Mitochondrial DNA and microsatellite analyses of the genetic status of the presumed subspecies *Cervus elaphus montanus* (Carpathian red deer)

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The possibly distinct Carpathian red deer was compared genetically to other European populations. We screened 120 red deer specimens from Serbia, the Romanian lowland and the Romanian Carpathians for genetic variability using 582 bp of the mitochondrial control region and nine polymorphic nuclear microsatellite loci. The study aimed at a population genetic characterization of the Carpathian red deer, which are often treated as a distinct subspecies (*Cervus elaphus montanus*). The genetic integrity of the Carpathian populations was confirmed through the haplotype

distribution, private alleles and genetic distances. The Carpathian red deer are thus identified as one of the few remaining natural populations of this species, deserving special attention among game and conservation biologists. The history of the populations studied, in particular the introduction of Carpathian red deer into Romanian lowland areas in the 20th century, was reflected by the genetic data. *Heredity* (2004) **93**, 299–306. doi:10.1038/sj.hdy.6800504
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Introduction

Subspecific designations of populations have an important bearing on conservation biology and management plans (O'Brien and Mayr, 1991) although the subspecies concept is partly arbitrary since it is based upon 'diagnostic morphological characters' (Mayr, 1963, p 348), which makes it impossible to consider subspecies to be real evolutionary units (Mayr, 1942, 1963). From a population biological point of view, it is genetic integrity rather than taxonomic status that renders populations worthy to be treated as evolutionary and conservation units.

The European red deer (*Cervus elaphus*) has been undergoing human influences such as translocations, habitat fragmentation and selective hunting throughout Europe for many centuries (Lowe and Gardiner, 1974; Hartl *et al.*, 2003). As a consequence, genetic boundaries between populations have been blurred and few stocks are historically indigenous. In Italy, for example, the only native red deer population is that from the Mesola sanctuary in the Po delta area (Geist, 1998; Lorenzini *et al.*, 1998). The Carpathian red deer are interesting in that they have been considered both to be indigenous (Nedic, 1940; Philipowicz, 1961) and to be a distinct subspecies, characterized morphologically by their grayish color, the absence of a dark mark on the rump patch, and the lack of a mane (Dobroruka, 1960; Groves and Grubb, 1987).

The first to ascribe Carpathian deer taxonomic status was Botezat (1903), and since then, the Carpathian red deer, when granted subspecific status, has been called *C. elaphus montanus* (but see Grubb, 2000, who presents evidence that the name *montanus* is taxonomically invalid). According to Dobroruka (1960), the geographic distribution of *C. elaphus montanus* ranges from the eastern Carpathians to southern Ukraine and the Crimea, while Groves and Grubb (1987) extend its northern range as far as the Baltic coast. The Carpathian red deer is thus considered to be geographically intermediate between *C. e. hippelaphus* in the west and *C. e. maral* in the east. No genetic analysis of this presumed subspecies has been carried out. Our study does not aim at corroborating any taxonomical designation but rather at elucidating the genetic status in terms of isolation and differentiation of the Carpathian red deer in order to test the hypothesis of its genetic integrity, and thus possibly identify another natural red deer stock in Europe. The molecular markers chosen are nine microsatellite loci and sequence data from the mitochondrial control region – both powerful tools for population genetic analyses (Avise *et al.*, 1987; Schlötterer and Pemberton, 1994; Goldstein and Schlötterer, 1999) which have been used in previous studies on red deer (eg Coulson *et al.*, 1998; Slate *et al.*, 2000; Randi *et al.*, 2001; Kuehn *et al.*, 2003; Zachos *et al.*, 2003). The mitochondrial control region is the noncoding and, with a large number of point mutations, insertions and deletions, it is the most variable part of the mitochondrial DNA (Moritz *et al.*, 1987). A combination of these two markers is a comprehensive approach that combines the highly polymorphic microsatellites whose high mutation rates (Weber and Wong, 1993; Hancock, 1999) allow for

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small-scale resolution of demographic events with mitochondrial lineages which are often used in analyses of phylogeographic events dating further back in time (eg *Avise et al*, 1987; *Randi et al*, 1998; *Barnes et al*, 2002).

Material and methods

Sampling

The study is based on 120 specimens of *C. elaphus* from 10 different locations in Serbia and Romania (abbreviations and sample sizes of mtDNA/microsatellite analyses given in parentheses): Bachka (BAC, 33/30), Banat (BAO: free-living animals, 13/13; BAF: animals from a fenced area, 8/8), Temishvar (TEM, 10/10), Arad (ARA, 15/12), Karansebesh (KAR, 1/0), Brashow (BRA, 12/10), Covasna (COV, 8/8), Harghita (HAR, 11/11), and Reghin (REG, 9/9) (Figure 1). From Karansebesh only one individual was available and was excluded from the population genetic analyses, but its mtDNA haplotype was included in the phylogenetic analysis. Muscle tissue was collected from culled animals during the hunting seasons 2000 and 2002 by local hunters and was stored at -20°C until further analysis.

The extinct Banat population was refounded in the 20th century (1938, 1943 and 1994) with 29 individuals of native Bachka red deer (*Bradvarovic et al*, 1994; *Bjedov et al*, 1997; *Bradvarovic*, 1997). Between 1960 and 1980, an introduction project of Carpathian red deer throughout Romania was carried out with the aim of replacing the Romanian lowland deer, which had been introduced there from game parks in Hungary, Austria and Czechoslovakia in the 18th and 19th century, with the stronger Carpathian ones in order to improve the quality of antler trophies (*Almasan*, 1988). Consequently, the populations in Karansebesh and Arad are a mixture of Carpathian and central European red deer. In Temishvar, on the other hand, the red deer are free from Carpathian

introgressions. There are no migration barriers within the Carpathians, but, according to local hunters' experience, there is no migration between the Carpathians and the Romanian lowland. Red deer are known to cross the border between the Banat region in Serbia and the adjacent region in Romania, and the Bachka population is in contact with stocks in Croatia (via the Danube which the animals are able to cross) and Hungary.

Mitochondrial DNA

Total genomic DNA of frozen muscle tissue was extracted by means of the Super QuikGene-Extraction kit (Genetic Analytical Testing Center). Amplification of the mitochondrial control region was performed using the primers Pro-L and Phe-Hb (cf *Zachos et al*, 2003). PCR amplification was conducted according to *Zachos et al* (2003). Conditions were as follows: 40 cycles of 75 s at 94°C , 90 s at 59°C and 75 s at 72°C , preceded by a 5 min initial denaturation at 95°C and followed by a 7 min terminal elongation at 72°C . PCR products were sequenced with an automatic sequencer (ABI Sequencer 373) using the forward primer Pro-L. Sequences were aligned manually using BioEdit version 5.0.9 (*Hall*, 1999). Haplotype and nucleotide diversities (*Nei*, 1987) were calculated using DnaSP3.51 (*Rozas and Rozas*, 1999). Relationships among haplotypes were visualized using neighbor-joining (based on Kimura-2-parameter distances), maximum likelihood and maximum parsimony methods as implemented in PHYLIP (*Felsenstein*, 1993). These three methods were chosen to check if the trees based on distances (neighbor-joining), character states (maximum parsimony, an algorithm constructing the tree with the minimum number of mutations) and likelihood approaches yielded consistent results. In order to carry out population comparisons, genetic distances after *Reynolds et al* (1983) were derived from haplotype frequencies using PHYLIP, and a neighbor-joining tree was constructed. Support for nodes was assessed by bootstrap resampling using 100 (maximum likelihood) or 1000 replicates. A Mantel-test was conducted with the software NTSYS-pc 1.80 (*Rohlf*, 1994) to test for a correlation between genetic and geographic distances. Contrary to absolute genetic differentiation as assessed by pairwise distances, *Wright's F-statistics* provides a means of hierarchical analysis of genetic diversity. We calculated the overall F_{ST} value, yielding the proportion of the total genetic diversity accounted for by the differentiation among populations, using *Arlequin* (*Schneider et al*, 2000). For the construction of mtDNA trees, a sequence of *C. alfredi*, a close relative of *C. elaphus*, was chosen as outgroup (<http://www.ncbi.nlm.nih.gov/>, accession number AF291891, *Randi et al*, 2001). Additional red deer sequences from *Randi et al* (2001, accession numbers AF291885-9) and *Zachos et al* (2003) were integrated into an extended analysis, which was conducted on the basis of 332 bp because this was the length of the sequences analysed by *Zachos et al* (2003).

Microsatellites

Microsatellite amplification was performed as described above except reaction volumes were $37.5\mu\text{l}$ and microsatellite-specific annealing temperatures ranging from 55 to 63°C were used. Nine polymorphic loci developed in a variety of other ungulates were analysed: BM888,

Figure 1 Geographic location of the populations studied in Serbia and Romania. Bachka, Banat, Arad, Temishvar and Karansebesh are lowland populations; Reghin, Harghita, Covasna and Brashow lie within the Carpathians. From Banat, two populations were studied, one free-ranging and one from a fenced area.

BM4208, BM4513 (Bishop *et al*, 1994), MAF35 (Swarbrick *et al*, 1991), MAF109 (Swarbrick and Crawford, 1992), INRA11 (Vaiman *et al*, 1992), OarCP26 (Ede *et al*, 1995), OarFCB304 (Buchanan and Crawford, 1993), and CSSM43 (Moore *et al*, 1994). Microsatellite loci were tested for linkage disequilibrium with GENEPOP 3.2 (Raymond and Rousset, 1995). Observed and expected heterozygosities as well as deviations from Hardy–Weinberg equilibrium and overall F_{ST} were calculated using Arlequin. To obtain a measure of allelic diversity (mean number of alleles per locus) independent of sample sizes, allelic richness values were calculated with FSTAT (Goudet, 1995). Private alleles (Slatkin, 1985) were counted and a population differentiation test (GENEPOP 3.2) and an assignment test based on individual genotypes (Paetkau *et al*, 1995) were carried out. In the assignment test, all individuals are assigned to the population of which their genotype is most typical. Incorrect assignments indicate little genetic differentiation between the respective populations. Population differentiation was further quantified with Nei's (1972) genetic standard distances D and chord distances (Cavalli-Sforza and Edwards, 1967), both calculated using PHYLIP. Correlation between genetic and geographic distances was tested as described above. Neighbor-joining and Fitch-Margoliash trees (1000 bootstrap replications) based on standard and chord distances were constructed with the PHYLIP package. An additional microsatellite tree based on seven of the

nine loci was constructed with data taken from Zachos *et al* (2003). The seven loci used were the same as those analysed by Zachos *et al* (2003).

Results

Mitochondrial DNA

A total of 120 individuals from 10 sampling sites were screened for variation in the mitochondrial control region. Sequencing of 582 bp revealed 36 variable sites (31 transitions, two transversions, and three deletions) and resulted in the detection of 14 putative haplotypes (Table 1). Haplotypes 14 and 9 were the most common sampled with overall frequencies of 46 and 24%, respectively. The former haplotype was found in the Serbian populations (where 53 out of 54 individuals showed this haplotype) and in Arad while the latter was confined to the four Carpathian populations in each of which it was by far the most common. Haplotype 4 was the most divergent, separated from the other 13 haplotypes by at least 18 mutations, and was found in only one individual from Reghin in the Carpathians. The four Carpathian populations yielded six haplotypes four of which were found nowhere else; the remaining two haplotypes also occurred in Arad (Table 1) where Carpathian deer were introduced in the second half of the 20th century (see above). Overall differentiation among all studied populations (F_{ST} value) was 61.65%, indicating considerable differentiation between populations. Haplotype diversities ranged from 0.000 to 0.810 and nucleotide diversities from 0.000 to 0.954% (Table 2), and in both cases the highest values were exhibited by the hybrid population from Arad. Among the Carpathian populations, net nucleotide diversities were very low, ranging from 0.000 to 0.134 while those between

Table 1 Distribution of mitochondrial haplotypes (HT)

	BAC	BAO	BAF	TEM	ARA	KAR	BRA	COV	HAR	REG	Σ
HT 1				7							7
HT 2					1				2		3
HT 3			1								1
HT 4										1	1
HT 5							1	1			2
HT 6				6	1						7
HT 7				2							2
HT 8				2							2
HT 9							6	6	9	8	29
HT 10					3		1				4
HT 11							4	1			5
HT 12	1										1
HT 13					1						1
HT 14	32	13	8		2						55
Σ	33	13	8	10	15	1	12	8	11	9	120

For population abbreviations see text. Note that haplotype 9, the most common in the Carpathian populations (BRA, COV, HAR, REG), is exclusive to this region.

Table 2 Haplotype (HD) and nucleotide (π) diversities for the red deer populations in Serbia and Romania

Pop	HD	π (%)
BAC	0.061	0.010
BAO	0.000	0.000
BAF	0.000	0.000
TEM	0.511	0.463
ARA	0.810	0.954
BRA	0.667	0.845
COV	0.464	0.461
HAR	0.327	0.451
REG	0.222	0.691

Table 3 Net nucleotide diversities (in %) between populations as a pairwise distance measure based on 582 bp of the mitochondrial control region

	BAC	BAO	BAF	TEM	ARA	BRA	COV	HAR	REG
BAC	—								
BAO	0.000	—							
BAF	0.000	0.000	—						
TEM	1.000	1.000	1.000	—					
ARA	0.468	0.468	0.468	0.419	—				
BRA	0.697	0.701	0.701	0.208	0.361	—			
COV	1.046	1.048	1.048	0.162	0.475	0.014	—		
HAR	1.162	1.162	1.162	0.199	0.471	0.134	0.000	—	
REG	1.279	1.280	1.280	0.215	0.545	0.125	0.000	0.000	—

Carpathian and non-Carpathian populations ranged from 0.162 to 1.280 (Table 3).

The phylogenetic trees based on the haplotypes were consistent irrespective of the underlying construction algorithm, but yielded no clear geographic pattern. The inclusion of sequences from other south European red deer populations (southern Spain, northern Italy, Sardinia, and Bulgaria, Zachos *et al*, 2003) as well as from single specimens from Spain, Norway, Italy, and Sardinia (Randi *et al*, 2001) did not change tree topology. The divergent haplotype 4, along with the two sequences from Spanish and Norwegian red deer taken from Randi

et al (2001), clustered with some of the Spanish and Sardinian samples from Zachos *et al* (2003) while all other haplotypes from Serbia and Romania formed a clade with the sequences from Italian and Bulgarian red deer (Figure 2).

When genetic distances derived from haplotype frequencies were used for tree reconstruction, the resulting topology was consistent with the geographic location of the respective populations (cf Figure 3). In particular, all four Carpathian populations clustered together. However, genetic and geographic distances between all populations under study (neglecting only the

Figure 2 Maximum parsimony consensus tree of the fourteen haplotypes found in this study (Haplo 1–14) combined with red deer sequences from the literature. Haplotypes represented by three capital letters are taken from Zachos *et al* (2003), numbers and abbreviations are the same as in the original publication (SSP = southern Spain; VDS = Val di Susa, Italy; TAR = Tarvis, Italy; SAR = Sardinia; BUL = Bulgaria). The sequences denoted *Cervus alfredi* (outgroup), *C. e. corsicanus* (Sardinia), *C. e. hispanicus* (Spain), *C. e. atlanticus* (Norway) and *C. e. hippelaphus* (Italy) are taken from Randi *et al* (2001, for gene bank accession numbers see text). Numbers at nodes denote support from 1000 bootstrap replications. Haplotypes 5 and 10 as well as 13 and 14 of this study are identical in this tree because the sequences had to be reduced to 332 bp to be comparable to those of Zachos *et al* (2003). The topology of this tree is consistent with the results gained from other tree reconstruction algorithms (neighbor-joining, maximum likelihood). For details, see text.

deer from the fenced area in Banat) did not show a statistically significant correlation (Mantel-test).

Microsatellites

All loci were polymorphic with between 16 and 37 alleles and there was no linkage disequilibrium after Bonferroni correction for multiple testing so that all loci were included in the analysis. The PCR-amplification for locus MAF109 failed in 10 out of 11 individuals of the Harghita population, suggesting the existence of a nearly fixed mutation in the primer binding region resulting in null alleles (Callen *et al*, 1993; Koorey *et al*, 1993; for

microsatellite null alleles in red deer cf Pemberton *et al*, 1995). In order to rule out the possibility of a bias due to these putative null alleles, further calculations were performed both with and without MAF109. Results were the same in both approaches and only analyses including MAF109 data are presented here.

Values of allelic diversity and allelic richness are given in Table 4. Out of 657 alleles 68 (10.4%), were private alleles. The proportion of private alleles in the Carpathian populations was very low (0.68–4.9%). When these four populations were combined, the proportion of private Carpathian alleles was 16.6% with one allele (152 at locus BM4513) occurring in all four populations. Overall expected and observed heterozygosities ranged from 0.79 to 0.90 and 0.47 to 0.62, respectively (Table 4). Significant deviations from Hardy–Weinberg-equilibrium, all due to an excess of homozygotes, occurred in all populations under study, in one (Bachka) for all nine loci. The test of population differentiation revealed significant differences between the populations ($P < 0.05$) and the assignment test yielded a proportion of correct assignments of 55%. Incorrect assignments occurred especially often among the four Carpathian populations, but only four out of 38 Carpathian individuals were assigned to a non-Carpathian population. Overall genetic differentiation (F_{ST} value) was 3.6%, a value much lower than that for mtDNA sequences, but this is due largely to the high number of alleles in microsatellites leading to a large amount of genetic diversity within populations. The pairwise genetic distances given in Table 5 clearly show that differentiation among the Carpathian populations is considerably lower than between the Carpathian and the other populations. The resulting neighbor-

Figure 3 Neighbor-joining tree based on Nei's standard distances derived from microsatellite allele frequencies. Numbers at nodes denote support from 1000 bootstrap replications. The neighbor-joining tree based on the mtDNA haplotype frequencies (distance values according to Reynolds *et al*, 1983) yielded a consistent topology. The geographic location of the populations (cf. Figure 1) is reflected by the dendrogram.

Table 4 Average (mean over all loci) values of allelic diversity (AD), allelic richness (AR), expected and observed heterozygosity (H_E and H_O) based upon the analysis of nine microsatellites

Pop.	AD	AR	H_E	H_O
BAC	12.8	1.813	0.82	0.53
BAO	7.3	1.784	0.81	0.57
BAF	5.4	1.748	0.79	0.61
TEM	8.5	1.867	0.90	0.58
ARA	9.3	1.869	0.90	0.47
BRA	7.5	1.830	0.87	0.50
COV	6.7	1.830	0.88	0.62
HAR	7.7	1.849	0.88	0.55
REG	6.3	1.834	0.87	0.52

Table 5 Nei's standard (below diagonal) and Cavalli-Sforza and Edwards' chord (above diagonal) distances between populations based upon allele frequencies of nine polymorphic microsatellite loci

	BAC	BAO	BAF	TEM	ARA	BRA	COV	HAR	REG
BAC	—	0.063	0.053	0.068	0.068	0.073	0.088	0.085	0.083
BAO	0.448	—	0.076	0.020	0.085	0.102	0.098	0.101	0.102
BAF	0.263	0.492	—	0.082	0.079	0.092	0.108	0.105	0.095
TEM	0.612	0.726	0.595	—	0.062	0.078	0.100	0.089	0.096
ARA	0.540	0.722	0.573	0.330	—	0.075	0.095	0.095	0.084
BRA	0.569	0.917	0.660	0.653	0.624	—	0.055	0.057	0.058
COV	0.855	0.928	1.053	0.988	0.879	0.458	—	0.060	0.075
HAR	0.767	1.028	0.858	0.691	0.787	0.383	0.414	—	0.055
REG	0.736	1.061	0.704	0.765	0.627	0.385	0.529	0.370	—

joining trees (Figure 3, the Fitch-Margoliash trees yielded very similar topologies and are not shown) confirm the genetic integrity of the Carpathian deer by clustering the four populations with very high bootstrap support. This topology is consistent with that based on the mtDNA haplotype frequencies in that it reflects the geographic location of the studied populations: the Carpathian and the Serbian populations are connected by the geographically intermediate populations of Arad and Temishvar. In accordance with the results obtained from the mtDNA sequences, there was no statistically significant correlation between genetic and geographic distances (Mantel-test). The trees obtained from the extended data set (including populations from Spain, mainland Italy, Sardinia, and Bulgaria, cf Zachos *et al*, 2003) did not reveal a close relationship between any of the populations of the two studies but yielded two distinct clusters, one comprising the populations of the present study, the other those from Zachos *et al* (2003).

Discussion

Genetic variability of the studied populations as revealed by haplotype and nucleotide diversity, allelic diversity and heterozygosity were within the range of values previously found for red deer (Martinez *et al*, 2002; Kuehn *et al*, 2003; Zachos *et al*, 2003). With the exception of Reghin, haplotype and nucleotide diversity were correlated. The Reghin population showed little haplotype diversity but a high nucleotide diversity, which is explained by the fact that one of the two haplotypes found in Reghin, haplotype 4, was rare but at the same time very divergent (leading to a high nucleotide diversity, which is not only based on the number of different haplotypes but also on their degree of difference). The striking deviations from Hardy-Weinberg equilibrium yielded by our microsatellite analysis could be a consequence of the relation between sample size and microsatellite variability: given the high allele numbers in the different populations, Hardy-Weinberg frequencies are *a priori* not very probable with sample sizes ranging from 8 to 30 animals per population. Another possible explanation is that migration occurs. As stated above, there are no migration barriers within the Carpathian region, and migration is known to take place between western Romania and the Banat as well as between Bachka, Hungary and Croatia. The population of Arad is remarkable in that it exhibits both the lowest observed and (together with Temishvar) the highest expected heterozygosities. Arad also shows the highest allelic richness and the second highest allelic diversity (second only to Bachka where the sample size is more than twice as high). Regarding mitochondrial DNA, Arad has the highest number of haplotypes (6 *vs* 1–4 in the other populations) and the highest haplotype and nucleotide diversity. Such a large genetic variability may well be a consequence of the different origins of the deer in Arad. The low observed heterozygosity and hence the excess of homozygotes could be the result of inbreeding in the recent past. This inbreeding may have only affected the highly variable microsatellite loci so far but not the more conservative mitochondrial DNA. Alternatively, the Arad population could be composed of different subpopulations (Wahlund effect).

The data presented here show that there are both congruences and incongruences between the mtDNA and microsatellite analyses. One of the most striking differences is the genetic variability of the Serbian red deer as represented by haplotype or nucleotide diversity *vs* allelic diversity and heterozygosity: while the latter was well within the range of the values for the other populations studied, mtDNA variability of Serbian red deer was practically nonexistent. The phylogenetic analysis of haplotypes revealed, contrary to the distribution analysis of microsatellite alleles, no geographic pattern. Interestingly, such a pattern emerged when the distribution of haplotypes was examined. These findings could be interpreted as evidence that the mtDNA haplotypes themselves (as opposed to their distribution) are too old to uncover the recent population biological processes in the area under study. The resolving power of microsatellites, which have a higher mutation rate than mtDNA, seems to be high enough to detect genetic variability even in populations without haplotypic variation as is exemplified by the Serbian populations of Bachka and Banat. While all deer analysed from Banat, both free-living and captive, showed the same haplotype as the Bachka deer (from which they are derived, see above), the genetic distances based on microsatellites were within the range of those among the Carpathian populations. Since mtDNA patterns are of purely maternal origin sex-specific differences in migration may also account for different patterns yielded by mitochondrial and nuclear markers. While this may partly be true for both Banat and Bachka where migration has been observed (see above), it is hard to believe that migration alone could explain the discrepancy between usual microsatellite distances on the one hand and 53 out of 54 Serbian red deer exhibiting the same mitochondrial haplotype on the other. Alternatively, it could be speculated that a selective sweep generated by an advantageous mutation in a mitochondrial gene almost led to the fixation of this particular haplotype although this scenario does not seem very probable.

As mentioned earlier, the populations in Karansebes and Arad contain both Carpathian and central European red deer while the Temishvar population is free from Carpathian introgressions. These historical data are in line with our population genetic findings: whereas the Carpathians share two haplotypes with Arad, they have none in common with Temishvar. The single individual from Karansebes showed a haplotype that was also found in Arad but not in the Carpathians, and this haplotype may be a legacy of the introductions from central Europe in the 18th and 19th century.

As outlined in the introduction, the main aim of the present study was to examine the status of the Carpathian red deer. Our data unequivocally confirm their genetic integrity, suggesting that they are indeed historically indigenous. The distance values based on both mitochondrial DNA (net nucleotide diversities) and microsatellites (standard and chord distances) among the four populations of Reghin, Harghita, Covasna and Brashov are lower than between the Carpathian and the other populations. The proportion of private microsatellite alleles of the four Carpathian populations pooled is 16.6%, that is, one out of six alleles is confined to this region. Haplotype 9 is exclusive to the region and

was found in 29 out of 40 individuals. Altogether, four out of six mitochondrial haplotypes were exclusive to the Carpathians, the remaining two only occurring in Arad where Carpathian deer have been introduced. Thus, the Carpathian red deer did not share any haplotypes with truly non-Carpathian populations. Incorrect assignments based on microsatellite genotypes often occurred among Reghin, Harghita, Covasna, and Brashow, whereas only four out of 38 Carpathian individuals were incorrectly assigned to a population outside the Carpathians. There is also a high bootstrap support for the Carpathian branch in the trees based on mitochondrial haplotype and microsatellite allele frequencies. The fact that, in spite of the clear geographic pattern displayed by the frequency distribution trees, there is no statistically significant correlation between genetic and geographic distances might be accounted for by the blurring of the original pattern through migration and the extensive introductions in Serbia and the Romanian lowland.

Regarding microsatellite variability as revealed by allelic diversity, allelic richness and heterozygosity, the Carpathian populations exhibited values similar to those found in the other populations studied. When compared to the endangered red deer population on Sardinia (allelic diversity = 3.2, $H_E = 0.52$, $H_O = 0.36$, Zachos *et al*, 2003), Carpathian deer showed much higher variability values. mtDNA variability, however, was lower on average than in the populations from southern Europe studied by Zachos *et al* (2003), including Sardinia (only the Brashow population had both a higher haplotype and a higher nucleotide diversity than the Sardinian deer), but mitochondrial variability of the Sardinian red deer was unexpectedly high given its population history and its isolation. Carpathian red deer thus show no signs of being threatened by genetic depletion in the near future. Nevertheless, whether they are granted subspecific rank or not, the Carpathian red deer represent one of the few remaining historically indigenous red deer stocks in Europe and as such deserve attention among game and conservation biologists – their genetic integrity should be preserved and any introgression from foreign stocks should be avoided.

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