Infection, Genetics and Evolution 12 (2012) 1020–1026

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Polymorphisms at MHC class II DRB1 exon 2 locus in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*)

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ARTICLE INFO

Article history:
Received 21 December 2011
Received in revised form 19 February 2012
Accepted 28 February 2012
Available online 8 March 2012

Keywords:
MHC DRB1 locus
Pyrenean chamois
Wildlife diseases
Balancing selection
Population dynamics

ABSTRACT

Chamois (*Rupicapra* spp.) are mountain ungulates from Southern and Central Europe and the Near East. A newly reported border disease virus (BDV) has affected the easternmost populations of Pyrenean chamois, leading to a dramatic population decrease that may drive to genetic variability loss. The Major Histocompatibility Complex (MHC) is a sensitive marker for genetic variation of populations: polymorphism on the MHC genes is affected both by pathogens and population dynamics and it is ecologically relevant, as depending on host-pathogen relationships and life history features. In the present study MHC class II DRB1 exon 2 variation was investigated in 81 Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) belonging to four populations. Haplotype analysis, population genetics statistics and network analysis were carried out, in order to analyze variability, phylogeography and genealogy, and the effects of geography and demographic trend. Twenty-nine haplotypes were identified, 26 of them newly described, with high Gene diversity (Gd). The variability observed in the easternmost populations of Pyrenean chamois showed a higher genetic diversity than that previously reported for other populations of Pyrenean and Cantabrian chamois (*Rupicapra pyrenaica parva*). The most frequent allele was *RupyDRB*∗15, previously undetected, which seems to play a significant role in genotyping the variability, suggesting a possible effect of positive selection.

1. Introduction

Chamois are medium-sized ungulates inhabiting mountain areas of Southern and Central Europe and the Near East, belonging to the *Caprinae* subfamily in the *Bovidae* Family (Shackleton, 1997; Crestanello et al., 2009). Currently, two chamois species are recognized: the Alpine chamois (*Rupicapra rupicapra*) and the Southern chamois (*Rupicapra pyrenaica*). The first includes seven subspecies (*cartusiana, rupicapra, tatrica, carpatica, balcanica, asiatica* and *caucasica*) and the latter includes three different subspecies: the Pyrenean chamois (*R. p. pyrenaica*), the Cantabrian chamois (*R. p. parva*), and the Abruzzo chamois (*R. p. ornata*). There are several differences between the two species, mainly morphological, e.g. body size and color of the coat, and behavioral differences concerning reproductive strategy, which would have prevented hybridization events in sympatric areas in the past, have been proposed (Nascetti et al., 1985; Catusse et al., 1996). Population dynamics of chamois are influenced by pathogen-derived epidemics. For instance, *R. p. parva* and *R. rupicapra* from the eastern Alps are severely affected by sarcoptic mange due to *Sarcoptes scabiei* var. *rupicaprae* (Rossi et al., 1995, 2007; Fernández-Moran et al., 1997; Pence and Ueckermann, 2002). This pathogen has not been so far detected in the Pyrenean chamois *R. p. pyrenaica* (Arnal et al., 2004), which is however exposed to different pathogens. As a matter of fact, recent studies carried out on Pyrenean chamois from northeastern Spain reported several outbreaks of disease associated to a border disease virus (BDV) affecting the easternmost populations of Pyrenean chamois from 2001 and now considered to be endemic. BVD is one of the four main viral species within the genus *Pestivirus* (Family Flaviviridae), which includes viruses that are major pathogens for cattle, sheep, pigs and wild ruminant species, with remarkable economic impact worldwide (Marco et al., 2007). The infection in the easternmost populations of Pyrenean chamois has leaded to a population decrease ranging from 40% to 85%, depending on the area, becoming endemic (Hurtado et al., 2004; Marco et al., 2007, 2008, 2009; Pioz et al., 2007). Such dramatic population decrease may drive to genetic variability loss. This fact can be even more important in mountain species, as chamois, inhabiting semi-isolated, fragile and fragmented habitats, due to low rates of colonization and reduced gene flow between populations (Brown, 2001). Genetic variation analyses at neutral and adaptive markers improve the understanding of natural events, geographic features, and dynamics of natural populations (Hartl and Clark, 2007). The Major Histocompatibility Complex (MHC) is a sensitive marker of genetic variation of populations; it is a multigene family of the vertebrate immune system

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http://dx.doi.org/10.1016/j.meegid.2012.02.017

comprising highly polymorphic loci (Klein, 1986) and containing the most variable functional genes described in vertebrates (Piertney and Oliver, 2006). The primary role of the MHC is to present foreign proteins to effectors cells of immune system; it includes two subfamilies: class I presents endogenously and intracellular pathogens derived peptides to cytotoxic lymphocytes T, class II presents bacteria and parasite- derived peptides to helper T cells. Genetic polymorphism on the MHC genes system may vary in time and space, since it is affected both by pathogens and population dynamics, like expansions or bottlenecks (Klein, 1986; Janeway et al., 2001). MHC genes are usually highly polymorphic and variation is mostly concentrated in the peptide binding region (PBR) (Hughes and Yeager, 1998). The DRB1 exon 2, encoding the PBR in β-1 domain, is probably the most polymorphic region of MHC class II (Apanius et al., 1997). The extensive polymorphism and unusual persistence of alleles at the MHC loci suggest the action of balancing selection, although they may be influenced also by sexual selection, intragenic recombination, demographic history and population structure (Bernatchez and Landry, 2003; Schaschl et al., 2006; Mona et al., 2008). The objective of this study is to investigate the genetic variability at MHC class II DRB1 exon 2 locus in the easternmost populations of Pyrenean chamois, and its relation with geography and demographic trends.

2. Materials and methods

2.1. Sample collection and PCR amplification

Skeletal muscle samples were collected between 1991 and 2008 from 81 hunted or found dead Pyrenean chamois in the National Hunting Reserves of Cadí, Alt Pallars-Aran, Freser-Setcases and Cerdanya-Alt Urgell, in the Eastern Pyrenees (Fig. 1). Serum samples of all the chamois had previously been tested for BDV (Marco et al., 2007, 2008). Twenty-seven chamois were diseased animals PCR-positive to BDV; 23 BDV-negative chamois showed other diseases (eleven affected by pneumonia, four affected by keratoconjunctivitis, one suffering both from pneumonia and keratoconjunctivitis, one with nervous disease, one infected by *Arcanobacterium pyogenes*, and other five chamois found dead where the cause of death could not be determined due to autolysis); the remaining 31 chamois were considered healthy after post-mortem examination. Genomic DNA was extracted from ethanol preserved tissues (70%) using the Wizard® Genomic DNA purification kit (Promega), according to manufacturer's protocol. Polymerase chain reaction (PCR) amplification of the exon 2 of the MHC class II DRB gene was performed using the two cattle primers HL030-HL031 and a semi-nested primer HL032, as previously described (Schaschl et al., 2004), including a negative control without genomic DNA in each amplification. Five microliter aliquots of the individual PCR products were separated by electrophoresis using agarose gels (1.5%) stained with ethidium bromide (0.4 μg/ml) and detected using ultraviolet transillumination. Positive amplicons were purified using SureClean Product Insert (Bioline), following the manufacturer's instructions, and shipped to MWG EurofinsDNA for sequencing.

The resulting electropherograms were first manually analyzed using Chromas Lite (www.technelysium.com.au) and aligned using Clustal_X (Thompson et al., 1997), as implemented in MEGA 4.1 (Tamura et al., 2007).

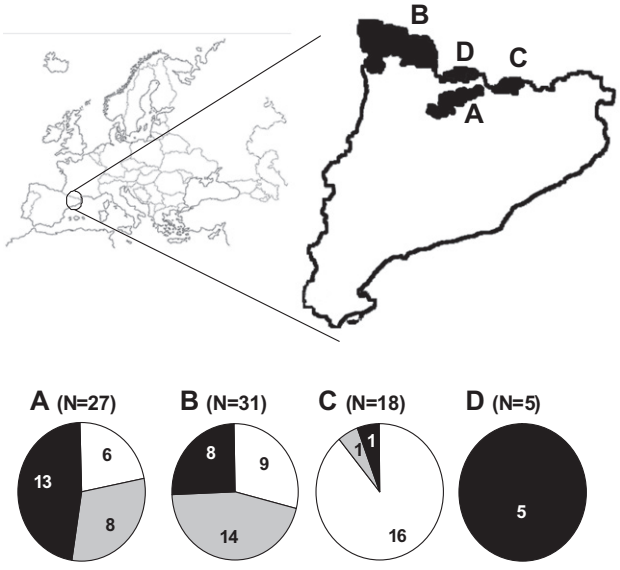

Fig. 1. Sampling areas, including the number of chamois sampled in each area. (A) Cadí; (B) Alt Pallars-Aran; (C) Freser-Setcases; (D) Cerdanya-Alt Urgell. White: healthy chamois; Gray: chamois with disease other than pestivirus; Black: chamois affected by pestivirus.

2.2. MHC haplotypes reconstruction, variability analyses and neutrality test

The correct assignment of polymorphic sites to one strand or to another of each individual (in-phase haplotypes), were inferred comparing sequences with thirteen haplotypes previously described for Pyrenean chamois (*RupyDRB*∗01–13 from Schaschl et al., 2005; Álvarez-Busto et al., 2007) obtained from GenBank (AY212149–57 and AY898752–55) using PHASE (Stephens et al., 2001) as implemented in DnaSP v5 (Librado and Rozas, 2009). In accordance with the proposed MHC nomenclature of non-human species (Klein et al., 1990), each haplotype was designated as the exon 2 alleles *Rupy*-DRB for Pyrenean chamois followed by a serial number (e.g., *Rupy*DRB∗01).

Haplotype polymorphism analysis was carried out using DnaSP v5 (Librado and Rozas, 2009). Population genetic data such as allelic frequencies, Gene diversity (Gd), number of polymorphic sites (S), mean number of pairwise differences (Pi), sample differentiation and departures from neutrality (Tajima's D test, Fu and Li's test) were evaluated using Arlequin 3.11 (Excoffier et al., 2005). Sample analysis was performed by grouping the specimens according to two criteria: geographic origin (Alt Pallars-Aran, Cadí, Cerdanya, and Freser-Setcases) and health status (healthy, pestivirus-affected, and affected by other diseases), as defined in Fig. 1 and in Table 2. Tajima's D test was repeated considering only the two populations which had a balanced representation of the three health status groups (Cadí and Alt Pallars-Aran). D and F neutrality test proposed by Fu and Li (1993) was also carried out on these two populations, to confirm results obtained by Tajima D test. The Exact Test of Sample Differentiation (Raymond and Rousset, 1995) was performed in order to detect differences in allele distribution among the abovementioned groups, excluding the Cerdanya population due to small sample size ($n = 5$). A median-joining network using Network software (Bandelt et al., 1999) and a statistic parsimony network using TCS software (Clement et al., 2000) were carried out to analyze phylogeographic and gene genealogy of the MHC class II DRB alleles.

3. Results

3.1. PCR results and haplotypes analysis

The size of PCR products of the DRB exon2 locus was around 300 bp. The total length of the sequences obtained from positive amplicons, after manual editing of sequences, was of 243 bp,