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Effects of an MHC-DRB genotype and allele number on the load of gut parasites in the bank vole *Myodes glareolus*

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Abstract

The major histocompatibility complex (MHC) genes code for the proteins responsible for pathogen recognition. The MHC class II DRB gene is multiplicated in the bank vole, *Myodes glareolus*, with different numbers of loci found in different individuals. Possessing large numbers of loci should increase the probability of pathogen recognition, but according to the optimality hypothesis, there is a cost of possessing too many MHC alleles. Using 454 technology, we determined the individual DRB allelic diversity and related it to the load of intestinal parasites in voles collected from three sites separated by a distance of 12 to 27 km. The analysis of six microsatellite loci revealed significant population structure (F_{ST} = 0.07). The sites differed significantly in the prevalence and abundance of nematode species as well. We found two significant associations between MHC alleles and the intensity of the infection with the most prevalent nematode, *Aspiculuris tetraptera*. One of these associations was population-specific. This result suggests that the directions of selection can differ between populations connected by a low level of gene flow, which may contribute to the maintenance of high DRB allele diversity. In accordance with the optimality hypothesis, individuals with an intermediate number of alleles carried the lowest number of nematode species and had the lowest prevalence of *A. tetraptera*. However, the intensity of infection with *A. tetraptera* was linearly and negatively associated with the number of alleles.

Keywords: 454 sequencing, bank vole, MHC, parasites

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Introduction

The major histocompatibility complex (MHC) genes code for the proteins responsible for presenting foreign peptides to T cells. MHC genes are the most polymorphic genes described in vertebrates, with the polymorphisms occurring predominantly at the residues involved in peptide binding (Hughes & Nei 1989; Brown *et al.* 1993; Bernatchez & Landry 2003). This variation presumably allows binding a variety of parasite-derived antigens and there is growing evidence for an association between MHC-types and susceptibility to disease (e.g. Hill *et al.* 1991; Kaufman & Wallny 1996; Thursz *et al.* 1997; Langefors *et al.* 2001; Carrington & Bontrop 2002; Froeschke & Sommer 2005; Harf & Sommer 2005). The mechanisms proposed to prevent fixation of advantageous MHC alleles include frequency-dependent selection (Snell 1968; Takahata & Nei 1990; Borghans *et al.* 2004) and heterozygote advantage (Doherty & Zinkernagel 1975). Frequency dependence arises because fast-evolving parasites are more likely to invade the bearers of common alleles (Trachtenberg *et al.* 2003), while heterozygosity allows for the presentation of a wider range of pathogen-derived peptides and thus better resistance to infection (Carrington & Bontrop 2002; Penn *et al.* 2002). Furthermore, resistance conferred by some MHC alleles may only be effective locally (Bonneaud *et al.* 2006b). If there is a

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moderate population structure, which allows for local adaptation but retains some gene flow among populations, the number of alleles segregating in a meta-population may be higher than expected under frequency-dependence or heterozygote-advantage operating within a large, unstructured population (Hedrick 2002).

Possessing a large number of alleles located at multiple loci should increase the probability that at least some of them will bind any pathogen-derived antigen and indeed MHC loci are often multiplicated. However, according to the optimality hypothesis, there is also a cost of possessing too many alleles that prevents an accumulation of too many MHC loci in the genome (Milinski 2006). Such costs may stem from a negative selection on self-reacting lymphocytes that are more likely to appear when individual MHC variation is high (Nowak *et al.* 1992; Woelfing *et al.* 2009). The prediction that the highest resistance to parasites is warranted by an optimal, rather than a maximal, number of alleles, has been confirmed in studies on sticklebacks (Wegner *et al.* 2003a) and sparrows (Bonneaud *et al.* 2006b). These studies relied on between-haplotype within-species variation in the number of MHC alleles. Such variation seems to be common in many species, but testing the 'optimality' hypothesis has been hindered by difficulties of genotyping complex, multilocus systems. However, this obstacle can be overcome using highly parallel next generation sequencing (NGS) technologies that provide sequences of all alleles present in a sample thus enabling efficient genotyping of multilocus systems of virtually any complexity (Babik *et al.* 2009).

Here, we use the 454 NGS technology to investigate MHC DRB variation and its associations with parasite load in bank voles, a species whose gut parasite fauna is well characterized (Bajer *et al.* 2001; Behnke *et al.* 2001a, b; Barnard *et al.* 2003; Bajer *et al.* 2005; Behnke *et al.* 2008). Our study had two goals. First, we aimed to investigate whether possessing particular MHC DRB alleles affect susceptibility to an infection with parasites and whether such effects are population-specific. Simultaneously, we used microsatellite loci to investigate the level of gene flow between the sites. Local differences in the selection pressures on DRB alleles coupled with limited gene flow could act to increase MHC diversity at the meta-population level, as hypothesized above. Second, we tested the prediction of the optimality hypothesis, that an intermediate number of alleles confers the highest resistance to parasites.

Methods

Sampling of hosts

Animals were collected in September 2005 in Mazury lakeland, north-eastern Poland. Post-glacial landscape of this area consists of mixed and coniferous forests, lakes, meadows and abandoned farmlands. The animals were captured in woods in three sites: Urwitałt N53º48.2', E21º39.7'), Tałty (N53º53.6', E21º33.0') and Pilchy (N53º42.2', E21º48.5'). The sites were set along a 27-km long transect from NW to SE, with Urwitałt situated in the middle and Pilchy and Tałty on the edges. The distance between Urwitałt and Tałty was 12.5 km and the distance between Urwitałt and Pilchy was 14.5 km. All sampling sites were situated close to lake shores in mature woods predominated by pines (*Pinus sylvestris*) with an admixture of silver birches (*Betula verucosa*), Norway spruces (*Picea abies*), occasional oaks (*Quercus robur*) and common alders (*Alnus glutinosa*). The shrub layer composed mostly of hazel (*Corylus avellana*) was patchy and rather dense. Voles were baited with grain and captured into wooden traps $25 \times 10 \times 10$ cm. Traps were checked every morning (8–9 am) and evening (6–7 pm). Sampling in all populations was performed by the same team at the following dates: Uwritałt 5–8 September, 106 traps, Pilchy 9–11 September, 130 traps, Tałty 11–13 September, 114 traps.

The captured animals were transported to the field station of Warsaw University where they were weighed and sexed on the basis of the urogenital distance. The juvenile animals were distinguished from the adults by weight and sexual maturity (Bajer *et al.* 2001; Bajer *et al.* 2005). The animals were killed and during necropsy, the alimentary tracts were removed and, if not analysed immediately, stored in 10% formaldehyde solution until examination. For molecular analyses, a part of an ear and/or the liver was cut and preserved in 75% ethanol. The protocol was approved by local Ethical Committee no. 1 in Warsaw, decision no. 280/2003.

Parasite identification

The intestines were separated into their anatomical parts (stomach, small intestine, caecum and colon) and were carefully examined under 2.5–4× magnification. All helminths were removed, counted and stored in 75% ethanol. If necessary, standard staining techniques were applied to reveal details of the parasite anatomy. Nematodes were preserved in 37 °C for 7 days in a 5% solution of glycerol in 70% ethanol and were then transferred onto a microscope glass into a drop of hot glycerine-gelatin and covered. Cestodes were fixed at least 24 h in AFA solution and then washed in 75% ethanol and stained with borax carmine. Species identification was based on the parasite morphology according to the original species descriptions given in Genov (1984) and Haukisalmi & Tenora (1993).

MHC genotyping and measures of polymorphism

A 122-bp (without primers) fragment of the bank vole MHC II DRB 2nd exon was amplified using fusion primers containing sequences of the primers MgDRBL and MgDRBR, which are located in conserved regions of the 2nd MHC II DRB exon (Babik *et al.* 2009). The forward fusion primer 5′-GCCTCCCTCGCGCCATCAG-NNNNNNGACAGAKACWTCTACAAYCRG-3′ was composed of the 454 amplicon A primer, a 6-bp tag (indicated with Ns), used to distinguish individuals and the MgDRBL sequence (underlined); the reverse fusion primer consisted of the 454 amplicon B primer and the MgDRBR sequence (underlined) 5′-GCCTTGCCAGCC-CGCTCAGTAGTTGTRTCTGCAGWAYGYGTCC-3′.

Polymerase chain reaction (PCR) was performed as described (Babik *et al.* 2009). The concentration of the PCR product was estimated through agarose gel electrophoresis, and PCR products were pooled into approximately equimolar quantities; we found that the performance of this approximate method is as satisfactory or better than that based on the Nanodrop measurements used previously (Babik *et al.* 2009). The resulting pools were purified using the MinElute PCR Purification Kit (QIAGEN). Purified pools were then sequenced as a part of a single 454 FLX run according to the 454 Amplicon Sequencing protocols provided by the manufacturer (Roche 454) at the Functional Genomics Center, Uni/ETH Zurich. Since only 35 tagged primers were used to genotype 132 bank voles, we analysed our samples in four lanes of an eight-lane run. The sequence determination was made using a GS Run Processor in the Roche 454 Genome Sequencer FLX Software Package 2.0.00.22. The performance of the sequencing run was gauged using known pieces of DNA introduced in the sequencing run as DNA Control Beads. On average, 95% of reads from DNA Control Beads matched the corresponding known sequences with at least 98% accuracy over the first 200 bases, which was above the typical threshold (80% matches of 98% accuracy over 200 bases). Sequences containing MgDRBL and reverse complement MgDRBR sequences were extracted from the multifasta files as described (Babik *et al.* 2009) and assigned to the respective individuals on the basis of the tag sequence. Reliable MHC genotyping requires efficient quality control to identify all of the true alleles (TA) and to eliminate artefacts produced during PCR and 454 sequencing (Babik *et al.* 2009). Throughout the text, we use for convenience the term 'allele' to designate unique sequence variants although this is not strictly correct, as these sequences represent multiple loci.

In the present study, we identified the TA based on empirically validated thresholds obtained from an independent experiment (details in Supporting Information). In that experiment, the DRB of each of the eighteen voles was amplified in two independent PCR reactions and all the amplicons were subjected to 454 sequencing. Alleles present in both replicates were accepted as the TA. For each individual, the frequency (measured as the fraction of all reads from the amplicon) of the least abundant TA was recorded. The maximum and mean of this distribution were taken as two thresholds in our two step genotyping procedure. In step one, the higher threshold (maximum frequency of the least abundant TA) was applied to filter out artefacts and retain the TA. Since step one is likely to be very conservative, in step two, we also examined less abundant sequences falling between the higher (the maximum) and the lower thresholds (the mean). Step two thus allowed identification of TA that were less abundant. However, only sequences which exhibited at least three substitutions to the most similar alleles from step one were considered TA. This condition is introduced to filter out artefacts that could have been produced from abundant (on the individual basis) TA due to the nonrandom nature of the 454 sequencing errors (Babik *et al.* 2009). To confirm that none of the TA we identified using these thresholds were artefacts, the PCR product derived from the individual with the highest detected number of alleles (11) was cloned and 119 clones were sequenced. Cloning is still regarded as the gold standard in MHC studies (Lenz & Becker 2008). All alleles classified as the TA but none of the sequences classified as artefacts on the basis of 454 were found in the clones (details in Supporting Information).

The thresholds identified in the experiment (see Supporting Information), which we used in the current study, were 4.9% of all reads per individual (but not less than five reads) in step one and 2.7% of reads in the second step. Consequently, all sequences that were present in at least 4.9% of the reads in a given individual were retained. In the second step, sequences with a frequency of 2.7–4.9% that differed by at least three substitutions from the most similar sequence identified in step one were identified and retained. The 2.7% limit is in fact very close to the 3% that was identified using a different approach as the threshold separating the TA from the artefacts (Babik *et al.* 2009).

In the final step of genotyping, the 'two PCR' criterion was applied to sequences identified through the two step procedure; to be included in the final genotype the allele must have been already reported (Axtner & Sommer 2007; Babik & Radwan 2007) or must have been detected in two independent PCR reactions in the present study. The two PCR criterion is a standard in MHC studies (e.g. Babik *et al.* 2005).

Our genotyping criteria are conservative, perhaps too conservative (see Supporting Information), however, because the current understanding of the nature and frequency of artefacts in MHC genotyping using 454 sequencing is far from complete, conservatism is necessary to avoid reporting artefacts.

It seems that not all DRB sequences in the bank vole are expressed (Axtner & Sommer 2007; Babik & Radwan 2007). We excluded from further analyses 13 alleles that grouped with those of the apparently nonexpressed cluster I of Axtner & Sommer (2007), as well as three other alleles showing clear signatures of nonfunctionality (frameshift and nonsense mutations) (Fig. S2, Supporting Information). The remaining 58 alleles were considered potentially functional.

Variation at neutral loci

Genetic variation at neutral loci was scored with seven microsatellites (Gockel *et al.* 1997; Gerlach & Musolf 2000; Barker *et al.* 2005): *MSCg*-4 (HEX), *MSCg*-6 (HEX), *MSCg*-7 (NED), *MSCg*-9 (FAM) *MSCg*-20 (NED), *MSCg*-24 (FAM) and *LIST*3-003 (NED). The names in parentheses refer to the fluorescent dye used to label the forward primer for each locus.

Microsatellite loci were amplified in two separate multiplex 10 μL reaction containing 1.0 μL of the 10 × PCR buffer (Fermentas), 2.25 mM $MgCl_2$, 0.05–0.2 μM of each primer and 0.5 U of *Taq* polymerase (Fermentas). Loci *MSCg*-4, 7, 9 and 20 were amplified in multiplex A with the following cycling scheme: 95 °C for 2 min followed by 31–33 cycles of: 30 s at 94 °C, 90 s at 57 °C and 60 s at 72 °C and a final elongation of 20 min at 72 °C. The remaining loci were amplified in multiplex B with the same cycling scheme, but the number of cycles was reduced to 29. The PCR products were electrophoresed on an ABI 3130xl analyser. GENEMAPPER 4.0 was used for genotyping.

The conformance of the allele frequencies with Hardy–Weinberg expectations for all loci in all populations was tested by applying the exact tests of Guo & Thompson (1992) in GENEPOP 4.0 (Rousset 2008). Locus *MSCg*-6, which showed a consistent deficit of heterozygotes suggesting the presence of null allele(s) in high frequency, was excluded from further analyses. Differences in the allele frequencies between populations were assessed with Markov chain Monte Carlo approximations of Fisher exact tests (Raymond & Rousset 1995) in GENEPOP. The overall F_{ST} was computed with the method of Weir & Cockerham (1984) and its significance was tested by bootstrapping over loci in FSTAT (Goudet 2001). Pairwise F_{ST} values were computed in GENEPOP. The sequential Bonferroni procedure was applied where appropriate to keep the Type I error level at $\alpha \leq 0.05$ (Rice 1989).

Statistical analyses

We used two measures of parasite load: (1) prevalence, measured as presence/absence of a given species of worm; and (2) intensity, measured as the number of worms of a given species per individual. Prevalence was analysed using generalized linear models with a binomial error distribution and a logit link function. In models of infection intensity, we employed a quasipoisson, rather than a Poisson error distribution to deal with overdispersion, and a log link function. The significance of the terms was tested based on the change in deviance resulting from their deletion to the chi-square distribution (Crawley 2007). All analyses were performed with the R statistical package (R Development Core Team 2008).

The association between the number of DRB alleles carried by individuals and the total species richness (number of all parasite species present in one host) was estimated using a generalized linear model with a quasipoisson error and log link function. We also analysed the effects of the number of alleles separately on the load of each of the most common parasites, namely *Aspicularis tetraptera*, *Heligmosomum mixtum* and *Heligmosomoides glareoli*, using a generalized linear model with a quasipoisson or a binomial error distribution for intensity and prevalence, respectively. The distribution of the latter two was limited to particular sampling sites (*Heligmosomum mixtum* was present in sites Urwitałt and Tałty, and *Heligmosomoides glareoli* was exclusively found in Pilchy), i.e. in the sites where one heligmosomoidid species occurred the other was absent and vice versa; therefore, the calculations concerning those species were restricted to samples from their respective sites. The models also included additional explanatory variables that could influence the parasite load: sampling site, host sex, host maturity, and the presence of putative antagonistic species (*A. tetraptera* vs. *Heligmosomoididae*) since the occurrence of one worm may decrease the chance of successful infection by another species (Christensen *et al.* 1987). In our data set, the number of *A. tetraptera* was negatively correlated with the number of *Heligmosomum mixtum* (Spearman's $r = -0.398$, $n = 78$, $P < 0.001$) and *Heligmosomoides glareoli* (Spearman's $r = -0.286$, $n = 54$, $P < 0.05$).

The effect of the DRB alleles on parasite prevalence was calculated using generalized linear models with the most common alleles as predictors. As the overall number of alleles was very high (58 potentially functional alleles), we preselected the fifteen most frequent alleles (occurring in more than 10% of individuals) and then excluded the less common alleles from each pair of significantly correlated frequent alleles. The correlation between alleles was tested by using permutation tests

of linkage disequilibria in ARLEQUIN 3.11 (Excoffier *et al.* 2005) with 10 000 permutations; individual alleles were coded as binary loci. The type I error was controlled through the Bonferroni procedure. This resulted in a set of nine alleles for the analyses. One of them (*Mygl-DRB*054*) was frequent in the Pilchy site (occurred in 13 individuals) but absent in other sites so it was fitted only in the models concerning *Heligmosomoides glareoli* (a parasite present exclusively in this site). Moreover, to reduce the number of variables to the recommended level of about 10% of the number of individuals (Neter *et al.* 1996), in models concerning *Heligmosomoides glareoli* in Pilchy we fitted only the five most frequent alleles. We additionally fitted sampling site, host sex, host maturity and occurrence of an antagonistic species to all models. As populations may vary with respect to the association between host and parasite genotypes, we fitted second order interactions between the population and the alleles. Since we tested a number of alleles, the probability of detecting a significant association with any of these alleles was increased. Therefore, the results were considered statistically significant if the analyses of the associations between the DRB alleles and the parasite load had *P*-value of 0.00625 or 0.01 for the analyses with the eight and five most common alleles respectively.

Results

Parasite load

We captured 132 bank voles: 54 at Pilchy (13 adult females, 5 adult males, 14 immature females and 22 immature males), 27 at Tałty (9 adult females, 4 adult males, 4 immature females and 10 immature males) and 51 at Urwitałt (16 adult females, 8 adult males, 12 immature females and 15 immature males). We identified seven species of intestinal helminths, one cestode (*Catenotaenia henttoneni*) and six nematodes (*Mastophorus muris*, *Heligmosomum mixtum*, *Heligmosomoides glareoli*, *Aspiculuris tetraptera*, *Syphacia petrusewiczi, Aonchoteca annulosa*). The majority of voles (83.4%) carried at least one intestinal helminth; however, single-species infections were the most common (59.8%). We found that 28 (21.2%) of the hosts harboured two parasite species and only three voles (2%) were infected by three parasites.

A. tetraptera was the most prevalent (38%), but *H. mixum* was most prevalent in Urwitalt (80% hosts infected). Four species, namely *C. henttoneni*, *M. muris*, *S. petrusewiczi* and *A. annulosa*, occurred in <10% of the voles and were not included in further analyses, except for the analysis of the individual species richness.

Parasites inhabiting the stomach (*M. muris*) and the small intestine (*C. henttoneni, H. mixtum, H. glareoli*) generally occurred in lower numbers than parasites present in the caecum and the colon (*A. tetraptera, S. petrusewiczi, A. annulosa*) (Table 1). The small number of *S. petrusewiczi* and *A. annulosa* was probably caused by the high intensity of *A. tetraptera* infections because these species are known competitors (Christensen *et al.* 1987).

MHC diversity

Amplification primers were identified in 45 777 reads, which corresponds to an average of 344.2 (SD = 199.7) reads per amplicon. Final genotypes were based on 35 126 reads (77% of the initial number) and the mean coverage at this stage was 266.1 reads (SD = 158.3, range 84-1057). The lack of correlation between the number of reads per individual and the number of detected alleles (Fig. S1, Supporting Information) indicates that the coverage was sufficient for reliable genotyping. Originally we identified 74 alleles (GenBank Accession Numbers for new alleles GQ901811–863), but 16 were removed from analysis because they were deemed potentially not functional (see Materials & Methods and Fig. S2, Supporting Information). A total of 58 potentially functional alleles were included in the analyses. The mean number of potentially functional alleles per individual was 4.75 (SD = 1.42, range 2–8). In the Pilchy site, we identified 41 alleles, in Tałty 26, and in Urwitałt 35. The number of unique alleles for particular sampling sites was high: 16 alleles occurred exclusively in Pilchy, five in Tałty and five in Urwitałt. However, the distribution of the eight most frequent alleles included in the models did not differ significantly between the sampling sites (Friedman test, $\chi^2 = 1.86$, d.f. = 2, $P = 0.393$), and their frequencies are given in Table S1 (Supporting Information).

Associations between MHC alleles and parasite load

As we expected, there was a significant effect of the presence of antagonistic parasitic species. The risk of infection by *Aspiculuris tetraptera* was lower when a host was already infected by one of the Heligmosomoidae species (23.6% vs. 55.7%). Similarly, voles were less often infected by *H. glareoli* when *A. tetraptera* was present (85% vs. 29.1%). The infection intensity of *A. tetraptera* was significantly lower when either *H. mixtum* or *H. glareoli* was present (1.9 vs. 16.5 worms/host) (Table 2).

The prevalence of *A. tetraptera* was higher in mature hosts than in juveniles (47.3% vs. 32.4%), and adults also suffered from more abundant infections (32.4 vs.15.7 worm/host). The effect of sex was significant only in the case of *H. glareoli*, where females were infected more often (59.2% vs. 29.6%) and on average harboured more worms (2.8 vs. 0.7 worms/host).

Table 1 The helminth burden in bank voles from three sampling sites

	Localization	Prevalence (%)					Infection intenstity (worms/infected host)				
		Pilchy, $n = 54$	Talty, $n = 27$	Urwitalt, $n = 51$	Overall	G test (χ^2 and P)	Urwitatl, $n = 51$	Pilchy, $n = 55$	Talty, $n = 30$	Overall	Kruskal-Wallis test (χ^2 and P)
Catenotaenia henttonem	Small intestine	3.7	3.7	15.6	8.3	11.383**	1.25 (1–2)	1.0 (1)	1.0 (1)	1.2 (1–2)	5.973 ns
Mastophorus muris	Stomach	9.2	6.7	0	5.3	0.1956ns	0	2.66 (1–8)	1.0 (1)	2.2 (1–8)	0.115 ns
Heligmosomum mixtum	Small intestine	0	23.3	80.3	36.1	29.234***	4,54 (1–18)	0	1.71 (1–4)	4.1 (1–18)	24.599***
Heligmosomoides glareoli	Small intestine	44.4	0	0	18.0	—	0	3.92 (1–27)	0	3.9 (1–27)	—
Aspiculuris tetraptera	Caecum and colon	46.3	70	11.7	38.3	50.664***	3.0 (1–11)	25.32 (1–210)	23.43 (1–141)	21.9 (1–210)	34.293***
Syphacia petruszewiczii	Caecum and colon	0	3.3	0	0.7	—	0	0	2.0 (2)	2 (2)	—
Capillaria sp.	Small intestine	0	1	0	2.2	—	0	0	2,0 (1–3)	2 (1–3)	—

Prevalence is expressed as a percent of the animals infected. The tests for differences between sampling sites include only cases of non-zero prevalence (i.e., sites where a parasite was not present were omitted).
Significance codes *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

After correcting for the number of alleles tested, we found a significant population-specific effect of the allele *Mygl-DRB*024* on the intensity of infection with *A. tetraptera* (Table 2). In the Pilchy site, animals carrying this allele had fewer worms than animals without it, but in Tałty, the association was the opposite (Fig. 1). We also found a significant effect of the allele *Mygl-DRB*028* on the infection intensity of *A. tetraptera* (Table 2). The average infection intensity in individuals carrying this allele was lower than in the remaining individuals (respectively, 5.1 (range 1–70) vs. 10.8 (range 1–210) worm/host).

Individual MHC diversity and parasite load

The number of alleles significantly affected the prevalence and intensity of infection with *Aspiculuris tetraptera* (Table 3). The relationship between the number of alleles and prevalence was not linear, as only a quadratic term was significant (Table 3, Fig. 2a). However, in the model for infection intensity, only the linear term was significant and the parasite load in animals with a high number of alleles was lower than in animals with few alleles (Fig. 2b). For other parasite species, neither a linear nor a quadratic term was significant (Table 3). We also found a significant, quadratic relationship between the number of alleles and the number of parasite species infecting an individual, with the lowest number of parasite species in individuals with the intermediate number of DRB alleles (Table 4; Fig. 3).

Microsatellite variation

Genotype frequencies in six of the seven microsatellite loci did not show significant departures from the Hardy–Weinberg equilibrium after the Bonferroni adjustment. Only these six loci were used to quantify the population differentiation. The overall differentiation was substantial as evidenced by the overall $F_{ST} = 0.066$ (95% CI 0.046–0.089) and the highly significant tests of differentiation in allele frequencies ($P < 0.0001$). The overall average effective number of migrants per generation estimated from the F_{ST} under the assumption of the equilibrium island model is 3.5 (95% CI 2.6–5.2). The F_{ST} between pairs of populations ranged from 0.038 to 0.086 which corresponds to 2.7 to 6.4 effective migrants per generation. All the pairwise test of the population differentiation were highly significant ($P < 0.0001$).

Discussion

Using the next generation sequencing (NGS)-based genotyping method we were able to resolve multilocus

Table 2 Results of generalized linear models for investigating the impact of DRB alleles on parasite prevalence and infection intensity

	Prevalence									Infection intensity								
	A.tetraptera			*H. mixtum*			*H. glareoli*			*A.tetraptera*			*H. mixtum*			*H. glareoli*		
	Δ dev	**d.f.**	***P***	**Δ dev**	**d.f.**	***P***	**Δ dev**	**d.f.**	***P***	**Δ dev**	**d.f.**	***P***	**Δ dev**	**d.f.**	***P***	**Δ dev**	**d.f.**	***P***
Site	**12.916**	2	**0.002**	**10.155**	**1**	**0.001**	—	—	—	**381.42**	**2**	**0.001**	**27.303**	**1**	**0.005**	—	—	—
Host maturity	**3.943**	1	**0.047**	3.127	1	0.077	0.157	1	0.692	**370.46**	**1**	**<0.001**	10.596	1	0.077	1.37	1	0.58
Host sex	0.026	1	0.873	0.043	1	0.836	**4.867**	1	**0.027**	0.009	1	0.96	3.580	1	0.304	**22.435**	1	**0.025**
Other helminth	**4.189**	1	**0.041**	0.002	1	0.961	**5.363**	1	**0.021**	**394.29**	**1**	**<0.001**	3.114	1	0.338	4.641	1	0.308
*Mygl-DRB**024	0.046	1	0.831	0.010	1	0.922	0.287	1	0.592	97.35	1	0.11	2.598	1	0.381	7.633	1	0.191
*Mygl-DRB**028	3.226	1	0.072	1.445	1	0.229	—	—	—	**234.31**	1	**0.004**	3.898	1	0.284	—	—	—
*Mygl-DRB**029	1.525	1	0.217	2.284	1	0.131	1.190	1	0.275	9.21	1	0.56	14.525	1	0.038	11.488	1	0.109
*Mygl-DRB**030	1.546	1	0.214	0.086	1	0.769	0.410	1	0.552	1.16	1	0.84	0.135	1	0.842	8.687	1	0.163
*Mygl-DRB**041	1.835	1	0.176	1.473	1	0.225	—	—	—	81.0	1	0.09	0.070	1	0.886	—	—	—
*Mygl-DRB**044	0.007	1	0.934	0.006	1	0.939	1.261	1	0.261	10.59	1	0.54	0.419	1	0.725	17.14	1	0.05
*Mygl-DRB**047	0.365	1	0.546	0.023	1	0.881	—	—	—	2.92	1	0.75	0.272	1	0.777	—	—	—
*Mygl-DRB**054	—	—	—	—	—	—	2.049	1	0.152	—	—	—	—	—	—	1.305	1	0.589
popul:*Mygl-DRB**024	0.989	2	0.610	0.028	1	0.866	—	—	—	**445.95**	2	**<0.001**	9.269	1	0.770	—	—	—
popul:*Mygl-DRB**028	6.246	2	0.044	2.792	1	0.095	—	—	—	0.96	2	0.98	0.245	1	0.780	—	—	—
popul:*Mygl-DRB**029	0.364	2	0.833	1.508	1	0.219	—	—	—	25.64	2	0.59	3.298	1	0.305	—	—	—
popul:*Mygl-DRB**030	3.806	2	0.149	2.166	1	0.141	—	—	—	134.74	2	0.06	2.935	1	0.333	—	—	—
popul:*Mygl-DRB**041	0.775	2	0.679	0.389	1	0.533	—	—	—	32.77	2	0.51	3.154	1	0.316	—	—	—
popul:*Mygl-DRB**044	1.807	2	0.405	0.055	1	0.815	—	—	—	6.41	2	0.88	0.240	1	0.782	—	—	—
popul:*Mygl-DRB**047	2.450	2	0.294	0.006	1	0.936	—	—	—	16.03	2	0.72	0.003	1	0.975	—	—	—

Terms significant after the Bonferroni correction for the number of tested alleles are given in bold.

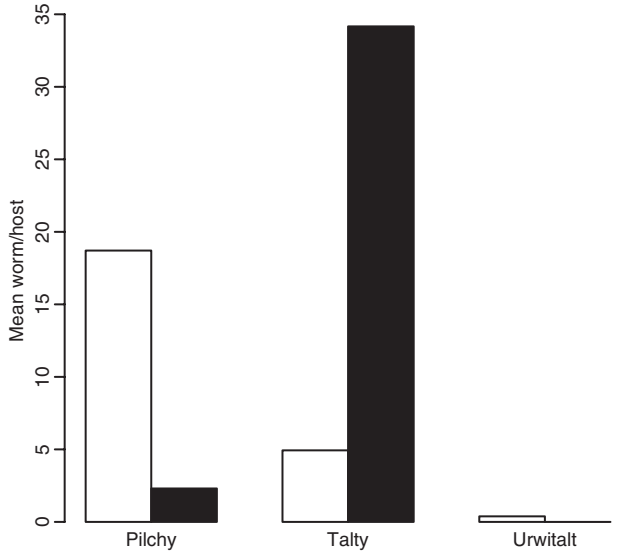

Fig. 1 The effect of the interaction between the *Mygl-DRB*024* allele and the population on the intensity of infection with *A. tetraptera*. Bars represent mean numbers of worms/individual. Solid bar—allele present, open bar—allele absent.

DRB genotypes in bank voles and test the optimality hypothesis for the number of MHC alleles. Diversity of DRB sequences detected in the current study was higher than previously reported (Axtner & Sommer 2007; Babik & Radwan 2007), which is probably the consequence of the use of the new bank vole-specific primers located in the conserved portions of the DRB 2nd exon (details in Supporting Information).

We found evidence supporting optimality hypothesis at two levels. First, we found that individuals with an intermediate number of DRB alleles were burdened with the lowest number of parasite species (Fig. 3). Second, we found that individuals with an intermediate number of alleles were the most resistant to the most prevalent gut parasite, the pinworm nematode *Aspiculuris tetraptera*. Heavy pinworm infections, such as those recorded in our study, can cause adverse effects on hosts, including enteritis (Hendrix 1998). We also performed analyses on two other common nematode species, *Heligmosomum mixtum* and *Heligmosomoides glareoli*, but found no significant effects. However, the *Heligmosomoididae* species did not occur in all three populations, so the number of individuals entered into analyses was lower than for *A. tetraptera*. Thus, the nonsignificant results for *Heligmosomoididae* may simply reflect the lower power of these analyses, rather than the weak selection preassure exerted by this parasites. Indeed, the *H. mixtum* has been shown to increase mortality of *M. glareolus* under poor quality food (Haukisalmi & Henttonen 2000).

Table 3 Associations between the number of DRB alleles carried by a host and the load of the three most common parasites

	Prevalence									Infection intensity								
	A. tetraptera			*H. mixtum*			*H. glareoli*			*A. tetraptera*			*H. mixtum*			*H. glareoli*		
	Δ dev.	d.f.	*P*	**Δ** dev.	d.f.	*P*	**Δ** dev.	d.f.	*P*	**Δ** dev.	d.f.	*P*	**Δ** dev.	d.f.	*P*	**Δ** dev.	d.f.	*P*
Sampling site	**20.283**	2	**<0.001**	**13.246**	1	**<0.001**	—	—	—	**416.74**	2	**<0.001**	**41.153**	1	**<0.001**	—	—	—
Host maturity	**4.936**	1	**0.026**	1.716	1	0.190	0.002	1	0.966	**649.1**	1	**<0.001**	9.161	1	0.088	4.034	1	0.443
Host sex	0.009	1	0.924	0.091	1	0.763	3.348	1	0.067	0.10	I	0.95	0.984	1	0.575	22.365	1	0.071
Presence of antagonist species	3.156	1	0.076	0.038	1	0.845	4.350	1	0.037	**405.43**	1	**<0.001**	3.387	1	0.299	3.383	1	0.438
No. of alleles	0.672	1	0.412	0.921	1	0.337	0.144	1	0.704	**268.88**	1	**0.0018**	4.714	1	0.220	0.087	1	0.912
(No. of alleles)^2	**0.471**	1	**0.033**	0.040	1	0.842	0.012	1	0.914	4.94	1	0.67	0.182	1	0.809	0.570	1	0.663

While resistance to *A. tetraptera* was nonlinearly associated with the number of alleles, the intensity of infection decreased linearly with the number of alleles. The optimality hypothesis is based on the conjuncture that on one hand, a high diversity of alleles enables recognition of a large number of antigens, but on the other hand causes self-reacting with a higher proportion of lymphocytes during negative selection (Nowak *et al.* 1992). Higher proportions of deleted lymphocytes in individuals with a large number of alleles could consequently result in a limited ability of the immune system to resist infection (hence increased prevalence). But the intensity of the infection with each species could still be controlled in some limited extent, as nearly all parasite genotypes will be presented by one of the many MHC alleles. While this scenario could account for the quadratic association with prevalence and the linear association with infection intensity, it should be noted that the actual mechanism preventing evolution of the ever-increasing number of MHC alleles remains unknown, and the role of negative selection has been questioned (Borghans *et al.* 2003).

Optimal resistance to parasites at intermediate levels of individual MHC diversity was previously reported for sticklebacks (Wegner *et al.* 2003a, b). Our study provides another example of such an association, but for the first time in a mammal. A similar mechanism has been argued to be at work in house sparrows (Bonneaud *et al.* 2006a), where females with intermediate numbers of alleles laid the highest number of eggs and maintained large clutches after immune challenge, whereas the clutches of females with extreme numbers of alleles were reduced. However, a direct link of the optimal number of alleles with increased parasite resistance in house sparrows has not yet been demonstrated. NGS-based genotyping methods will allow the testing of this hypothesis in a variety of other, non-model species.

Another important finding of our study is the population-specificity of the association between MHC type and infection intensity (Fig. 1). Population specificity has been previously reported for MHC-malaria associations in humans (Hill 1998) and house-sparrows (Bonneaud *et al.* 2006b). These results imply that selection acting on MHC can dramatically differ in space, probably due to differences in the composition of parasitic species or parasite genotypes within the species. If gene flow between subpopulations is limited, then such diversifying selection can maintain a higher level of genetic variation at the population level compared to a panmictic situation (Christiansen 1974). Compared to the house sparrow study, where population structure was significant but the F_{ST} value was as low as 0.004 (Bonneaud *et al.* 2006a), we found more limited gene flow, with an F_{ST} value of 0.07, despite the close geographical distance between the subpopulations. This level of gene flow is more conducive to the maintenance of a higher level of genetic polymorphism (Christiansen 1974), and diversifying, population-specific selection of the MHC allele can thus provide an additional mechanism, apart from the heterozygote advantage and frequency-dependent selection that maintains high levels of MHC polymorphism. Spatial variation in MHC alleles composition, suggestive of geographical variation in selective pressure, has previously been reported in several species (e.g. Ekblom *et al.* 2007; Alcaide *et al.* 2008; Oliver *et al.* 2009).

Of course, not all the alleles associated with the level of infection need to show population-specific effects, and we also found an example of an allele (*Mygl-DRB**028) associated with an increased resistance to *A. tetraptera* across the populations. The association of the particular alleles with the prevalence of helminths was previously shown in several other rodent species: grass mouse *Rhabdomys pumilio* (Froeschke & Sommer 2005), yellow necked mouse *Apodemus flavicolis* (Meyer-Lucht & Sommer 2005) and hairy-footed gerbil *Gerbillurus paeba* (Harf & Sommer 2005).

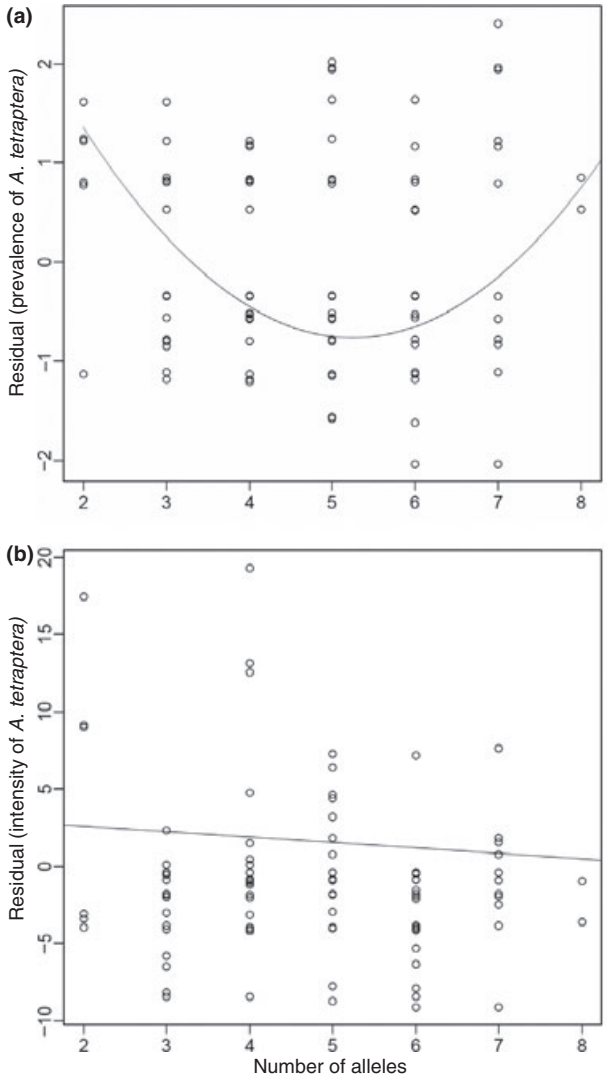

Fig. 2 The effect of the number of DRB alleles carried by a host on prevalence (a) and intensity (b) of infection with *A. tetraptera*. Prevalence and infection intensity are expressed as residuals from a generalized linear model including population, host maturity, host sex and the presence of an antagonistic parasite as covariates (Table 3). Trend line (a) $y = 4.75 - 2.16x + 0.21x^2$, (b) $y = 3.29 - 0.35x$.

Table 4 Effect of the number of DRB alleles on the number of parasite species infecting a host

	Multiple infections		
	Δ deviance	d.f.	*P*
Sampling site	0.182	2	0.785
Host maturity	**4.657**	1	**<0.001**
Host sex	1.157	1	0.080
No. of alleles	0.002	1	0.942
(No. of alleles)^2	**2.174**	1	**0.010**

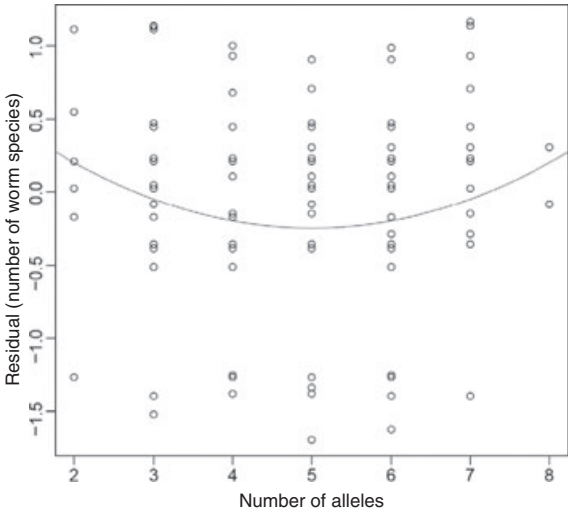

Fig. 3 The effect of the number of DRB alleles on the host parasite species richness (the number of parasite species per individual). Data points represent residuals from the generalized linear model including population, host maturity, host sex and the presence of an antagonistic parasite as covariates (Table 4). Trend line $y = 0.97 - 0.45x + 0.05x^2$.

Our analyses are likely to underestimate the number of associations between the DRB alleles and parasite load, as only the most common alleles could be entered into the statistical analysis. Further work should be done with larger sample sizes, including time-series to trace changes in the selective pressures on MHC alleles not only in space, but also in time. NGS-based genotyping now makes it possible to efficiently study complex, multilocus MHC genotypes, which are practically not possible to resolve using other methods.

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Conflicts of interest

The authors have no conflict of interest to declare and note that the sponsors of the issue had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Frequencies of individuals possessing the eight most common potentially functional alleles detected in the three populations of the bank vole.

Fig. S1 The relationship between the per individual coverage and the number of detected alleles. Both potentially functional and nonfunctional (see text) alleles are included.

Fig. S2 Diversity of the bank vole MHC II DRB sequences detected with primers MgDRBL and MgDRBR. The Neighbor Joining tree was constructed from the matrix of the Jukes-Cantor nucleotide distances; bootstrap values of 70% and above (1000 replicates) are shown.

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