayesian Structure analyses, however, were indicative of a low degree of overall differentiation in the Serbian population. A vagrant Austrian jackal that was also analyzed was genetically indistinguishable from its Serbian conspecifics.

**Keywords** *Canis aureus* · Serbia · Founder effect · Microsatellites · Mitochondrial DNA

## Introduction

The golden jackal (*Canis aureus* Linnaeus 1758) is one of the most widespread canid species, occurring in southeastern Europe, northern and eastern Africa, and in large parts of Asia eastward to Thailand (Demeter and Spassov 1993). The northern

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border of the European resident population is along the Danube in Romania and former Yugoslavia (Krystufek et al. 1997). Golden jackals are found in the Caucasus, Turkish Thrace, Greece, Bulgaria, Albania, along the eastern Adriatic coast, and in Romania; Serbia has recently been recolonized (Mitchell-Jones et al. 1999). Apart from Greece, where the jackals are on the decline and listed as vulnerable in the national Red List (Giannatos et al. 2005), the species has expanded its European distribution range, most notably in Bulgaria, where there was a 33-fold increase in the area inhabited by jackals between the 1960s and the 1980s and which now supports the largest jackal population in Europe (Genov and Wassilev 1989; Krystufek and Tvrtkovic 1990; Krystufek et al. 1997). Vagrant animals have repeatedly been recorded from northeastern Italy, Slovenia, Hungary, Austria, Slovakia, and Macedonia (Krystufek et al. 1997; Bauer and Suchentrunk 1995). The golden jackal generally seems to benefit from the extinction of local wolf populations, although in eastern Serbia both species occur sympatrically (Milenkovic 1987).

In Serbia, Milenkovic (1987) and Krystufek et al. (1997) reported two permanent populations, one in the plain of Srem and one in the northeastern part of the country (Negotin). From the Srem population, which probably established itself in the 1980s, vagrant jackals reached Hungary and Slovakia. The northeastern population is probably an offshoot of the Bulgarian range expansion (Krystufek et al. 1997; see also Milenkovic 1987). At present, however, these two populations cannot be considered disjunct (see also map in Mitchell-Jones et al. 1999), and it is possible that one gave rise to the other.

From the Late Pleistocene, there are no records of the golden jackal in Europe, and the only subfossil European jackals are four records from the Neolithic in Greece (Kitsos and Delphi) (Sommer and Benecke 2005). The jackal is thus a very young European element, but the hypothesis that the European population goes back to the introduction of jackals from northern Africa in the 15th century (Kühn 1935) is now generally rejected (Demeter and Spassov 1993).

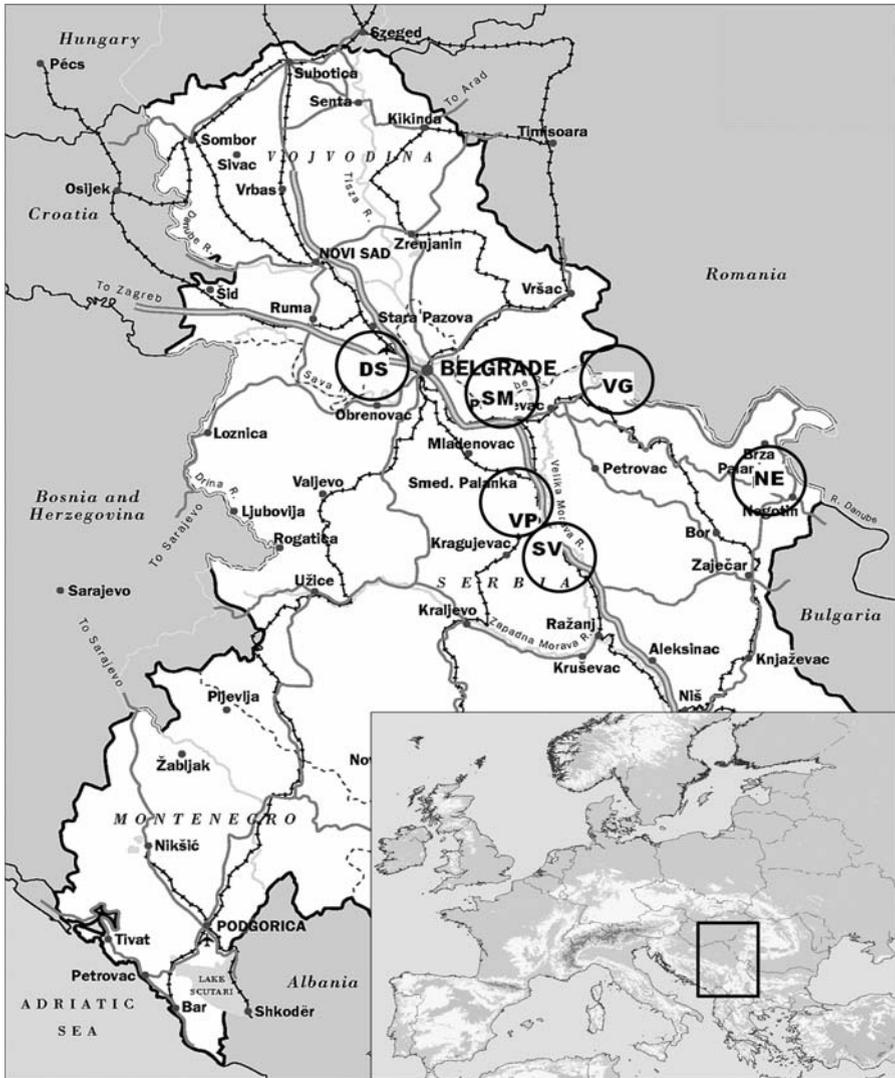
Despite the widespread occurrence of the golden jackal, as yet no population genetic study has been carried out on this species, except for the analysis of 18 specimens from Kenya, which, however, were used only as a comparison sample in a study on North American canids by Roy et al. (1994).

This study represents, to our knowledge, the first thorough analysis of genetic variability and differentiation in the golden jackal, aiming at (a) genetically characterizing the Serbian population and looking for a signature of the recent range expansion, which is expected to have resulted in low genetic diversity, and (b) looking for potential differentiation between Negotin (representing the northeastern population) and the plain of Srem or other Serbian jackals. The markers chosen, sequences of the mitochondrial control region and nuclear microsatellite loci, are known for their high intraspecific variability and have been used successfully in population and conservation genetic studies before (e.g., Hajji et al. 2007; Ben Slimen et al. 2007; Zachos et al. 2006, 2007a, b).

**Materials and Methods**

**Sampling**

Tissue samples from 121 legally culled golden jackals from six sample sites in Serbia were analyzed (Fig. 1): Donji Srem ( $n = 17$ ), Smederevo (15), Veliko Gradiste (28), Velika Plana (14), Svilajnac (15), and Negotin (32). The six sample sites cover both the northeastern (Negotin) and the plain of Srem (Donji Srem)



**Fig. 1** Geographic location of the sample sites analyzed in the present study of Serbian jackals. *DS* Donji Srem, *SM* Smederevo, *VG* Veliko Gradiste, *VP* Velika Plana, *SV* Svilajnac, *NE* Negotin

populations as mentioned by Milenkovic (1987) and Krystufek et al. (1997), as well as locations between the two sites. In addition, we had a tissue sample from a vagrant golden jackal from Scheiblingkirchen, about 60 km south of Vienna, Austria, which was also analyzed.

### Mitochondrial DNA

DNA was extracted from tissue samples using the Qiagen DNeasy Tissue Kit. Control region amplification was carried out with the H-primer DLH (5'-CCT GAAGTAAGAACCAGATG-3') and a modified L-primer LF15926F (5'-ATATA AAATACTTTGGTCTTGTAACC-3') (see Kirschning et al. 2007). PCR conditions were as described in Zachos et al. (2003), with a total reaction volume of 75  $\mu$ l MgCl<sub>2</sub> buffer, 3  $\mu$ l template DNA, and an annealing temperature of 55°C. In order to better cope with ambiguous sites, both strands were sequenced on an automated sequencer, and the control region sequences were aligned using the BioEdit 7.0 software (Hall 1999). Since we found only a single haplotype in all animals, no further statistical analyses were carried out.

### Microsatellites

Individual genotypes were established through amplification of eight polymorphic canine loci: 109, 123, 204, 225 (Ostrander et al. 1993), 374, 468, 502, and 622 (Ostrander et al. 1995). PCR conditions were as described in Zachos et al. (2003), using the annealing temperatures given by Ostrander et al. (1993, 1995). Length determination of the microsatellite alleles was conducted with an automated sequencer (MegaBace 1000) and the Genetic Profiler software. Since allelic diversity was generally rather low by usual microsatellite standards, we repeated the PCR and fragment length analysis each time we found a rare new allele, in order not to increase the number of alleles due to artifacts.

The eight loci were tested for pairwise linkage disequilibrium using the Genepop software (Raymond and Rousset 1995). We tested our data for the occurrence of null alleles, large allele dropout, and stutter bands with the Micro-Checker program (van Oosterhout et al. 2004) because these potential error sources are known to distort allele frequencies and estimates of heterozygosity (Pemberton et al. 1995; Wattier et al. 1998; Ewen et al. 2000). Allelic richness was calculated with the Fstat software (Goudet 1995) as a measure of allele diversity corrected for differences in sample sizes among populations. Observed and expected heterozygosity as well as deviations from Hardy–Weinberg expectation were calculated with Arlequin 3.0 (Excoffier et al. 2005). Arlequin was also used for the analysis of molecular variance (AMOVA). We calculated the overall fixation indices  $F_{ST}$  and  $R_{ST}$  (the latter being a microsatellite-specific analog of  $F_{ST}$  based on the stepwise mutation model), yielding the proportion of the total genetic diversity accounted for by the differentiation among (as opposed to variation within) populations. Pairwise population differentiation was calculated on the basis of pairwise  $F_{ST}$  and  $R_{ST}$  values. Pairwise genetic distances between populations were quantified with Nei's (1972) standard distances and the Cavalli-Sforza and Edwards (1967) chord

distances, both calculated with the Genetix program (Belkhir 2000). Finally, the genetic structure of the jackals was analyzed using two kinds of assignment procedures. First, we used the GeneClass2 software (Piry et al. 2004) to conduct classical assignment tests on individual genotypes, where individuals are assigned to the population of which their genotype is most typical. A large proportion of incorrect assignments thus indicates little genetic differentiation between the respective populations. Second, the Structure software (Pritchard et al. 2000) was used to infer the most probable number of genetic clusters without a priori definition of populations. This particularly suits our sampling scheme, as it is improbable that our sampling sites represent distinct populations. Therefore, classical population structure analysis using  $F$ -statistics might not be sufficient. We used the batch-run function to carry out a total of 80 runs, 10 each for one to eight clusters (i.e.,  $K = 1$  to  $K = 8$ ). The repetitions were run to see if there were deviations among the different runs for a fixed  $K$  and to calculate means and standard deviations. For each run the burn-in and simulation length was 250,000 and 1,000,000, respectively, and the admixture model and the option of correlated allele frequencies were chosen, as this configuration is most suitable in cases of subtle population structure (Falush et al. 2003). Since the log probability values for the different  $K$  values have been shown to be of little reliability in some cases, the more refined ad hoc statistic  $\Delta K$  was used to infer the real number of populations. Based on the rate of change in the log probability of data between successive  $K$  values (Evanno et al. 2005),  $\Delta K$  has been shown to be better at unveiling the correct number of genetic clusters. It is calculated as  $\Delta K = (lmL(K + 1) - 2mL(K) + mL(K - 1))/sdL(K)$ , where  $L(K)$  is the natural logarithm of the probability that  $K$  is the correct number of clusters,  $m$  is the mean, and  $sd$  is the standard deviation of the replicate runs for the same  $K$  value.

The significance level of 0.05 was adjusted using Bonferroni corrections (Rice 1989) whenever multiple tests were carried out.

## Results

The Serbian jackals were completely monomorphic for the mitochondrial control region sequences based on an alignment of 392 bp (which in some individuals had to be shortened to 326 bp), so both haplotype and nucleotide variability were zero. The same haplotype was also found in the Austrian jackal.

There was no significant linkage disequilibrium between any two of the eight loci; consequently, they were all included in subsequent multilocus analyses.

The Micro-Checker analysis yielded no signs of large allele dropout and only occasional hints at misidentification of alleles due to stutter bands (at locus 468 in Svilajnac, Negotin, and Veliko Gradiste, and at locus 123 in Negotin). Careful re-examinations of our electropherograms, however, made it obvious that no heterozygotes went unnoticed in these cases. A positive result for null alleles was found in the above mentioned cases with a positive signal for stutter bands and at locus 204 in Donji Srem. On the whole, these results make it improbable that our data have been distorted by one of these error sources.

The eight loci were all polymorphic and yielded between two and five alleles each. Some sample sites were monomorphic for single loci (Velika Plana, Donji Srem, Svilajnac, and Negotin for locus 225 and Svilajnac also for locus 123). Locus 225 was special in that it yielded four alleles, three of which, however, occurred only once (but were confirmed by repetitions of the PCR and the fragment length analysis). Genetic variability as estimated by allelic richness and observed and expected heterozygosity was very low (Table 1). A total of 31 different alleles was found, yielding an overall allele diversity of  $31/8 = 3.875$ ; the average expected heterozygosity over all six sample sites and for all individuals combined was 0.34 and 0.33, respectively. Statistically significant deviations from Hardy–Weinberg expectation were found only in Negotin (at loci 123 and 468) and in Veliko Gradiste (at locus 468), all due to an excess of homozygotes. When all jackals were pooled into a single total population, a significant heterozygote deficiency was found at three loci (123, 204, and 468), suggesting an excess of homozygotes due to substructuring (Wahlund effect; Wahlund 1928).

Overall  $F_{ST}$  was 0.071 and  $R_{ST}$  was 0.089, significantly different from zero, indicating that between 7% and 9% of the total genetic variation found was on the interpopulational level. Genetic differentiation and distances as assessed by pairwise  $F_{ST}$  values and chord distances are shown in Table 2. Pairwise  $R_{ST}$  values and standard distances yielded almost identical results and are not shown in detail. Only seven out of 15 pairwise  $F_{ST}$  comparisons were significant, and there is an almost exact match between  $F_{ST}$  values and chord distances regarding which comparisons were significant and which were not.

The assignment tests yielded concordant results irrespective of the algorithm chosen (Bayesian or frequency-based) or the running conditions: only 31.7–42.5%

**Table 1** Genetic variability in Serbian jackals derived from eight microsatellite loci

Population	$n^a$	Heterozygosity <sup>b</sup>		$N_{\lambda}^c$	AR <sup>d</sup>
		$H_O$	$H_E$		
Velika Plana	14 (14)	0.32	0.36	20	2.3
Donji Srem	17 (17)	0.29	0.35	22	1.6
Smederevo	14 (15)	0.26	0.32	21	2.4
Svilajnac	15 (15)	0.29	0.35	19	2.3
Negotin	32 (23)	0.23	0.29	21	2.2
Veliko Gradiste	28 (22)	0.32	0.34	22	2.4
Average	–	0.29	0.34	20.8	2.2
Total <sup>e</sup>	120 (106)	0.28	0.33	31	–

<sup>a</sup> Sample size; the number in parentheses refers to the sample size of the mtDNA analysis

<sup>b</sup> Observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosity, calculated over all eight loci

<sup>c</sup> Number of microsatellite alleles found

<sup>d</sup> Allelic richness (based on a minimum sample size of 10 individuals), calculated over all eight loci

<sup>e</sup> “Total” refers to the whole population, i.e., all individuals combined into one group

**Table 2** Genetic differentiation and distance between golden jackal sample sites based on allele frequencies of eight polymorphic microsatellite loci

Sample site	VP	DS	SM	SV	NE	VG
VP	–	0.180*	0.065	0.073	0.111*	0.077*
DS	0.189*	–	0.113*	0.082	0.061	0.059
SM	0.047	0.088*	–	0.042	0.091*	0.057
SV	0.076	0.043	0.010	–	0.058	0.043
NE	0.170*	0.016	0.088*	0.051	–	0.055*
VG	0.114*	0.043*	0.026	0.029	0.069*	–

Above the diagonal: Cavalli-Sforza and Edwards (1967) chord distance. Below the diagonal: Pairwise  $F_{ST}$  value. Significant values are marked with an asterisk (\*). Sample site abbreviations as in Fig. 1

**Table 3** Results of the Structure analysis

$K$	Mean $\ln(\text{Pr}) \pm \text{SD}^a$	$\Delta K^b$
1	$-1136.15 \pm 0.15$	–
2	$-1100.34 \pm 0.81$	109.91
3	$-1153.56 \pm 5.65$	8.23
4	$-1160.27 \pm 13.17$	3.63
5	$-1214.85 \pm 29.97$	0.40
6	$-1257.36 \pm 37.32$	1.40
7	$-1247.52 \pm 37.74$	0.42
8	$-1253.35 \pm 53.13$	–

<sup>a</sup>  $\ln(\text{Pr})$ , logarithm of the probability, calculated by the Structure software, that  $K$  is the correct number of populations. SD, standard deviation, calculated from 10 independent runs

<sup>b</sup> The ad hoc statistic  $\Delta K$  is not applicable for  $K = 1$ , and from the equation given in the methods section it is obvious that it cannot be calculated for the highest  $K$  number either (because data for  $K + 1$  are needed)

of the individuals were correctly assigned to their sample site, and none of the single sites was found to show high numbers of correct assignments.

The Structure analysis yielded a maximum probability for two clusters ( $K = 2$ ), and this was confirmed by the  $\Delta K$  statistic, which also showed a clear peak for  $K = 2$  (Table 3). Bear in mind, though, that  $\Delta K$  is not able to unveil the true  $K$  if  $K = 1$ . The two clusters both comprised single animals from each population, but those from Velika Plana were almost all assigned to the smaller cluster that otherwise harbored comparatively few individuals from the other populations.

The Austrian jackal showed only alleles common also in the Serbian jackals.

## Discussion

We analyzed 121 Serbian golden jackals and a single Austrian vagrant. Genetic variability was very low, as might be expected in a recently established population

at the margin of a species' distribution range. Unfortunately, very few data are available for direct comparison with our results. To our knowledge, the only study that includes golden jackals in a population genetic analysis is the one by Roy et al. (1994), who genotyped 18 golden jackals from Kenya at 10 microsatellite loci as an outgroup for comparison with North American canids. Four of these 10 loci were also used in the present study, and although we found fewer alleles at loci 123 (2 vs. 5) and 204 (4 vs. 6), the loci 109 and 225 yielded more alleles in Serbia than in Kenya (3 vs. 2 and 4 vs. 1, respectively), but three of our four alleles at locus 225 occurred only once. Given that our sample size was more than six times that of Roy et al. (1994), it becomes clear that Serbia is considerably less diverse than Kenya. Since the number of alleles found in the different Serbian populations varied only between 19 and 22, although sample sizes varied by a factor of more than two, it seems very likely that we did not miss any alleles in our sample.

In line with this picture, the Kenyan jackals yielded observed and expected heterozygosities much higher than those of their Serbian conspecifics ( $H_O$  0.41 vs. 0.29;  $H_E$  0.52 vs. 0.34). Still, the Kenyan jackals were less diverse than the North American gray wolves and coyotes analyzed in the same study, so golden jackals might generally harbor less genetic variability than other canids. In a recent study, Kirschning et al. (2007) analyzed mitochondrial control region sequences in a similarly large sample of Serbian red foxes ( $n = 110$ ). This study also had a similar geographic scope, but the foxes yielded nine different haplotypes, in contrast to only one found in the jackals, which, of course, also reflects the much longer history and the larger effective population size of the fox in this region. European gray wolves, although having undergone severe persecution, also show considerably higher genetic variability, and this also holds for the strongly bottlenecked Italian population, which, based on 18 microsatellite loci, still yielded observed and expected heterozygosities of 0.44 and 0.49, respectively (Lucchini et al. 2004).

Lack of mtDNA control region variability has been observed in a range of populations from different taxa, among them Italian wolves (Lucchini et al. 2004), brown bears from the Apennines (Zachos et al. 2008), and the relict red deer from Mesola in Italy (Hmwe et al. 2006), but in all these cases nuclear diversity as assessed by microsatellites was considerably higher than in the jackals of the present study. Yet, interspecific comparisons of genetic diversity have to be viewed with caution, on account of possible species-specific differences. With regard to genetic variability in golden jackals, it can be summarized that the Serbian jackals showed very low diversity, but this may be due partly to a generally low level of variability in this species.

With regard to differentiation, we did not find evidence of significant differences between the Srem and the northeastern populations (represented by Negotin). On the whole, there was very little differentiation among the Serbian jackals. Still, the jackals were not completely homogeneous genetically, as evidenced by the significant overall and some pairwise  $F_{ST}$  and  $R_{ST}$  values (see Table 2, in particular, comparisons involving Velika Plana), as well as the Wahlund effect in the combined sample and the Structure and  $\Delta K$  results, which both favored two genetic groups over one. It is interesting that Velika Plana, and not Negotin in the northeast, was most differentiated from the other samples in the Structure analysis. Knowledge

about the golden jackal in Serbia does not offer a straightforward explanation for this, but since the degree of differentiation was low, a random effect based on sampling error cannot be excluded.

The analysis of the Austrian vagrant jackal showed that it was genetically indistinguishable from the Serbian animals with respect to both mtDNA and microsatellites. This is interesting because it has been assumed that the golden jackals found in Italy, Slovenia, and Austria came from Istria in northwestern Croatia (Krystufek et al. 1997). Either this does not hold for the Austrian specimen that we analyzed (in this case, it most likely immigrated from Serbia, perhaps via Hungary), or our results indicate that golden jackals across the whole northern Balkans display a high amount of genetic uniformity. Further analyses of jackals from other countries in southeastern Europe are needed to answer this question.

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