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Wolf-dog hybridization in Croatia

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ABSTRACT

Wolf-dog hybridization is considered as one of the main threats for wolf conservation since the admixture and introgression of domestic genes may disrupt local adaptations and threaten the long term survival of wild wolf populations. We investigated the occurrence of wolf-dog hybridization in Croatia by analyzing a panel of 12 autosomal microsatellite markers using Bayesian admixture tests, and assessed its directionality by the use of maternally and paternally inherited markers in combination with morphometric data and morphological features. A systematic analysis of morphologic features and morphometric data was used to rank the studied individuals into either phenotypic wild-type wolves or suspected hybrids. By combining Bayesian assignment results with phenotypic features, we set three thresholds which differentiated wolves from hybrids with maximized hybrid detection and a minimized chance for false positive hybrid identification. On the basis of phenotype, out of 176 wild canids, 157 (89.2%) were categorized as wolves and 19 (10.8%) as suspected hybrids. On the basis of the Bayesian admixture tests and phenotype together, five (2.8 percent) animals were classified as wolf-dog hybrids, four of them as backcrosses with wolves, and one as a backcross with a dog. Mitochondrial DNA suggested that all hybrids originated from the mating of female wolves and male dogs. Two male hybrids had Y chromosome haplotypes common to both wolves and dogs, while the other two had wolf private Y chromosome haplotypes. One wolf had a dog Y-haplotype, indicating a past introgression of dog genes. All hybrids were found in Dalmatia, where wolves settled recently, and where they live close to humans, with a high rate of human-caused mortality. These conditions are considered as favorable for wolf-dog hybridization. However, we found a low hybridization prevalence in Croatia, which is nonetheless expected to persist as long as the conditions favoring its occurrence are met. The ecological, sociological, conservation and management implications of hybrid occurrence are yet to be determined.

Key words: wolf; dog; *Canis lupus*; hybridization; Croatia; Bayesian admixture analysis; microsatellites; parental markers

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Introduction

Wild canids (genus *Canis*) can naturally hybridize amongst themselves (WAYNE et al., 1997) and also with domestic dogs (*C. lupus familiaris*), which were domesticated from gray wolves (*C. lupus*) in a process which started more than 27 000 years ago (SKOGLUND et al., 2015). The divergence of dogs from wolves was completed between 11000 to 16000 years ago (FREEDMAN et al., 2014). Wolf-dog hybridization is considered to be one of the main threats to wolf conservation, since the admixture of domestic genes may disrupt local adaptation (VILÀ and WAYNE, 1999) and thus threaten the long term survival of wild wolf populations (BOITANI, 2003). The chances for wolf-dog hybridization are considered to be higher in areas where wolves live close to settlements with a high density of dogs (BLANCO et al., 1992), in situations when wolves are heavily victimized (RANDI et al., 2000), or during the expansion phase of the wolf population (LORENZINI et al., 2014). One of the first extensive studies of wolf-dog hybridization did not find widespread hybridization in European gray wolf populations in contrast to the rather high prevalence (8-17%) of hybrids in Ethiopian wolves (VILÀ and WAYNE, 1999). Sexual asymmetry (the prevalence of female gray wolf over male dog hybrids), and physiological and behavioral differences between gray wolves and dogs have been recognized as reasons for the low detected prevalence of hybrids, particularly when maternally inherited markers (mitochondrial DNA) were analyzed (VILÀ and WAYNE, 1999). The advances in research techniques brought increasing evidence that hybridization between gray wolves and dogs exists in Europe in Bulgaria, Serbia, Latvia and Estonia (RANDI et al., 2000; MILENKOVIĆ et al., 2006; HINDRIKSON et al., 2012) and particularly in Italy (RANDI and LUCCHINI, 2002; LORENZINI et al., 2014; RANDI et al., 2014). Such findings may render wolf-dog hybridization to be one of major wolf conservation challenges, not only in Italy, but also in wolf populations in other countries with a similar history, status and ecological conditions for wolves, such as Croatia.

Wolves inhabited the whole of Croatia until the end of 19th century, but systematic eradication confined them to mountainous areas of Gorski kotar and Lika (about 10000 km^2), with numbers declining to only 30-50 individuals in the late 1980s (FRKOVIĆ and HUBER, 1992). At the beginning of 1990s, the Croatian wolf population started to grow and reached about 200 individuals by 2010, expanding to areas of Dalmatia, Banovina and Učka, comprising about 18.000 km^2 in total (KUSAK and HUBER, 2010a). In spite of the legal protection since 1995 (ŠTRBENAC et al., 2005), a monitoring program revealed a high proportion of human caused mortality in Croatian wolves (HUBER et al., 2002; KUSAK and HUBER, 2010b). Wolf management plans recognize hybridization between protected wolves and domestic dogs as a possible threat, but marginal management attention was dedicated to this phenomenon since its actual impact on the Croatian wolf population still remains poorly known (ŠTRBENAC et al., 2010).

This study was thus planned in order to investigate if wolf-dog hybridization really occurs in Croatia and if it does, what the prevalence is of hybrids in the wild population, their spatial distribution and the main directionality of hybridization events.

Materials and methods

We collected 176 samples of wolves (W) or suspected wolf-dog hybrids (SH) from Gorski kotar (GK; n = 42), Lika (L; n = 46), Dalmatia (DA; n = 80), Banovina (B; n = 2), the Panonian part of Croatia (P; n = 1) and Bosnia & Herzegovina (B&H; n = 5, close to the border with Dalmatia), in the period from 1996 to 2011 (Fig. 1). Samples from Banovina and Panonia were merged with samples from Gorski kotar, while samples from B&H were joined to samples from Dalmatia. We also collected 49 samples of dogs (D) from the same areas where wolf samples were collected. All samples were collected as part of the wolf monitoring program (ŠTRBENAC et al., 2010) when dead wolves (traffic accidents, shot legally and illegally, dead due to diseases) and wolves captured alive for telemetry tracking (KUSAK et al., 2005; KUSAK, 2010) were externally examined and measured for 23 body parameters, as in PLATIŠA et al. (2011). The body measurements of the wolves and suspected hybrids were compared with the average measurements of wolves of the same age and gender category determined by PLATIŠA et al. (2011) from 149 "pure" (i.e. without any apparent morphological deviations) Croatian wolves. All measurements that differed more than 1SD from the average for corresponding age and gender category were considered morphometric differences. The phenotypic anomalies (morphometric differences and morphological deviations) which we considered in distinguishing wolves from suspected hybrids were: 1) absence of black stripes on the front legs in combination with at least one more atypical features, 2) connected posterior ends of the 3rd and 4th digits on the paws, in a combination with at least one more atypical feature, 3) flat forehead, i.e. almost no "step" between the cranium and nose, 4) short or long head 5) long and narrow nose, 6) length asymmetry between the upper and lower jaws, 7) presphenoid bone on the base of the skull without lateral "wings", but simple and spiky as in dogs, 8) slightly concave muzzle (snout), i.e. saddle-like, 9) too small or too wide a distance between the left and right upper and/or left and right lover canines, 10) long ears, 11) small paws, 12) small body mass, and then, as in RANDI et al. (2014), 13) the presence of dewclaw, 14) dog-like body shape (a combination of short legs with rounded, barrel-like body), 15) atypical coat coloration including black, yellow, patchy or completely white coat and claws (albinism).

The DNA of wolves, suspected hybrids and dogs was extracted, PCR-amplified and genotyped for twelve autosomal microsatellites (STR) and four Y-linked STR haplotypes, following the methodology described in FABBRI et al., 2014. Partial sequences (280 bp) of the hypervariable part of the mitochondrial DNA control-region (mtDNA CR1) were obtained using the PCR primers CR1 and CR2R, as described in GOMERČIĆ et al., (2010). Procedures for aligning and collapsing identical mtDNA sequences, and matches with sequences in GenBank, were done as in FABBRI et al. (2014).

The software STRUCTURE v2.3.4 (FALUSH et al., 2007) was used to assign samples to the wolf or dog group, and to identify genetic signals of hybridization or introgression of domestic genes in the wolf population. The models implemented in STRUCTURE are based on Bayesian statistics. The software, inferring population structure, assigns individuals to clusters, independent of any prior non-genetic information. The run parameters used in STRUCTURE were the following: "*admixture*" and independent allele frequency "*I*" models, without any prior population information, assuming K from 1 to 5. Three independent runs were done for each K using 400,000 of the Monte Carlo Markov chain (MCMC) and discarding the first 40,000 of burn-ins. The ΔK statistics were used to identify the highest rate of increase in the posterior probability LnP(D) of the data between each consecutive K. Following RANDI et al., 2014, the power of admixture analyses to detect F_1 and F_2 hybrids, first and second generation of backcrosses, given the number of markers and the level of genetic differentiation between parental populations (estimated by F_{st}), was assessed by simulation (VÄHÄ and PRIMMER, 2006). We randomly selected two groups of parental genotypes (43 wolves and 43 dogs with individual q_i values >0.980) to generate 200 individuals in each parental (PD and PW), F_1, F_2 and backcross (BC_1D, BC_2D, BC_1W, BC_2W) genotype classes with the software HYBRIDLAB v1.0 (NIELSEN et al., 2006). Hybrid genotypes were created by random sampling alleles from their frequency distributions in the parental populations, assuming neutrality, linkage equilibrium and random mating. Then the simulated genotypes were used to carry out admixture analyses with STRUCTURE v.2.3.4 and the qi values for the different simulated hybrid classes were compared with the sampled individuals.

The simulated genotypes were analyzed in the assignment procedure running STRUCTURE as follows: 1) Only parental populations (PW, PD, n = 400 individuals), $K = 2$, "*admixture*" and "*I*" models; 2) Parental populations and all 6 classes of hybrid genotypes (n = 1600), $K = 2$, "*admixture*" and "*I*" models; 3) Parental populations and all 6 classes of hybrid genotypes (n = 1600), $K = 2$, "*population information" (POPINFO)* and "*I*" models.

To identify wolf-dog hybrids we used three thresholds predetermined by the results obtained using simulated hybrid genotypes. To maximize hybrid detection but minimize false positives (wolves erroneously identify as hybrids) we fixed the following rules for the chosen thresholds: 1) lower qi values observed in parental simulated populations; 2) the highest qi in F_2 hybrids; 3) lower qi values observed in the parental wolf population PW (obtained analyzing only Parental populations in STRUCTURE), to be used for individuals with only phenotypic anomalies. Spatial distribution analysis and data representation of the collected samples were done with the use of ArcGIS Desktop ver. 10.3 (ESRI Inc.).

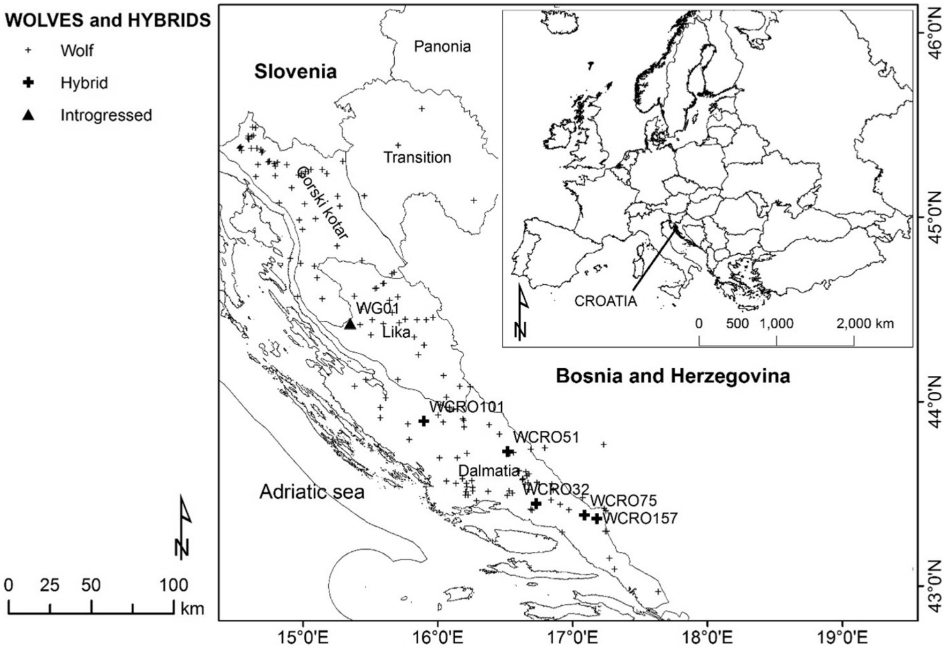

Fig. 1. Spatial distribution of wolves and wolf-dog hybrids found in Croatia in the period from 1996 to 2011. All genetically determined hybrids were found in Dalmatia, while one animal from the Lika region showed a dog Y-haplotype but no other genetic (12-STR, mtDNA CR1) or phenotypic signals of hybrid origin, and therefore was classified as a wolf with introgressed dog genes.

Results

The analysis of phenotypic anomalies allowed us to identify 157 (89.2%) of the 176 wild canids (78 females, 95 males and three of undetermined gender) as wolves and 19 (10.8%) as suspected hybrids. The most frequent anomaly was atypical coat color (n = 11), then the absence of black stripes on the front legs (n = 5), followed by a long head (n = 4), long ears (n = 4), flat forehead (n = 3), while connected 3rd and 4th digits, short head, saddle shape nose, and small body mass were found twice, and small paws, dog-like body shape, small distance between canines, asymmetrical jaws and dog-like presphenoid bone, were found once (Table 1). These phenotypic anomalies were found in different combinations in different animals. The phenotypic anomalies found were further illustrated by photographs of some of the suspected hybrids (Fig. 2 and Fig. 3) and determined hybrids (Fig. 4).

Table 1. Suspected wolf-dog hybrids and one wolf with introgressed dog genes, found in Croatia in 1996-2011, with a description of the phenotypic anomalies detected. Genetic identification of wolf-dog hybrids and suspected hybrids.

Animal ID[1]	Sex	Phenotypic anomalies found	Phenotype[2]	Structure[3] (q_w and IC)	Y-STR[4]	mtDNA CR1[5]	IDhy[6]
WCRO051	M	No black stripes on front legs, dark hairs on the back, too small (32 kg) for a three year old male	Suspected hybrid	0.281 (0.000-0.779)	YH05	WCR03	BCD
WCRO032	M	No black stripes on front legs, 3rd and 4th digits connected at posterior ends on all four legs. Upper canine distance 3.9cm, lover canines distance 3.7 cm only!	Suspected hybrid	0.770 (0.309-1.000)	YH05	WCR03	BCW
WCRO075	F	Atypical coloration of hair, rather black head and body. Black patchy areas, particularly on hind legs.	Suspected hybrid	0.834 (0.432-1.000)	NA	WCR01	BCW
WCRO101	M	Hair red-brownish, tips of hair black, black stripe on front legs present, head long and narrow, long ears (11cm), flat forehead, muzzle (snout) slightly concave.	Suspected hybrid	0.848 (0.391-1.000)	YH16	WCR06	BCW
WCRO157	M	Longer ears (11.4 and 11.5 cm), smaller paws (front: 8.6 and 8.1 cm; hind 7.5 and 7.7) for a male wolf in the second year of life.	Suspected hybrid	0.919 (0.545-1.000)	YH16	WCR01	BCW
WG01	M	Nothing unusual	Wolf	0.990 (0.930-1.000)	YH05	WCR06	IG
W-Bijeli	M	Albino, white hair and white claws	Suspected hybrid	0.974 (0.797-1.000)	missing	WCR03	W
WCRO007	M	Small body mass (28 kg) for three year old male wolf	Suspected hybrid	0.994 (0.971-1.000)	YH18	WCR02	W
WCRO043	F	Lateral wings on presphenoid bone only 1mm long.	Suspected hybrid	0.997 (0.986-1.000)	NA	missing	W
WCRO052	M	No black stripes on front legs, flat forehead.	Suspected hybrid	0.997 (0.988-1.000)	YH20	WCR06	W
WCRO058	F	No black stripes on front legs, 3rd and 4th digits connected at posterior ends on all four legs. Asymmetric upper and lower jaw.	Suspected hybrid	0.995 (0.977-1.000)	NA	WCR02	W

[1] ID = sample identification; [2] Phenotype = phenotype information on the basis of morphological features and morphometric data; [3] STRUCTURE individual proportion of assignment in Structure admixture analyses to the wolf cluster; [4] Y-STR = Y linked STR detected in males (NA = not available because the individual was a female); [5] mtDNA CR1 = mitochondrial DNA control region 1 haplotype; [6] IDhy = final identification of each sample as: BCW (back cross with wolf), BCD (back cross with dog), IG (introgressed dog) and W (wolf).

Table 1. Suspected wolf-dog hybrids and one wolf with introgressed dog genes, found in Croatia in 1996-2011, with a description of the phenotypic anomalies detected. Genetic identification of wolf-dog hybrids and suspected hybrids. (continued)

Animal ID[1]	Sex	Phenotypic anomalies found	Phenotype[2]	Structure[3] (q_w and IC)	Y-STR[4]	mtDNA CR1[5]	IDhy[6]
WCRO069	F	Short legs, rounded body, short muzzle	Suspected hybrid	0.986 (0.908-1.000)	NA	WCR03	W
WCRO071	M	Black hairs on back and legs, black stripes on front legs rather wide. Head long and narrow, and without forehead.	Suspected hybrid	0.996 (0.979-1.000)	YH11	WCR06	W
WCRO119	M	Light yellow hair, no black stripes on front legs, long narrow head, muzzle saddle concave, long ears (13.4 and 12.8 cm).	Suspected hybrid	0.995 (0.976-1.000)	YH16	WCR06	W
WCRO127	F	Too small mass (22.5 kg) for a female wolf 1.5 years old.	Suspected hybrid	0.991 (0.945-1.000)	NA	WCR01	W
WCRO152	M	Albino, white hair, white claws, blue eyes. Shot in the same area where W-Bijeli (also albino) was shot 4 years earlier.	Suspected hybrid	0.992 (0.953-1.000)	missing	WCR03	W
WCRO160	M	Dark (almost black) hair particularly on head, back and distal parts of legs, long head (30 cm).	Suspected hybrid	0.995 (0.976-1.000)	YH31	WCR03	W
WCRO162	F	Short head (24 cm), long ears (11.5 cm and 11.7 cm), yellow hair.	Suspected hybrid	0.993 (0.966-1.000)	NA	missing	W
WCRO170	M	Dark to black hair on head, back and distal part of legs, grey thorax, abdomen and proximal part of legs.	Suspected hybrid	0.990 (0.943-1.000)	YH31	WCR03	W
WCRO184	F	Short head (24.2 cm).	Suspected hybrid	0.989 (0.930-1.000)	NA	WCR06	W

[1] ID = sample identification; [2] Phenotype = phenotype information on the basis of morphological features and morphometric data; [3] STRUCTURE individual proportion of assignment in Structure admixture analyses to the wolf cluster; [4] Y-STR = Y linked STR detected in males (NA = not available because the individual was a female); [5] mtDNA CR1 = mitochondrial DNA control region 1 haplotype; [6] IDhy = final identification of each sample as: BCW (back cross with wolf), BCD (back cross with dog), IG (introgressed dog) and W (wolf).

Fig. 2. Suspected wolf-dog hybrids, but genetically classified as wolves, found in Croatia in the period 1996-2011. Combinations of phenotypic anomalies shown include: albinism (W-Bijeli and WCRO152), head long and narrow, and without forehead (WCRO71), light yellow hair, no black stripes on front legs, long narrow head, muzzle saddle concave, ears 13.4 and 12.8 cm long (WCRO119).

Out of 49 examined dogs (D), 25 (51.0%) were males, and out of 157 phenotypic wolves 95 (60.5%) were males, while for three (1.9%), gender was not determined. Based on their phenotype, out of 95 wild male canids, 83 were classified as wolves (W), while 12 were classified as suspected hybrids (SH). For 18 males (D = 3, W = 13, SH = 2) Y-STR was not determined. The distribution of Y-STR for the remaining 105 males is shown in Table 2, and compared with the findings of the same Y-STR haplotypes found by other studies in dogs, wolf-dog hybrids and in other wolf populations in Europe and Russia (SUNDQVIST et al., 2001; IACOLINA et al., 2010; RANDI et al., 2014).

A total of ten Y-STR haplotypes were found in dogs from Croatia, and eight of them were private, i.e. not found in wolves or suspected hybrids from Croatia. Three Y-STR haplotypes were found as new in dogs (Table 2). In groups of phenotypic wolves and suspected hybrids, eleven and six different Y-STR haplotypes were found, respectively.

Phenotypic wolves and suspected hybrids carried nine private Y-linked STR haplotypes. The haplotypes common to dogs and wolves were YH05 and YH08. Haplotype YH05 was the most common in dogs (n = 11), but it was found in one phenotypic wolf and in two suspected hybrids, while YH08 was found eleven times in phenotypic wolves and only once in dogs.

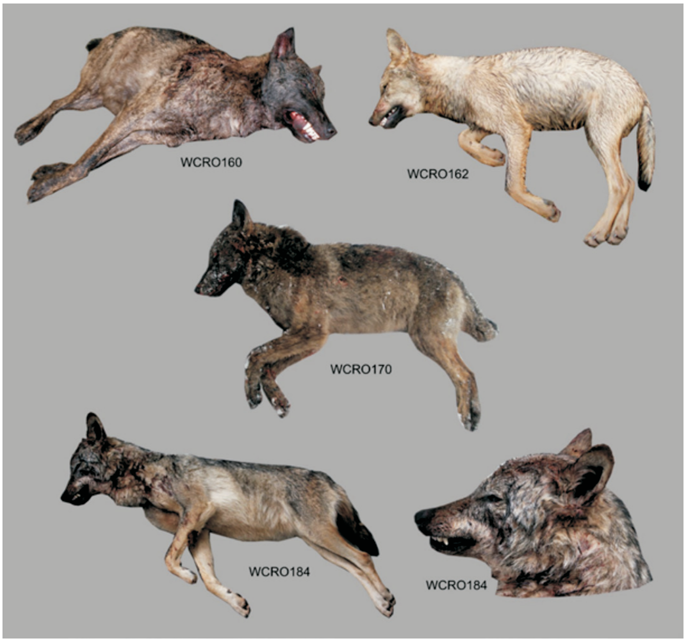

Fig. 3. Suspected wolf-dog hybrids, but genetically classified as wolves, found in Croatia in the period 1996-2011. Combinations of phenotypic anomalies shown include: dark (almost black) hair particularly head, back and distal parts of legs (WCRO160 and WCRO170), plus 30 cm long head (WCRO160), short head (24 cm), long ears (11.5 cm and 11.7 cm), yellow hair, all found in WCRO162, while short head (24.2 cm) was found as the only deviation in WCRO184

Mitochondrial DNA control region 1 haplotypes were determined for 42 dogs, 135 phenotypic wolves and for 17 suspected hybrids. Four different mtDNA CR1 haplotypes were found in wolves and the same four haplotypes were also found in suspected hybrids. Dogs had 15 different mtDNA CR1 haplotypes and all of them were private, i.e. there was no any mtDNA CR1 haplotype common between the groups of dogs and wolves/suspected hybrids (Table 3).

The eight genotypic classes simulated in HYBRIDLAB were identified by STRUCTURE with *K* = 2. The lower *q*i values observed in the parental populations were:

parental wolves (PW) qi = 0.78; parental dogs (PD) qi = 0.721, whereas the highest values observed in hybrid populations were: first generation hybrids (F_1) qi = 0.765; second generation hybrids (F_2) qi = 0.907; first generation backcrosses with dogs (BC_1D) qi = 0.688; second generation backcrosses with dogs (BC_2D) qi = 0.729; first generation backcrosses with wolves (BC_1W) qi = 0.953 and second generation backcrosses with wolves (BC_2W) qi = 0.944. After running only parental populations in STRUCTURE (with $K = 2$, without *a priori* information, *Admixture* and *I* models), the qi values ranged between 0.930 and 0.998 in PW (IC 90% = 0.654-1.000) and between 0.905 and 0.998 in PD (IC 90% = 0.560-0.993) (Fig. 5).

Table 2. Frequencies of Y-linked microsatellite haplotypes in the wolf (W), dog (D) and suspected hybrid (SH) sample groups. In parenthesis is the total number of analyzed males. Detection of haplotypes in previous studies indicated in the last column: D = dogs, H = hybrids, WHR = Croatian wolves, WBS = wolves from Baltic States, WCP = wolves from western Carpathians, WSC = Scandinavian wolves, WF = Finland wolves, WR = Russian wolves, NEW = new haplotype found in this study in dogs.

Y haplotype ID(1)	D (22)	W (73)	SH (10)	Previous studies(2)
YH02	1			NEW
YH06	2			D
YH07	1			WF, WBS, WR
YH24	1			D
YH25	1			NEW
YH29	1			NEW
YH32	1			WHR
YH34	2			D
YH05	11	1	2	D, H, WBS
YH08	1	11		WHR, WSC, WBS
YH09		10		WHR
YH19		1		WHR
YH30		1		WHR
YH33		1		WHR, WBS
YH11		15	1	WCP, WHR, WF, WBS, WR
YH16		9	3	WCP, WHR, WBS, WR
YH18		2	1	WF, WBS, WR
YH20		20	1	WHR
YH31		2	2	WCP, WHR,
Total haplotypes	10	11	6	
Private haplotypes	8	9		

[1] - Haplotype identifications as named in (RANDI et al., 2014). [2] - Y-STR haplotypes found in other studies (SUNDQVIST et al., 2001; IACOLINA et al., 2010; RANDI et al., 2014)

Table 3. Frequencies of mtDNA CR1 haplotypes in dogs (D), wolves (W) and suspected hybrids (SH) groups. The number of analyzed individuals is in parenthesis.

mtDNA CR1 HAPLOTYPES*	D (42)	W (135)	SH (17)
D01	7		
D02	7		
D03	8		
D04	1		
D05	1		
D06	4		
D07	3		
D08	1		
D09	1		
D10	1		
D11	2		
D12	1		
D13	3		
D14	1		
D15	1		
WCR01		50	3
WCR02		26	2
WCR03		12	7
WCR06		47	5
Total haplotypes	15	4	4
Private haplotypes		4	

* mtDNA CR1 haplotypes identifications as named in (RANDI et al., 2000; GOMERČIĆ et al., 2010).

We used the simulated data to infer information about the power of admixture analyses to detect F_1, F_2 hybrids, the first and second generation of backcrosses, given the number of markers. Considering the STRUCTURE results obtained with the simulated classes of hybrid genotypes, choosing a threshold of 0.78 (lower *q*i values observed in parental populations) all F_1 offspring would be correctly identified as hybrids, but we would miss 4% of F_2 hybrids, then also 34% of BC_1W and 42% of BC_2W. Choosing more conservative threshold *q*i = 0.90 (the highest *q*i for F_2 hybrids), we would correctly identify 100% of F_1, F_2 hybrids and 96% BC_1W and 94% BC_2W, but we would confuse the 13.5% of wolves as hybrids (false positive as in RANDI et al., 2014. However, any PW showing a *q*i value ≤0.90, running STRUCTURE using only parental populations and using "*population information POPINFO*" model the 99% of PW was correctly identified by both 0.90 and 0.93 *q*i thresholds. Therefore, a threshold of 0.93 was chosen to be used only for classification of individuals with the phenotypic anomalies found.

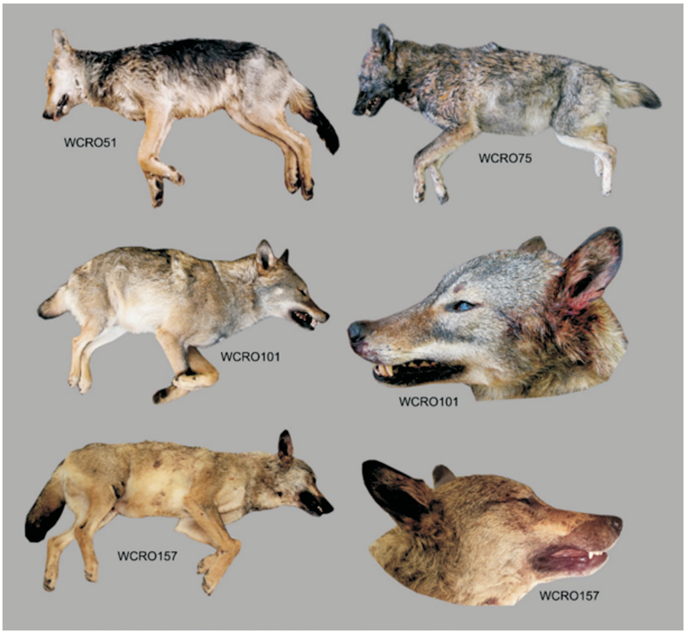

Fig. 4. Wolf-dog hybrids found in Croatia in the period 1996-2011. Combinations of phenotypic anomalies shown include: no black stripes on front legs, dark hairs on the back, too small (32 kg) for a three year old male (WCRO51), then atypical coloration of hair, rather black head and the body with black patchy areas, particularly on hind legs (WCRO75), head long and narrow, long ears (11 cm), flat forehead, muzzle (snout) slightly concave (WCRO101) and longer ears (11.4 and 11.5 cm), with small paw length (front: 8.6 and 8.1 cm; hind 7.5 and 7.7) for a male wolf in the second year of life (WCRO157).

Results from STRUCTURE analyses (K = 1-5, *Admixture* and I models, using Evanno methods), applied to the real sample data, showed that the optimal clustering of samples was obtained with $K = 2$ (LnP(D) = -8197,23). This divided samples into two distinct clusters corresponding to wolves and dogs, with an average membership proportion of $Q_W = 0.990$; IC 90% = 0.948-1.000 and $Q_D = 0.988$; IC 90% = 0.928-1.000. Genotypes were assigned to two clusters with an individual proportion of admixture q_W ranging from 0.77 to 0.998, and q_D from 0.895 to 0.997. Suspected hybrids showed a lower Q_W = 0.941and q_W ranged from 0.281 to 0.998 (Fig. 6).

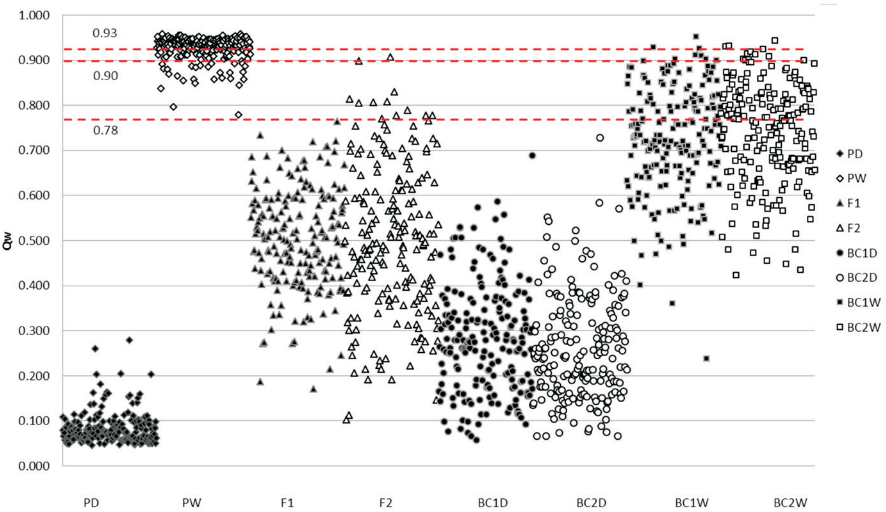

Fig. 5. Eight genotypic classes as the output of simulation in HYBRIDLAB classified by STRUCTURE with K = 2. PD = parental dogs; PW = parental wolves; BC_1D, BC_2D = first and second generation backcross with dog; BC_1W, BC_2W = first and second generation backcross with wolf.

Hybrids identification. The output of STRUCTURE showed that at a threshold of *q*i = 0.78 two individuals were identified as hybrids: WCRO032 and WCRO051, both classified as suspected hybrids on the basis of phenotypic anomalies. Two more individuals (phenotypically suspected hybrids WCRO075, WCRO101) were also identified as genetic hybrids, with a threshold of 0.90. Finally, using a *q*i = 0.93 (the lowest *q*i value observed in PW running only parental populations) one more individual (WCRO157) was identified as a wolf-dog hybrid. The detection of hybrid individuals was supported by the IC intervals, i.e. in all genetic hybrids the inferior interval was always <0.60. Four hybrids were identified as backcrosses with wolves, while one was identified as a backcross with dogs, but all without the determination of the generation of backcrossing. Four determined hybrids were males, while one was female, but all of them had mtDNA sequences previously found in wolves and not found in dogs from Croatia, i.e. all of the hybrids found were descendants of a female wolf. One individual (WG01) showed a dog Y-haplotype but not any other genetic (12-STR, mtDNA CR1) or phenotypic signal of hybrid origin. This indicates past introgression of dog genes. The remaining 14 suspected hybrids were identified as wolves, considering all three genetic criteria used in the analysis. All five (2.8 percent) identified hybrids were found in the Dalmatia region, while the animal with introgressed dog genes was found in captivity in the Lika region (Fig. 1).

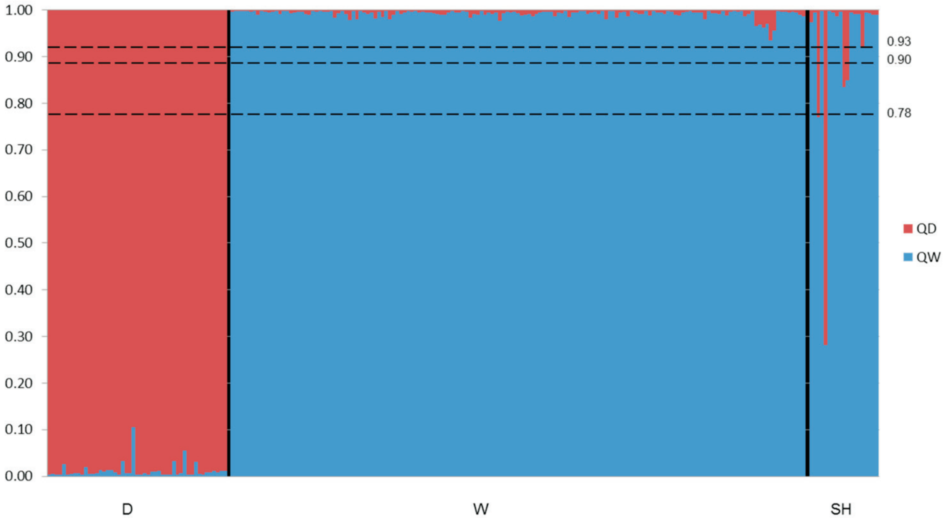

Fig. 6. Posterior probability assignments of dog (D), wolf (W) and suspected hybrid (SH) in samples genotyped at 12 STRs obtained by STRUCTURE analysis using "*admixture*" and "*I*" models without prior population information, K = 2. Wolf and suspected hybrid individuals were classified on morphometric data and morphological features. Each individual is represented by a vertical bar fragmented in K sections of specific length, according to their proportion of membership in each genetic cluster. The horizontal dotted black lines represent the three chosen thresholds (0.78; 0.90, and 0.93).

Discussion

Deviations from the "standard" gray wolf phenotype are sometimes used as a proof of hybridization (MILENKOVIĆ et al., 2006), but in most studies they are just considered the first indication of the possible introgression of dog genes, which are then tested by further genetic analyses (ANDERSONE-LILLEY et al., 2002; CIUCCI et al., 2003; CANIGLIA et al., 2013; KHOSRAVI et al., 2013). We used a combination of 15 distinctive phenotypic features, with three recommended (RANDI et al., 2014) biparental and uniparental classes of genetic markers, to maximize the likelihood of hybrid detection, while minimizing the probability of false hybrids, given the number of autosomal STRs used. The results of STRUCTURE procedures were influenced by the number of populations, by the degree of admixture among them, and the number of autosomal microsatellites used. The shortcoming of the possible identification of false positive hybrids (RANDI et al., 2014) with the use of only 12 autosomal microsatellites, was mitigated by the use of a higher *qi* threshold for animals with phenotypic deviations. The use of 12 microsatellite STRs was not informative enough for identification of the generations of admixture, they only allowed us to determine if an identified hybrids was a backcross with wolves (BCW), a backcross with dogs (BCD) or a wolf with a past dog gene introgression (IG). The only introgression we found (in WG01) was determined on the basis of the Y chromosome haplotype. The use of phenotypic deviations alone would not be reliable for hybrid detection. Fourteen animals with phenotype deviations were genetically determined as wolves, including two dark and two albino individuals (Table 1. Suspected wolf-dog hybrids and one wolf with introgressed dog genes, found in Croatia in 1996-2011, with a description of the phenotypic anomalies detected. Genetic identification of wolf-dog hybrids and suspected hybrids., Fig. 2 and Fig. 3), while introgressed dog genes were found in one of the phenotypic wolves (WG01).

Two Y-STR haplotypes (YH05 and YH08) were shared among wolves, suspected hybrids and dogs (Table 2). The YH05 haplotype was the most common in dogs, but was found also in two wolves and one putative hybrid (Table 2). In RANDI et al. (2014), YH05 is recognized as a private haplotype found in dog samples of different origin (Italian feral dogs, local dog breeds and Czechoslovakian wolf-dog breed). In IACOLINA et al. (2010) YH05 (named H3) was the most common in dogs but it was also found in five hybrid wolf-dog individuals. The only evidence of YH05 in wolves comes from SUNDQVIST et al. (2001) who found the same haplotype (named L) in four male wolves from Baltic States. In our study, two suspected hybrids carrying YH05 (WCRO32 and WCRO51) were confirmed as hybrids also by their low *qw*, while a third animal with YH05 (WG01) did not show any other (phenotypic, STR or mtDNA CR1) signs of hybridization. These findings could suggest that (1) YH05 is a rare haplotype present in the Croatian wolf population, as in other wolf populations (SUNDQVIST et al., 2001), or (2) YH05 came from a dog and it is a sign of ancient introgression. The haplotype YH08 was found to be quite common in the Croatian wolf population, but the same haplotype was found in one male village dog which showed no apparent traces of admixture with wolves ($q_d = 0.995$ CI 90% = 0,976-1,000). Haplotype YH08 has been also found in other European wolf populations (Scandinavia and Baltic States) and named C in SUNDQVIST et al. (2001), but never before in dogs. These findings could suggest that (1) YH08 is a rare haplotype also present in domestic dogs; (2) YH08 is private haplotype present in wolf populations, while the dog carrying YH08 was the result of an ancient introgression from the wolf population. If the second hypothesis is true, then this interbreeding and consequent gene introgression included a male wolf and a female dog, which is a possible, but extremely rare event, proven to have happened in only two cases in the world so far: Vancouver Island (MUÑOZ-FUENTES et al., 2010) and Latvia (HINDRIKSON et al., 2012). The common direction of hybridization includes female wolves and male dogs (RANDI et al., 2000; ANDERSONE-LILLEY et al., 2002; VILÀ et al., 2003; VERARDI et al., 2006) and this was reconfirmed by this study. All five proven hybrids shared mtDNA CR1 found in the phenotypic and genotypic wolves determined in this study, and those previously found for wolves in Croatia (GOMERČIĆ et al., 2010; SINDIČIĆ et al., 2011). Since mtDNA is inherited by the maternal line only, it is clear that all hybrids found originated from mating between a female wolf and a male dog. Evidence of the opposite direction of hybridization, although it is a rare event (HINDRIKSON et al., 2012), was possibly found in one wolf (WG01), which carried an introgressed dog Y-STR haplotype (YH05). The finding of YH08 in one of the investigated dogs could be more evidence of mating between a male wolf and a female dog. While there are several possible explanations for mating between female wolves and male dogs, the only explanation for the rare opposite direction of mating hypothesized by HINDRIKSON et al. (2012) assumes a low density of female wolves and a high density of stray dogs.

The occurrence of wolf-dog hybrids only in the south of Croatia (Dalmatia, Fig. 1) can be understood by consideration of the history and current status of the wolf population in Croatia, with the accepted conditions for wolf-dog hybridization. Systematic eradication of wolves after WWII caused the range of the wolf population to shrink, with numbers declining to 30-50 individuals in late 1980s (FRKOVIĆ and HUBER, 1992), including their temporary disappearance from Dalmatia. Fortunately, this smal number of wolves were never isolated from the rest of the Dinaric-Balkan wolf population and the bottleneck effect was not as severe as in some other European wolf populations (ARBANASIĆ et al., 2013; FABBRI et al., 2014). At the beginning of the 1990s, the number of wolves started to grow, with the expansion of their range back to Dalmatia and some other parts of Croatia (KUSAK and HUBER, 2010a). Wolves in Dalmatia live in close proximity to humans (KUSAK et al., 2005), largely depending on human food sources (ŠTRBENAC et al., 2005), which causes human animosity toward wolves (MAJIĆ and BATH, 2010) and consequent high human-caused mortality rates (KUSAK and HUBER, 2010b). Since the chances for wolf-dog hybridization increase when wolves live close to settlements with a high density of dogs (BLANCO et al., 1992), as well as in situations when wolves are heavily victimized (RANDI et al., 2000) or during the expansion phase of the wolf population (LORENZINI et al., 2014; RANDI et al., 2014), it seems that the prevalence of hybrids found in Dalmatia (five out of 85, 5.9%) was surprisingly low, considering that all the listed conditions for hybridization were met. VILÀ and WAYNE (1999) summarized that hybridization between gray wolves and domestic dogs is unusual and even if it happens, the offspring of male dogs and female wolves may rarely survive because male dogs provide limited parental care, rendering such events insignificant for the genetic composition of the gray wolf population. However, examples of introgression of dog genes into wolf populations, such as the presence of black wolves in North America (ANDERSON et al., 2009) and Italy (CANIGLIA et al., 2013), has been proven to persist in wolf populations, since it gives higher life expectancy to individuals carrying these genes, resulting in positive selection under the current environmental changes (COULSON et al., 2011). Two dark haired suspected hybrids in this study did not show genetic signs of hybridization, but they were not checked for melanistic mutation at the *K* locus, determining black coloration in wolves (ANDERSON et al., 2009). The presence of YH05 haplotype was found as the only sign of the past introgression of dog genes into the Dinaric wolf population, but even if introgression happens more often than could be detected with the methodology used, the ecological importance of this process remains largely unknown. The only study of trophic overlap between wolves and free-ranging wolf-dog hybrids (BASSI et al., 2017) proved that hybrids can live as wolves and be equal competitors for food as pure wolves with each other. With such diverse and unpredictable effects of hybridization, it is difficult to render unambiguous legislative, conservation and management decisions. The Convention on International Trade in Endangered Species (CITES, Conf. 10.17, Rev. Cop14) gives the same level of protection to hybrids as to wild species. Croatian nature conservation law (Official Gazette 80/13) lists equally wolves and their hybrids as strictly protected. Evidence of past hybridization and introgression of dog genes, such as black, yellow or patchy coat coloration, dewclaw (CIUCCI et al., 2003), dental anomalies etc., raises question if such animals should be removed (lethal or live removal) from the population, and if so, how and under which conditions (LESCUREUX and LINNELL, 2014). As proven by this study, distinguishing wolves from hybrids by their external appearance is unreliable, and even if distinction is done genetically, as pointed out by LESCUREUX and LINNELL (2014), the question is where to set the threshold of (un)acceptable introgression. More studies are needed on the frequency of hybridization events and the circumstances resulting in wolf-dog hybrids, in addition to studies on the behavior and ecology of hybrids. These studies are necessary to support sound conservation and management decisions. The prevalence of hybridization in Croatia was found to be lower compared to Italy (2.8 *vs.* 6.5 percent; LORENZINI et al., 2014), but as LORENZINI et al. (2014) warned, the proportion of hybrids, and consequently the perception of how severe the hybridization is, depends on the detection power of the marker set and the threshold selected for assigning genotypes. It is possible that with the use of a larger number of autosomal microsatellites, we would achieve different results. Nevertheless, hybridization between wolves and dogs exists in Croatia and is expected to persist as long as the conditions favoring its occurrence are met. The ecological, sociological and conservation implications of hybridization are yet to be determined and considered in the future management of the species.

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SAŽETAK

Križanje vukova i pasa jedna je od glavnih prijetnji očuvanju vukova jer se miješanjem i introgresijom gena domaćih životinja mogu narušiti lokalne prilagodbe te tako ugroziti dugoročni opstanak populacija vukova u prirodi. Istražili smo pojavu križanja vukova i pasa u Hrvatskoj analizom 12 autosomskih mikrosatelitskih markera Bayesian testom primjese te odredili smjer hibridizacije određivanje markera nasljeđivanih i po majčinskoj i po očinskoj liniji, u kombinaciji s određivanjem morfometrijskih i morfoloških svojstava. Sustavna analiza morfoloških svojstava i morfometrijskih podataka korištena je za rangiranje istraživanih jedinki, bilo kao fenotipske divlje tipove vukova bilo kao sumnjive na križanost. Kombiniranjem rezultata Bayesian statistike s fenotipskim obilježjima postavili smo prag za razlikovanje vukova od križanaca, koji povećava vjerojatnost određivanja križanaca sa smanjivanje vjerojatnost za pogrešno pozitivna određivanja križanaca. Temeljem fenotipa od 176 divljih kanida 157 (89,2 %) bilo je kategorizirano kao vukovi, dok je 19 (10,8 %) bilo svrstano u sumnjive križance. Temeljem Bayesian testa primjese pet (2,8 %) životinja bilo je svrstano u križance vuka i psa, četiri od njih određena su kao unatražno križani s vukovima, dok je jedan bio unatražno križan sa psom. Mitohondrijska DNK pokazala je da su svi križanci bili rezultat parenja vučice sa psom. Dva su muška križanca na Y-kromosomu imala haplotipove i od vukova i od pasa, dok su druga dva imala Y-kromosomske haplotipove dosad utvrđene samo kod vukova. Jedan je vuk imao pseći Y-kromosomski haplotip što je uputilo na introgresiju psećih gena kod njega. Svi su križanci pronađeni u Dalmaciji, gdje su se vukovi nedavno proširili te gdje žive blizu ljudi, s visokom stopom smrtnosti uzrokovane od ljudi. Te se okolnosti inače smatraju pogodujućima za nastanak križanaca vukova i pasa. Ipak, pronađena učestalost pojave križanaca bila je niska, ali i za očekivati je da će se zadržati sve dok postoje uvjeti koji pogoduju nastanku križanaca. Ekološke, sociološke te značajke hibridizacije za zaštitu i upravljanje populacijom vukova tek treba utvrditi.

Ključne riječi: vuk; pas; *Canis lupus*; križanje; Hrvatska; Bayesijska analiza primjese; mikrosateliti; roditeljski markeri