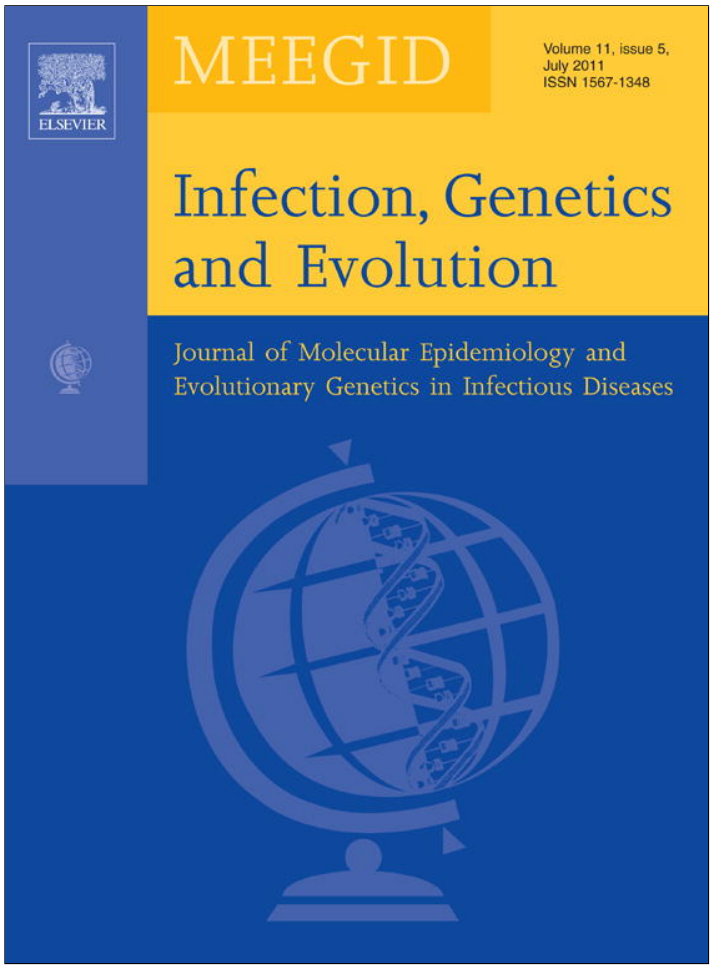

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Genetic diversity of the class II major histocompatibility *DRA* locus in European, Asiatic and African domestic donkeys

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ABSTRACT

The major histocompatibility complex (MHC) genes coding for antigen presenting molecules are the most polymorphic genes in vertebrate genome. The MHC class II *DRA* gene shows only small variation in many mammalian species, but it exhibits relatively high level of polymorphism in Equidae, especially in donkeys. This extraordinary degree of polymorphism together with signatures of selection in specific amino acids sites makes the donkey *DRA* gene a suitable model for population diversity studies. The objective of this study was to investigate the *DRA* gene diversity in three different populations of donkeys under infectious pressure of protozoan parasites, *Theileria equi* and *Babesia caballi*. Three populations of domestic donkeys from Italy ($N = 68$), Jordan ($N = 43$), and Kenya ($N = 78$) were studied. A method of the donkey MHC *DRA* genotyping based on PCR-RFLP and sequencing was designed. In addition to the *DRA* gene, 12 polymorphic microsatellite loci were genotyped. The presence of *Theileria equi* and *Babesia caballi* parasites in peripheral blood was investigated by PCR. Allele and genotype frequencies, observed and expected heterozygosities and F_{IS} values were computed as parameters of genetic diversity for all loci genotyped. Genetic distances between the three populations were estimated based on F_{ST} values. Statistical associations between parasite infection and genetic polymorphisms were sought. Extensive *DRA* locus variation characteristic for Equids was found. The results showed differences between populations both in terms of numbers of alleles and their frequencies as well as variation in expected heterozygosity values. Based on comparisons with neutral microsatellite loci, population sub-structure characteristics and association analysis, convincing evidence of pathogen-driven selection at the population level was not provided. It seems that genetic diversity observed in the three populations reflects mostly effects of selective breeding and their different genetic origins.

1. Introduction

The amount of genetic diversity has been associated with the ability to adapt to environmental changes and with the potential to evolve (Reed and Frankham, 2003). Immune functions represent one of major components of an organism's fitness and determine the potential for evolutionary interactions with pathogens or with other species (Lazzaro and Little, 2009). Diversity of genes important for immune functions may be associated with resistance and susceptibility to pathogens (Trowsdale and Parham, 2004; Tibayrenc, 2007). The major histocompatibility complex (MHC) is a cluster of linked genes playing a central role in the presentation of antigenic peptides to T lymphocytes (Klein, 1986). The MHC genes are the most polymorphic genes in the vertebrate genome. Their high polymorphism seems to be maintained by balancing selection, predating speciation events and reflecting the co-evolution of hosts with their pathogens (Bernatchez and Landry, 2003). The mechanisms maintaining the genetic diversity and the role of pathogens have not yet been completely clarified. Empirical evidence for pathogen-driven selection on MHC genes is based on the population diversity analysis and on associations with pathogens (Spurgin and Richardson, 2010). For this purpose, specific model populations living in specific areas and exposed to various pathogens can be studied.

The family Equidae is a suitable model for studying diversity, selection and evolution of the MHC genes (Janova et al., 2009). It is

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a rapidly evolving and variable group composed of a single genus, *Equus*, with a relatively well-documented history of evolution (Bowling and Ruvinsky, 2000). Domestic, captive and free ranging equid populations are available for different types of studies. Domestication of wild asses occurred probably 6000 years ago in Northeastern Africa (Rossel et al., 2008). Analysis of mitochondrial DNA of modern donkeys revealed two highly divergent phylogenetic groups, suggesting existence of two maternal origins of the domestic donkeys from two distinct wild populations, the Nubian (*Equus africanus africanus*) and the Somali (*Equus africanus somaliensis*) wild asses (Beja-Pereira et al., 2004; Kimura et al., 2011). The domestic donkey is a suitable model equid species for diversity study. It exists in various populations in different geographical areas, often naturally exposed to infectious pathogens.

The horse major histocompatibility complex (*ELA* or *Eqca*) is located on the horse chromosome (ECA) 20. The equine and human MHCs have a similar genomic organization with class I, II and III regions (Gustafson et al., 2003). The class II genes of Equidae have been extensively characterized and high level of exon 2 sequence variation was observed (Albright-Fraser et al., 1996; Fraser and Bailey, 1998; Horin and Matiasovic, 2002; Brown et al., 2004; Janova et al., 2009). For population diversity studies, a reliable method of individual genotyping is needed. Due to the extensive variation in the class II *DQA*, *DRB* and *DQB* genes, individual genotyping of these genes in Equids is not available or it is of limited value (Fraser and Bailey, 1998; Diaz et al., 2001; Horin and Matiasovic, 2002; Janova et al., 2009).

While exon 2 *DRA* alleles generally exhibit if ever only small variation in mammalian species (e.g. Yuhki et al., 2003), extensive polymorphism even of *DRA* genes has been reported in Equidae. The sequence variations are mainly located in exon 2 coding for the extracellular antigen binding domain. Current knowledge of the donkey MHC is only fragmentary. The donkey MHC (*Eqas*) contains probably a single *DRA* locus with seven *DRA* alleles identified so far (Albright-Fraser et al., 1996; Brown et al., 2004; GenBank accession numbers FJ487912, HM165492). Effect of positive selection on exon 2 *DRA* sequences was reported (Janova et al., 2009).

Availability of various donkey populations living in different climatic conditions and with different levels of general and health care, relatively, but not extremely high level of polymorphism in a single locus, with signatures of selection in specific amino acid sites, makes the donkey *DRA* gene a suitable model for population diversity studies. Similarly to other equids, donkeys are affected by plethora of infectious diseases. Among them, the piroplasmids, apicomplexan intracellular protists represent valuable model pathogen, as they apparently co-evolved with their hosts and exhibit remarkable pathogenicity. Equine and donkey piroplasmosis is an often fatal, tick-borne disease of equids caused by *Theileria equi* and *Babesia caballi* (Bruning, 1996).

The objective of this study was to investigate, based on individual genotyping, the *DRA* gene diversity in three different populations of donkeys under infectious pressure of equine piroplasms.

2. Materials and methods

2.1. Animals

The genetic diversity was studied in three populations of domestic donkeys. Italian donkeys belonged to the Martina Franca breed. It is an ancient native breed of Apulia (southern Italy), characterized by extraordinary sturdiness, frugality and adaptation to rocky ground. The genetic uniqueness of this breed lies in its adaptation to enzootic tick-borne pathogens typically found in Apulia (Rizzi et al., in press). Unrelated donkeys selected from 12 farms ($n = 68$) were used in this study. Jordanian donkeys ($n = 43$) were sampled from several rural localities in western Jordan, characterized by hot semi-arid and arid climate; all sampled animals belonged to the local breed. The third population were local African donkeys owned by semi-nomadic pastoralists of Turkana and Samburu tribes living in an arid environment in Northern Kenya ($n = 78$). All donkeys were under permanent risk of infection by tick-transmitted piroplasmids. In contrast to Italian donkeys, where basic veterinary care is available, no therapeutic and/or prophylactic measures were ever taken in the Asian and African donkeys.

2.2. Assessment of genetic diversity

The MHC *DRA* genetic diversity was compared to diversity in 14 microsatellite loci. Distribution of genotype and allelic frequencies, expected heterozygosities within populations, population structure and associations with a common pathogen were investigated for both types of loci, i.e. *DRA* and microsatellites.

2.3. Genetic diversity within populations, neutrality tests

In all donkeys, individual genotypes were determined for the MHC *DRA* locus and the microsatellite loci. In all loci analyzed, genotype and allelic frequencies, expected, observed and unbiased expected heterozygosities and the corresponding *P*-values were computed using GENETIX v. 4.05 (http://www.genetix.univ-montp2.fr/genetix/genetix.htm) and Arlequin v. 3.11 (Excoffier et al., 2005; http://cmpg.unibe.ch/software/arlequin3/). Ewens-Watterson, Tajima's D and Fu's F_S tests were used for analyzing neutrality of the *DRA* locus by Arlequin.

2.4. DRA genotyping

Blood for DNA extraction was collected by jugular venipuncture. Two methods for genomic DNA extraction were used, due to different methods of fixation of blood samples collected in different climatic conditions. In Italian donkeys, genomic DNA was extracted from EDTA-fixed peripheral blood, using the NucleoSpin blood kit (Macherey-Nagel, Duren, Germany). In Jordanian and Kenyan donkeys, a standard phenol–chloroform extraction from ethanol-fixed blood samples was used.

Amplification of the 307 bp long product was carried out with standard primers Be3 and Be4 (Albright-Fraser et al., 1996). The extent of exon 2 *DRA* sequence variation in all populations was prescreened by single strand conformation polymorphism analysis (SSCP) as described previously (Janova et al., 2009). Individual PCR-SSCP patterns were sequenced by 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were aligned by using the BioEdit sequence alignment editor (Hall, 1999) with known *DRA* alleles. A new *E. asinus DRA* allele (accession number HM165492), submitted to GenBank after we had completed the analysis, was not included. Heterozygote genotypes recognized based on double peaks were resolved manually by subtracting known alleles identified as specific SSCP patterns. No new allele was identified in the groups studied. The nomenclature suggested by Janova et al. (2009) was used for designing the *DRA* alleles.

Based on the exon 2 *DRA* sequences, a PCR-RFLP genotyping system was developed. Digestion of PCR products with restriction enzymes BsaJI, NlaIII, AciI, and Cac8I produced fragments of specific length (Table 1) that could be distinguished by capillary electrophoresis (MCE-202 MultiNA, Shimadzu Corporation, Kyoto, Japan). The combination of restriction sites allowed identification of 4 alleles, *Eqas-DRA*0101*, *Eqas-DRA*0201*, *Eqas-DRA*0401* and *Eqas-DRA*0501*. The remaining alleles, *Eqas-DRA*0301* and *Eqas-DRA*0601*, could be identified by subsequent sequencing and

Table 1
Predicted DNA fragment size (bp) for PCR-RFLP detection of *Eqas* alleles.

	BsaJI	NlaIII	AciI	Cac8I
*Eqas-DRA*01*	73,228,6	128,119,60	157,150	184,123
*Eqas-DRA*02*	307	247,60	157,150	184,123
*Eqas-DRA*03*	73,228,6	247,60	157,150	184,123
*Eqas-DRA*04*	73,228,6	247,60	157,150	307
*Eqas-DRA*05*	73,228,6	247,60	307	184,123
*Eqas-DRA*06*	73,228,6	247,60	157,150	184,123

manual editing of heterozygous sequences. As the recently reported allele *Eqas-DRA*0701* could not be involved in the set-up, it could not be distinguished with this genotyping system.

2.5. Microsatellites

Fourteen microsatellites from the horse parentage test (Lee and Cho, 2006) were amplified in a standard multiplex PCRs using fluorescent-labeled primers and analyzed using automated sequencer ABI Prism310 (Applied Biosystems, Foster City, CA, USA) as described (Glowatzki-Mullis et al., 2006). 12 microsatellite loci, including one X-linked marker (Lex003), were polymorphic in the populations analyzed.

2.6. Population structure

F_{IS} and F_{ST} values for the entire donkey group and for its three sub-populations and their *P* values were computed by Arlequin (based on Weir and Hill, 2002) for both types of loci.

2.7. Model pathogen: blood parasites Theileria equi and Babesia caballi

The blood parasites *Theileria equi* and *Babesia caballi* occurring in all three populations were selected as model pathogens for association studies. Both pathogens were diagnosed by PCR from DNA extracted from peripheral blood of all donkeys analyzed. Amplification of a specific fragment of the piroplasmid SSU rRNA gene was performed as described elsewhere (Sloboda et al., 2010). Based on the results of PCR, donkeys were classified as "double positive", *Theileria equi* or *Babesia caballi* positive or negative. The values of pathogen prevalence were 77.94; 26.19 and 88.46% in the Italian, Jordanian and Kenyan donkeys, respectively.

2.8. Association analysis

Associations between infection with one or both pathogens and *DRA* and microsatellite polymorphisms were investigated using standard chi-square and/or Fisher's exact tests with Bonferroni corrections for multiple comparisons. For *DRA* alleles, the *P* value computed was corrected for six/five alleles identified in the respective populations, while for microsatellite loci; corrections for 12 polymorphic loci analyzed were made. The associations were analyzed within the three populations, within the whole group of donkeys analyzed and within the group of Kenyan and Jordanian donkeys with the same allele pool.

Table 2
Frequencies of *DRA* alleles.

N[a]	Italy	Jordan	Kenya
	68	42	78
f (*Eqas-DRA*01*)	0.096	0.107	0.032
f (*Eqas-DRA*02*)	0.199	0.381	0.468
f (*Eqas-DRA*03*)	0.198	0.143	0.083
f (*Eqas-DRA*04*)	0.007	0.083	0.244
f (*Eqas-DRA*05*)	0.500	0.072	0.077
f (*Eqas-DRA*06*)	0.000	0.214	0.096

[a] Number of individuals.

3. Results

The numbers of *DRA* alleles and their frequencies, the values of heterozygosity found in the populations studied are in Tables 2 and 3. In Jordanian and Kenyan donkeys, six *DRA* alleles were found, while in Italian donkeys the allele *Eqas-DRA*0601* was missing. The highest value of expected heterozygosity was found in Jordanian donkeys, while their observed heterozygosity was lowest from the group. Departure from H–W equilibrium due to the excess of *DRA* homozygotes was observed in the Jordanian population.

The data on microsatellite diversity are in Table 4. Two loci *HMS1* and *ASB17* were monomorphic. In the remaining loci, numbers of alleles ranged from 2 (*HMS6*) to 12 (*AHT4*). The numbers of alleles were smaller in Italian donkeys (average number per locus 4.42), while in Jordanian and Kenyan donkeys the numbers of alleles per locus were similar (6.83 and 6.75, respectively).

The F_{IS} values for *DRA* and microsatellite loci are also shown in Table 3. All F_{IS} values were positive except the *DRA* locus in Kenyan donkeys. The F_{ST} pair-wise comparisons among populations for *DRA* and microsatellites are in Table 5. In both types of loci, the genetic distances between the Jordanian and Kenyan populations are smaller than distances between either of them and the Italian group. The F_{ST} values for *DRA* and microsatellites were comparable. In neutrality tests, only a significant Ewens–Waterson neutrality test *P* value for the Jordanian population was found ($F_{obs} = 0.235$, $F_{exp} = 0.417$, $P = 0.041$).

Marginal *P* values were found for associations of *Eqas-DRA* with *Theileria equi* infection in the merged group of Jordanian and Kenyan donkeys (Table 6). A statistically significant difference ($P < 0.004$) in numbers of *Theileria equi* PCR-positive donkeys between *Eqas-DRA*0401* and *Eqas-DRA*0601* carriers was observed

Table 3
Comparison of genetic diversity of *DRA* locus and average values of 12 polymorphic microsatellite loci.

		N_{all}[a]	H exp.	H n.b.	H obs.	F_{IS} (CI 95%)[b]	HWE *P* value/range of *P* values
Italy	*DRA*	5.00	0.662	0.667	0.603	0.097 (−0.02 to 0.206)	0.294
(	MS	4.42	0.569	0.574	0.555	0.033 (0.004 to 0.052)	0.132–0.933
N = 67)							
Jordan	*DRA*	6.00	0.765	0.774	0.548	0.295 (0.147 to 0.359)	0.000
(*N* = 43)	MS	6.83	0.697	0.705	0.623	0.119 (0.084 to 0.155)	0.02–0.785
Kenya	*DRA*	6.00	0.699	0.703	0.769	−0.095 (−0.176 to 0.012)	0.572
(*N* = 78)	MS	6.75	0.691	0.695	0.645	0.072 (0.045 to 0.106)	0.010–0.866

H exp., expected heterozygosity; H n.b., non-biased expected heterozygosity; H obs., observed heterozygosity.
[a] Mean number of alleles per loci.
[b] 95% confidence interval.

Table 4
Genetic diversity of 11 autosomal microsatellite loci in three domestic donkey populations.

	Number of alleles			H exp.			H n.b.			H obs.			*P* value		
	Italy *N* = 67	Jordan *N* = 43	Kenya *N* = 78	Italy	Jordan	Kenya	Italy	Jordan	Kenya	Italy	Jordan	Kenya	Italy	Jordan	Kenya
AHT004	3	12	10	0.609	0.774	0.780	0.614	0.783	0.785	0.671	0.786	0.705	0.381	0.301	0.369
VHL020	3	3	5	0.642	0.605	0.664	0.646	0.612	0.669	0.776	0.605	0.654	0.134	0.785	0.866
AHT005	7	11	11	0.773	0.873	0.825	0.779	0.885	0.831	0.776	0.769	0.859	0.603	0.051	0.628
ASB023	5	5	6	0.662	0.791	0.781	0.667	0.801	0.786	0.657	0.833	0.718	0.132	0.617	0.150
HMS006	2	4	4	0.430	0.447	0.328	0.434	0.452	0.330	0.388	0.349	0.303	0.404	0.254	0.321
HTG006	4	4	4	0.254	0.654	0.687	0.256	0.663	0.691	0.227	0.657	0.718	0.412	0.020	0.232
CA425	4	9	7	0.515	0.704	0.717	0.519	0.713	0.722	0.552	0.595	0.680	0.579	0.136	0.440
HMS002	3	6	6	0.419	0.637	0.661	0.422	0.644	0.665	0.493	0.619	0.590	0.180	0.597	0.010
HMS003	4	4	3	0.640	0.546	0.606	0.645	0.555	0.610	0.609	0.531	0.526	0.487	0.109	0.059
HTG010	6	8	7	0.605	0.773	0.646	0.609	0.783	0.650	0.597	0.833	0.654	0.933	0.621	0.643
HTG007	7	10	10	0.620	0.862	0.875	0.625	0.873	0.881	0.522	0.791	0.885	0.135	0.224	0.491

H exp., expected heterozygosity; H n.b., non-biased expected heterozygosity; H obs., observed heterozygosity.

Table 5
Genetic distances (pairwise F_{ST}) determined for *MHC-DRA*/microsatellite loci.

	Italy	Jordan	Kenya
Italy	0		
Jordan	0.154/0.132	0	
Kenya	0.191/0.152	0.029/0.041	0

(Table 7). No associations of *DRA* alleles or genotypes with homozygosity/heterozygosity were found. No associations for microsatellite loci were found.

4. Discussion

The extent of *Eqas DRA* polymorphism was primarily determined by sequencing. In Equids, individual *DRA* genotyping based on single strand conformation polymorphism analysis (PCR-SSCP) (Albright-Fraser et al., 1996), reference-strand-mediated conformational analysis (RSCA) (Brown et al., 2004) or pyrosequencing (Diaz et al., 2008) was reported. Our approach using PCR-RFLP and sequencing proved to be another feasible approach for assessing individual variation in the donkey *DRA* locus. It is rapid and efficient; there is no need for reference sequences like in RSCA and SSCP. However, pre-screening with PCR-SSCP was used for avoiding the risk of loss of non-recognized alleles potentially present in the populations studied.

Five *DRA* alleles seem to be equally common in donkeys analyzed so far. The sixth and seventh allele, *Eqas-DRA*0601*, *Eqas-DRA*0701* were only recently added to GenBank (FJ487912, HM165492) and there is no information on their population frequencies. *DRA* frequencies observed by Brown et al. (2004) in 23 donkeys (origin not specified) were similar to those found in this study in the Jordanian group. *Eqas-DRA*0601* and *Eqas-DRA*0701* were not known at this time and they were not identified by the authors in the group analyzed. In two populations studied here, all known *DRA* alleles were found with exception of *Eqas-DRA*0701*. It could not be distinguished by the PCR-RFLP system used. However, we have observed no unexpected PCR-SSCP patterns in the groups analyzed and the sequence data have not suggested existence of additional alleles. We thus believe that if this allele was present in our populations, it must be very rare and could not influence the results obtained and their interpretation.

In the Italian group, where *Eqas-DRA*0601* was not found, observed frequency of another allele, *Eqas-DRA*0401*, was also very low. This population thus seems to be less diverse in *DRA* than the two other groups. Similar results were found for expected *DRA*

Table 6
Associations of *Eqas DRA* with *Theileria equi* infection.

Population/allele associated	Positive		Negative		*P* uncorrected	*P* corrected
	Absolute *f*[a]	Relative *f*[a]	Absolute *f*[a]	Relative *f*[a]		
Jordan/*Eqas-DRA*03*	6/16	0.375	6/68	0.088	0.009	0.053
Jordan, Kenya/*Eqas-DRA*04*	34/154	0.221	9/86	0.105	0.010	0.061
Jordan, Kenya/*Eqas-DRA*06*	15/154	0.097	18/86	0.209	0.010	0.058

Associations of individual *DRA* alleles with *Theileria equi* infection were computed for the Jordanian population and for the merged group of Kenyan and Jordanian donkeys. Absolute frequencies show number of *Theileria equi* positive/negative individuals carrying a specific allele out of total numbers of *Theileria equi* positive/negative individuals. Odds ratios could not be calculated due to null frequencies.
[a] Frequency.

Table 7
Distribution of susceptibility- and resistance-associated alleles (*Eqas-DRA*04* and *Eqas-DRA*06*, respectively) in Jordanian and Kenyan donkeys.

	Infected		Non-infected	
	Absolute *f*[a]	Relative *f*[a]	Absolute *f*[a]	Relative *f*[a]
*Eqas-DRA*04* but not *Eqas-DRA*06* carriers	24/30	0.80	6/30	0.20
*Eqas-DRA*06* but not *Eqas-DRA*04* carriers	9/22	0.41	13/22	0.59

$P = 0.0038$

Absolute frequencies show numbers of animals carrying only resistance- or susceptibility-associated alleles, respectively, among infected and non-infected donkeys.
[a] Frequency.

heterozygosities. The Italian population showed lowest values of expected *DRA* heterozygosity. Calculations of expected and unbiased expected heterozygosities produced similar results due to the high numbers of donkeys. The most diverse in this parameter was the Jordanian group, showing at the same time the lowest values of observed *DRA* heterozygosity. The values of expected *DRA* heterozygosity observed in our donkeys (0.66–0.77) are higher than values observed for the same MHC class II locus in horses, 0.27–0.65 (Diaz et al., 2008), and roughly correspond to values for human HLA class I loci observed by Prugnolle et al. (2005) in populations living in environments with moderate level of pathogen-richness.

The differences observed can be explained by several different factors, including different origins of the populations under study, different approaches to selective breeding as well as by pathogen-driven selection. They suggest that populations living under "natural" conditions, i.e. with no selective breeding, virtually no veterinary care and no preventive measures, like vaccinations, are slightly more diverse in this MHC locus than the Italian breed, subject to selective breeding and elementary veterinary care. In Kenyan and Jordanian donkeys, no pedigree information was available, and random sample collection could not eliminate the risk of including relatives into the group analyzed. In Italian horses, pedigree data were available. Due to selective breeding, parental half-sibs could not be completely eliminated from the study. However, in terms of parentage, the group analyzed was a representative population sample.

Comparison with microsatellites showed that the Italian population was less diverse even in these neutral loci both in terms of heterozygosity values and especially of mean numbers of alleles (Table 3). In contrast to *Eqas DRA*, there was no discrepancy between observed and expected heterozygosities in Jordanian donkeys. The reasons for the departure from H–W equilibrium and from neutrality observed for the *DRA* locus in the Jordanian population remain unclear. Due to the absence of pedigree data, excess of homozygotes in this population cannot be interpreted properly. The lower extent of genetic diversity in both types of loci seems to be due to selective breeding applied in Italian donkeys with controlled pedigree. The mean expected microsatellite heterozygosity values ranging between 0.57 and 0.71, similar to those reported in Spanish (0.66) and Croatian (0.66–0.70) domestic donkeys (Aranguren-Mendez et al., 2001; Ivankovic et al., 2002) are in agreement with this assumption. In terms of neutral variation, genetic diversity of African and Arab donkeys studied was not strikingly different from the European domestic donkey populations. However, the two non-European populations were more similar to each other than to Italian donkeys in both types of loci as expressed by F_{ST} values.

MHC polymorphism can be maintained by balancing selection (Hedrick, 1999). Effects of pathogen-driven selection on the MHC locus can be observed at the population level as differences between MHC and neutral loci, population substructure and associations with pathogens (Spurgin and Richardson, 2010). The population structure estimated based on F_{ST} values, similar for microsatellite and MHC loci, did not indicate effects of selection, similarly to the results of neutrality tests for the *DRA* locus. The pathogen richness in the areas concerned could not be estimated due to lack of systemic data on disease prevalence in the populations studied. The only pathogens comparable in the populations were piroplasmids. Associations between protozoan infections and MHC were repeatedly reported in humans and domestic animals (Gray and Gill, 1993; Stear and Wakelin, 1998; Gilbert et al., 1998). Protozoan parasites were also shown to drive human genetic diversity (Pozzoli et al., 2010). Here, only marginal associations between *Eqas DRA* and infection were observed. This may be partially due to relatively small numbers of donkeys studied. However, significant differences in *Theileria equi* infection among *Eqas-DRA*0401* and *Eqas-DRA*0601* carriers were observed in two populations closely related in terms of genetic distances and genetic diversity, living in similar arid environment with the same tick vectors (e.g. *Rhipicephalus*, *Hyalomma*). Effects of MHC on protozoan infections could be biologically plausible (Luder et al., 2009), but biases in candidate-gene association studies leading to false positive results should always be considered (Campbell and Rudan, 2002). No association with microsatellites was found. This is in agreement with the assumption of neutrality, although for some microsatellites, associations with disease due to linkage disequilibrium with other loci could be found, even in Equids (Horin et al., 2004).

The data collected from these three special populations thus allowed us to characterize the extent of genetic diversity in the *Eqas DRA* locus. Extensive *DRA* locus variation characteristic for Equid MHC was found. The results showed differences between populations both in terms of numbers of alleles and their frequencies as well as variation in expected heterozygosity values. At the molecular level, we could identify effects of positive selection on the *DRA* locus (Janova et al., 2009). In this study however, despite the fact that the pathogens selected are endemic in all three areas of origin and their long-term interaction with the host populations might be assumed, we could not provide convincing evidence for pathogen-driven selection at the population level. Based on comparisons with neutral microsatellite loci, population sub-structure description and results of association studies, it seems that the results reflect effects of selective breeding and different genetic origins of the populations studied rather than effects of pathogen-driven selection.

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