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Short Communication

High genetic diversity and low population structure in red foxes (*Vulpes vulpes*) from CroatiaAna Galov^a, Magda Sindičić^{b,*}, Tibor Andreanszky^c, Snježana Čurković^b, Danko Deždek^d, Alen Slavica^b, Günther B. Hartl^e, Bastian Krueger^e^a Division of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia^b Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia^c Croatian Veterinary Institute, Department Rijeka, Podmurvice 29, 51000 Rijeka, Croatia^d Croatian Veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia^e Zoological Institute, Christian-Albrechts-University, Olshausenstraße 40, 24118 Kiel, Germany

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ABSTRACT

The red fox (*Vulpes vulpes*) is a highly adaptable omnivorous mammal distributed across all continents on the northern hemisphere. Although the red fox is present throughout Europe, where it plays an important ecological and socio-economic role not only as a game species but also as a rabies reservoir, few studies have examined its population-level mitochondrial DNA variability. In this study, 27 mitochondrial DNA control region haplotypes were identified in 229 red fox samples taken from four regions in Croatia. Haplotype diversity of Croatian red foxes (0.901) was found to be among the highest of all European red fox populations studied to date. Genetic differentiation among regions was quite low, and statistically significant estimates of differentiation were obtained only when comparing the population from the peninsular region of Istria with the three continental populations. It seems that landscape barriers like rivers and small mountains do not restrict gene flow among foxes in the continental part of Croatia, while the combination of a narrow land bridge and altitudes exceeding 1000 m limit fox migration between Istria and the rest of the continent. Better understanding of small-scale population structure will require analysis of highly variable nuclear markers like microsatellites.

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Distributed across all continents on the northern hemisphere, from the Arctic Circle to North Africa, Central America, and the Asiatic steppes, the red fox (*Vulpes vulpes*) has the widest geographical range of any member of the order Carnivora (Macdonald and Reynolds, 2008). This highly adaptable omnivorous species also inhabits urban areas, and despite constant hunting pressures its spring population in Europe (excluding Russia) is estimated at up to one million (Mitchell-Jones et al., 1999). The red fox is an important game species, and it is usually treated as a pest due to its supposedly negative impact on small game populations and its ability to spread numerous diseases, primarily silvatic rabies.

The red fox population in Europe shows no phylogeographic structuring on a wide spatial and temporal scale (Fрати et al., 1998; Teacher et al., 2011; Edwards et al., 2012; Kutschera et al., 2013). Analysis of mitochondrial DNA suggests that foxes in Eurasia survived the last glacial maximum as a single, large, interbreeding population (Teacher et al., 2011; Edwards et al., 2012; Kutschera

et al., 2013), but several lines of evidence suggest survival in southern refugia (detailed discussion in Kutschera et al., 2013). In modern samples evidence of isolation by distance, reduced levels of connectivity and local adaptations were found in some regions (Teacher et al., 2011; Kutschera et al., 2013). Southern European regions generally show less connectivity than northern regions (Edwards et al., 2012). Frати et al. (1998) found little genetic structuring in recent red fox populations in southern Europe and Israel, and Gachot-Neveu et al. (2009) reported similar findings for red foxes in France. Kirschning et al. (2007) analyzed the mtDNA control region of red foxes from Serbia and showed no population differentiation in accordance with the physical geography of the study area.

The red fox is an autochthonous species in Croatia, where populations are found throughout the continental part as well as on several larger islands such as Krk and Rab. Detailed population data are unavailable, but population density has been estimated at about 0.7 animals per km² (Slavica et al., 2010). The fox hunting season lasts all year, and 10–12,000 animals have been hunted annually over the last decade (Slavica et al., 2010). As little is known about red foxes in Croatia, the aim of our study was to investigate the mitochondrial DNA control region variability and genetic structure of this population.

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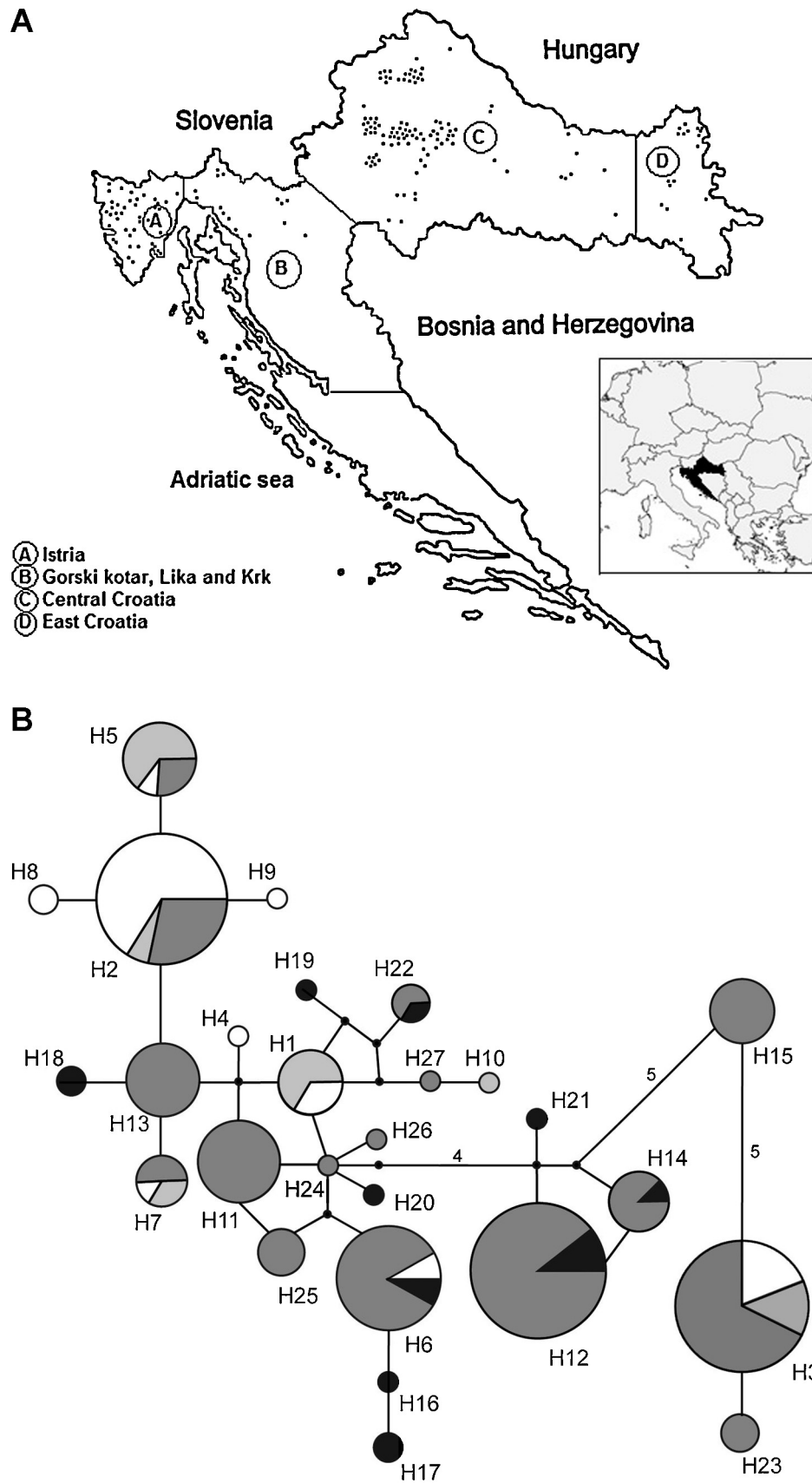


Fig. 1. (A) Geographic map of Croatia with black dots indicating sampling locations. (B) Median joining network based on mitochondrial DNA control region sequences. Circles represent haplotypes, with size proportional to relative frequencies. Sectors within circles are proportional to the frequency of each haplotype observed in each population. Populations are identified by different colors (white, Istria; light gray, Gorski kotar and Lika; dark gray, Central Croatia; black, East Croatia). All lines represent one mutational step except where indicated, with branch lengths proportional to the number of mutations. Small black circles indicate extinct or unsampled haplotypes.

Tissue samples (liver, kidney, muscle) were taken from 229 red foxes (*Vulpes vulpes*) in Croatia between 2009 and 2011 after the animals had been killed in traffic accidents or culled as part of regular hunts. Samples originated from four regions: Istria (41), Gorski kotar and Lika (including one sample from Krk island) (23), Central Croatia (149) and East Croatia (16) (Fig. 1A). DNA was extracted using a commercially available kit (DNeasy Blood & Tissue Kit, Qiagen) following the manufacturer's instructions. The mitochondrial DNA control region was amplified using primers DLH (5'-CCTGAAGTAAGAACCAGATG-3') and LF15926F (5'-ATATAAAATACTTTGGTCTTGTAACC-3') and thermal cycling according to Kirschning et al. (2007). PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega), and the control region was sequenced using the LF15926F primer and an 3730 XL DNA Analyzer (Applied Biosystems). Sequence alignment was performed using Clustal W (Thompson et al., 1994) implemented in BioEdit software (Hall, 1999), and alignments spanning 364 base pairs (bp) were checked manually.

Haplotype frequencies and haplotype divergence were calculated using the program Arlequin 3.1 (Excoffier et al., 2005). The same program was used to estimate haplotype and nucleotide diversity (\pm SE) according to Nei (1987), and to estimate genetic differentiation between pairs of putative populations based on F_{ST} values. A Mantel test for matrix correlation between genetic distance [$F_{ST}/(1 - F_{ST})$] and the logarithm of geographic distance was performed using Isolation by Distance Web Service software (Jensen et al., 2005). Phylogenetic relationships among the haplotypes identified were visualized using a median-joining network generated using Network 4.6.1.0. (Fluxus Technology; Bandelt et al., 1999). To evaluate the phylogenetic position of Croatian red foxes in the context of other Eurasian populations, 102 publicly available unique red fox haplotypes were included in the analysis (Valière et al., 2003; Statham et al., 2005; Arnason et al., 2006; Kirschning et al., 2007; Aubry et al., 2009; Teacher et al., 2011; Yu et al., 2012; Kutschera et al., 2013). Alignments were based on 300-bp overlap, except the alignment with 32 late Pleistocene and early Holocene sequences from Teacher et al. (2011), which was based on 270-bp overlap. Modern sequences shorter than 300 bp and ancient sequences shorter than 270 bp were not included in the analysis.

A 364-bp mtDNA control region fragment amplified in this research corresponds to positions 15,405–15,767 of the complete red fox mitochondrial genome (AM181037, Arnason et al., 2006, JN711443, Yu et al., 2012). Among 229 red fox samples from Croatia, a total of 31 polymorphic sites were identified (27 transitions, 1 transversion and 3 indels), yielding 27 haplotypes. These haplotypes showed 1–15 pairwise differences (mean 6.4) that resulted in sequence divergences of 0.27–4.12%. The greatest difference (15 bp) was observed between haplotypes H23 and H10 and between H23 and H17. The most frequent haplotypes were H12 (16.6%), H3 (16.2%), H2 (15.3%) and H6 (10%); none of the haplotypes identified in this study was present in all four populations sampled (Appendix A). Ten of the 18 haplotypes that were exclusive to a single population were represented by one individual. Three haplotypes were exclusive to Istria, eight to Central Croatia, six to East Croatia, and only one to the Gorski kotar and Lika region (Appendix A). The overall haplotype diversity (H) of all analyzed samples was 0.901 ± 0.008 , while nucleotide diversity (Π) was 0.018 ± 0.009 (Table 1). Haplotype diversity varied between regions, with the highest haplotype diversity found among 16 samples from East Croatia and the lowest diversity found among samples from Istria. Nucleotide diversity was relatively high, with the lowest value again occurring in Istria.

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Genetic differentiation was quite low between pairs of regions, with F_{ST} values ranging from -0.003 to 0.141 (Table 1). Statistically significant ($p < 0.05$) estimates of differentiation were obtained between the Istria population and each of the three continental populations. Overall F_{ST} was 0.074 ($p < 0.05$), indicating that 7.4% of the total genetic variance was inter-populational, while 92.6% was intra-populational. The hypothesis of no relationship between genetic and geographic distance could not be disregarded, as the Mantel test yielded non-significant results (correlation coefficient $r = 0.3947$, $p = 0.2873$).

The median-joining network is shown in Fig. 1B. The most frequent haplotypes (H2, H3, H6 and H12) were not closely associated and were observed in at least two different regions. No clusters reflected geographic origin.

We identified potential matches between our haplotypes and several modern haplotypes from Poland (H1 and HF677248; H2 and HF677250; H12 and HF677252), Germany (H11 and HF677218) and Russia (H16 and HF677203) (Kutschera et al., 2013). Our haplotype H6 matches sequence HF677224 found in Germany and Poland, while our haplotype H13 matches sequence HF677232 found in Germany and Switzerland (Kutschera et al., 2013). Furthermore, our haplotypes H20 and H24 appear to be identical to ancient samples from France (JN232514) and England (JN232513) (Teacher et al., 2011). Median joining network based on a 270 bp fragment of the mitochondrial DNA control region in 540 red foxes samples from Holarctic (115 unique haplotypes) is depicted in Appendix B. Sixteen sequences from Teacher et al. (2011) and Valière et al. (2003) were not included as they were too short or too many nucleotides were unidentified.

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The present study aims to close the gap between the ecological and socio-economic importance of European red foxes and the scarcity of population-level studies on their control region variability. A total of 27 mtDNA control region haplotypes were defined in 229 red fox samples from Croatia. The haplotype diversity of Croatian red foxes (0.901) is comparable to that of geographically widely distributed lineages like the Holarctic lineage (0.964), and it is higher than that of regionally restricted lineages like the Japanese Hokkaido II lineage (0.6) or the North American Nearctic lineage (0.742) (Kutschera et al., 2013). Likewise, haplotype (0.901) and nucleotide (0.018) diversities of Croatian red foxes correspond to those values found in red fox samples from all over Europe (0.93 and 0.019, respectively). They are among the highest found so far in any European red fox population (Edwards et al., 2012). Only populations from Germany (0.92), France/Switzerland (0.95) and Sweden (0.91) have higher haplotype diversities. Such a high level of haplotype diversity in the Croatian red fox population indicates a solid population with a large effective population size in the past and present, which is in line with previous studies suggesting a large and stable population (Janicki et al., 2007). Although populations from Croatia and France have the highest nucleotide diversity (0.018 both), this diversity remains quite low compared with that of the entire Holarctic lineage (0.047, Kutschera et al., 2013), which is in line with the suggestion of historic bottleneck and subsequent expansion of red fox in Europe (Edwards et al., 2012). In addition, although high genetic diversity may identify Croatia as one of the Last Glacial Maximum refugia, northern populations like those in Germany and Sweden also exhibit similarly high diversities, which together with haplotype sharing among different European regions and time periods is congruent with the suggestion that foxes were present across Europe throughout the last glaciation (Edwards et al., 2012).

Even though microsatellite analyses are necessary for better understanding of possible gene flow among regions, our results

Table 1
Genetic variability of red fox samples from Croatia and pairwise F_{ST} values.

Region	No. samples	No. haplotypes	Haplotype diversity (H)	Nucleotide diversity (Π)	Pairwise F_{ST}		
					Istria	Gorski kotar Lika	Central Cro
All samples	229	27	0.901 ± 0.008	0.018 ± 0.009			
Istria	41	9	0.659 ± 0.074	0.011 ± 0.006			
Gorski kotar and Lika	23	6	0.810 ± 0.043	0.015 ± 0.008	0.06953 [*]		
Central Croatia	149	16	0.881 ± 0.012	0.018 ± 0.009	0.12689 [*]	0.01527	
East Croatia	16	9	0.914 ± 0.052	0.016 ± 0.009	0.14115 [*]	0.01984	−0.00259

^{*} $p < 0.05$.

suggest that landscape barriers like rivers and small mountains in central and eastern Croatia might not restrict gene flow among foxes in the continental part of Croatia. This is not surprising given that red foxes in northern Serbia, which has similar landscape characteristics as central and eastern Croatia, show a similar lack of population differentiation (Kirschning et al., 2007). This is also consistent with weak phylogeographic structuring on much larger geographic scales, such as within the Holarctic lineage (Kutschera et al., 2013). The geography of the peninsular region of Istria, in contrast, seems to separate red foxes from Istria and the three continental populations. Samples from Istria showed the lowest haplotype and nucleotide diversity, and Istria was the only Croatian region to show significant pairwise population differentiation from the other populations sampled (Table 1). The land bridge that connects Istria to the continent is dominated by the Učka and Čičarija mountains, the highest peaks of which rise above 1250 m. Foxes in Croatia are scarcely observed at heights above 700 m, due to limited food sources (Janicki et al., 2007). It seems that the combination of a narrow land bridge approximately 70 km wide and altitudes exceeding 1000 m high limit the migration of foxes between Istria and the rest of the continent.

Our study has shown Croatian red foxes to exhibit the same high haplotype diversity found in large and stable populations. The haplotype diversity of this population was found to be among the highest of all European red fox populations studied to date. Populations from four regions of Croatia showed low levels of genetic differentiation, with significant differences found only between the population from Istria and the three continental populations. More detailed insights into the small-scale population structure of European red foxes will require analysis of highly variable nuclear markers like microsatellites.

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