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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