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Genetic diversity of the toll-like receptor 2 (TLR2) in hare (*Lepus capensis*) populations from Tunisia

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ABSTRACT

Toll-like receptors (TLRs) are a major group of proteins that recognize molecular components of infectious agents, known as pathogen associated molecular patterns (PAMPs). The structure of these genes is similar and characterized by the presence of an ectodomain, a signal transmembrane segment and a highly conserved cytoplasmic domain. The latter domain is homologous to the human interleukin-1 receptor (IL1R) and human IL-18 receptor (IL-18R) and designated TIR domain. The latter domain of the *TLR* genes was suggested to be very conservative and its evolution is driven by purifying selection. Variability and evolution of the TIR sequences of *TLR2* gene were studied in three hare populations from Tunisia with different ecological characteristics (NT–North Tunisia with Mediterranean, CT–Central Tunisia with semi-arid, and ST–South Tunisia with arid climate). Sequencing of a 372 bp fragment of TIR2 revealed 25 alleles among 110 hares. Twenty variable nucleotide positions were detected, of which 7 were non-synonymous. The highest variability was observed in CT, with 16 polymorphic positions. In ST, only 4 polymorphic nucleotide positions were detected with all diversity values lower than those recorded for the other two populations. By using several approaches, no positive selection was detected. However, evidence of purifying selection was found at two positions. The logistic models of the most common TIR2 protein variant that we run to examine whether its occurrence was affected by climatic variation independent of the geographic sample location suggested only a longitudinal effect. Finally, the mapping of the non-synonymous mutations to the inferred tertiary protein structure showed that they were all localized in the different loop regions. Among all non-synonymous substitutions, three were suggested to be deleterious as evidenced by PROVEAN analysis. The observed patterns of variability characterized by low genetic diversity in ST might suggest that the TIR region was more affected, than other markers, by genetic drift or/and that these patterns were shaped by different selective pressures under different ecological conditions. Notably, this low diversity was not detected by other (putatively neutral) microsatellite markers analysed in the course of other studies. But low diversity was also found for two MHC class II adaptive immune genes. As expected from functionally important regions, the evolution of the TIR2 domain is mainly driven by purifying selection. However, the occurrence of deleterious non-synonymous substitutions might highlight the flexible evolution of the *TIR* genes and/or their interactions with other proteins.

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1. Introduction

The adaptive and the innate immunity are the two parts of the mammalian immune system. The adaptive immune system is based on molecules corresponding to MHC antigens, T-cell receptors, B-cell receptors and antibodies. However, the innate immunity provides the first line of defence against infection [1] and constitutes a set of disease-resistance mechanisms that are not specific to a particular pathogen but that include cellular and molecular components that recognize classes of molecules peculiar to frequently encountered pathogens [2]. Among these components, Toll-like receptors (TLRs) are a major group of proteins that recognize molecular components of infectious agents, known as pathogen associated molecular patterns (PAMPs) [2]. To date, 13 TLRs have been described in mammals, although it has been shown that not all species contain this full component of receptors [3]. The structure of these genes is similar and characterized by the presence of an ectodomain, a signal transmembrane segment and a highly conserved cytoplasmic domain homologous to the human interleukin-1 receptor (IL1R) and human IL-18 receptor (IL-18R) and designated TIR domain [4,5]. In mammals, TIR domains are involved in mediating interactions in the Toll-like receptor and interleukin-1 signalling pathways [6]. Among *TLR* genes, *TLR2* is located on the outer membrane and forms a dimer complex with TLR1 or TLR6 to recognize peptidoglycans, lipoproteins or lipoteichoic acid of Gram-positive bacteria [7,8]. This gene is widely expressed across species and recognises the greatest number of PAMPs, detecting components from bacteria, viruses and fungi [9,10]. TLR2 was suggested to exhibit high levels of polymorphism in several mammal species [11–13].

Evolutionary patterns of genes of the innate immune system are still under intense debate. The classical view considers this polymorphism of the evolutionary ancient *TLR* genes to be strongly optimized by natural selection and, therefore, should evolve under purifying selection [14]. Indeed, several point mutations affecting *TLR* genes were suggested to alter the immune response [15] or to increase susceptibility to infection in sheep [16] and in humans [17,18]. However, recent studies [19,20] have suggested that *TLR* genes involved in pathogen recognition are evolving in direct response to pathogen-mediated selective pressures. Evidences of adaptive substitutions were observed in bovine TLR2 and TLR5 [12,21], in TLR4 in primates and birds [22,23], and in TLR2 in birds [19] and in sheep [13].

Hares from Tunisia are found across the whole country along a steep ecological gradient ranging from a Mediterranean humid climate in the north down to a Saharan climate in the south. Population genetic data on these hares that were based on nuclear and mitochondrial DNA markers (microsatellites, transferrin intron sequences, mtCR1 sequences) indicated relatively high levels of gene flow and high genetic diversity [24,25]. However, variability of the adaptive immunity MHC class II genes showed more spatial partitioning than the supposedly neutral microsatellite markers, parallel to strong positive selection on these immune genes [26]. Moreover, the observed pattern of positive selection followed climatic variation across the country suggesting occurrence of different pathogen pressures in the different ecoregions. In this study, we examined the level of genetic diversity of the TIR domain of the *TLR2* gene of the innate immune system in hares from three regions in Tunisia with two very different climates (NT–North Tunisia, with Mediterranean climate, and ST–South Tunisia, with arid Saharan climate) and one region between these two regions (CT–Central Tunisia, a transition zone with semi-arid climate). We aimed to investigate the level of genetic diversity within and among populations and to compare diversity patterns of TIR2 sequences to the earlier results from other markers [24–26]. In addition, we intended to test whether the observed pattern of diversity has been shaped by neutral or selective processes and if climatic differences may affect the occurrence of protein variants. We looked also for evidence of positive and purifying selection at single codons of the analysed sequences. Finally, the tertiary structure of TIR2 encoded proteins was predicted using computational program and homology modelling methods.

2. Material and methods

2.1. Samples

A total of 110 hares were collected by hunters at fifteen locations in Tunisia across a distance of less than 500 km between the northern Mediterranean seaboard with Mediterranean climate and high annual rainfall (ca. 916 mm) and the arid northern parts of the Saharan desert with less than 100 mm annual rainfall. Localities and sample size of these specimens are shown in Fig. 1 along with assignment of localities to the three regions NT–North Tunisia, CT–Central Tunisia, ST–South Tunisia. Those three regions were operationally considered three populations.

2.2. DNA amplification and typing via next-generation sequencing

Protocols used for DNA extraction are described in previous publications [25,27,28]. We targeted a 372 bp fragment of Toll like receptor 2 corresponding to the Toll-interleukin-1 receptor domain protein (TIR2) in a total of 110 hare specimens. Briefly, library preparation was performed by firstly amplifying each sample using the primer pair 5′-ATGCGTTCGTGTCCTACAGC-3′ and TLR-R 5′-CTCAAGTTCCCCCAGAACC-3′. A second round of PCRs was carried out to attach unique DNA barcodes to all samples and achieve compatibility with Illumina's MiSeq flow cell. PCR products were then purified, and after the quality and quantity of PCR products were estimated, all samples were pooled and sent to the Microsynth (AG) for sequencing on an Illumina Miseq using 2×250 bp chemistry.

Initial TIR2 sequence data processing was achieved as outlined in Biedrzycka et al. [29] and Sebastien et al. [30] using the different amplicon sequencing analysis tools available at: http://www.evobiolab.biol.amu.edu.pl/amplisat/.

2.3. Analysis of polymorphism and genetic differentiation

DNA polymorphism within populations (haplotype diversity h, nucleotide diversity π, and mean number of

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