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Differentiating between Y chromosome sequences in Croatian canids - short communication

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ABSTRACT

Detecting the hybridization between species is important for evolutionary studies of speciation processes, conservation biology and management, but we still lack a general empirical perspective of hybridization problems in canids. Hybridization may occur between many species of the canid family, sometimes threatening the survival of endangered species or populations. Mitochondrial DNA control region haplotypes can be used to discriminate between Croatian wolves, dogs and jackals, and the goal of our research was to identify species specific Y chromosome haplotypes that could be used in detection of paternal origin in possible hybrids. We analyzed three non-overlapping Y chromosome fragments of the grey wolf, golden jackal and dog in the total length of 1,898 base pairs. Two haplotypes were identified, one shared among grey wolf and dog, and one specific golden jackal haplotype. We did not find polymorphic sites that could be used to distinguish the paternal line in wolf - dog hybrids, but six polymorphic sites were identified that can be used to discriminate golden jackal from grey wolf and domestic dog.

Key words: Canidae, *Canis aureus*, *Canis lupus*, *Canis lupus familiaris*, Y chromosome

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Introduction

In mammals, the Y chromosome is transmitted from fathers to sons and Y-specific haplotypes can provide data about the paternal line. However several studies report lower than expected levels of Y chromosome diversity (HELLBORG and ELLEGREN, 2004) and only a few studies (with the exception of primates) report Y chromosome variation within and between natural populations (SUNDQVIST et al., 2001; HAILER and LEONARD, 2008).

Detecting the hybridization between species is important for evolutionary studies of speciation processes, conservation biology and management (VILÁ et al., 2003a). Also, identifying the directionality of hybridization is essential for a proper understanding of the dynamics of this process in natural populations, and separate analysis of both maternally and paternally inherited markers is important (HAILER and LEONARD, 2008; GODINHO et al., 2011). Development of molecular markers has enabled identification of hybrids in natural populations, but in a case of hybridization between closely related taxa, like canids, identifying individual hybrids may be particularly problematic. Most recent canid hybridization studies have combined the use of mitochondrial DNA (mtDNA), autosomal and Y chromosome microsatellite markers.

During the past decade the grey wolf (*Canis lupus*) and golden jackal (*Canis aureus*) populations have been expanding in several European countries (BOITANI, 2003; ARNOLD et al., 2012). Higher population density is increasing the frequency of encounters between wild canids and free-ranging or feral dogs, especially in peripheral areas of wolf distribution (GODINHO et al., 2011). Genetic analysis has detected grey wolf and dog hybrids in several European countries (VILÁ and WAYNE, 1999; RANDI et al., 2000; ANDERSONE et al., 2002; RANDI and LUCCHINI, 2002; VILÁ et al., 2003b; VERARDI et al., 2006; RANDI, 2008) and there are first indications about golden jackal hybrids (Bošković, personal communication).

MtDNA control region haplotypes can be used to discriminate between Croatian wolves and dogs (GOMERČIĆ et al., 2010; SINDIČIĆ et al., 2011). Furthermore, the only golden jackal haplotype found so far in Europe (ZACHOS et al., 2009) can be easily differentiated from wolf and dog haplotypes. MtDNA cannot provide any information about paternal line, so Y-chromosome data can be applied as paternal analogues to mtDNA.

The goal of our research was to investigate Y chromosome variations among the golden jackal, grey wolves and dogs in Croatia. Further, we wanted to identify species specific Y chromosome haplotypes that could be used in detection of paternal origin in possible hybrids between any of those canids.

Materials and methods

Grey wolf (*Canis lupus*) and golden jackal (*Canis aureus*) tissue samples were collected from animals killed in traffic accidents or culled during regular hunting management in Croatia. Blood samples of mixed breed dogs (*Canis lupus familiaris*) were collected during routine clinical examinations at the Faculty of Veterinary Medicine of the University of Zagreb. Ten samples from each of the three canids were analyzed. DNA was extracted with a commercial kit (ChargeSwitch® gDNA Tissue Kit Invitrogen) following the manufacturer's procedure. Three non-overlapping Y chromosome fragments were chosen for analysis, based on grey wolf and dog Y chromosome sequences published by DING et al. (2011). Among their data we identified polymorphic sites that were species specific and could be used for discrimination between canid species. The Y chromosome 1,897 base pair (bp) long fragment 21 published by DING et al. (2011) had three possible species specific polymorphic sites (positions 536, 645 and 1455) and 1,814 bp-long fragment 24 had one possible species specific polymorphic site (position 1318).

PCR reaction was carried out on a Veriti 96 Well Thermal Cycler (Applied Biosystems), in a total volume of 20 µl using Platinum® PCR SuperMix (Invitrogen) (consisting of *Taq* DNA polymerase with Platinum® *Taq* antybody, 22 mM Tris-HCL (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 µM dNTP mix), 5-10 ng of DNA and 0.2 mM of each primer. Primers (NATANAELSSON et al., 2006) and protocols for PCR are described in Table 1. After purification with ChargeSwitch®PCR Clean-Up Kit (Invitrogen), amplified fragments were sequenced with an ABI3730x1 DNA Analyzer (Applied Biosystems).

Sequence alignment was performed using Clustal W (THOMPSON et al., 1994) implemented in BioEdit software (HALL, 1999) and alignments were manually proofed.

Results

We analyzed three non-overlapping Y chromosome fragments of grey wolf, golden jackal and dog, in the total length of 1,898 bp. Our fragment 21 f2 corresponds to positions 130-793 of the entire fragment 21 sequences from DING et al. (2011), while our fragment 21 f5 corresponds to positions 1120-1705. When compared to the sequence of the entire fragment 24 from DING et al. (2011), our fragment 24 f4 corresponds to positions 1112-1759.

None of the possible species specific polymorphic sites (positions 536, 645 and 1455 of fragment 21, and position 1318 of fragment 24) proved discriminant for our canid species of interest (Tables 2 and 3). Wolf and dog Y chromosome fragment 21 sequences from DING et al. (2011) can be distinguished based on loci 536 and 1455, but neither Croatian wolf and dog nor golden jackal sequences can be distinguished based on this polymorphic site. Wolf and dog fragment 24 sequences from Croatia are identical to dog haplotypes from DING et al. (2011), and this polymorphic site cannot be used to

Table 1. Primers (NATANAELSSON et al., 2006), PCR primer annealing temperature, PCR product length and GenBank accession numbers for each of the analyzed Y chromosome fragments.

Fragment	Primers	Annealing tem. °C	PCR product length (bp)	GenBank accession number
21f2	21f2 (5'-GGATAGAATGCAGGAGAGGG-3') 21r6 (5'-GGTGCCCCAAGAACTAATTC-3')	55	664	JX220168, JX220169
21f5	21f5 (5'-ATTTCCCATCAAGTGTGTCCT-3') 21r4 (5'-GAAATAGGCCACGAAACCAG-3')	58	586	JX220170, JX220171
24f4	24f4 (5'-ATGAATCCTGCAACCAACCTC-3') 24r2 (5'-TGCGGGTATTGGTAGGCTC-3')	59	648	JX220172, JX220173

Table 2. Polymorphic site identified among sequences of Y chromosome fragment 21 f2 from this study and sequences deposited in the Genbank. Position numbers refer to the aligned sites of 1,897 bp-long fragment 21 published by DING et al. (2011). Only variable sites are shown, dots represent identity with the first haplotype.

GenBank No	Species	Reference	536	566	645
JX220168	*C. lupus*, *C. l. familiaris*	this study	G	T	C
JX220169	*C. aureus*	this study	.	.	.
DQ973639, DQ973657, DQ973675, DQ973783, DQ973693, DQ973711, DQ973729, DQ973747, DQ973765, DQ973801, HQ389378, HQ389379, HQ389380, HQ389384	*C. l. familiaris*	Natanaelsson et al. (2006); Ding et al. (2011)	.	.	.
HQ389381	*C. l. familiaris*	Ding et al. (2011)	.	C	.
HQ389382	*C. lupus*	Ding et al. (2011)	A	.	.
HQ389383	*C. latrans*	Ding et al. (2011)	.	.	T

Table 3. Polymorphic site identified among sequences of Y chromosome fragment 21 f5 from this study and sequences deposited in the Genbank. Position numbers refer to the aligned sites of 1,897 bp-long fragment 21 published by DING et al. (2011). Only variable sites are shown, dots represent identity with the first haplotype and dashes denote deletions.

GenBank No	Species	Reference	1136	1137	1138	1298	1368	1405	1455	1581
JX220170	*C. lupus, C. l. familiaris*	this study	T	T	A	T	G	C	T	-
JX220171	*C. aureus*	this study	.	.	.	.	T	.	.	T
DQ973639, DQ973657, DQ973675, DQ973783, DQ973693, DQ973711, DQ973729, DQ973747, DQ973765, DQ973801, HQ389379, HQ389381, HQ389384	*C. l. familiaris*	Natanaelsson et al. (2006); Ding et al. (2011)	.	.	.	.	.	.	.	-
HQ389378	*C. l. familiaris*	Ding et al. (2011)	.	.	.	.	.	A	.	-
HQ389380	*C. l. familiaris*	Ding et al. (2011)	-	-	-	.	.	.	.	-
HQ389382	*C. lupus*	Ding et al. (2011)	.	.	.	.	.	.	C	-
HQ389383	*C. latrans*	Ding et al. (2011)	.	.	.	C	.	.	C	-

Table 4. Polymorphic site identified among sequences of Y chromosome fragment 24 from this study and sequences deposited in the Genbank. Position numbers refer to the aligned sites of 1,814 bp-long fragment 24 published by DING et al. (2011). Only variable sites are shown, dots represent identity with the first haplotype.

GenBank No	Species	Reference	1318	1371
JX220172	*C. lupus, C. l. familiaris*	this study	C	T
JX220173	*C. aureus*	this study	.	.
DQ973640, DQ973658, DQ973676, DQ973694, DQ973712, DQ973730, DQ973748, DQ973766, DQ973784, DQ973802, HQ389385, HQ389387, HQ389390	*C. l. familiaris*	Natanaelsson et al. (2006), Ding et al. (2011)	.	.
HQ389386	*C. l. familiaris*	Ding et al. (2011)	.	A
HQ389388	*C. lupus*	Ding et al. (2011)	T	.
HQ389389	*C. latrans*	Ding et al. (2011)	.	.

discriminate either between wolf and dog samples from Croatia or between golden jackal and wolf/dog samples.

However, among the three investigated Y chromosome fragments, we identified six additional polymorphic sites that discriminate Croatian golden jackal samples from grey wolf and domestic dog samples (Table 5). They consist of five substitutions and one insertion/deletion. Fragment 21 f2 had two polymorphic sites, fragment 21 f5 had three and fragment 24 f4 had one polymorphic site. One obtained haplotype is shared between the grey wolf and dog, while the other is unique for golden jackals (Table 5).

Table 5. Polymorphic sites identified within the two Y chromosome haplotypes. Position numbers refer to the aligned sites of each fragment separately. Only variable sites are shown with dashes denoting deletions.

Fragment	21 f2		21 f5			24 f4
Locus	135	188	249	336	462	639
C. lupus and C. l. familiaris haplotype	A	A	G	T	-	G
C. aureus haplotype	G	T	T	C	T	A

Discussion

We still lack a general empirical perspective of hybridization problems in canids, and more detailed genetic studies using a variety of genetic markers and in different populations are necessary to conclusively address the issue of wolf-dog hybridization, to understand its directionality and frequency of occurrence (VILÁ et al., 2003b; GODINHO et al., 2011). Until recently only 3,200 bp of the non-repetitive dog Y-chromosome sequence were deposited in GenBank, but in 2006 NATANAELSSON et al. (2006) have identified 24,159 bp of dog Y-chromosome sequence to be used for population genetic studies. DING et al. (2011) analyzed 14,437 base pairs (bp) of Y chromosome DNA sequence in 151 dogs, 12 wolves and 2 coyotes. Dog samples were collected worldwide, while wolf samples came from China, America and Scandinavia. Among their data, we identified several polymorphic sites that were specific for wolves and had the potential to be used as markers for dog-wolf hybridization. But when we analyzed 1,898 bp of Y chromosome in dog and wolf samples from a single country (Croatia), we did not find polymorphic sites that could be used to distinguish the paternal line in wolf - dog hybrids. However, six polymorphic sites were identified that can be used in golden jackal research. Knowing the golden jackal species biology, its demography in Europe and the overwhelming evidence of hybridization among canid species, the possibility of jackal - dog or even jackal - wolf hybrids cannot be neglected.

Given the interest of evolutionary biologists in disentangling paternal and maternal genetic lineages, the currently published work dealing with developing and employing

Y-specific markers probably represents only a small portion of the studies which have aimed to find male-specific polymorphism. There appears to be an obvious bias in that studies that fail to develop such markers or which show no or very little variation are more difficult to get published (GREMINGER et al., 2010). In our study we confirmed low Y chromosome variation in canid species from Croatia. Contrary to the Y chromosome, the mtDNA control region expressed greater variability, with 6.0% polymorphic sites among dog haplotypes and 3.9% polymorphic sites among wolf haplotypes (SINDIČIĆ et al., 2011).

Although we did not find polymorphic sites that could be used to distinguish between dogs and wolves, which are considered as the same species, we did find polymorphic sites that could be used in future research of possible golden jackal hybrids.

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SAŽETAK

Otkrivanje hibridizacije među vrstama važno je za evolucijska istraživanja procesa nastajanja vrsta, konzervacijsku biologiju i upravljanje populacijama, no još uvijek nemamo empirijske podatke o problemu hibridizacije kod porodice pasa. Hibridizacija se javlja među mnogim vrstama iz porodice pasa, što ponekad predstavlja opasnost opstanku ugroženih vrsta ili populacija. Na temelju haplotipova kontrolne regije mitohondrijske DNK mogu se razlikovati vukovi, psi i čagljevi iz Hrvatske, te je cilj našeg istraživanja bio utvrditi vrsno specifične haplotipove Y kromosoma koji se mogu koristiti u otkrivanju očinske linije kod mogućih hibrida. Istražili smo tri nepreklapajuća slijeda Y kromosoma vuka, psa i čaglja u dužini od 1898 parova baza. Utvrdili smo prisutnost dvaju haplotipova, jedan zajednički vuku i psu, te jedan haplotip specifičan za čaglja. Nismo pronašli polimorfna mjesta koja se mogu koristiti za istraživanje očinske linije kod hibrida vuka i psa, no pronašli smo šest polimorfnih mjesta pomoću kojih se mogu razlikovati čagljevi od vuka i psa.

Ključne riječi: kanidi, *Canis aureus*, *Canis lupus*, *Canis lupus familiaris*, Y kromosom