Susceptibility to *Puumala virus* infections in *Myodes glareolus*: what can we learn from *Mhc* class II gene polymorphism ?[1]

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Running title : Mhc genes and *Puumala* infection in *M. glareolus*

Summary

We used experiments and population surveys to analyse the influence of two *Mhc* class II genes (*Dqa* and *Drb*) on *Puumala virus* (PUUV) infection success in bank voles (*Myodes glareolus*). We found no significant associations between infection success and *Mhc* alleles during PUUV transmission experiment. By contrast, we revealed significant genetic differentiation between PUUV seronegative and seropositive bank voles trapped in the wild, at the *Drb* gene only, and mostly for Fennoscandian localities. The absence of genetic differentiation at neutral microsatellites confirmed the important role of selective pressures in shaping *Drb* patterns. Selection could be directly driven by PUUV or, in absence of obvious experimental evidence, indirectly mediated by other parasites. Furthermore, the diversity of associations observed between *Drb* alleles and PUUV serology suggested processes of local adaptation. Therefore, we recommend that future PUUV / *M. glareolus* immunogenetic studies combine whole parasite communities and geographic variation information.

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Puumala virus (PUUV) is a rodent-borne enveloped RNA virus, belonging to the genus *Hantavirus* (Bunyaviridae). In humans, it is responsible of a mild hemorrhagic fever with renal syndrome (nephropatia endemica NE, Lundkvist & Niklasson, 1992). The symptoms vary between human patients with different levels of severity (Lahdevirta, 1971; Mustonen *et al.*, 1994). Genetic studies have revealed the influence of Human Leucocyte Antigen (*Hla*) haplotypes on the severity of PUUV infection in humans (Plyusnin *et al.*, 1997; Mustonen *et al.*, 1996).

The bank vole, *Myodes glareolus*, is the main reservoir species of PUUV in Europe. PUUV infection in bank voles is chronic (Haderstam *et al.*, 2008) and asymptomatic (Meyer & Schmaljohn, 2000). Rodents were therefore considered as healthy carriers of the virus (Yanagihara *et al.*, 1985). However, recent studies have revealed that the infection decreased the winter survival of infected individuals (Kallio *et al.*, 2007). Besides, field surveys and experiments indicate that some variation exists among bank voles in their probability of being infected with PUUV (e.g. Deter *et al.*, 2008a; Olsson et al., 2002). Experimental infections designed to test the modes of PUUV transmission have also shown that the infectivity varied among bank voles (Hardestam *et al.*, 2008; Kallio *et al.*, 2006). This variability could be related to immunogenetic background. First, a large number of genes that encode for proinflammatory, antiviral, Major Histocompatibility Complex (MHC, equivalent to HLA in humans), Ig, and T cell marker proteins are upregulated in female compared with male Norway rats following an infection with *Seoul hantavirus* (SEOUV, Klein *et al.*, 2004). This upregulation leads to an increased ability of females to control SEOUV replication, and we can assume that it could also prevent infection in the first place. Second, an epidemiological survey has revealed associations between *Mhc* alleles and PUUV patterns of infection in wild bank vole populations (Deter *et al.*, 2008b). However in this study, uninfected bank voles might never have been exposed to PUUV, and therefore it is not sure that these alleles directly influence susceptibility to *Puumala*. Altogether, these preliminary observations raise questions about the existence of genetic factors underlying the variability of bank vole susceptibility to PUUV infections. In this context, *Mhc* genes deserve particular attention as their polymorphism seems to influence the ability of humans and rodents to control hantavirus replication (Klein *et al.*, 2004; Mäkelä *et al.*, 2002; Plyusnin *et al.*, 1997; Terajima *et al.*, 2004). They could mediate differences in the efficiency to prevent hantavirus infection.

Our study focused on two *Mhc* class II genes, *Dqa* and *Drb*. First, we used the bank vole material from the experiment of Kallio *et al.* (2006). We aimed to determine if variability of infectivity, which is determined by RT-PCR, could result from different *Mhc* haplotypes in recipient bank voles. We included the 101 recipient voles of the study (referred as S1 dataset), or we selected a sub-sample (S2) of 62 voles corresponding to those known to have been exposed to a viral infecting dose. Indeed, even if voles were uninfected, their bedding was shown to infect other bank voles in the next steps of the experiment. Second, we looked for associations between *Mhc* haplotypes and PUUV infection status in wild populations. At a local geographical scale, the balance between evolutionary forces, including neutral processes and parasite-mediated selection, may strongly determine the frequencies of *Mhc* haplotypes. We compared five localities that differed in their level of PUUV prevalence. Three corresponded to areas of high PUUV

prevalence (Ilmajoki (Finland), Konnevesi (Finland) and Våsterbotten (Sweden)) and two others corresponded to lower PUUV prevalence (Ardennes: Elan (France) and Beaumont (Belgium)). In areas of high prevalence, we expected a strong selective pressure imposed by PUUV on bank vole. Therefore, *Mhc* haplotypes should be highly associated with PUUV susceptibility. This would result in strong genetic differentiation at *Mhc* genes between PUUV-seronegative and seropositive voles. Finally, we also expected differences in PUUV lineages to drive local adaptation processes (e.g. Asikainen *et al.*, 2000; Johansson *et al.*, 2008). By consequence, *Mhc* haplotypes associated with PUUV infection should depend on the localities studied.

Individual blood samples were screened for IgG anti-PUUV antibodies using an IgG immunofluorescence assay (IFA) as described earlier by Kallio-Kokko *et al.* (2005). We have included about 30 individuals per locality, with about half of PUUV seropositive voles. For each vole, we kept a piece of tissue stored in ethanol 96%. Genomic DNA was extracted using the DNeasy 96 kit (Qiagen) according to manufacturer's instructions. We amplified the complete exon 2 of the *Mhc* class II gene *Dqa* (*Dqa*-exon 2) following the protocol described by Bryja *et al.* (2006) using fluorescently labelled primers (forward by 6'-FAM and reverse by HEX) and 35 cycles of denaturation / annealing / extension. Single strand conformation polymorphism (SSCP) analyses of PCR products were then performed by capillary electrophoresis (CE) on MegaBACE 1000 DNA Analysis System (Amersham Biosciences) following Bryja *et al.* (2005). The electropherograms were aligned and analyzed with the software MegaBACE Genetic Profiler 1.5 (Amersham Biosciences). Two alleles (Clgl-*DQA**08 and Clgl-*DQA**35 GenBank under accession nos.EU371603 and EU371614) differed only in one bp and exhibited undistinguishable CE-SSCP patterns. We thus applied the restriction fragment length polymorphism (RFLP) test based on HphI and PdmI enzymatic restrictions to discriminate these alleles, even in the presence of other alleles (Male *et al.*, submitted). Note that for technical problems, seronegative vole samples from Våsterbotten have unfortunately not been genotyped at the *Dqa*-exon 2 gene. We amplified the exon 2 of the *Mhc* class II gene *Drb* (*Drb*-exon 2) using the 454 GS FLX (Roche) plateform. We used the primers JS1 (forward 5'-GAGTGTCATTTCTACAACGGGACG-3') and JS2 (reverse 5'-GATCCCGTAGTTGTGTYTGCA-3') defined by Schad *et al.* (2005). The combination of tags in the reverse and forward-primers formed unique barcode identifiers and allowed the reassignation of each of the thousands of sequences obtained from the 454 pyrosequencing run to a unique individual. A stepwise bioinformatic procedure had been developed to provide individual genotypes (see details in Galan *et al.*, In revision). The *Drb*-exon 2 sequences were edited and aligned in BioEdit Sequence Alignment Editor (Hall, 1999) using ClustalX Multiple Alignment. Nomenclature of the bank vole sequences follows Klein *et al.* (1990). The sequences are available in GenBank under the following accession numbers: XXXX-XXX. Human *Homo sapiens* (Human M17236; AM109973), sheep *Ovis aries* (OvAr1, M33304; AY230000) and pig *Sus scrofa* (PigM29938; NM001113695) sequences were used as outgroups in phylogenetic analyses. MEGA 4 software (Tamura *et al.*, 2007) was employed to construct a phylogenetic tree of the *Drb*-exon2 gene using the neighbour-joining (NJ) algorithm and Kimura-2-parameters distance. A bootstrap analysis (1,000 replicates) was performed to determine the reliability of the branching. We confirmed that *Drb*-exon2 was duplicated in *M. glareolus* (Axtner & Sommer, 2007) although phylogenetic reconstructions did not enable us to assign all alleles to one or

another copy of the gene (supplementary material). However, we identified different alleles as belonging to cluster I (Axtner *et al.*, 2007).

In natural populations, *Mhc* genetic differentiation between PUUV seronegative and seropositive bank voles could result from neutral processes, such as strong familial structure, or from selective ones. To discriminate the influence of these forces, we compared the genetic patterns observed at *Dqa*-exon 2 and *Drb*-exon 2 genes with those obtained from microsatellites, which are supposed to be neutral (Luikart *et al.*, 2003). Therefore we genotyped the bank vole samples corresponding to natural populations at 17 microsatellite loci using the primer sets developed by Rikalainen *et al.* (2008) except for primers (Cg1F11, Cg17C9, Cg2F2, Cg4F9, Cg1G12) which have been redesigned. Genotyping was carried out using an ABI3130 automated DNA sequencer with labelling of the 5' end forward primer with fluorescent dye and separated in two sets (Set 1: FAM : Cg15F7, Cg10A11, Cg1F11; HEX : Cg2F2, Cg13G2, Cg16E4; NED: Cg13F9, Cg3A8; Set 2: FAM : Cg16A3, Cg13C12, Cg17C9; HEX: Cg4F9, Cg3F12, Cg14E1; NED: Cg6G11, Cg2C5, Cg12A7). Four microsatellites (Cg3F12, Cg2F2, Cg17C9, Cg3F9) showed a significant deviation from Hardy-Weinberg Equilibrium within population due to null alleles and were removed from the analyses.

We first performed multivariate analyses using ADE-4 software (Thioulouse *et al.*, 1997) to determine the *Mhc* alleles that best discriminated among infected / noninfected groups within the experimental dataset or within the five localities sampled. The discriminant function analysis (DFA) was used to maximize the variance between designated groups (serology*locality) while keeping the intra-group variance constant (Jombart *et al.*, 2009). The significance of the ratio of these two values was estimated using 10,000 permutation tests. For each allele, we estimated the relative risk (RR) following Haldane (1956) and we tested the association with vole serological status using Fisher exact tests and Bonferroni sequential corrections. Next, we tested the conformity to Hardy-Weinberg equilibrium for each microsatellite locus and locality using the Markov chain methods implemented in GENEPOP v3.3 (Raymond & Rousset, 1995). We looked for signatures of selection using Analyses of Molecular Variance (Amova) in ARLEQUIN v3.0 software (Excoffier *et al.*, 2005). We estimated the proportion of variance and S-statistics (S_{ct}, S_{sc}, S_{st}) at three hierarchical levels of subdivision: among the five localities sampled; among the serological groups within each locality and within each serological group. The significance of these parameters was evaluated using 10,000 permutations. Amovas were performed independently for microsatellites on one hand and *Drb* cluster I on the other hand. The detection of significant contrasted patterns of genetic differentiation at *Drb* with regard to microsatellites would reveal the action of selection on *Drb* (e.g. Spurgin & Richardson, 2010).

No polymorphism could be detected at *Dqa*-exon2 gene on the 101 bank voles corresponding to the experimental infection (S1, S2). Besides, we found seven *Drb* alleles. The discriminant analyses performed on the S1 and S2 datasets were not significant (S1: p = 0.741, S2: p = 0.675). Fisher exact tests revealed no significant differences in infection status distribution whatever the *Drb* allele and the dataset considered (Table 1), although the relative risks associated with *Drb**0101 were high (S1: RR = 2.87, p = 0.008; S2: RR = 4.82, p = 0.062). These results indicated that the two *Mhc* class II alleles did not play a major role in the differences of susceptibility to infection observed in this experiment. However, it should be

noticed that this laboratory stock of bank voles had been maintained in captivity for 20 years. Therefore inbreeding and loss of genetic variability could have prevented the detection of genetic associations. Besides, note that while other individual factors such as gender, weight and sexual maturity did not seem to explain differences in susceptibility to PUUV here (Kallio *et al.*, 2006), it was likely that the existence of variability further detected on PUUV S segment sequences could have mediated the observed variability in infection success (Sironen *et al.*, 2008).

We performed the discriminant analyses on the natural population dataset considering 124 bank voles (57 seropositive ones) and 7 alleles (3 others had frequencies lower than 5%) for *Dqa* gene, and 130 voles (68 seropositive ones) and 11 alleles (22 others had frequencies lower than 5%) for *Drb* gene. Both analyses revealed significant differences among serology*locality groups (*Dqa*: $p < 10^{-4}$; *Drb*: $p < 10^{-4}$; Fig.1). Regarding *Dqa* gene, the two first discriminant factors, which represented respectively 47.7% and 24.6 % of the total inertia, only revealed geographic oppositions between the Finnish and the Ardennes populations, whatever the serological status of individuals (Fig. 1a). Again, this result did not support the influence of the *Dqa* exon 2 gene on the probability of bank voles to be infected with PUUV. The *Drb* gene gave a different pattern as the first axis of the discriminant analysis (29.7% of the total inertia) well separated seropositive bank voles of the Ardennes from those of Ilmajoki (Fig. 1b). This was explained by the allele *Drb**0008 which was not found in the Ardennes populations and exhibited a high RR in Ilmajoki (RR = 3.73, Fisher exact test: $p = 0.069$), and by the allele *Drb**0014, which was not found in Ilmajoki but exhibited a high RR in the Ardennes (Beaumont: RR = 6.44, Fisher exact test $p = 0.098$; Elan: RR = 2.39, Fisher exact test $p = 0.232$, Fig. 1c). The second axis (25.2% of the total inertia) also discriminated seropositive bank voles sampled in Konnevesi and Våsterbotten from all voles of the Ardennes. The allele *Drb**0003 mostly explained this pattern (Fig. 1c) as it was associated with high RR in Våsterbotten (RR = 2.95, Fisher exact test $p = 0.047$) and Konnevesi (RR = 1.8, Fisher exact test $p = 0.500$), whereas it was not found in the Ardennes. As expected, PUUV seronegative and seropositive voles were more significantly discriminated in Fennoscandia than in the Ardennes (Fig. 1b).

Genetic analyses based on microsatellites markers and *Drb* cluster I indicated significant differentiation among localities, whatever the loci considered (Table 2). Besides, no significant molecular variance was observed between serological groups on the basis of microsatellites ($S_{sc} = 0.00124$; $p = 0.374$) whereas it was significant when considering *Drb* cluster I ($S_{sc} = 0.03998$; $p = 0.001$).

This immunogenetic study of wild bank vole populations revealed clear associations between some *Drb*–exon2 alleles and PUUV serological status. This genetic differentiation between seronegative and seropositive bank voles within localities was not explained by neutral processes, as reflected by the absence of differentiation detected at microsatellites. It is likely that it was mediated by selective pressures acting on *Drb*–exon2 gene. As expected, the selective pressure mediated by PUUV on *Drb* gene seemed stronger in Fennoscandia than in the Ardennes. We therefore concluded that the associations between *Drb*–exon2 alleles and PUUV serological status observed in the wild could be directly driven by PUUV itself. However, as the experimental infection reported no obvious direct role for *Drb*–exon2 gene in PUUV infection

success, we could not exclude the possibility that other parasites present in the environment mediated the patterns observed. Actually, biological antagonistic or synergistic interactions between parasites have previously been shown to lead to indirect associations between susceptibility/resistance and *Mhc* gene alleles (review in Hartgers & Yazdanbakhsh, 2006). Several candidates including helminths and mites could be considered in the future (e.g. in Deter *et al.*, 2008b). Finally, our study confirmed that *Drb*–exon2 alleles associated with PUUV serological status differed among localities. Again, this pattern could also reflect geographic variations of the parasite community interacting with PUUV. This would result in a geographic mosaic of coevolutionary processes affecting *Mhc* gene polymorphism (see for review Spurgin & Richardson, 2010). Understanding the impact of such local adaptation processes on PUUV infectivity, with regard to *M. glareolus* population genetics or phylogeography, now deserves further investigations.

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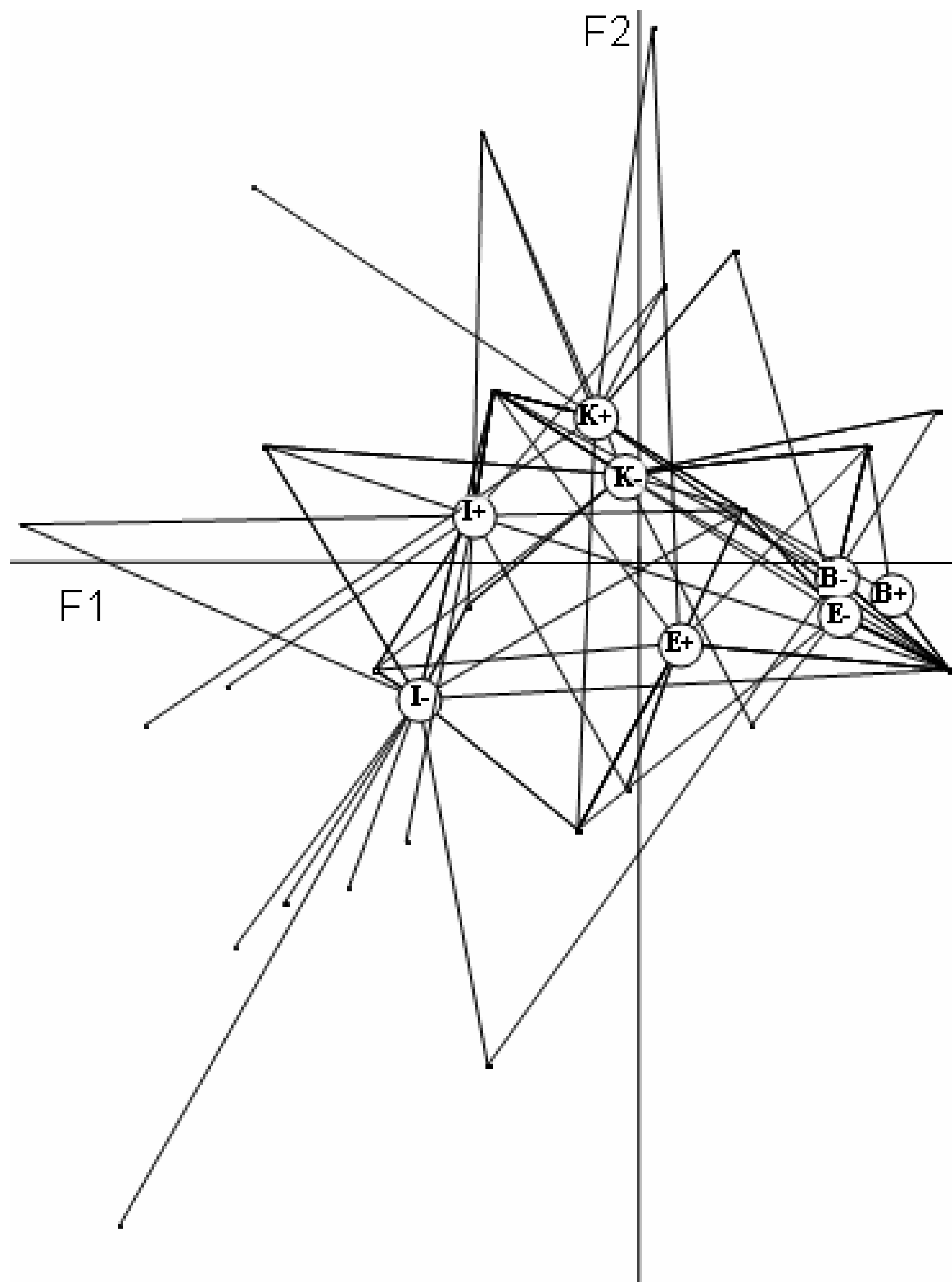
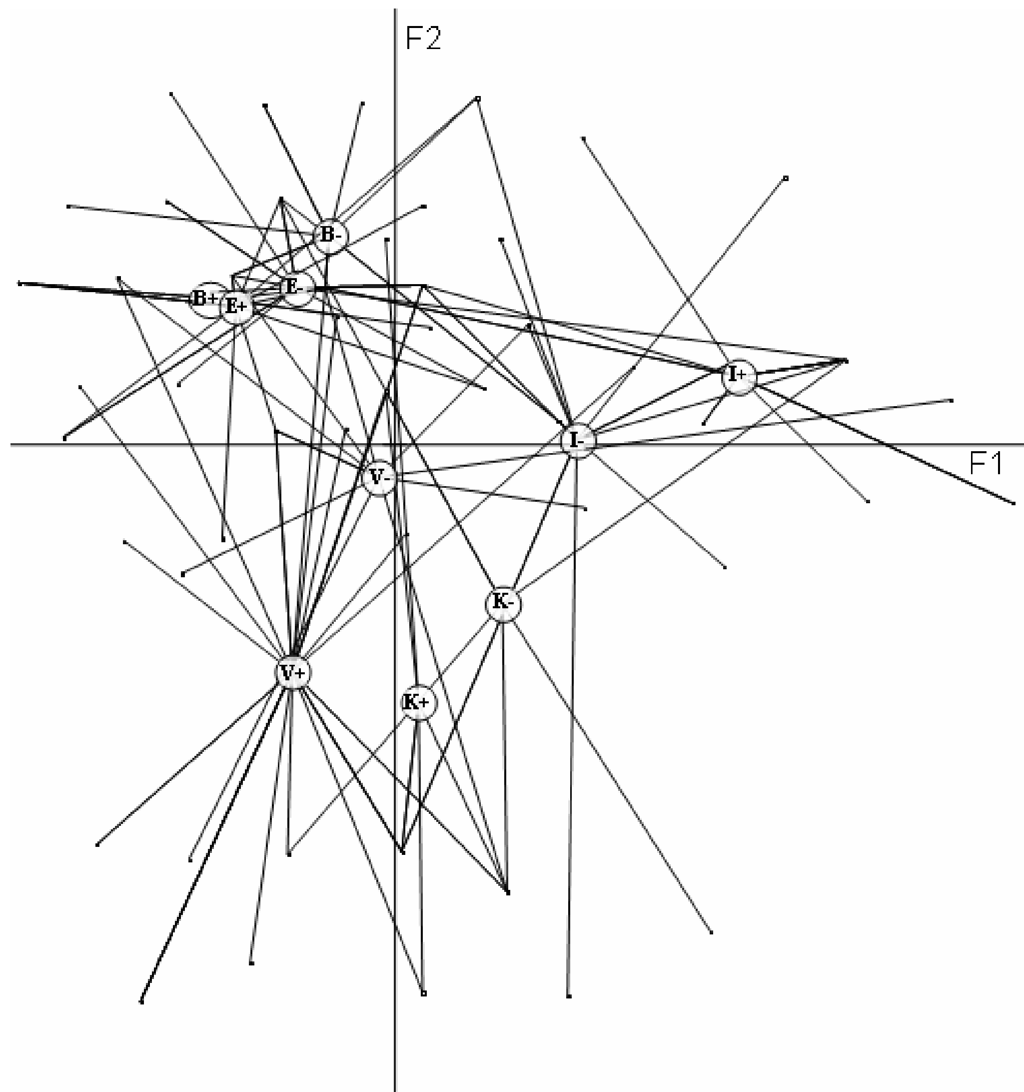
Fig. 1. Discriminant analyses performed on *Mhc* class II gene polymorphism with regard to PUUV serological status and locality. a) and b) respectively indicate *Dqa* and *Drb* exon 2 gene results. c) represents the discriminant scores for *Drb* exon 2 gene. Letters correspond to localities sampled (B = Beaumont (Ardennes), E = Elan (Ardennes), I = Ilmajoki (Finland), K = Konnevesi (Finland) and V = Våsterbotten (Sweden)). – and + respectively correspond to PUUV seronegative and seropositive bank voles. Circles represent the barycentre of each PUUV*locality group.

a)



b)



c)

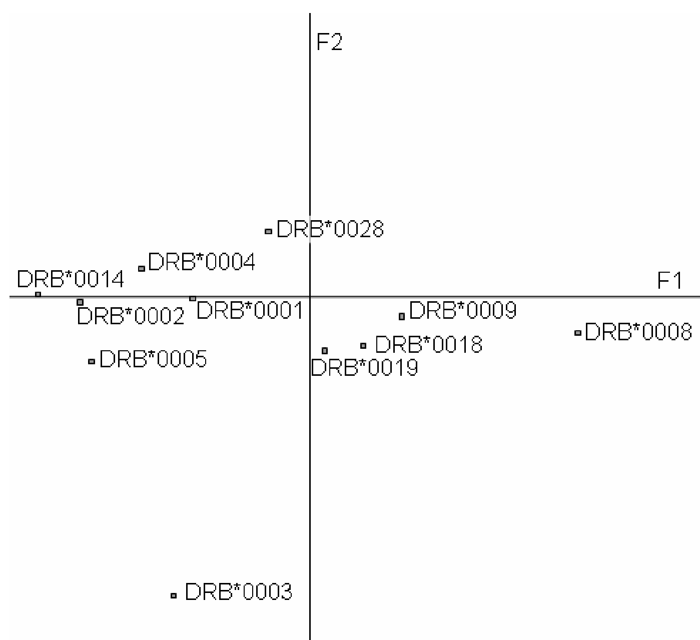


Table 1. Probability tests and relative risks (RR, Haldane 1956) associated with PUUV infections and *Drb* alleles for voles corresponding to Kallio *et al.*'s experiment (2006). We considered the whole dataset (S1) or only individuals known to have been exposed to infected bedding (S2).

		Drb alleles						
		*DRB**0001	*DRB**0016	*DRB**0050	*DRB**0052	*DRB**0101	*DRB**0152	*DRB**0147
S1	Fisher exact test	0.495	0.127	0.571	0.454	0.086	0.609	0.316
	RR	1.113	0.566	0.993	0.866	2.876	0.961	6.619
S2	Fisher exact test	0.557	0.227	0.462	0.567	0.062	0.524	0.516
	RR	0.919	0.608	1.210	1.056	4.824	1.612	2.904

Table 2. Results of the analyses of molecular variance (Amova) based on 14 microsatellites (a) or on cluster I of *Drb*-exon2 (b) for *M. glareolus* sampled in five localities.

a)

	Variance component	**Statistics**	***P* value**
Among localities	0.410	$S_{CT} = 0.06985$	$<10^{-6}$
Among serogroup within localities	0.006	$S_{SC} = 0.00124$	0.374
Among individuals within serogroups	5.464	$S_{ST} = 0.07100$	$<10^{-6}$

b)

Among individuals within serogroups	0.414	$S_{ST} = 0.09706$	$<10^{-6}$
	Variance component	**Statistics**	***P* value**
Among localities	0.027	$S_{CT} = 0.05946$	0.002
Among serogroup within localities	0.017	$S_{SC} = 0.03998$	0.001

Supplementary material. Neighbour-Joining phylogeny for 33 sequences of *Drb*-exon2 gene from our data set. Human *Homo sapiens* (AM109973), sheep *Ovis aries* (AY230000) and pig *Sus scrofa* (NM001113695) sequences are used as outgroups. Bootstrap values (> 50) are indicated at each node and were obtained using 1,000 replicates.