Zoological Studies **49**(6): 816-823 (2010)

Zoological Studies

High Genetic Variability of the Grey Wolf (*Canis lupus* L.) Population from Croatia as Revealed by Mitochondrial DNA Control Region Sequences

Tomislav Gomerčić[1,*], Magda Sindičić[1], Ana Galov[2], Haidi Arbanasić[2], Josip Kusak[1], Ivna Kocijan[2], Martina Đuras Gomerčić[1], and Đuro Huber[2]

[1]*Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, HR-10000 Zagreb, Croatia*
[2]*Faculty of Science, University of Zagreb, Rooseveltov trg 6, HR-10000 Zagreb, Croatia*

(Accepted June 10, 2010)

Tomislav Gomerčić, Magda Sindičić, Ana Galov, Haidi Arbanasić, Josip Kusak, Ivna Kocijan, Martina Đuras Gomerčić, and Đuro Huber (2010) High genetic variability of the grey wolf (*Canis lupus* L.) population from Croatia as revealed by mitochondrial DNA control region sequences. *Zoological Studies* **49**(6): 816-823. Declines of grey wolf (*Canis lupus* L.) populations in Europe began some 250 yr ago, eventually leading to the almost-complete eradication of wolves from Western Europe. As a consequence, the majority of the remaining populations today exhibit unique haplotypes. A population in the Dinaric Mountains survived the eradication, and represents the closest stable wolf gene pool to the Western Europe. The grey wolf in Croatia reached the edge of extinction in the beginning of the 1990s, while during the last 10 yr, an increase in the population size was observed, and in 2008, it was estimated at around 200 individuals. We analyzed a 281 bp portion of the mitochondrial DNA control region of 91 grey wolf samples from Croatia. Totally, 4 haplotypes with 11 polymorphic sites were identified, with a haplotype diversity of 0.711 ± 0.018. One haplotype is new and has heretofore not been registered in any grey wolf population worldwide. The Croatian wolf population has one of the highest levels of mtDNA variability recorded to date, and probably retains a significant proportion of the genetic diversity of the formerly widespread and continuous European wolf population. Therefore, the Croatian wolf population is a valuable source of genetic diversity, which through immigration could help restore populations with reduced variability. http://zoolstud.sinica.edu.tw/Journals/49.6/816.pdf

Key words: Grey wolf, *Canis lupus*, Control region mtDNA, D-loop, Croatia.

The decline of grey wolf (*Canis lupus* L.) populations in Europe began some 250 yr ago (Breitenmoser 1998). By the end of the 19th century, human prosecution, decreases in natural prey, and habitat fragmentation (Delibes 1990) caused the almost-complete eradication of wolves from Western Europe, except for isolated populations in Italy (Ciucci and Boitani 1991, Boitani 1992) and the Iberian Peninsula (Blanco et al. 1992), while large wolf populations remained in Eastern Europe. The wolf population in the Dinaric Mountains remained, at the border between extinct populations in the west and surviving ones in the east, and represents the closest stable wolf gene pool to Western Europe. The grey wolf in Croatia reached the edge of extinction in the late 1980s (Huber et al. 1999 2002), and at the beginning of the 1990s, the population was estimated at around 50 individuals (Kusak 2002). The population became legally protected in 1995, and a National Wolf Management Plan was implemented in 2005. During the last 10 yr, an increase in the population size was observed, and in 2008, it was estimated at around 200 individuals, occupying 17,468 km^2

*To whom correspondence and reprint requests should be addressed. Tel: 385-1-2390158. Fax: 385-1-2390158.
E-mail:tomislav.gomercic@vef.hr

(32.4% of the total Croatian landmass) (Oković 2008). Radiotelemetric research confirmed connectivity between wolves in Croatia and neighboring Slovenia and Bosnia and Herzegovina (Štrbenac et al. 2005, Štrbenac et al. 2008).

Before the human-caused population fragmentation, wolves in Europe showed no phylogeographical structuring (Vilà et al. 1999, Randi et al. 2000), while recent population bottlenecks and geographical isolations have greatly reduced genetic variability (Wayne et al. 1992, Randi et al. 1995), raising many questions about wolf population genetics (Ellegren 1999, Vilà et al. 1999, Flagstad et al. 2003). Mitochondrial (mt) DNA is a standard population genetic tool and is often used in population genetic analyses of mammals (Grobler et al. 2005, Hartl et al. 2005, Lorenzini et al. 2005, Nies et al. 2005, Lebarbenchon et al. 2006, Kirschning et al. 2007, Gu et al. 2008). MtDNA was analyzed for several grey wolf populations (Vilà et al. 1997 1999, Randi et al. 2000, Lucchini et al. 2002 2004, Valiere et al. 2003), and several phylogenetic and dog-wolf hybridization studies were carried out based on wolf mtDNA (Vilà and Wayne 1999, Andersone et al. 2002, Randi and Lucchini 2002, Ciucci et al. 2003, Verginelli et al. 2005).

This paper presents the results of population genetic analyses of the Croatian grey wolf using mtDNA and provides new insights into grey wolf diversity in the region. A study by Lucchini et al. (2004) revealed the presence of 2 mtDNA control region haplotypes in 28 samples from Croatia. Six grey wolf samples from Croatia were included in phylogeographic research done by Vilà et al. (1999), also confirming the presence of the same 2 haplotypes. The purpose of this study was to investigate the possibility that more mtDNA control region haplotypes are present in the Croatian grey wolf population that had not been detected in previous studies due to smaller sample sizes and to determine and compare the mtDNA variability of Croatian wolves with other wolf populations.

MATERIALS AND METHODS

Sampling

In total, 91 samples of wild grey wolves from Croatia were collected and analyzed. Samples were collected as a part of the research and monitoring of the wolf population, carried out by the Biology Department of the Faculty of Veterinary Medicine, Univ. of Zagreb. In 1996-2006, 79 muscle samples were taken during necropsies of animals killed by traffic, hunting, poaching, or disease, while 12 blood samples were from animals live-captured by the same team for radiotracking studies. Samples originated from the Gorski kotar, Lika, and Dalmatia regions, covering the entire wolf range in Croatia. Four samples that originated from Bosnia-Herzegovina were from very close to the Croatian border (Fig. 1), so they were treated as part of the same population.

Molecular analysis

Prior to analysis, muscle samples were stored in 96% ethanol at -20°C, while blood samples were kept in vacutainers with EDTA. DNA was extracted using a Promega Wizard Genomic Purification Kit (Promega, Madison, WI, USA). The control region was amplified with CR1 (5'-CCACTATCAGCACCCAAAGC-3') and CR2R (5'-CCCGGAGCGAGAAGAGG-3') primers designed by Palomares et al. (2002). The total reaction volume for the polymerase chain reaction (PCR) was 25 μL, containing 150-250 ng of genomic DNA, 1x QIAGEN Multiplex PCR Master Mix (Qiagen, Hilden Germany) (consisting of QIAGEN Multiplex PCR buffer at a final concentration of 3 mM $MgCL_2$, dNTP mix, Q solution, and HotStart Taq DNA polymerase), and 0.2 μM of each primer (Gomerčić 2009). The reaction was carried

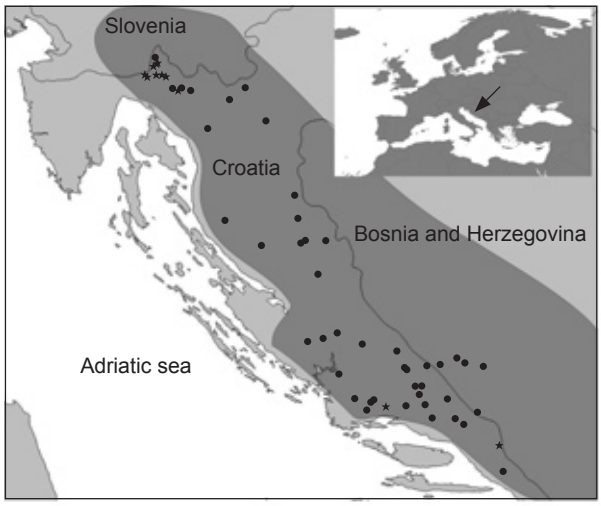

Fig. 1. Locations where dead wolves were found (●) and locations where wolves were captured for radiotracking (★). The darker area on the map indicates the wolf range in the broader area.

out on a GeneAmp PCR System 2700 (Applied Biosystems, Foster city, California, USA) using the following cycling parameters: 15 min at 95°C, then 35 cycles of 40 s at 94°C, 50 s at 55°C, and 60 s at 72°C, with a final extension for 10 min at 72°C. After purification with Wizard® SV Gel and a PCR Clean-Up System kit (Promega), the control region was sequenced with an ABI3730x1 DNA Analyzer (Applied Biosystems).

Statistical analysis

Sequence alignment was performed using Clustal W (Thompson et al. 1994), implemented in BioEdit software (Hall 1999), and alignments were manually proofed. Aligned sequences comprising 281 base pairs (bp) were analyzed. Haplotype frequencies and distances between haplotypes were calculated using the program Arlequin 3.1 (Excoffier et al. 2005). The same program was used to estimate haplotype and nucleotide diversities (± S.E.) according to Nei (1987). To evaluate the phylogenetic position of Croatian grey wolves in the context of other grey wolf populations, 44 sequences (comprising 27 unique mtDNA haplotypes) from GenBank were included in the analysis. Due to the various lengths of sequences, the phylogenetic analysis was based on a 224 bp fragment of the mtDNA control region. Mega 4 (Tamura et al. 2007) was used to construct a Neighbor-joining (NJ) unrooted tree, computed using the Kimura 2-parameter distance of a nucleotide substitution model. The confidence of each branch was generated through bootstrap resampling with 500 replicates.

RESULTS

We analyzed a 281 bp portion of the mtDNA control region of 91 grey wolf samples from Croatia. Totally, 4 haplotypes with 11 polymorphic sites (3.9%) were identified (Table 1). One of the polymorphic sites is a result of an insertion/deletion, while the others resulted from a substitution.

Haplotype WCRO1 was the most common, being found in 34 (37.4%) individuals. Haplotypes WCRO2 and WCRO6 were found in 25 (27.50%) individuals each. The rarest haplotype, WCRO3, was only found in 7 (7.7%) individuals (Table 1). Haplotype diversity (H) of the Croatian wolf population was 0.711 ± 0.018, while the nucleotide diversity (Π) was 0.018 ± 0.0096.

Distances between individual haplotypes, based on the number of different nucleotides between them, are shown in table 2. The 4 haplotypes showed 1-9 pairwise differences resulting in sequence divergences of 0.36%-3.20%. Haplotypes WCRO1 and WCRO6 differed the most, with 9 different nucleotides. The mean number of pairwise differences was 4.934 ± 2.424.

Comparison of 4 Croatian mtDNA haplotypes found in this study with 44 worldwide grey wolf mtDNA sequences from GenBank revealed potential matches between haplotypes. Croatian

Table 1. Four mitochondrial control region haplotypes found in the Croatian grey wolf population. Eleven polymorphic sites were identified within the 281 bp sequence. Dots represent identity with haplotype WCRO1 and dashes denote deletions. Potential matches with GenBank sequences are provided (sequences are based on 224 bp fragments of the mtDNA control region sequences, due to various lengths of GenBank sequences)

Haplotype name with GenBank accession number	43	62	71	90	132	148	158	162	173	180	189	No. of individuals	Haplotype frequency	Potential matches with GenBank sequences
WCRO1 GU059550	T	T	-	A	T	T	T	C	T	A	A	34	0.37	w5 from the Alps (AF338 807), w3 from Bulgaria (AF115689)
WCRO2 GU059551	·	·	-	G	C	·	C	T	·	G	·	25	0.27	w4 from the Alps (AF338 806), w9 from Bulgaria (AF11569)
WCRO3 GU059552	·	C	C	·	C	C	C	T	C	·	G	7	0.08	w2 from the Alps (AF338 804), w16 from Bulgaria (AF115701)
WCRO6 GU059555	C	C	C	·	C	C	C	T	C	·	G	25	0.27	no match

haplotypes were grouped most closely with haplotypes from Bulgaria and the Alps. Haplotype WCRO3, with an 8% frequency in Croatia, was identical to haplotype w2 from the Alps (AF338804) and w16 from Bulgaria (AF115701). Haplotype WCRO1, with a 37% frequency in Croatia, was identical to w5 from the Alps (AF338807) and w3 from Bulgaria (AF115689). Haplotype WCRO2, with a 27% frequency in Croatia, was identical to w4 from the Alps (AF338806) and w9 from Bulgaria (AF11569). Croatian haplotype WCRO6 (with a 27% frequency) is new and so far has not been registered in any grey wolf population worldwide. We deposited these sequences in GenBank (accession nos.: GU059550, GU059551, GU059552, and GU059555). The NJ tree illustrating the phylogenetic relationship among wolf mtDNA haplotypes registered in GenBank and haplotypes from this research was generally poorly resolved, and the majority of branches lacked substantial support (bootstrap values were generally low). Furthermore, it indicated an absence of a clear geographical pattern in the distribution of wolf haplotypes (Fig. 2).

DISCUSSION

The majority of extant wolf populations in Western Europe exhibit unique haplotypes, as genetic drift has caused random fixation of their genotypes (Vilà et al. 1999). A single mtDNA haplotype was confirmed in Italy by Randi et al. (1995 2000) and Lucchini et al. (2002 2004). Valiere et al. (2003) also confirmed presence of this unique haplotype in Italy and discovered its presence in France and Switzerland, indicating the natural return of wolves in France and Switzerland from an expanding Italian population. Ellegren et al. (1996) discovered the presence of 1 mtDNA haplotype in wild wolves in Sweden. Flagstad et al. (2003) confirmed the presence of this unique haplotype, but also discovered 2 new haplotypes that were present in the population in the period 1950-1979, as a result of new immigrants from the East. Flagstad et al. (2003) found that mtDNA variability of the Scandinavian population, which went through a dramatic population decline and virtually became extinct, was significantly lower than that of Finish wolves, which are a part of a larger eastern population. Andersone et al. (2002) confirmed the presence of 2 haplotypes in the Estonian wolf population. Vilà et al. (1997) investigated 162 samples from 13 European countries, finding 10 haplotypes in total. Vilà et al. (1999) expanded their research on 259 samples from 30 countries worldwide, confirming the presence of 34 different haplotypes. The highest number of different mtDNA haplotypes was found in Saudi Arabia: 5 haplotypes in 7 samples; while Greece (n = 7), Russia (n = 4), and Mongolia (n = 8) had 4 haplotypes each. Randi et al. (2000) investigated 150 grey wolf samples from Europe, and found 20 mtDNA haplotypes, with the highest variability among 25 Bulgarian samples (6 haplotypes). Jedrzejewski et al. (2005) confirmed 4 mtDNA haplotypes in the wolf population in Bialowieza Primeval Forest (Poland, Belorussia), and concluded that a high immigration rate was the most probable explanation for the high genetic diversity of Bialowieza wolves. Pilot et al. (2006) found that most local populations in Eastern Europe had more than 1 mtDNA haplotype, and most haplotypes were widely distributed.

A review of this data confirmed that the Croatian wolf population has high genetic diversity of mtDNA. Before the human-caused population fragmentation, wolves in Europe showed no phylogeographical structuring (Vilà et al. 1999, Randi et al. 2000). Populations were connected by high gene flows which allowed long-range diffusion of mtDNA haplotypes (Vilà et al. 1999, Randi et al. 2000). The present differentiation of wolf populations is a result of past admixture and present restricted gene flow, combined with the influence of environmental and ecological factors, such as temperature and prey composition (Pilot et al. 2006). In spite of population decline in the 1980s (Huber 2002), Croatian wolves probably retain a significant portion of genetic diversity of the formerly widespread and continuous European wolf population, and the notion was suggested by Randi et al. (2000) for Bulgarian wolves. Vilà et al. (1999) found that the haplotype marked as

Table 2. Genetic distances between haplotype pairs, stated as the number of different nucleotides (under the diagonal) and sequence divergences (above the diagonal)

	WCRO1	WCRO2	WCRO3	WCRO6
WCRO1		0.0178	0.0285	0.0320
WCRO2	5		0.0249	0.0285
WCRO3	8	7		0.0036
WCRO6	9	8	1	

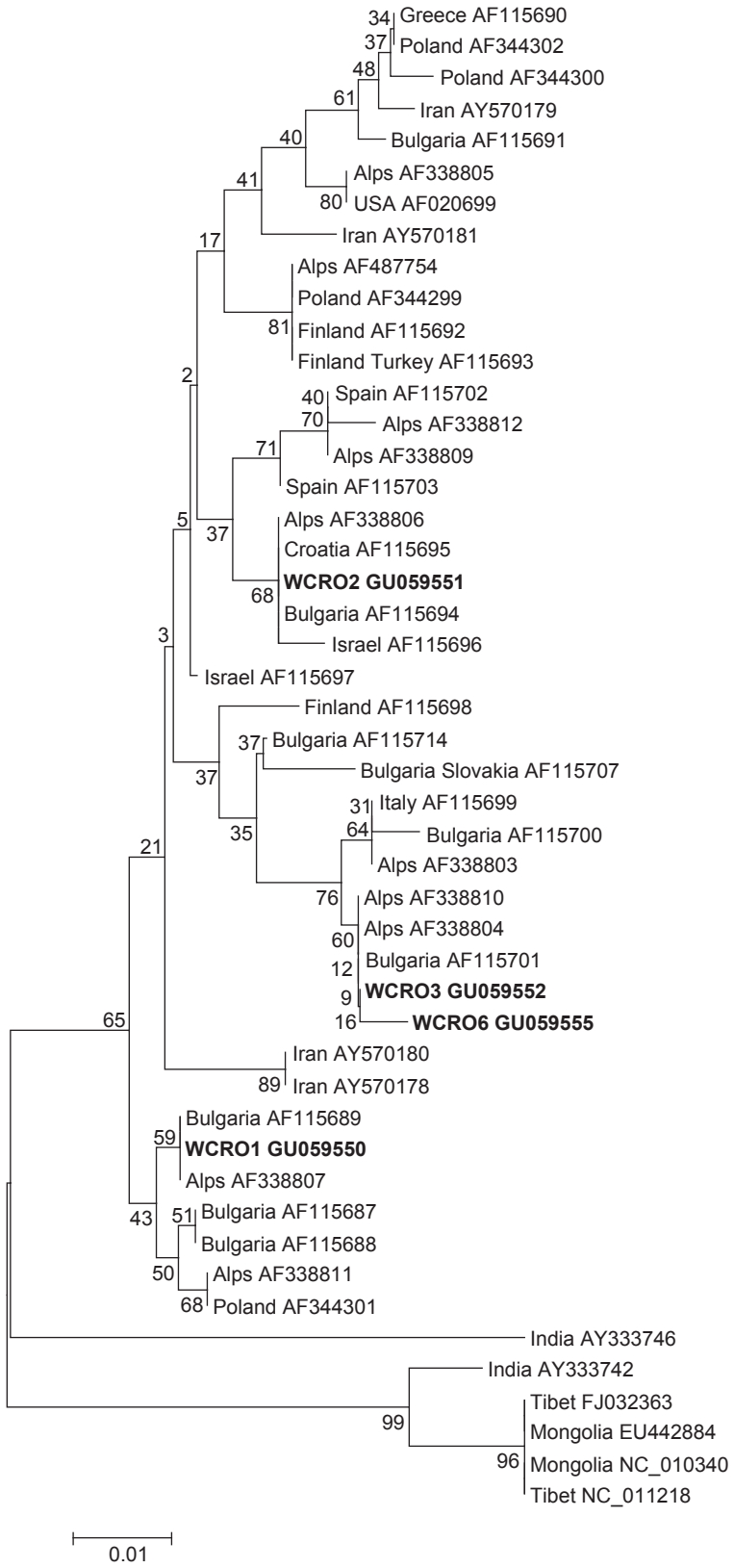

Fig. 2. Neighbor-joining original tree, computed using a nucleotide substitution model with Kimura 2 parameter distances, illustrating the phylogenetic relationship among wolf mtDNA haplotypes registered in GenBank. Croatian wolf haplotypes are indicated by WCRO1, WCRO2, WCRO3, and WCRO6. Bootstrap values from 500 iterations are indicated at the branches.

lu-3 is shared among Croatia, Portugal, Greece, Sweden, European Russia, and Turkey. This result also confirms that before the fragmentation, wolf mtDNA haplotypes were shared between geographically distant populations, and that nowadays, populations with the highest mtDNA variability, such as Bulgarian and Croatian wolf populations, conserve some of those haplotypes. This was also corroborated by our research which showed that three of 4 haplotypes found in the Croatian population are shared by wolves from Croatia, Bulgaria, and the Alps. Vilà et al. (1999) and Pilot et al. (2006) found no clear geographical pattern in the distributions of haplotypes. Our NJ tree (Fig. 2), which illustrates the phylogenetic relationship among wolf mtDNA haplotypes registered in GenBank and haplotypes from this research, also indicated the absence of clear geographical patterns in the distributions of wolf haplotypes.

Using data of Randi et al. (2000), we calculated the nucleotide diversity of 25 Bulgarian grey wolf mtDNA control region sequences of 546 bp in length (6 haplotypes) to be 0.0026. Pilot et al. (2006) found 21 mtDNA haplotypes among 643 wolf samples from 10 Eastern European countries, with a nucleotide diversity of 0.017. The nucleotide diversity of 91 mtDNA control region sequences of Croatian wolves analyzed in this research, among which 4 haplotypes were found, was higher (0.018 ± 0.0096) than that of Bulgarian and Eastern European wolves, confirming the high genetic variability of wolves from Croatia. Vilà et al. (1999) found that 259 samples from 30 countries worldwide had 34 wolf haplotypes, and their nucleotide diversity was 0.026 ± 0.014, which was higher than the nucleotide diversity of Bulgarian (Randi et al. 2000), eastern European (Pilot et al. 2006), and Croatian samples (this research). This comes as no surprise as Vilà et al. (1999) estimated the nucleotide diversity of the worldwide grey wolf mtDNA control region.

Wolves in Croatia are a part of a bigger Dinaric population, with a continuous distribution and documented gene flow among Slovenia, Croatia, and Bosnia-Herzegovina (Štrbenac et al. 2005 2008), and probable continuous gene flow down to Serbia, Montenegro, Albania, Macedonia, Greece, and Bulgaria. The Dinaric population is estimated to be 5000 individuals (Linnell et al. 2007). A decrease in the size of the Croatian wolf population in the 1980s probably was not strong enough to affect the population genetic variability, and even though there are reports of severely reduced ranges at that time, connectivity still existed with Slovenian and Bosnian-Herzegovian (Frkovic and Huber 1992) wolves, enabling gene flow. Furthermore, in the 1990s, during the Homeland war in Croatia, human populations from large areas were exiled, opening up suitable habitat for wolf recolonization. Empirical data suggest that the immigration rate may increase in cases where the density of wolves is low (Pulliainen 1980, Ballard et al. 1987), and the existence of neighboring populations may be of considerable importance to restoring small wolf populations on the verge of extinction (Flagstad et al. 2003). The results of our study have direct implications for grey wolf conservation in Europe. They show that the Croatian wolf population is a valuable source of genetic diversity, which through immigration could help restore populations with reduced variability, such as the Italian one. It is therefore necessary to preserve and enhance the existing connectivity between wolf populations of Southern and Eastern Europe in order to maintain gene flow among them and ensure a genetic foundation for the long-term stability of these populations.

It was observed for several taxa, including Italian wolves (Lucchini et al. 2004), that nuclear diversity assessed by microsatellites was considerably higher than mtDNA diversity. Thus research on Croatian grey wolves should be expanded to include microsatellites, not only to reveal variability but also to investigate paternal ancestry as female wolf-dog hybrids cannot be revealed by maternally inherited mtDNA. Furthermore, evolutionarily relevant and adaptive processes within and between populations can only be reflected by coding genes, and in vertebrates, growing evidence suggests that genetic diversity is particularly important at the level of the major histocompatibility complex (MHC) (Sommer 2005, Yang et al. 2007 2010). Thus, research on grey wolves in Croatia should also be expanded to analyze variations in MHC genes, which could reveal the adaptive potential of this population.

REFERENCES

Andersone Z, V Lucchini, E Randi, J Ozolins. 2002. Hybridisation between wolves and dogs in Latvia as documented using mitochondrial and microsatellite DNA markers. Mamm. Biol. **67:** 79-90.

Ballard WB, JS Whitman, CL Gardner. 1987. Ecology of an exploited wolf population in south-central Alaska. Wildl. Monogr. **98:** 1-54.

Blanco J, S Reig, L de la Cuesta. 1992. Distribution, status and conservation of the wolf *Canis lupus* in Spain. Biol. Conserv. **60:** 73-80.

Boitani L. 1992. Wolf research and conservation in Italy. Biol. Conserv. **60:** 125-132.

Breitenmoser U. 1998. Large predators in the Alps: the fall and rise of man's competitors. Biol. Conserv. **83:** 279-289.

Ciucci P, L Boitani. 1991. Viability assessment of the Italian wolves and guidelines for the management of the wild and captive populations. Ric. Biol. Selvaggina **89:** 1-58.

Ciucci P, V Lucchini, Boitani, E Randi. 2003. Dewclaws in wolves as evidence of admixed ancestry with dogs. Can. J. Zool. **81:** 2077-2081.

Delibes M. 1990. Status and conservation needs of the wolf in the Council of Europe member states. Nat. Environ. Ser. Strasbourg, **47:** 1-46.

Ellegren H. 1999. Inbreeding and relatedness in Scandinavian grey wolves *Canis lupus*. Hereditas **130:** 239-244.

Ellegren H, P Savolainen, B Rosen. 1996. The genetical history of an isolated population of the endangered grey wolf *Canis lupus*: a study of nuclear and mitochondrial polymorphisms. Phil. Trans. R. Soc. B **351:** 1661-1669.

Excoffier L, G Laval, S Schneider. 2005. Arlequin vers. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinf. Online **1:** 47-50.

Flagstad O, CW Walker, C Vilà, AK Sundqvist, B Fernholm, AK Hufthammer et al. 2003. Two centuries of the Scandinavian wolf population: patterns of genetic variability and migration during an era of dramatic decline. Mol. Ecol. **12:** 869-880.

Frkovic A, D Huber. 1992. Wolves in Croatia: baseline data. *In* C Promberger, W Schröder, eds. Wolves in Europe - status and perspectives. Ettal, Germany: Munich Wildlife Society, pp. 67-69.

Gomerčić T. 2009. Genetic diversity of gray wolf (*Canis lupus*) in Croatia. Dissertation, Univ. of Zagreb, Zagreb, Croatia. (in Croatian with English summary)

Grobler JP, DM Pretorius, K Botha, A Kotze, EM Hallerman, BJ Van Vuuren. 2005. An exploratory analysis of geographic genetic variation in southern African nyala (*Tragelaphus angasii*). Mamm. Biol. **70:** 291-299.

Gu XM, SY He, L Ao. 2008. Molecular phylogenetics among three families of bats (Chiroptera: Rhinolophidae, Hipposideridae, and Vespertilionidae) based on partial sequences of the mitochondrial 12S and 16S rRNA genes. Zool. Stud. **47:** 368-378.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. **41:** 95-98.

Hartl GB, FE Zachos, K Nadlinger, M Ratkiewicz, F Klein, G Lang. 2005. Allozyme and mitochondrial DNA analysis of French red deer (*Cervus elaphus*) populations: genetic structure and its implications for management and conservation. Mamm. Biol. **70:** 24-34.

Huber Đ, J Kusak, A Frković, G Gužvica. 2002. Causes of wolf mortality in Croatia in the period 1986-2001. Vet. Arhiv. **72:** 131-139.

Huber Đ, J Kusak, D Kovačić, A Frković, J Radović, Ž Štahan. 1999. Temporary wolf management plan for Croatia. Zagreb, Croatia: State Institute for Nature Protection.

Jedrzejewski W, W Branicki, C Veit, I Međugorac, M Pilot, AN Bunewich et al. 2005. Genetic diversity and relatedness within packs in an intensely hunted population of wolves *Canis lupus*. Acta Theriol. **50:** 3-22.

Kirschning J, FE Zachos, D Cirovic, IT Radovic, SS Hmwe, GB Hartl. 2007. Population genetic analysis of Serbian red foxes (*Vulpes vulpes*) by means of mitochondrial control region sequences. Biochem. Genet. **45:** 409-420.

Kusak J. 2002. Conditions for life of wolves (*Canis lupus*) in Croatia. Dissertation, Univ. of Zagreb, Zagreb, Croatia. (in Croatian with English summary)

Lebarbenchon C, F Poitevin, C Montgelard. 2006. Genetic variation of the weasel (*Mustela nivalis*) in Corsica based on mitochondrial control region sequences. Mamm. Biol. **71:** 164-171.

Linnell J, V Salvatori, L Boitani. 2007. Guidelines for population level management plans for large carnivores. Large Carnivore Initiative for Europe report prepared for the European Commission. Rome, Italy. Large Carnivore Initiative for Europe

Lorenzini R, R Fico, S Mattioli. 2005. Mitochondrial DNA evidence for a genetic distinction of the native red deer of Mesola, northern Italy, from the Alpine populations and the Sardinian subspecies. Mamm. Biol. **70:** 187-198.

Lucchini V, E Fabbri, F Marucco, S Ricci, L Boitani, E Randi. 2002. Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. Mol. Ecol. **11:** 857-868.

Lucchini V, A Galov, E Randi. 2004. Evidence of genetic distinction and long-term population decline in wolves (*Canis lupus*) in the Italian Apennines. Mol. Ecol. **13:** 523-536.

Nei M. 1987. Molecular evolutionary genetics. New York: Columbia Univ. Press.

Nies G, FE Zachos, GB Hartl. 2005. The impact of female philopatry on population differentiation in the European roe deer (*Capreolus capreolus*) as revealed by mitochondrial DNA and allozymes. Mamm. Biol. **70:** 130-134.

Oković P. 2008. Report about the status of wolf population in Croatia in 2008. Zagreb, Croatia: State Institute for Nature Protection. (in Croatian)

Palomares F, JA Godoy, A Piriz, SJ O'Brien, WE Johnson. 2002. Fecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx. Mol. Ecol. **11:** 2171-2182.

Pilot M, W Jedrzejewski, W Branicki, WE Sidorovich, B Jedrzejewska, K Stachura, SM Funk. 2006. Ecological factors influence population genetic structure of European grey wolves. Mol Ecol **15:** 4533-4553.

Pulliainen E. 1980. The status, structure and behaviour of populations of the wolf (*Canis l. lupus* L.) along the Fenno-Soviet border. Ann. Zool. Fennici **17:** 107-112.

Randi E, F Francisci, V Lucchini. 1995. Mitochondrial DNA restriction-fragment-length monomorphism in the Italian wolf (*Canis lupus*) population. J. Zool. Syst. Evol. Res. **33:** 97-100.

Randi E, V Lucchini. 2002. Detecting rare introgression of domestic dog genes into wild wolf (*Canis lupus*) populations by Bayesian admixture analyses of microsatellite variation. Conserv. Genet. **3:** 31-45.

Randi E, V Lucchini, MF Christensen, N Mucci, SM Funk, G Dolf, V Loeschke. 2000. Mitochondrial DNA variability in Italian and East European wolves: detecting the consequences of small population size and hybridization. Conserv. Biol. **14:** 464-473.

Sommer S. 2005. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. Frontiers Zool. **2:** 16.

Štrbenac A, Đ Huber, J Kusak, A Majić-Skrbinšek, A Frković, Ž Štahan et al. 2005. Wolf management plan for the Republic Croatia. Zagreb, Croatia: State Institute for Nature Protection.

Štrbenac A, Đ Huber, J Kusak, P Oković, M Sindičić, J Jeremić et al. 2008. Large carnivore conservation in Croatia bulletin. Zagreb, Croatia: State Institute for Nature Protection.

Tamura K, J Dudley, M Nei, S Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software vers. 4.0. Mol. Biol. Evol. **24:** 1596-1599.

Thompson JD, DG Higgins, TJ Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. **22:** 4673-4680.

Valiere N, L Fumagalli, C Gielly, B Miquel, ML Lequette, J Poulle et al. 2003. Long-distance wolf recolonization of France and Switzerland inferred from non-invasive genetic sampling over a period of 10 years. Anim. Conserv. **6:** 83-92.

Verginelli F, C Capelli, V Coia, M Musiani, M Falchetti, L Ottini et al. 2005. Mitochondrial DNA from prehistoric canids highlights relationship between dogs and South-east European wolves. Mol. Biol. Evol. **22:** 2541-2551.

Vilà C, IR Amorim, JA Leonard, D Posada, J Castroviejo, F Petrucci-Fonseca et al. 1999. Mitochondrial DNA phylogeography and population history of the grey wolf *Canis lupus*. Mol. Ecol. **8:** 2089-2103.

Vilà C, P Savolainen, JE Maldonado, IR Amorim, JE Rice, RL Honeycutt et al. 1997. Multiple and ancient origins of the domestic dog. Science **276:** 1687-1689.

Vilà C, RK Wayne. 1999. Hybridization between wolves and dogs. Conserv. Biol. **13:** 195-198.

Wayne RK, N Lehman, NW Allard, RL Honeycutt. 1992. Mitochondrial DNA variability of the grey wolf: genetic consequences of population decline and habitat fragmentation. Conserv. Biol. **6:** 559-569.

Yang WC, LS Chou, JM Hu. 2007. Molecular characterization of major histocompatibility complex class II DQB and DRG genes in bottlenose dolphins (*Tursiops truncatus* and *T. aduncus*) from the Western Pacific. Zool. Stud. **46:** 664-679.

Yang WC, LS Chou, JM Hu. 2010. Phylogenetic analyses of MHC class II genes in bottlenose dolphins and their terrestrial relatives reveal pathogen-driven directional selection. Zool. Stud. **49:** 132-151.