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Multiple parasites mediate balancing selection at two MHC class II genes in the fossorial water vole: insights from multivariate analyses and population genetics

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Abstract

We investigated the factors mediating selection acting on two MHC class II genes (*DQA* and *DRB*) in water vole (*Arvicola scherman*) natural populations in the French Jura Mountains. Population genetics showed significant homogeneity in allelic frequencies at the *DQA1* locus as opposed to neutral markers (nine microsatellites), indicating balancing selection acting on this gene. Moreover, almost exhaustive screening for parasites, including gastrointestinal helminths, brain coccidia and antibodies against viruses responsible for zoonoses, was carried out. We applied a co-inertia approach to the genetic and parasitological data sets to avoid statistical problems related to multiple testing. Two alleles, Arte-DRB-11 and Arte-DRB-15, displayed antagonistic associations with the nematode *Trichuris arvicolae*, revealing the potential parasite-mediated selection acting on *DRB* locus. Selection mechanisms acting on the two MHC class II genes thus appeared different. Moreover, overdominance as balancing selection mechanism was showed highly unlikely in this system.

Introduction

Major histocompatibility complex (MHC) genes are ideal candidates for the study of selection in natural populations (Bernatchez & Landry, 2003). The way in which their extreme polymorphism evolved remains a matter of debate (review in Piertney & Oliver, 2006). Balancing selection has frequently been detected on these genes at the macroevolutionary timescale, as shown by studies of substitution patterns or trans-species polymorphism (e.g. Musolf *et al.*, 2004; Bryja *et al.*, 2006). Evidence has recently been found for selection acting over the microevolutionary timescale, based on comparisons of population genetic structures for MHC and neutral genes (e.g. Landry & Bernatchez, 2001; Charbonnel & Pemberton, 2005; but see Seddon & Ellegren, 2004). Contemporary selection can also be detected by investigating associations between MHC alleles and infection status. Parasite-mediated balancing selection (PMBS) has long been suspected because of the key immune function of these genes (foreign peptide recognition triggering specific immune response, Hughes & Yeager, 1998; Delves *et al.*, 2006) and the ubiquitous nature of this immune function in different taxa (Klein & O'Huigin, 1994). Associations have thus frequently been reported in the context of human infectious diseases (Hill *et al.*, 1991; Thursz *et al.*, 1995; Carrington *et al.*, 1999) or for a single parasite species in wild host populations (Briles *et al.*, 1977; Langefors *et al.*, 2001; Bonneaud *et al.*, 2006). This approach may not meet the requirements of evolutionary studies, due to the difficulties involved in selecting a relevant, single, parasite species. Moreover, evidence suggests that multiple parasites are required to drive

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MHC polymorphism (McClelland *et al.*, 2003; Wegner *et al.*, 2003). Given this constraint, associations between MHC genes and infection have been explored focusing on parasite richness or burden (Prugnolle *et al.*, 2005; Schad *et al.*, 2005). This approach is more satisfactory but is also subject to certain limitation, as such indices of parasite pressure do not reflect the particular selection mediated by each parasite species (especially as concerns antagonistic effects between species, e.g. Meyer *et al.*, 1994). The associations between MHC alleles/genotypes and the specific composition of the parasite community have, to our knowledge, never been studied.

In this study, we aimed to determine whether balancing selection was mediated by multiple parasites and to identify the parasite species acting on MHC polymorphism. The fossorial water vole (*Arvicola scherman*, Arvicolinae, Cricetidae) is a relevant model for exploring this question, because it suffers from only moderate numbers of macroparasite infections (Cerqueira *et al.*, 2007), making it possible to carry out almost exhaustive screening. We considered gastrointestinal helminths, brain coccidias and viral agents of zoonoses. MHC class II genes were studied, as they encode proteins that present exogenous antigens to T helper cells, and are therefore involved in the recognition of extracellular parasites (Hughes & Yeager, 1998). Experiments on MHC class II-deficient mice have shown that MHC class II genes are also involved in immunity against viruses (Apanius *et al.*, 1997). *DQA* and *DRB* are the most studied MHC class II genes in small mammals (Charbonnel *et al.*, 2006). Both genes were included in this study, and the second exon of these genes was studied, as it encodes the peptide-binding site (PBS, in humans: Brown *et al.*, 1993; and in voles: Bryja *et al.*, 2006). Associations between host genetics and parasite load have already been reported for the *DRB* gene in several wild populations (Froeschke & Sommer, 2005; Schad *et al.*, 2005; Schwensow *et al.*, 2007). In Arvicolinae (voles and lemmings), evidence has been obtained for both historical and contemporary balancing selection acting on *DQA* and *DRB*, using phylogenetic and population genetic methods (Bryja *et al.*, 2006, 2007; Oliver & Piertney, 2006).

The specific objectives of this study were to explore associations between genetics (MHC class II *DQA* and *DRB* genes, in terms of alleles and heterozygosity, and global genomic heterozygosity) and parasitism (parasite species and parasite richness) in natural populations of water vole. Multivariate analyses made it possible to consider each parasite species as a potential agent mediating selection while minimizing type I statistical errors due to multiple testing. Detecting associations would reveal the potential action of parasites as selective pressure. It would also help in discriminating between two possible mechanism of PMBS: (i) negative frequency dependence (or rare allele advantage, Doherty & Zingernagel, 1975), which requires associations between parasites and specific alleles; and (ii) overdominance (or heterozygote advantage, Takahata & Nei, 1990), which assumes associations with MHC heterozygosity or genomic heterozygosity (in the latter case, a mechanism of sexual selection would also be involved, Penn, 2002). We also used intrapopulation genetic analyses to detect balancing selection acting in the history of populations, and to evidence heterozygote excess, an argument favouring the hypothesis of overdominance.

Materials and methods

Species, sampling design and parasite screening

The fossorial water vole (*A. scherman*) is an arvicoline species that typically inhabits grasslands and experiences multiannual density cycles in various regions of France and Switzerland (Saucy, 1994). We sampled water vole populations at six sites in eastern France (Nozeroy, Jura, Franche-Comté, France, Fig. 1), during three successive years (2003, 2004 and 2005). A group of animals sampled from a single site in a particular year is referred to as a population. Only three populations are similar to those studied by Bryja *et al.* (2007): A = 03, B = 05, C = 06, in 2003. Sampling was carried out in October, as the prevalence levels of most viruses or helminth parasites of mammals globally increase from spring to autumn (e.g. Haukisalmi *et al.*, 1988). This has been confirmed in these water vole populations considering viruses (*Cowpox virus*, Deter *et al.*, unpublished data) and nematodes (*Trichuris arvicolae* and *Syphacia nigeriana*, Deter *et al.*, 2007a). Autumn sampling is thus expected to reflect the highest potential pressure exerted by parasites and pathogens on water vole populations. The areas sampled each covered about 2 ha and were separated by 4 to 13 km. We used 80 Sherman live traps per site, but only one live trap per colony (i.e. nest), to prevent the sampling of related individuals. This led to the capture of about 20 animals per site per day of sampling. If more than 24 voles were trapped, we simply took a tail sample from the additional animals for genetic analyses and then released them. Voles were brought to the laboratory and housed individually for 1 week in standardized conditions for the need of physiological experiments (Charbonnel *et al.*, 2008). Voles were then killed by cervical dislocation, as recommended by Mills *et al.* (1995), weighed and dissected. Only sexually mature individuals were retained for the analysis. Pregnant females and females that gave birth in the laboratory were excluded from the analyses. Toe tissue was kept in 95° ethanol for the extraction of DNA. Blood samples were taken from the heart and centrifuged. Serum samples were screened for IgG antibodies against *Puumala virus* (PUUV, *Hantavirus*), *Cowpox virus* (CPXV, *Orthopox virus*) and *Lymphocytic choriomeningitis virus* (LCMV, *Arenavirus*), by

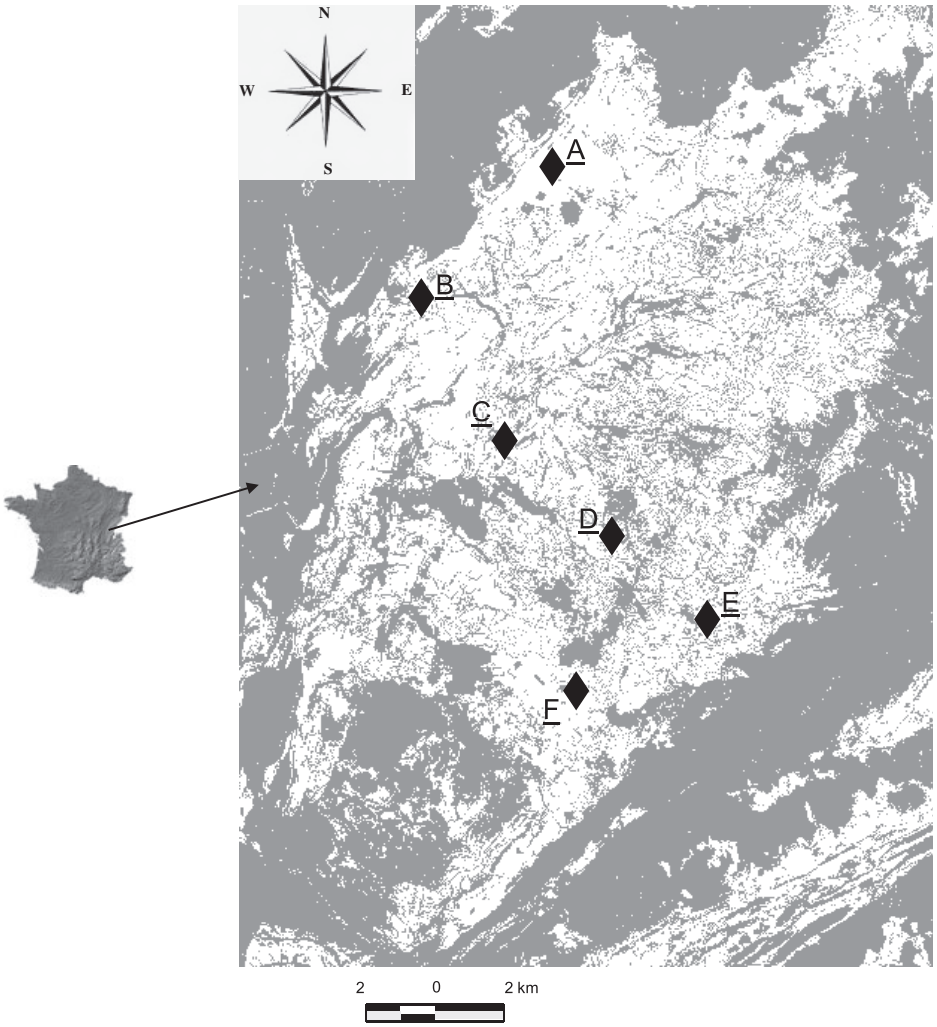

Fig. 1 Location of the sampling sites. On the left, situation of the Jura Mountains (Franche-Comté) in France. On the right, map of the Nozeroy canton with the six sampling sites indicated.

means of immunofluorescence assays (IFA), as described by Kallio-Kokko *et al.* (2005). Brains were crushed between slides and checked under a microscope for the presence of *Frenkelia* cysts (Coccidia, Apicomplexa: Sarcocystidae). Helminths from the liver and the body cavity were identified during dissection of the vole. Finally, the digestive tracts were stored in 95° ethanol before dissection. Helminths were identified under the microscope. The diseases caused by helminths are generally more dependent on infection intensity than on infection status (May & Anderson, 1979). We therefore counted the individuals detected for each helminth species when possible. Parasite richness is expressed as the number of parasite species detected per vole. We considered both helminth richness (R_h) and total richness (R_p), which included viruses and coccidia.

MHC class II gene and microsatellite genotyping

Genomic DNA was extracted from a single vole toe, using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, using 400 μL of elution buffer.

We amplified the complete exon 2 of *DQA* and part of the exon 2 of *DRB* for genotyping, as described in Bryja *et al.* (2005, 2007). Primers were fluorescently labelled with different dyes (FAM, HEX or NED). Capillary electrophoresis-single strain conformation polymorphism (CE-SSCP) analysis was performed on a MegaBACE 1000® DNA analysis system (Amersham Biosciences, Buckinghamshire, UK) (see details on the method in Bryja *et al.*, 2005). The *DQA* gene is duplicated in water voles and the second copy (*DQA2*) is not present in all individuals (see Bryja *et al.*, 2006). We identified two

alleles at this locus by cloning and sequencing, but these alleles could not be distinguished by CE-SSCP. We therefore decided to consider only the polymorphism of *DQA* duplication, namely the presence/absence of *DQA2*.

All individuals were genotyped at nine polymorphic autosomal microsatellite loci specifically developed in *A. scherman*, as described by Berthier *et al.* (2004). This made it possible to infer the effects of neutral evolutionary processes on MHC gene polymorphism.

Detecting genetic/parasitological associations using co-inertia analyses

We used multivariate analyses to investigate relationships between: (i) an MHC class II genetic matrix including, for each individual screened, the presence/absence for each allele of the *DQA1* and *DRB* genes, *DQA1* and *DRB* heterozygosity (0 = homozygote and 1 = heterozygote), the presence/absence of *DQA2*, microsatellite heterozygosity (0 = low level of heterozygosity and 1 = high level of heterozygosity); and (ii) a parasitological matrix including, for each individual screened, the abundance of each parasite species and parasite richness indices (R_h and R_p).

We analysed jointly all vole individuals providing from 15 samplings. Indeed, genetic differentiation among them is expected to be very low in this system as the sites sampled are geographically very close (Fig. 1), and as sampling was carried out during the 'increase' and 'outbreak' phases of the cycle (years 2003–2004–2005, see Charbonnel *et al.*, 2008), when the effective size of water vole populations was very high (Berthier *et al.*, 2006; Bryja *et al.*, 2007). However, as spatial structure may increase the probabilities of missing or identifying false parasitological/genetic associations, we checked for genetic structure among years and localities, computing global F_{ST} estimates over all microsatellites, and testing global genetic differentiation using GENEPOP 3.3 software (Raymond & Rousset, 1995).

Multivariate methods have been widely used in ecology, particularly for the study of species/environment relationships (Dolédec & Chessel, 1994), but have not previously been used in studies of genetic associations. Considering the large number of genetics and parasitological variables included here, more conventional univariate statistics would rely on hundreds of tests and would thus require corrections for multiple testing. The acceptable type I error in all univariate statistical tests would thus be very low ($< 10^{-4}$) and such a method would be very conservative. In this way, multivariate approach revealed particularly relevant, avoiding the increase of type I errors. We carried out co-inertia analysis (ACO), searching for axes that maximize the covariance between the row coordinates of two matrices (Dray *et al.*, 2003). We chose this method as it can be used with all types of variables and is robust to correlation between variables from a given matrix (Dray *et al.*, 2003). This analysis involves two steps. Each matrix is first analysed independently – the MHC genetic matrix by correspondence analysis (CA) and the parasitological matrix by principal component analysis (PCA, correlation matrix), using the row weights of the CA. These analyses can be displayed on factorial maps, to evidence particular distributions of alleles or of parasites potentially affecting the interpretation of associations between genetics and parasites. The global relationship between the two matrices was then estimated from the co-inertia (vectorial correlation) of the matrices. Relationships between the two matrices were assessed by comparing the co-inertia estimated from the real data set with the co-inertia distribution estimated after 1000 permutations of the parasitological matrix lines. Particular associations were detected by eye from the distribution of genetic and parasitological variables on the ACO factor map. Variables located in a given direction with respect to the origin were considered to be positively associated, whereas those located in the opposite direction were considered to be antagonistic. Variables close to the origin were considered to be unrelated to the variables of the other matrix. These associations were then tested statistically, using a cross-validation approach (Draper & Smith, 1981; see, e.g. Hallgren *et al.*, 1999). We randomly split the data into two independent data sets and carried out two independent ACO. Associations between genetic and parasitological data were considered significant if they were observed in both data sets. This approach also prevents from corroborating associations, which would only be artefacts of population structure. The relative risk (RR) associated with these genetic variables of interest was estimated as described by Haldane (1956). All multivariate analyses were carried out with ADE-4 software (Thioulouse *et al.*, 1997).

According to the frequency-dependent selection hypothesis, we expected to find positive or negative associations between the presence of specific MHC alleles and parasite species presence/abundance. Negative associations may be interpreted as indicating alleles conferring resistance to the parasite species, whereas positive associations indicate susceptibility to the parasite species. Alternatively, in situations of overdominance, negative associations should be observed between estimation of genetic diversity (heterozygosity at MHC genes, at microsatellites or MHC locus duplication) and specific infection or parasite richness.

Within-population genetic analyses

As demography affects all the genome, whereas selection acts on a single locus, comparisons of patterns for MHC genes and microsatellites may provide evidence for balancing selection (Cavalli-Sforza, 1966; Luikart *et al.*, 2003). We first tested for linkage disequilibrium for each pair of loci, including nine neutral microsatellite loci, *DQA1* and *DRB*, in each population. We used exact tests

and Markov chains, as implemented in GENEPOP (Raymond & Rousset, 1995). Corrections for multiple tests were performed using the false discovery rate (FDR) approach, as described by Benjamini & Hochberg (1995). We then described genetic diversity for the *DQA1* and *DRB* genes, and for all the microsatellites considered together, in each vole population. We calculated expected and observed heterozygosity (H_E and H_O), using GENETIX software (Belkhir *et al.*, 1996–2004) and the unbiased estimator $\hat{f}$ of Wright's inbreeding coefficient F_{IS}, as described by Weir & Cockerham (1984), using FSTAT 2.9.3 (Goudet, 1995).

Homogeneity in allelic frequencies is expected not only for loci under balancing selection (Watterson, 1978), but also for all the genome after a recent demographic bottleneck (Watterson, 1986). Such demographic events are likely to occur in water vole populations, which periodically decline to low densities (Saucy, 1994; Charbonnel *et al.*, 2008). Under balancing selection, a significant deviation from mutation–drift equilibrium in MHC genes is expected, whereas microsatellite loci would be at equilibrium. For each locus, we tested for deviation of gene diversity from equilibrium using BOTTLENECK 1.2.02 program (Piry *et al.*, 1999), simulating 10 000 data sets. Contrary to Ewens–Watterson test (Watterson, 1978), this test allows a variety of mutation model (Piry *et al.*, 1999). We used an infinite allele mutation (IAM) model for MHC genes and a generalized stepwise mutation (GSM) model for microsatellites (with the variance of the geometric distribution fixed at 0.36, Estoup *et al.*, 2001). Sequential Bonferroni corrections (Rice, 1989) were used to correct for multiple testing.

If overdominance occurs, selection should result in an excess of heterozygotes at MHC genes (Doherty & Zingernagel, 1975; Garrigan & Hedrick, 2003), with the other loci at equilibrium. This heterozygote excess might be detected through the demonstration of Hardy–Weinberg disequilibrium at MHC class II genes, with equilibrium at microsatellites (disequilibrium at all loci would reflect outbreeding, which may be MHC independent). An exact test of heterozygote excess was performed, using the Markov chain methods implemented in GENEPOP 3.3 to test this hypothesis.

Results

Data collection

We sampled 302 water voles for parasite screening and 367 for population genetic analyses. In 2005, the three sites located in the north of the study area had such low population densities that it was not possible to trap any animals over a period of several days.

We tested for antibodies against PUUV, CPXV and LCMV. Seroprevalence results are shown in Table 1. Two coccidian species were observed in the brain (*Frenkelia microti* and *Frenkelia glareoli*), four nematodes (*T. arvicolae*, *S. nigeriana*, *Aonchotheca* sp. and *Eucoleus bacillus*) and four adult cestodes (*Anoplocephaloides dentata*, *Paranoplocephala gracilis*, *Paranoplocephala omphalodes* and *Arostrilepis cf*

Table 1 List of viruses and macroparasites identified in the 302 water voles screened.

	Species	Code	N_p	Prevalence
Viruses				
Hantavirus	Undetermined	PUUV	8	2.61
Orthopox virus	*Cowpox virus*	CPXV	60	19.60
Arenavirus	*Lymphocytic choriomeningitis virus*	LCMV	12	3.92
Coccidia				
Sarcocystidae	*Frenkelia glareoli*	Cocc-Fg	44	14.38
	Frenkelia microti	Cocc-Fm	7	2.28
Helminths				
Nematoda	*Syphacia nigeriana*	Nem-Sn	85	27.77
	Trichuris arvicolae	Nem-Ta	132	43.14
	Aonchotheca sp.	Nem-Asp	27	8.82
	Eucoleus bacillus	Nem-Eb*	1	0.33
Cestoda larvae	*Ecchinococcus multilocularis*	Cest-Em	26	8.49
	Taenia taeniaeformis	Cest-Tt	56	18.3
	Taenia crassiceps	Cest-Tc*	1	0.33
Cestoda adults	*Anoplocephaloides dentata*	Cest-Ad*	1	0.33
	Paranoplocephala gracilis	Cest-Pg	4	1.31
	Paranoplocephala omphalodes	Cest-Po	73	23.86
	Arostrilepis cf horrida	Cest-Ah	27	8.82

Nem-, Cest and Cocc- refer to nematodes, cestodes and coccidia respectively. N_p is the number of infected (macroparasites, protozoans) or seropositive (viruses) animals identified in the whole data set. Prevalence estimates are presented as percentages. An asterisk indicates parasites not included in co-inertia analyses because their prevalence was too low.

horrida) were detected in the digestive tract. Two larval cestodes were observed in the liver (*Echinococcus multilocularis* and *Taenia taeniaeformis*) and one in the body cavity (*Taenia crassiceps*). Detailed prevalence values are shown in Table 1. Individual helminth richness ranged between 0 and 5 and total parasite richness between 0 and 7. As the helminths *E. bacillus*, *T. crassiceps* and *A. dentata* were each found in only one individual (Table 1), we could not include these species in the association analyses.

We identified five alleles at *DQA1* and 12 alleles at *DRB* in the 367 water voles screened for DNA analyses. All these alleles have previously been detected, cloned and sequenced in water voles (Bryja *et al.*, 2006, 2007). Two *DRB* alleles (Arte-DRB-12 and Arte-DRB-14) were found at very low frequencies (< 0.01) and so could not be included in association analyses.

Co-inertia analyses

Global genotypic differentiation over years and localities was low (F_{ST} = 0.017, P < 0.001), especially compared with other *A. sherman* studies in closed localities (F_{ST} estimates ranged between 0.015 and 0.037; Berthier *et al.*, 2006; Bryja *et al.*, 2007).

Co-inertia analysis was first carried out for the 302 individuals screened for both the 19 genetic (five *DQA1* alleles, 10 *DRB* alleles, *DQA1*, *DRB* and microsatellite heterozygosity, presence of *DQA2*) and 15 parasitological variables (three viruses, two coccidias, eight helminth species, helminth and total parasite richness). The binary variable reflecting individual microsatellite heterozygosity was obtained using the proportion of microsatellite locus being heterozygote. This variable thus makes out as 'high level of heterozygosity' the individuals having at least eight heterozygous microsatellites out of nine (21% of the individuals). As the choice of the threshold may influence the results, we also performed this analysis considering as 'highly heterozygote' the individuals presenting at least six heterozygous loci (76.8% of the individuals).

The CA based on the genetic table (Fig. 2) revealed that the two first axes accounted for 26.1% of the total variance (14.0% F1 and 12.1% F2). The first axis (F1) is structured by the alleles Arte-DQA-06 and Arte-DRB-09 opposed to Arte-DQA-07, Arte-DRB-16 and Arte-DRB-10. The second axis (F2) mainly opposes Arte-DRB-11 to Arte-DRB-13 (and Arte-DRB-15 to a lesser extent). Neither heterozygosity nor duplication makes a major contribution to the structure of this data set. The first two dimensions of the PCA performed on the parasitological table (Fig. 3) accounted for 29.4% of the total variance (18.4% F1 and 11.0% F2). The first axis is structured by the two parasite richness indices: R_p and R_h. The second axis opposes *Orthopox virus* (CPXV) and *Arenavirus* (LCMV) and *F. glareoli* (Cocc-Fg) to the two nematodes located in the caecum, *T. arvicolae* (Nem-Ta) and *S. nigeriana* (Nem-Sn).

We found no overall relationship between the genetic and parasitological matrices (global co-inertia = 0.112, P = 0.84), although this does not exclude the existence of particular associations. Similar results were obtained when this ACO was carried out with each MHC gene independently (*DQA1*: P = 0.170 and *DRB*: P = 0.328).

The two first axes of the ACO accounted for 46.3% of the variance shared between the two matrices (31.2% F1 and 15.1% F2). The genetic variables were plotted on the F1 × F2 ACO factor map (Fig. 4a), and four alleles were identified as principally responsible for co-inertia. All were alleles of the *DRB* gene (F1: Arte-DRB-11, Arte-DRB-15 and F2: Arte-DRB-07, Arte-DRB-11, Arte-DRB-16). The heterozygosity of *DQA1*, *DRB* and microsatellites, and the presence of *DQA2*, were located very close to the origin on the co-inertia factor maps (Fig. 4a), indicating the absence of linkage between these four variables and parasitism. When considering as 'high level of heterozygosity' the individuals having at least six heterozygote loci, similar results were observed, confirming that microsatellite heterozygosity is not associated with parasitism. Richness indices, the nematode *T. arvicolae* and, to a lesser extent, the two viruses CPXV and LCMV, were all involved in co-inertia (Fig. 4b), and were previously found to structure parasitological data in the PCA. The cestodes *P. omphalodes* and *A. horrida*, which were not identified

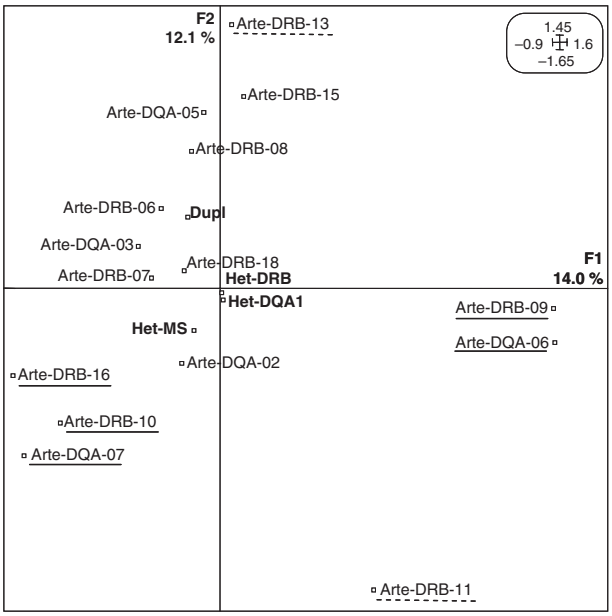

Fig. 2 Correspondence analysis (CA) of the genetic table including 19 variables and 302 voles. The variables *DQA1* and *DRB* alleles coded by name, heterozygosity of *DQA1* (Het-DQA1), *DRB* (Het-DRB) and microsatellites (Het-MS) and *DQA* duplication (Dupl) are projected on the F1 × F2 map. Variables reflecting genetic diversity are shown in bold and those structuring the data set are underlined using solid (F1) or dotted (F2) lines.

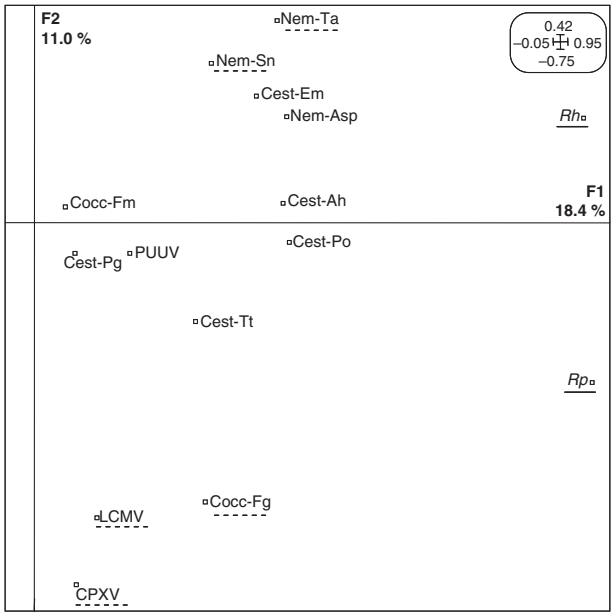

Fig. 3 Principal component analysis (PCA) of the parasitological table including 15 variables and 302 individuals. The variables are projected on the F1 × F2 correlation circle. *Nem-*, *Cest-* and *Cocc-* correspond to nematodes, cestodes and coccidia respectively. The next two letters refer to the code used to identify species (see Table 1). Viruses are coded using CPXV for *Orthopox virus*, LCMV for *Lymphocytic choriomeningitis virus* and PUUV for *Hantavirus* (undetermined species). R_h and R_p correspond to helminth and total parasite richness respectively. Variables structuring the data are underlined: with solid lines for variables structuring F1 and dotted lines for those structuring F2.

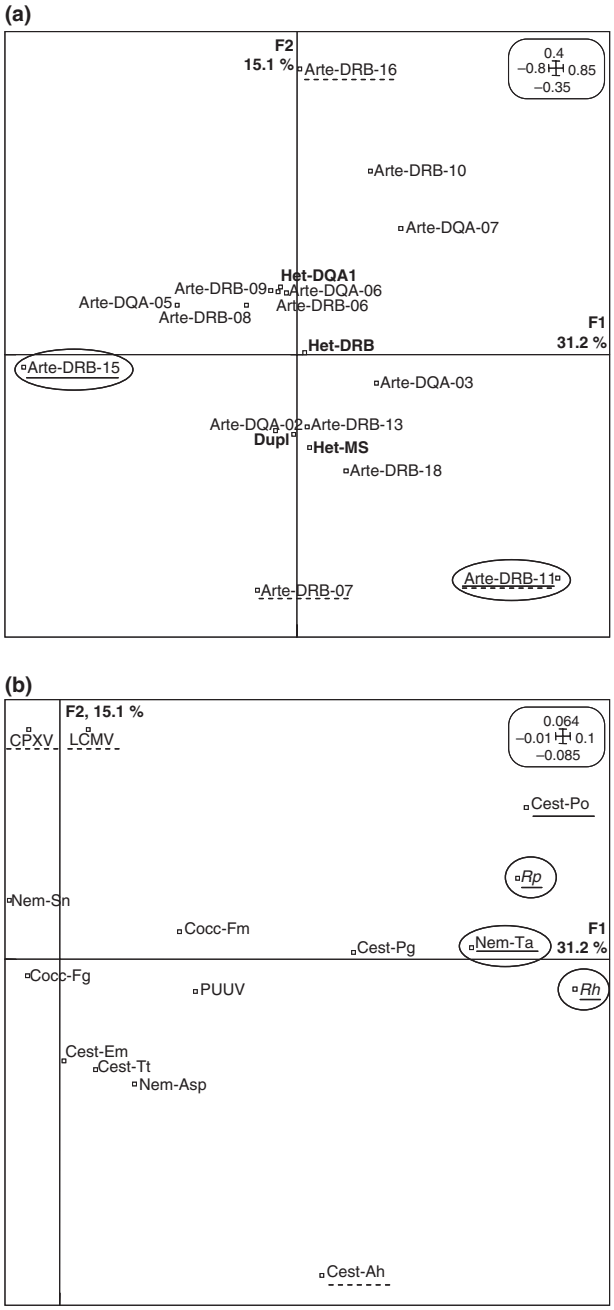

Fig. 4 Co-inertia analysis (ACO) of the genetic and parasitological matrices. Projection on common ACO axes of (a) the genetic variables and (b) the parasitological variables, encoded as in Figs 2 and 3 respectively. Variables located in a given direction relative to the origin may be considered to be positively associated (susceptibility), whereas those located in the opposite direction may be considered to be negatively associated (resistance). Associations between genetic and parasite variables visually detected on the F1 axis are underlined with solid lines, whereas those detected on the F2 axis are underlined with dotted lines. Associations shown to be significant by cross-validation are surrounded. Variables reflecting genetic diversity are indicated in bold.

as important in the PCA, strongly structured the co-inertia (first and second axes respectively).

Superimposing the genetic and parasitological variables on this ACO factor map revealed positive (negative) associations on the first axis between Arte-DRB-11 (Arte-DRB-15) and richness indices, *P. omphalodes* and *T. arvicolae*. Another positive association detected on both axes concerned Arte-DRB-11 and *A. horrida*. The associations detected on the second axis were less important, due to the smaller proportion of the variance accounted for by this axis. Associations were observed between the two viruses CPXV and LCMV and the Arte-DRB-16 and Arte-DRB-10 (positive associations) and Arte-DRB-07 and Arte-DRB-11 (negative) alleles.

We randomly split the data set into two independent data sets, to highlight nonrandom genetic/parasitological associations (Supplementary material). We only confirmed the positive (negative) associations between Arte-DRB-11 and richness indices (R_h and to a lesser extent R_p) and *T. arvicolae* and the negative association between Arte-DRB-15 and these factors. The ACO analyses performed with the *DRB* locus only also revealed this strong opposition between Arte-DRB-11 and Arte-DRB-

15 (Fig. 5). Individuals carrying Arte-DRB-11 are 1.5 and 6.2 times more likely than other individuals to be infected with at least three parasites or with high loads of *T. arvicolae* respectively (Table 2). Alternatively, individuals with the Arte-DRB-15 allele have a lower risk of infection with at least three helminths or parasites (by factors of 0.3 and 0.5 respectively). None of the individuals carrying Arte-DRB-15 was infected with high loads of *T. arvicolae* (Table 2). Note that *T. arvicolae* intensity was significantly correlated with parasite richness (Spearman, $S = 3.1 \times 10^6$, $P < 10^{-8}$, $\rho = 0.326$) and helminth richness ($S = 2.5 \times 10^6$, $P < 10^{-8}$, $\rho = 0.456$). When removing *T. arvicolae* from the richness estimates (new variables R_p-T, and R_h-T), these relationships disappeared (R_p-T: $S = 5.0 \times 10^6$, $P = 0.14$, and R_h-T: $S = 4.5 \times 10^6$, $P = 0.81$). We also performed ACO using R_p-T and R_h-T instead of R_p and R_h. These two variables and *T. arvicolae* abundance still appeared associated with the alleles Arte-DRB-11 and Arte-DRB-15 (data not shown). However, only the associations with the nematode could be confirmed through cross-validation.

The other associations found with the whole data set were not confirmed by cross-validation method. Some associations were not observed, regardless of the subset of data considered. This was the case for the association between Arte-DRB-11 (positive) or Arte-DRB-15 (negative) and *P. omphalodes*, or Arte-DRB-10 (positive) and LCMV or CPXV. Other associations were observed in only one of the two data sets. This was true for the positive associations between Arte-DRB-07 or Arte-DRB-11 and *A. horrida* (data set 2), and for the positive associations between Arte-DRB-16 and LCMV or CPXV (data set 1).

Population genetics

We performed 815 tests to analyse the linkage disequilibrium between genes (55 pairs of loci × 15 populations minus 10 pairs of loci as one microsatellite was monomorphic in one population). After FDR correction, four tests (0.48%) remained significant. If these remaining significant comparisons were stochastic (random) events, 0.48% of the 55 pairs of loci would correspond to 0.26 cases. Nevertheless, three of the four significant comparisons included *DQA1* and *DRB*, suggesting weak linkage between these two genes.

Significant deviation from mutation–drift equilibrium (Table 3) was detected for *DQA1* locus in five populations after Bonferroni correction (all 15 populations had $P < 0.05$). For *DRB* locus, only one population stayed significant after Bonferroni correction (four had $P < 0.05$). Considering microsatellite loci, the mean number of population exhibiting deviation from mutation–drift equilibrium ($P < 0.05$) was 3.4, ranging from zero (AT3) to eight (AT25). However, *P*-values were closed to 0.05

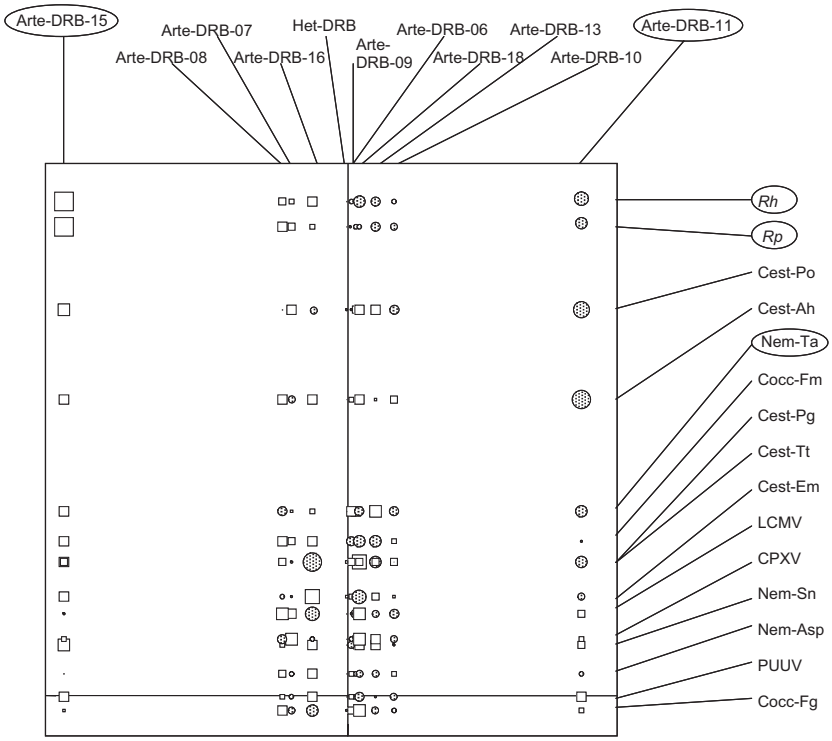

Fig. 5 Associations between *DRB* and parasitological variables projected on the F1 axis of the co-inertia analysis (ACO) based on *DRB* only. This first axis accounts for 33.1% of the total variance. Grey circles indicate positive associations and white squares, negative associations. The size of the symbol indicates the strength of the association. Variables involved in previously confirmed associations are surrounded.

Table 2 Correspondence table between alleles and parasitological variables selected by co-inertia analyses.

	Arte-DRB-11		Arte-DRB-15	
	Presence	Absence	Presence	Absence
Helminth richness R_h				
Presence				
≥ 3	8	36	1	43
1 or 2	28	168	12	184
Absence	3	59	10	52
RR ($R_h \geq 3$)	1.498		0.282	
Parasite richness R_p				
Presence				
≥ 3	14	64	3	75
1 or 2	22	170	13	179
Absence	3	29	7	25
RR ($R_p \geq 3$)	1.475		0.485	
Trichuris arvicolae intensity, *Nem-Ta*				
Presence				
≥ 6	3	3	0	6
1–5	8	69	4	73
Absence	28	191	19	200
RR (*Nem-Ta* ≥ 6)	6.25		0	

The number in each cell indicates sample size. Parasite richness and *T. arvicolae* distributions are given both for individuals with and without each genetic factor identified (Arte-DRB-11 and Arte-DRB-15). RR corresponds to the relative risk associated with each allele, estimated as described by Haldane (1956).

(Table 3) and no microsatellite locus differed significantly from mutation–drift equilibrium after Bonferroni correction, whatever the population considered. In this way, *DQA1* locus, but not *DRB* locus, strongly differed from neutral microsatellites in allelic frequency.

Whatever the gene considered, no population deviated from Hardy–Weinberg equilibrium, revealing an absence of significant heterozygosity excess (Table 3).

Discussion

This is the first study, to our knowledge, to have explored the potential roles of both parasite species and parasite burden as selective pressures acting on immune genes. An approach combining multivariate statistics and population genetics was applied to a genetic data set, including polymorphism of two MHC class II genes and nine microsatellites, and an almost exhaustive parasitological data set.

Evidence of selection

Evidence of balancing selection acting on both MHC class II genes first came from the extensive polymorphism found within the small study area (five *DQA1* alleles and 12 *DRB* alleles in a 100 km^2 area). Moreover, we observed contrasted patterns of genetic diversity at MHC genes and neutral microsatellite markers. After correction for multiple testing, no population deviated from demographic neutrality for microsatellites. The weak impact of demographic bottlenecks on neutral genetic diversity is consistent with the results of a previous study of *A. scherman* populations (Berthier *et al.*, 2006). On the contrary, homogeneous allelic frequencies (Watterson, 1978) were found in five of 15 populations for the *DQA1* gene and in only one population for the *DRB* gene. In this way, *DQA1* locus patterns strongly deviated from mutation–drift equilibrium neutral results, whereas it was not really the case for *DRB*. This locus-specific (*DQA1*) homogeneity in allelic frequencies can therefore be attributed to balancing selection (Cavalli-Sforza, 1966; Luikart *et al.*, 2003).

We found no significant global co-inertia between the genetic and parasitological matrices, but this is not surprising given the difficulties related in the literature to find MHC-parasite associations (Hedrick & Kim, 2000). This result is not inconsistent with PMBS if: (i) some important parasites, such as bacteria or viruses, were not analysed; (ii) other genes influence parasite burden (see Charbonnel *et al.*, 2006 for examples of genes likely to be associated with macroparasites); (iii) various strains of parasites are different enough that different alleles might confer resistance, and the genetic diversity of parasites should be included in such studies (e.g. Webster & Woolhouse, 1998; Moore *et al.*, 2002); or if (iv) only a few main associations drive MHC polymorphism.

Associations between two specific MHC alleles, parasite load and parasite species were found. One allele, Arte-DRB-15, was negatively associated with parasite richness (mainly helminth richness R_h) and with the presence of the nematode *T. arvicolae*. An antagonistic allele, Arte-DRB-11, was positively associated with the same parasitological variables. These associations were weak, but the large sample size used in this study made it possible to confirm the observed associations using cross-validation. Nevertheless, correlation between parasites involved in associations could lead to spurious associations. Here, parasite and helminth richness were correlated with *T. arvicolae* intensity as a consequence of their definition (sum of each parasite presence/absence, an effect which may be intensified by the fact that *T. arvicolae* is the most prevalent parasite). When removing *T. arvicolae* from parasite richness definition, associations with these new variables appeared in the whole data set but were not confirmed through cross-validation. In this way, we confirmed the association of two alleles (Arte-DRB-11 and Arte-DRB-15) with the nematode *T. arvicolae* (confirmed though cross-validation). The association of the same alleles with parasite richness could be direct, or alternatively, could be driven by *T. arvicolae* associations. These findings highlight the relevance of considering each parasite species separately to reveal

Table 3 Detection of overdominance and balancing selection in water vole populations: six sampling sites followed for 3 years.

			Heterozygote excess (F_{IS})			Mutation–drift neutrality tests		
Year	Site	*N*	*DQA1*	*DRB*	9 MS	*DQA1*	*DRB*	Significant microsatellite loci
2003	A	30	−0.077 (0.150)	−0.110 (0.140)	0.065 (0.871)	0.009	0.231	AT9 (P = 0.018) AT25 (P = 0.030) AT19 (P = 0.048)
	B	30	−0.095 (0.249)	−0.004 (0.497)	0.018 (0.930)	0.020	**0.002**	AT13 (P = 0.017) AT22 (P = 0.015) AT9 (P = 0.028) AT25 (P = 0.028)
	C	26	0.224 (0.980)	0.022 (0.476)	−0.002 (0.509)	0.005	0.145	AT2 (P = 0.026) AT9 (P = 0.034)
	D	27	0.132 (0.878)	0.120 (0.868)	0.004 (0.463)	**0.000**	0.034	AT13 (P = 0.047) AT25 (P = 0.029)
	E	25	0.166 (0.810)	0.006 (0.435)	−0.030 (0.187)	0.024	0.100	AT13 (P = 0.024) AT19 (P = 0.032)
	F	28	−0.095 (0.316)	−0.107 (0.143)	0.043 (0.958)	**0.002**	0.152	AT24 (P = 0.029)
2004	A	20	0.215 (0.875)	0.164 (0.937)	0.019 (0.951)	0.047	0.114	AT9 (P = 0.005) AT25 (P = 0.005)
	B	22	0.103 (0.774)	0.079 (0.930)	0.064 (0.975)	0.041	0.183	AT13 (P = 0.018) AT19 (P = 0.010)
	C	22	0.103 (0.844)	−0.077 (0.187)	0.057 (0.659)	**0.001**	0.134	AT9 (P = 0.021) AT19 (P = 0.025)
	D	23	0.215 (0.980)	0.025 (0.511)	0.028 (0.786)	0.017	0.006	
	E	21	0.341 (0.997)	−0.128 (0.090)	0.038 (0.801)	0.009	0.144	AT23 (P = 0.016) AT25 (P = 0.022) AT19 (P = 0.040)
	F	22	0.091 (0.714)	−0.101 (0.094)	−0.025 (0.398)	**0.003**	0.004	AT23 (P = 0.048)
2005	D	23	0.174 (0.866)	−0.093 (0.243)	−0.018 (0.518)	0.008	0.447	AT13 (P = 0.043) AT25 (P = 0.028)
	E	24	0.157 (0.959)	0.044 (0.640)	−0.080 (0.015)	0.006	0.097	AT23 (P = 0.033) AT13 (P = 0.029) AT25 (P = 0.014)
	F	24	0.343 (0.998)	−0.129 (0.085)	0.016 (0.779)	**0.002**	0.163	AT25 (P = 0.008) AT19 (P = 0.037)

N is the number of voles screened for genetic analyses. Estimates of $F_{IS,}$ are given separately for each type of marker. Probabilities of conformity to Hardy–Weinberg equilibrium (H1 heterozygote excess), obtained from exact tests, are indicated in brackets. Results of mutation–drift neutrality tests (Wilcoxon sign test probabilities) are provided, for each MHC gene (IAM), for each microsatellite only if it is significant (GSM). *P*-values are considered significant after Bonferroni correction are indicated in bold.

the true variable with which MHC alleles are associated.

Associations between parasitism and MHC polymorphism reveal PMBS only if the parasite species can affect host fitness (Little, 2002). Most of the parasite species studied here were known to affect host fecundity or mortality (e.g. Feore *et al.*, 1997 for *Cowpox virus*, Fichet-Calvet *et al.*, 2004 for *Frenkelia*, Vervaeke *et al.*, 2006 for *Echinococcus*). In particular, a recent laboratory study showed that *T. arvicolae* affects fecundity in another vole, *Microtus arvalis* (Deter *et al.*, 2007b). These associations between MHC and parasitism therefore provide only indirect evidence of selection acting on *DRB* or on another closely related gene. Associations between *DRB* genotypes and fitness would provide direct evidence of selection, but such data are difficult to obtain (but see De Eyto *et al.*, 2007 for impact on survival, Jäger *et al.*, 2007 on reproductive parameters and Sauermann *et al.*, 2001 on reproductive success).

Insights into selection mechanisms

Whatever the individual estimator considered (*DQA1*, *DRB* or microsatellites heterozygosity or *DQA* duplication), allelic diversity was not involved in the co-inertia between genetic and parasitological matrices. Thus, being heterozygous or having a supplementary copy of one gene may not affect the susceptibility of these water voles to parasites. Several other studies reported no effect on parasite burden of MHC heterozygosity (e.g. Paterson *et al.*, 1998; Schad *et al.*, 2005; Schwensow *et al.*, 2007; but see Froeschke & Sommer, 2005), nor of whole genome heterozygosity (e.g. Ortego *et al.*, 2007; but see Coltman *et al.*, 1999). Furthermore, none of the water

vole populations studied deviated from Hardy–Weinberg equilibrium, whatever the locus considered. Thus, none of the approaches used provided any evidence of overdominance in these populations. These results support the idea emerging from empirical (see references above) and theoretical (Borghans *et al.*, 2004; De Boer *et al.*, 2004) studies that particular alleles may be more important for disease resistance than heterozygosity (Bernatchez & Landry, 2003; Piertney & Oliver, 2006).

Associations between single alleles and parasite load would be expected under the negative frequency dependence hypothesis. Here, we report weak associations between two particular alleles of the *DRB* gene and the nematode species *T. arvicolae*. The low water vole genetic differentiation observed over years and localities and the cross-validation approach confirmed that these associations were not artefacts of spatial structure. These two *DRB* alleles seem to play antagonistic roles in the susceptibility of voles to parasites. Similar results were previously obtained concerning the resistance of sheep to gastrointestinal nematodes (Paterson *et al.*, 1998). We notice that these two *DRB* alleles encode proteins whose amino-acid sequences differ by 10 amino acids, nine of which seem to be involved in peptide recognition (Bryja *et al.*, 2007).

Confirming frequency dependence mechanism in this system would require a temporal monitoring of allelic frequencies, revealing fluctuations (Little, 2002; e.g. Dybdahl & Lively, 1998). However, as associations are weak and parasite effects on fitness are low, selection is expected to be soft, and this monitoring would need to be very long to allow observation of parasite-driven genetic change. On the other hand, the vole populations of the Jura Mountains are particularly likely to undergo spatio-temporal variations in selective pressures (Hedrick, 2002) as they go through marked density cycles (Saucy, 1994; Charbonnel *et al.*, 2008). Bryja *et al.* (2007) investigated this hypothesis and detected variations in selection patterns, both in space (local adaptation detected in one population during the low-density phase of the cycle) and in time (the population structure observed during the low-density phase disappeared at high density, when balancing selection homogenized allelic frequencies). Spatio-temporal variations in selective pressures are thus another, nonexclusive, possible mechanism of balancing selection likely to mediate selection in these populations.

Contrasted patterns of selection for two MHC class II genes

Within rodents, the precise chromosomal position of MHC class II genes is known only for the laboratory mouse (Blake *et al.*, 2003) and rat (Gunther & Walter, 2001), in which the *DQA* and *DRB* genes are tightly linked. We found a weak signal indicating linkage disequilibrium between *DQA1* and *DRB* genes and the action of different selection pressures on these two genes. The *DQA* gene seemed to play a lesser role in genetic/parasitological associations than *DRB*. Parasite-mediated selection would mostly act on *DRB* in the present generation (Garrigan & Hedrick, 2003). By contrast, homogeneity in allelic frequencies was observed mostly for the *DQA1* gene. This pattern reflects balancing selection acting on this gene over the history of the population (Garrigan & Hedrick, 2003). Bryja *et al.* (2007) obtained similar results for neighbouring populations of *A. scherman*: a strong signature of balancing selection on *DQA1* and limited evidence for such selection on *DRB*. These results challenge the common idea that the patterns observed at one locus can be used as an indicator of all MHC patterns (Bryja *et al.*, 2007; and references therein), and encourage the simultaneous study of several MHC genes, although such studies remain scarce (but see Aguilar *et al.*, 2004). This would provide new insight into the potential effects of different selective pressures acting on MHC genes, now that recombination within the MHC is increasingly widely recognized to be common (e.g. Richman *et al.*, 2003; Schaschl *et al.*, 2005).

Conclusions

This study proposes a new approach to detect associations between immunogenetic factors and parasitism. This question has frequently been tackled in the field of evolutionary biology over the last 10 years, but most studies have been subject to major flaws. First, they consider the relationship between MHC and either one species or global parasite load independently. Second, they do not take into account the multiple tests used to investigate all possible associations, strongly increasing the risk of type I error. Simultaneous evaluation of the influences of each parasite species on each MHC variable was made possible by applying multivariate co-inertia approach. This revealed particularly relevant as genetic associations with global parasite load may ultimately be due to only one parasite species. This method permits to analyse data sets containing numerous variables (several genes and numerous parasite species as in this study) and as it has no size limitation, this approach could also be used to study intraspecific parasite data. Nevertheless, rare alleles and parasite would challenge the utilization of cross-validation for confirming the significance of such rare associations observed over a whole data set. Possible solutions would be: (1) to pool alleles in supertypes (Schwensow *et al.*, 2007) and parasites in functional families; or (2) to enlarge sample size, but spatio-temporal variations of parasite pressure may prevent confirmation of previous results (e.g. Bonneaud *et al.*, 2006).

However, correlative field studies of MHC/parasite associations will always show main limitations as many confounding factors are likely to influence parasite burden, e.g. (1) host genetic background because parasite susceptibility is thought to be influenced by many genes

(Woolhouse *et al.*, 2002); (2) probability of exposure, as a result of differences in dispersion rate (Deter *et al.*, 2007a) for example; and (3) condition-based effects (Little, 2002), in particular immune status may be highly variable between and within individuals. Experimentation remains the only way to get free from these confounding effects, and thus to enhance the probability to detect MHC/parasites associations and to confirm the importance of immunogenetics on susceptibility to parasites. Nevertheless, this type of correlative field study would still be an important prerequisite to extract genetic and parasitological variables that will have to be tested through such experimental infestations.

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Supplementary material

The following supplementary material is available for this article:

Appendix S1 Co-inertia analyses of the genetic and parasitological matrices split two independent data sets, each corresponding to 151 individuals.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1420-9101.2008.01563.x

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