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Original investigation

Substantial functional diversity accompanies limited major histocompatibility complex class II variability in golden jackal (*Canis aureus*): A comparison between two wild *Canis* species in Croatia

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ABSTRACT

The golden jackal (*Canis aureus*) is one of the less studied carnivores and research on its major histocompatibility complex (MHC) variability is just at its early stages. MHC genes encode cell-surface receptors that serve to bind and present antigens to T cells, which is essential to initiating specific immunological responses in vertebrates. In this paper we present for the first time patterns of genetic diversity and natural selection on MHC class II DLA-DRB1, DQA1 and DQB1 loci in the golden jackal using samples from two geographically distinct regions in Croatia and further compare them to the values found in its congener grey wolf (*Canis lupus*). Diversity of golden jackals at all three loci was markedly lower than that of grey wolves (allelic richness values were 4, 2 and 3 in jackal versus 11.9, 6.6 and 10.2 in wolves for DRB1, DQA1 and DQB1, respectively) and can be attributed to a genetic drift rather than to the lack of historical positive selection. The finding of high evolutionary distances (16.3% for DRB1 and 8.5% for DQB1) and a substantial number of codons predicted to be under the influence of positive selection (11 for DRB1 and 9 for DQB1) suggests that the investigated golden jackal population still contains considerable functional diversity necessary for the presentation of varied foreign peptides. In contrast to neutral genetic variation, our results suggest that the Dalmatian population has a higher MHC diversity than the Slavonian population, casting doubt on its supposed isolation and calling for a more extensive investigation of the MHC variability of southern Balkan jackal populations.

Introduction

Croatia is among the European countries where two wild canid species coexist: the grey wolf (*Canis lupus*) and the golden jackal (*Canis aureus*). Though belonging to the same genus, these two species differ radically in population history and ecology. While the grey wolf, as the top predator, was widespread throughout Europe until approximately 250 years ago (Randi et al., 2000), the golden jackal has traditionally been restricted to the continent's southeast, i.e. the Balkan region (Trouwborst et al., 2015). However, over the last few decades, golden jackals have tended to occupy new territories in Europe, expanding northward and westward. Appearing in areas where they have not been recorded before, jackals have been recently sighted as far west as Switzerland and as far north as Estonia (Trouwborst et al., 2015). They possess great potential to colonise a wide range of different habitats and ecosystems and easily adjust to human-modified landscapes, particularly agricultural regions (Lanszki et al., 2010). Contrary to that, the wolf population in Europe dropped dramatically, due to human persecution and forest clearing (Randi and Lucchini, 2002). The grey wolf no longer exists in central and western Europe while isolated populations remain in the Iberian, Apennine, Balkan and Scandinavian peninsulas and in Eastern Europe (Large Carnivore Initiative for Europe, web reference). In Croatia, after an extermination program that was conducted throughout the 20th century, wolves came to the verge of extinction. However, today, the wolf is legally protected in Croatia and the population has recovered (Kusak and Huber, 2010).

Golden jackals in Croatia inhabit geographically distinct regions separated by the highland of Bosnia and Herzegovina: Dalmatia (eastern Adriatic coast) and Slavonia (the eastern continental lowland part of Croatia) (Fig. 1). Golden jackals were reported for the first time in Croatia in southern Dalmatia as far back as at the end of the 15th century, where they have been continuously occurring

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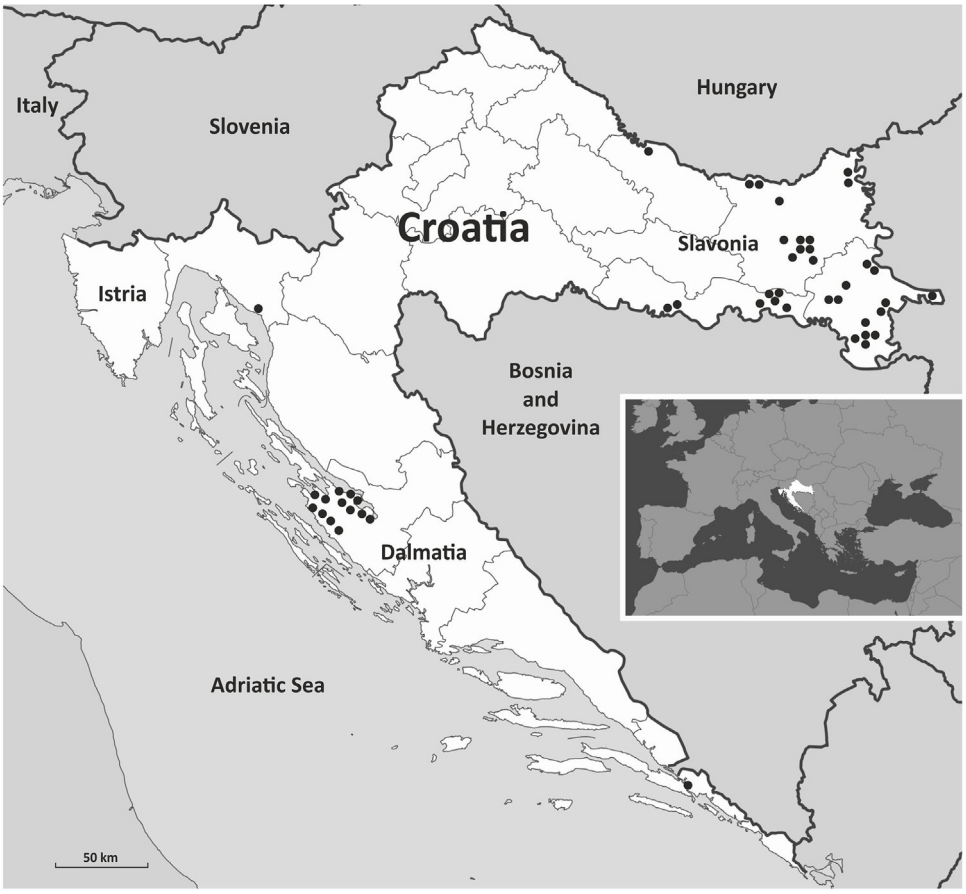

Fig. 1. The locations of sampled golden jackal individuals (black dots) used in the research.

since (Kryštufek and Tvrtkovič, 1990), but in the 1980s they started to spread north, reaching the Istrian peninsula, Slovenia and Italy. Contrary to that, in Slavonia, golden jackals have been recorded for the first time at the beginning of the last century. Since then, only sporadic sightings of jackals were reported by local hunters and no observations were made for decades in the second half of the 20th century. After that time, the first kill was recorded in 1986. Currently, golden jackals are rapidly expanding in this region, presumably due to immigration from Bulgaria, Romania and Serbia (Giannatos, 2004). Populations from Dalmatia and Slavonia are geographically separated and genetically distinct (Fabbri et al., 2014).

MHC genetic region represents immune genes that encode cell-surface receptors that serve to bind and present antigens to T cells, which is essential to initiating a specific immunological response in vertebrates. The extreme variability at MHC loci enables the recognition of a wide spectrum of antigens, which further influences individual and population fitness and the ability to face and confront different pathogens and cope with environmental challenges (Sommer, 2005). MHC currently represents the best system available in vertebrates to investigate how natural selection can promote local adaptation at the gene level (Bernatchez and Landry, 2003). Most polymorphism in MHC class II genes occurs in exon 2, which encodes for the receptor's peptide-binding region (Hughes and Nei, 1989).

In this investigation we report the genetic diversity and evolutionary indices of the exon 2 of DLA-DRB1, DLA-DQA1 and DLA-DQB1 MHC class II loci in golden jackals from Croatia. DLA-DRB1/DQA1/DQB1 haplotypes found in 50 Croatian samples were reported in Galov et al. (2015) for the purpose of identifying golden jackal-dog hybrids, and in this study four more samples were analysed. The main goal of our study was to determine the level of allelic diversity and the influence of long-term selection during evolutionary history on three investigated loci. We also intended to investigate whether genetic difference among the Slavonian (continental) and the Dalmatian (coastal) populations, which was indicated by neutral loci (Fabbri et al., 2014), would be reflected in MHC data. Further, we aimed to compare our results with those obtained for wolves from Croatia. We were particularly intrigued by the small number of alleles found in jackals in comparison with wolves and wanted to estimate if and to what extent the functional diversity of MHC alleles in golden jackals was reduced compared to grey wolves. Additionally, due to trans-species polymorphism, which is typical for MHC genes and assumes very similar or even identical alleles in closely related species (Bernatchez and Landry, 2003), we were interested in finding out if some alleles and three-locus haplotypes are shared between golden jackals and grey wolves and what the phylogenetic relationships among MHC class II alleles in these two species are.

Material and methods

For this study we considered 50 golden jackals used in the study from Galov et al. (2015) and four additional golden jackal individuals. Laboratory work which included sequencing and cloning for the new DLA-DQB1 allele, and haplotype inference were as described in Galov et al. (2015). In brief, the primers used to amplify exon 2 were: for DLA-DRB1 forward DRBF (Kennedy et al., 2005) and reverse DRB1R (Wagner et al., 1996b); for DLA-DQA1 forward DQAin1 and reverse DQAIn2 (Wagner et al., 1996a); for DLA-DQB1 forward DQB1BT7 (Wagner et al., 1996a) and reverse DQBR3, ACCTGGGTGGGGAGCCCG (Galov et al., 2015). The primer DQBR3 was designed for golden jackal genotyping since the primer

DQB1B from the study on dogs (Kennedy et al., 2002) proved to be unreliable for DLA-DQB1 locus genotyping in jackals (approximately half of jackal samples failed to amplify with the DQB1BT7 and DQB1B primer pair). The final set of primers seemed optimal for reliable genotyping of golden jackals as we did not detect basic population genetic signatures of unreliable primers (Babik, 2010), e.g. excess of homozygotes (deviation from HWE was not detected on any locus in this investigation; tested using the exact test as implemented in the program Arlequin v.3.5; (Excoffier and Lischer, 2010)), presence of multiple types of homozygotes or failure to obtain any PCR product in a number of samples. Amplifications were performed using a touchdown PCR protocol consisting first of 95 °C for 15 min, followed by 14 touchdown cycles comprising of 95 °C for 30s, the annealing temperature for 1 min and 72 °C for 1 min. Annealing temperatures were set initially at 62 °C for DLA-DRB1, 54 °C for DLA-DQA1 and 73 °C for DLA-DQB1, then reduced by 0.5 °C in each cycle. This was followed by 20 cycles of 95 °C for 30 s; 55 °C (DRB1), 47 °C (DQA1) or 66 °C (DQB1) for 1 min and 72 °C for 1 min. A final extension step was carried out at 72 °C for 10 min. The final data set consisted of a total of 54 golden jackal samples: 35 from Slavonia (continental) and 19 from Dalmatia (coastal region). For grey wolf analyses, we used data from our previous research on Croatian wolves (Arbanasić et al., 2013). We made a comparison with wolves because the two species belong to the same genus, but also the genotypic data we had on wolves enabled estimation of allelic richness and, therefore, a comparison of the number of alleles independent of sample size (54 jackals and 72 wolves). The following methods were used for analyses.

Molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013), and they included a number of nucleotide variable sites, the number of amino acid unique sequences, nucleotide evolutionary distances, Codon-based Z-test of positive selection and phylogenetic analysis. To construct phylogenetic trees, we used the maximum-likelihood method through 1000 bootstrap replicates and considered only alleles found in wild canid species to avoid vast and complex trees as more than a hundred alleles have been listed for DQB1 and DRB1 loci in canids. Allelic richness as a measure of the number of alleles independent of sample size for each locus and population (golden jackal and grey wolf) was estimated using the rarefaction method implemented in FSTAT version 2.9.3 (Goudet, 2002). To detect a positive selection, besides Codon-based Z-test provided by MEGA, we used OmegaMap software. Both tests assess non-synonymous rate (dN) to synonymous rate (dS) ratio (dN/dS = ω). The limitation of the Codon-based Z-test is that it calculates the average dN/dS ratio across the entire sequence, even though the selection is unlikely to act across a gene and is thought to influence particular codon sites. Therefore we used OmegaMap program (Wilson and McVean, 2006) to detect positively selected individual codons. Moreover, as recombination events, which are presumed to occur frequently within MHC region (Richman et al., 2003; Schaschl et al., 2006, 2005), can influence selection analysis tests, OmegaMap estimates the recombination rate and detects selection in the presence of recombination. OmegaMap assumes population sampling and we performed analysis separately for golden jackal and grey wolf genotypes. The analysis was run using the following settings. Mutation rate (μ) and the transition/transversion rate ratio (κ) were adjusted to follow an inverse distribution with starting values 0.1 and 3.0, respectively. Recombination rate (ρ) and selection parameter (ω) were adjusted to follow inverse distribution in the range from 0.01 to 100 and 0.01 to 20, respectively. For each codon ω was set independently and ρ was set to a block-like model with 10 codon blocks. A specified list of random orderings was generated by the program Order included with OmegaMap. For each locus and population, two independent analyses with 500,000 iterations were run and then summarised using the Summarise program included within OmegaMap.

Additionally, we employed the codon usage method to discern between convergent evolution and trans-species polymorphism. We analysed whether the amino-acid residues shared between wolf and jackals were coded with identical codons. Namely, if long-living allelic lineages are maintained across species, codons remain the same. Conversely, if convergent evolution is involved, the same amino-acid residues might be coded by different codons.

Results

On four individuals that were genotyped for this study, one new DLA-DQB1 allele and three new DLA-DRB1/DQA1/DQB1 three-locus haplotypes were identified. The new allele was named by the DLA Nomenclature Committee as DLA-DQB1*00806 (GenBank accession number KT954182) and was found in the Dalmatian population as a single copy (Table 1). Combined with previously published data on 50 golden jackal individuals (Galov et al., 2015), a total of four DLA-DRB1, two DLA-DQA1 and three DLA-DQB1 exon 2 unique nucleotide sequences were identified in golden jackals from Croatia. Among them three DLA-DRB, one DLA-DQA and all three DLA-DQB sequences were detected as typical for golden jackals, not having been identified in other canid species so far (Table 1). Allele DRB1*00901 was found in more than 25 dog breeds (Kennedy et al., 2007b) and in North American grey wolves (Kennedy et al., 2007a) while allele DQA1*00402 was previously identified in the Ethiopian wolf (*Canis simensis*) (Kennedy et al., 2010) and various dog breeds, namely Middle Asian Shepherd Dog, Volfdog, Husky, Pomeranian and Tanzanian Mongrel (Kennedy et al., 2007b). Identified alleles formed seven three-locus DLA-DRB1/DQA1/DQB1 haplotypes some of which appeared to be typical for the Dalmatian region (Table 1). Two haplotypes from Dalmatia were detected as single copies. Each of the seven haplotypes carried at least one exclusive jackal allele, so all three-locus haplotypes that we inferred can be considered typical for golden jackals (Table 1). A list of each animal with its haplotypes is found in the Supplementary Table S1 in the online version at DOI: 10.1016/j.mambio.2016.11.010.

Diversity data for the golden jackal and the grey wolf are presented in Table 2. The most of the wolf data were published in Arbanasić et al. (2013), except for allelic richness, which was computed for this study. Allelic richness values for all three loci and evolutionary distance for DLA-DQB1 locus were considerably lower in jackals than in wolves (allelic richness values were approximately three times lower, while nucleotide distance for DLA-DQB1 locus was almost four times lower). Evolutionary distances for DLA-DRB1 and DLA-DQA1 loci did not follow that pattern and showed identical or similar values in jackals and wolves. Only five variable nucleotide sites were found at the DLA-DQA1 of golden jackal sequences (Table 2), but their small number was expected since only two alleles were identified on the locus. However, the number of variable nucleotide sites found at the loci encoding the beta chains was rather large in golden jackals (30 at the DLA-DRB1 and 29 at DLA-DQB1), which was unexpected since the number of alleles found at both loci was still quite small (only four at the DRB1 and three at the DQB1). Furthermore, 5, 30 and 29 variable nucleotide sites were distributed across 3, 20 and 17 codons which further resulted in 3, 19 and 16 variable amino acid positions at the DQA1, DRB1 and DQB1 loci, respectively. Finally, all golden jackal alleles coded for different deduced amino acid sequences (Table 2). Those findings reflect the high level of non-synonymous substitutions and show the importance of genetic polymorphisms for immunological competence in golden jackals.

Significant excess of non-synonymous over synonymous substitutions detected with Codon-based Z-test that estimates average

Table 1
DLA-DRB1/DQA1/DQB1 haplotypes found in 54 golden jackals in Croatia (35 Slavonian and 19 Dalmatian) presented for the regions.

	DRB1	DQA1	DQB1	Absolute number and (frequency) of haplotypes			h
				per region		total	
				Slavonia	Dalmatia		
Haplotypes found in both regions (Slavonia + Dalmatia)				(2n = 70)	(2n = 38)	(2n = 108)	
	00901	00402	02305	30 (27.8)	21 (19.4)	51 (47.2)	15
	13001	00402	02305	29 (26.9)	2 (1.9)	31 (28.7)	6
	13101	03001	06801	10 (9.3)	5 (4.6)	15 (13.9)	2
	13101	00402	02305[a]	1 (0.9)	1(0,9)	2 (1.8)	
Haplotypes found exclusively in Dalmatia	**04503**	00402	02305		7 (6.5)	7 (6.5)	1
	13001	00402	**00806**[a]		1 (0.9)	1 (0.9)	
	13001	03001	06801[a]		1 (0.9)	1 (0.9)	
No. of alleles per locus	4	2	3				
No. of determined haplotypes						7	

Alleles in bold were only found in Dalmatia.
To date, underlined alleles have only been found in golden jackals.
h = number of animals that were homozygous for the haplotype.
[a] Three-locus haplotypes identified in this study.

Table 2
Comparison of allelic diversity observed in the analysis of 54 golden jackals and 72 grey wolves in Croatia. Data for wolves published in Arbanasić et al. (2013) are shown in italics.

Diversity parameters	species	MHC class II loci		
		DLA-DRB1	DLA-DQA1	DLA-DQB1
No. of alleles/No. of unique amino acid sequences	golden jackal	4/4	2/2	3/3
	grey wolf	*13/12*	*7/7*	*11/11*
Nucleotide distance	golden jackal	16.3%(JC + G)	2% (JC)	8.5% (JC + G)
	grey wolf	*16.3% (JC + G)*	*1.8% (JC)*	*32.1% (JC + G)*
No. of variable nucleotide sites	golden jackal	30	5	29
	grey wolf	*48*	*11*	*39*
Allelic richness	golden jackal	4	2	3
	grey wolf	*11.9*	*6.6*	*10.2*

JC = Jukes–Cantor nucleotide substitution model, G = gamma distribution shape parameter.

Table 3
Codon sites under positive selection with posterior probability of at least 95% identified by OmegaMap (Wilson and Mc Vean, 2006) on MHC class II loci in golden jackals and grey wolves from Croatia.

Locus	Species/No. of selected codons	Codons influenced by positive selection
DLA-DRB1	golden jackal/11	**6**, **8**, **23**, **25**, 27, **32**, **58**, **66**, **69**, **73**, **81**
	grey wolf/16	4, 5, **6, 8**, 21, **23**, **25**, **32**, 42, 52, **58**, 62, **66**, **69**, **73**, **81**
DLA-DQA1	golden jackal/2	29, **64**
	grey wolf/4	50, 63, **64**, 77
DLA-DQB1	golden jackal/9	**8**, **25**, **52**, **58, 62**, **66**, **80, 84**, 85
	grey wolf/13	**8**, 23, **25**, 32, **52**, **58**, **62**, 65, **66**, 69, 70, **80**, **84**

Codons that match the both species are marked in bold. Underlined codon sites are typical for golden jackal alleles.

dN/dS value across all codon sites indicated the acting of positive selection at all three loci in golden jackal (Supplementary Table S2 in the online version at DOI: 10.1016/j.mambio.2016.11.010). The analysis of positive selection acting on individual codons showed that the number of detected codons was smaller in golden jackals than in wolves (Table 3). However, that discrepancy was noticeably smaller than the discrepancy in allelic richness between the two species. The majority of codons detected to be under the influence of positive selection in golden jackals form a subset of codons that were predicted to be under the influence of positive selection in grey wolves. There are only three exceptions—codons identified only in jackals: codon 27 at DLA-DRB1 locus, codon 29 at DLA-DQA1 locus, and codon 85 at DLA-DQB1 locus. Furthermore, amino acids that are present on variable sites in golden jackal sequences represent the subset of amino acids that occur at corresponding amino acid positions in wolf sequences. There are only a few variable sites in golden jackal sequences with typical amino acid residues; one among DLA-DRB and two among DLA-DQA protein sequences (data not shown). Codon usage analysis revealed identical codons for corresponding amino acid residues between species at all tree loci (data not shown) and confirmed shared ancestry. Trans-species polymorphism is further obvious from the phylogenetic trees (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.mambio.2016.11.010). Phylogenetic reconstruction at all three loci analysed in this study followed the pattern which is typical for polymorphic MHC genes. Namely, alleles from different species are not grouped but intermingled across the tree while branches are generally supported with low bootstrap values. At all three loci, golden jackal alleles identified in this study were scattered across the tree implying their divergence.

Discussion

In the present study the patterns of genetic diversity and natural selection on MHC class II loci are analysed for the first time in the golden jackal. From this allelic richness data it is obvious that the

variability of golden jackals in Croatia at all three loci was markedly lower than that of grey wolves (4 DLA-DRB1, 2 DLA-DQA1 and 3 DLA-DQB1 in jackal versus 11.9 DLA-DRB1, 6.6 DLA-DQA1 and 10.2 DLA-DQB1 in wolves, Table 2) and comparable to those found in the endangered Ethiopian wolf (*Canis simensis*) where 4 DLA-DRB1, 2 DQA1 and 5 DQB1 alleles were detected in 99 individuals (Kennedy et al., 2010). Further, golden jackal variability is also very low if compared to the other investigated mammal species, e.g. 17 DLA-DRB1 alleles were found in 194 North American grey wolves (Kennedy et al., 2007a), six DLA-DQA alleles were identified in 30 African elephants (Archie et al., 2010), while 10 DLA-DQA and 12 DLA-DQB alleles were found in 50 bottlenose dolphins (Arbanasić et al., 2014). The small number of alleles found in this investigation is consistent with results obtained on neutral loci from the Serbian golden jackal population which showed a very low variability (Zachos et al., 2009) and with results and suggestions from Fabbri et al. (2014) that golden jackals from south-eastern Europe underwent a historical population contraction to restricted refuge areas, followed by a recent rapid demographic expansion. In contrast, extensive variability of MHC class II loci was reported on a small sample of golden jackals from East and South Africa and Israel, where 6 DRB1, 5 DQA1 and 6 DQB1 alleles were found in only 9, 11 and 13 individuals, respectively (Marsden, 2010). The limited variation that we found in this study can be attributed to a genetic drift rather than to the lack of historical positive selection since a positive selection was confirmed at all three analysed loci (Supplementary Table S2 in the online version at DOI: 10.1016/j.mambio.2016.11.010 and Table 3). This is in accordance with the assumption that on the short timescale, MHC variation is shaped predominately by demographic processes rather than by selection (Radwan et al., 2010).

Due to its role in eliciting an immunological response to various pathogens, variability on MHC genes is essential for population vitality while the loss of variation may render populations more susceptible to infections. However, some studies showed that populations survived and increased in number in spite of limited MHC variation (Radwan et al., 2010). There is a hypothesis that population survival might not be compromised by the reduction of MHC variation if the alleles retained are functionally highly divergent (Radwan et al., 2010). This is in line with the suggestion from Hedrick et al. (2002) that species threatened with extinction tend to retain highly divergent allelic variants. Currently, the expanding golden jackal population in Europe is not considered threatened, but based on investigations on neutral (Fabbri et al., 2014; Zachos et al., 2009) and MHC loci variability (this study), there are indications of a genetic drift. Although a reduction of diversity is found in golden jackals in Croatia, the standing alleles are notably divergent, suggesting that they represent diverse allelic lineages that originated a long time ago. We can assume that a relatively high divergence among alleles compensates for the small number of alleles present in the population, thus maintaining functional diversity. The same pattern that includes reduced variability and high divergence on MHC loci has been described in literature (Hedrick et al., 2000; Radwan et al., 2007; Babik et al., 2005). This is consistent with the mechanism of divergent allele advantage (Wakeland et al., 1990) where dissimilar MHC alleles are selectively maintained in population for their ability to present a broad variety of antigen peptides.

Both of the selection tests that we used, the one that calculates substitutions over the whole sequence (MEGA) and the maximum-likelihood method that allows the dN/dS ratio to vary among codon sites (OmegaMap), confirmed that a positive selection shaped the allelic polymorphism at all three investigated loci in the golden jackal (Supplementary Table S2 in the online version at DOI: 10.1016/j.mambio.2016.11.010 and Table 3). Due to a significant difference in allelic richness between golden jackals and wolves, we intended to assess to what extent the functional ability of MHC class II loci is reduced in golden jackals. For that purpose we compared the number of codons predicted to be under the influence of positive selection between species. While the number of alleles per locus was approximately three times smaller in golden jackals than in wolves, the number of codons predicted to be under the influence of positive selection was not substantially smaller (Table 3). This finding, together with the finding of high allelic divergence, suggests that the investigated golden jackal population might still contain the considerable functional diversity necessary for the presentation of varied foreign peptides.

The research on MHC diversity has been conducted on a majority of the species of the genus Canis. Most of the research was focused on domestic dogs (Kennedy et al., 2007b), while wild populations included red wolf and coyote (Hedrick et al., 2002), grey wolf (Arbanasić et al., 2013; Kennedy et al., 2007a; Seddon and Ellegren, 2004, 2002), Ethiopian wolf (Kennedy et al., 2010) and Mexican wolf (Hedrick et al., 2000). A considerable number of MHC alleles identified in those studies enabled us to conduct a phylogenetic analysis of golden jackal alleles and those of related species. One of the main characteristics of MHC class II alleles is that they are retained for a long evolutionary time, often across speciation events, presumably due to the action of balancing selection (Sommer, 2005). Consequently, alleles from related species may be more similar than alleles within the same species, the phenomenon known as trans-species polymorphism (TSP). TSP has been reported in many MHC studies (Bernatchez and Landry, 2003). Nevertheless, similar allelic variants in different species might also arise by convergent evolution, which is expected to result from the similar pathogenic selection pressure. Several cases of convergent evolution at the MHC genes have been reported, e.g. between baiji and finless porpoise, (Xu et al., 2008) and between striped skunks and raccoons (Srithayakumar et al., 2012). However, when considering possible convergent evolution at the MHC genes between the golden jackal and the grey wolf, it should be emphasised that some ecological factors differ markedly between those two species. Wolves are carnivores that prey on animals while jackals are omnivores that primarily utilise easily accessible human-derived food sources, including agricultural plots and human waste (Lanszki et al., 2010). Further, while wolves inhabit mostly wildland areas, jackals readily reside in human shaped environment. Therefore, we might assume somewhat different pathogenic selection pressures acting on the two species and, together with the finding of TPS and the results of the codon usage method in this investigation, exclude convergent evolution as a cause of similar allelic variants in the two species. However, two interspecific alleles identified in our sample, namely DRB1*00901 and DQA1*00402 (Table 1), found previously in dogs, could have theoretically arisen from interspecific hybridisation between the golden jackal and the domestic dog. Hybridization yielding viable offspring can occur between all species of the genus Canis (Marsden et al., 2009) and golden jackal-dog fertile hybrids have been reported in the recent study of Galov et al. (2015). Also, interspecific exchange of MHC alleles as an adaptive process was already recorded in some studies, e.g. in two species of newts (Nadachowska-Brzyska et al., 2012). Nevertheless, we believe that the possibility of common alleles that originated from hybridisation events is not likely because we found that although alleles may be shared between the species, the three-locus haplotypes are specific for the species (this study; Galov et al., 2015). The MHC genetic region is known for a high level of linkage disequilibrium, which results in the inheritance of allelic combinations (haplotypes) that provide effective protection against certain pathogens. It is thought that the preservation of haplotypes is maintained by a selective pressure that occurs over the history of a population (Garrigan and Hedrick, 2003), where particular allelic combinations are conserved.

We found different levels of MHC diversity in Slavonian (continental) and Dalmatian (coastal) populations, namely, two alleles and three haplotypes were exclusively found in Dalmatia (Table 1). This is in line with results of Fabbri et al. (2014) on neutral loci, which showed that the Dalmatian population is genetically differentiated from other Balkan golden jackal populations, including that of Slavonia (Fabbri et al., 2014). It was explained by historical reasons since the Dalmatian population represents an old population that has been present for centuries, while the Slavonian population is presumed to have colonised this area relatively recently from eastern territories like Bulgaria, Romania and Serbia (Giannatos, 2004). Furthermore, the study of Fabbri et al. (2014) evidenced the lowest levels of allelic richness and the heterozygosity of the Dalmatian population in relation to other populations, which was interpreted by a suggestion that jackals in Dalmatia might have experienced a demographic bottleneck in the past and have survived in isolation (Fabbri et al., 2014). The population from Samos island in Greece had levels of neutral genetic diversity comparable to that from Dalmatia, explained also by isolation and genetic drift (Rutkowski et al., 2015). In contrast to neutral genetic variation, our results suggest that the Dalmatian population has a higher MHC diversity than the Slavonian population since two additional alleles and three additional haplotypes are found in the Dalmatian population compared to the Slavonian population, while no exclusive haplotypes are found in the latter (Table 1). This indication of a higher level of MHC diversity in Dalmatia than in Slavonia is further emphasised by a smaller sample set analysed from Dalmatia (only 19 individuals were analysed from Dalmatia in contrast to 35 individuals from Slavonia). Inconsistency between neutral loci and MHC variability has been recorded in several studies where restricted diversity on neutral genetic markers was accompanied by high diversity on MHC loci (Aguilar et al., 2004; Schad et al., 2004). It is proposed that balancing selection might counteract the loss of MHC diversity despite low variability on neutral markers (Sommer, 2005). However, to draw more reliable conclusions on the MHC variability of the Dalmatian population, it is crucial to analyse more individuals from that region, especially since one allele and two haplotypes exclusive for the Dalmatian population were identified only as single copies, i.e. as representing rare genetic variants (Table 1). Nevertheless, rare MHC alleles derived by negative frequency-dependent selection are commonly present in populations (Sommer, 2005). Rare-allele advantage hypothesis predicts that low-frequency allelic variants of MHC genes confer resistance to pathogens that adapt to most common host genotypes. Yet, in bottlenecked populations rare MHC alleles may be lost due to a strong random drift (Radwan et al., 2010). Therefore, the possible presence of exclusive but rare genetic variants in the Dalmatian population casts doubts on its isolation, which was proposed by Fabbri et al. (2014). This calls for a more extensive investigation on the genetic diversity of the golden jackal, which would include samples from the southern Balkans such as Albania, Macedonia and Greece. Finally, MHC analysis could be suitable for elucidating the origin of golden jackal populations that spread over Europe. Namely, MHC-based population differentiation according to the geographic distribution has been found in studies on wild populations (Ekblom et al., 2007; Loiseau et al., 2009; Cammen et al., 2011). It is congruent with diversifying the selection where heterogeneous selective pressures promote local adaptation and sustain MHC allele's variability according to geographic origin. As golden jackals expand their range and become hosted in new European regions, we assume that more genetic research will be carried out in the near future, including research on the adaptive genetic variability of various jackal populations and further comparison with related species.

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