Evidence for selection maintaining MHC diversity in a rodent species despite strong density fluctuations

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Abstract Strong spatiotemporal variation in population size often leads to reduced genetic diversity limiting the adaptive potential of individual populations. Key genes of adaptive variation are encoded by the immune genes of the major histocompatibility complex (MHC) playing an essential role in parasite resistance. How MHC variation persists in rodent populations that regularly experience population bottlenecks remains an important topic in evolutionary genetics. We analysed the consequences of strong population fluctuations on MHC class II DRB exon 2 diversity in two distant common vole (*Microtus arvalis*) populations in three consecutive years using a high-throughput sequencing approach. In 143 individuals, we detected 25 nucleotide alleles translating into 14 unique amino acid MHC alleles belonging to at least three loci. Thus, the overall allelic diversity and amino acid distance among the remaining MHC alleles, used as a surrogate for the range of pathogenic antigens that can be presented to T-cells, are still remarkably high. Both study populations did not show significant population differentiation between years, but significant differences were found between sites. We concluded that selection processes seem to be strong enough to maintain moderate levels of MHC diversity in our study populations outcompeting genetic drift, as the same MHC alleles were conserved between years. Differences in allele frequencies between populations might be the outcome of different local parasite pressures and/or genetic drift. Further understanding of how pathogens vary across space and time will be crucial to further elucidate the mechanisms maintaining MHC diversity in cyclic populations.

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Introduction

Many species frequently display cyclic fluctuations in population size driven by internal or external drivers. In Holarctic regions, cycles of microtine rodents are affecting the entire ecosystems. The factors that drive microtine cycles remain unresolved and are widely debated in the literature (Andreassen et al. 2013; Eccard et al. 2011; Stenseth 1999; Tkadlec and Stenseth 2001; Yan et al. 2013). Suggested factors include internal density or phase dependency (Andreassen et al. 2013; Eccard et al. 2011; Stenseth 1999) and/or external factors such as seasonality or climate (Tkadlec and Stenseth

2001; Yan et al. 2013). Parasites and diseases may also represent strong drivers of rodent cycles (Pedersen and Greives 2008; Smith et al. 2008; Soveri et al. 2000), as they influence individual fitness and survival. Independent of the mechanism, population cycles often result in changes in population genetic structure including a loss of genetic diversity due to genetic drift. This may further lead to reduced individual fitness and a reduction in the adaptive potential of the population (Radwan et al. 2010).

Key genes of adaptive variation with high fitness relevance are encoded by the major histocompatibility complex (MHC) involved in the adaptive immune response. MHC genes encode molecules that bind selectively to antigens and present them to T-cells, which induce further immune reactions (Brown et al. 1993). The large diversity of MHC alleles detected in most vertebrate species is thought to be maintained mainly by pathogen-mediated selection, which operates on the MHC by heterozygote advantage (Doherty and Zinkernagel 1975; Penn et al. 2002) and/or can vary at the local scale in space and time ('fluctuating selection') and/or exhibit cyclical selection between host and pathogen ('negative frequency-dependent selection') (Sommer 2005, Spurgin and Richardson 2010). Moreover, MHC alleles usually are very divergent and show a high genetic distance at the amino acid level in the parts of the sequence which encode for the functionally important antigen-binding sites (ABS) (Piertney and Oliver 2006; Sommer 2005). Changes at these amino acids of the ABS may thereby result in changes in antigen-binding capability and subsequently in the recognition abilities of the MHC molecule (Brown et al. 1993). However, how this functionally important MHC variation persists in vertebrate populations that regularly experience population bottlenecks remains an important topic in evolutionary genetics and conservation (Winternitz et al. 2014). Studies so far provided controversial results. Whereas several studies revealed reduced MHC allele diversity in populations with decreased population sizes (Radwan et al. 2010), others reported high MHC diversity compared to neutral markers even in bottlenecked populations probably due to the stronger effect of selection processes than genetic drift (Sommer 2005; Hedrick and Hurt 2012; Oliver and Piertney 2012).

Microtine rodents provide an ideal system to investigate genetic diversity and selection processes in natural cyclic populations. Evidence for selection mechanisms acting on MHC variability in microtine rodents has been controversial. Recent studies detected a MHC heterozygote superiority against multiple parasites in a natural population of water voles (*Arvicola terrestris*; Oliver et al. 2009), but reported no strong evidence for parasite-mediated selection in cyclic populations of montane voles (*Microtus montanus*) despite relatively high neutral and MHC diversity (Winternitz et al. 2014). A comparison between cyclic and stable rodent species suggested that species that frequently undergo population bottlenecks had lower allelic richness than stable species (Winternitz and Wares 2013).

In this study, we investigate the power of selection on MHC variability using common voles (*Microtus arvalis*) as study organisms because they are well known for their pronounced natural annual density fluctuations (Boyce and Boyce 1988; Eccard and Herde 2013). Due to their short generation time and high number of offspring, population sizes can rise rapidly and population cycles are commonly observed (e.g. Delattre et al. 1999; Imholt et al. 2014). Thus, it is a highly suitable organism to investigate the variability of genes throughout natural population crashes. The only previous MHC study in this species focussed on MHC class II DQA (Bryja et al. 2006), a locus usually less variable than DRB genes (Sommer 2005).

Specifically, we analysed the MHC diversity of two distinct *Microtus arvalis* populations in three consecutive years. We (1) characterised the MHC class II DRB exon 2 for the first time in this species using a next-generation sequencing approach and (2) analysed allele diversity and frequency pattern in space and time. We hypothesised that in naturally fluctuating populations, genetic diversity is reduced, due to regular crashes in population size and associated neutral forces. The resulting consequences mainly depend on the power of selection versus genetic drift (Sommer 2005; Oliver and Piertney 2012). (A) If genetic drift dominates selection, different MHC alleles will remain in different populations over time. On the other hand, (B) if pathogen-driven selection is strong enough, the same alleles will be conserved among populations with varying allele frequencies across space and time due to distinct local parasite pressures.

Methods

Study species and trapping sites

Our study species, the common vole (*Microtus arvalis*, Pallas 1779), occurs all over Europe (Baláž 2010) and is believed to be the most abundant mammal in Europe (Gauffre et al. 2007). The common vole inhabits a wide range of open habitats including agricultural landscape, subalpine zones, diffuse wood vegetation and grasslands (Baláž 2010). It is a small mammal with maximum body weight of 51 g in males and 42 g for non-pregnant females (Stein 1958). The species is promiscuous and iteroparous, and multiple paternities in one litter are common (Borkowska and Ratkiewicz 2010).

In 2010, 2011 and 2012, we caught *Microtus arvalis* in the proximity of two field stations run by the University of Potsdam/Germany. Both study sites consist of meadows and shrubs and harbour distinct vole populations, which are located within the same geographical region, but at 80-km distance

from each other. One field site ('Potsdam') is located in Potsdam-Bornim, Brandenburg, Germany (N 52° 26′ 22″, E 13° 0′ 44″). The other field site ('Guelpe') in Guelpe, Brandenburg, Germany (N 52° 44′ 01″, E 12° 12′ 41″), is situated in a floodplain (ca. 2 km long and 200–500 m wide) surrounded by arms of the river *Havel* with regular flooding. It includes a 1-ha dwelling mound around the research station and a sand dune ridge serving as a refuge for voles during flooding. At the Potsdam site, populations may crash due to internal or external biotic reasons (such as diseases, parasites, food shortage, predation pressure, e.g. Andreassen et al. 2013), while in Guelpe population, declines might primarily be due to flooding as an external, abiotic factor.

Common vole densities are commonly indexed in captures per 100 trap nights (TN). Common voles in grassland can reach densities of >1000 individuals per hectare (e.g. Boyce and Boyce 1988; Hušek et al. 2013); however, trapping indices from different literature sources are difficult to compare, since they vary with trap type and trap spacing. We used multiple capture life traps in lines with 10-m spacing. Since we started monitoring in 2009, the index at both sites has ranged between 0 and 150 voles/100 TN (2009–2015), reaching a bottleneck of 0 voles/100 TN after a major flood event at the Guelpe site in 2013. A comparable flood was recorded in 2002. This indicates that bottlenecks due to flooding are common in this population. In 2010, 2011 and 2012, during years of tissue sampling for this study, trapping indices were >13, 93 and 96 voles/100 TN at the Guelpe site and 24, 12 and 70 voles/100 TN at the Potsdam site, respectively.

We used metal life traps (Ugglan special No. 2, Grahnab, Sweden, with shrew exit to prevent the capture of shrews, see Eccard and Klemme 2013). Traps were baited with oat flakes and apples to provide sufficient food and water resources. Traps were checked at least twice a day, around 8 a.m. in the morning and between 6 and 8 p.m. in the evening. Animals were sexed, weighed, sampled and either released directly at the trapping location or brought to the laboratory for further behavioural experiments (Eccard and Herde 2013; Herde and Eccard 2013). Small ear punches (max. 1 × 3 mm) for genetic analyses were taken and stored in 100 % ethanol at −20 °C. Voles were captured under permission of the Landesumweltamt Brandenburg (reference number RW-7.1 24.01.01.10).

DNA sampling

Genomic DNA from samples collected in 2010 was extracted by using the NucleoSpin® Tissue Kit (Machery-Nagel, Düren, Germany). We followed the manufacturer's protocol and eluted DNA in two steps by 80–130 μl of BE buffer depending on the size of the tissue sample. Genomic DNA from samples collected in 2011 and 2012 was isolated using the First-DNA all-tissue DNA extraction Kit (Genial GmbH,

Troisdorf, Germany). By following the manufacturer's instructions, tissue samples were dissolved for at least 3 h at 65 °C until samples were lysed completely. The DNA was eluted in 50–250 μl sterile water depending on the size of the DNA pellet. Extracted DNA was stored at −20 °C.

Primers and 454 pyrosequencing

We established species- and target-specific primers for the amplification of a 149-bp fragment of the MHC class II DRB exon 2 of common voles based on sequences derived from Sanger sequencing and an initial first 454 pyrosequencing test run (unpublished data). Fusion primers (see Supplemental Table 1) for the 454 pyrosequencing runs consisted of adaptor sequences at the 5′ end (adapter A, forward: CGTATCGCCTCCCTCGCGCCA and adapter B, reverse: CTATGCGCCTTGCCAGCCCGC) and a key sequence (TCAG). We created 18 forward and 18 reverse individual sequence combinations called multiplex identifiers (MIDs) to identify each PCR amplicon. The fusion primers ended with the target-specific primers at the 3′ end (forward: ACGCAGCGCGTGCGGTTTCTGG (MarvJA3), reverse: CYGGTAGTTGTRYCTGCAG (MarvAS2)).

To genotype the MHC of our samples, we conducted two further 454 pyrosequencing runs. For the second run, two independent PCR amplifications, i.e. replicates, were performed for each individual ($N = 156$ voles) using a definite MID combination to increase the reliability of our data (Sommer et al. 2013). We included the same PCR amplifications in a third run together with a repeated subsample, i.e. one 96-well plate ($N = 95$ animals), to check for consistency in library preparation, amplification efficiency and allele calling. All 50-μl amplification mixes contained 7–20 ng DNA, 5.0 μl 10× *FastStart Buffer* with 18 mM MgCl$_2$ (Roche Applied Science, Mannheim, Germany), 0.4 μM of each primer, 0.2 mM of each dNTP and 1.25 U *FastStart High Fidelity Taq Polymerase* (Roche Applied Science). The thermal profile was set to 3 min at 94 °C followed by 35 cycles of 15 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C. PCR ended with a final elongation at 72 °C for 8 min. Per PCR plate, we used one negative control with an individual MID combination to ensure no occurrence of any contaminations. The amplicons were purified using *Agencourt AMPure beads* (Agencourt Bioscience, Beverly, USA). We followed the manufacturer's manual by using 30 μl of PCR product with 30 μl *AMPure beads*. Purified PCR products were quantified by the Quant-*iT PicoGreen dsDNA Assay Kit* (Invitrogen, Karlsruhe, Germany). We diluted each amplicon separately to 1×10^7 molecules per microlitre and pooled them for further analysis. For the emulsion PCR (emPCR) of the pooled amplicons, we used the *GS FLX Titanium SV emPCR Kit (Lib-A)* with *GS FLX Titanium emPCR Filters* (Roche Diagnostics GmbH) (0.1 cpb) and the *GS FLX Titanium emPCR Breaking Kit*

(Roche Diagnostics GmbH) following the manufacturer's protocol. The enriched library amplicons were sequenced on a Roche 454 GS Junior instrument (Roche Diagnostic GmbH).

454 read analysis

454 read quality and chimera filtering, downstream analysis and subsequent MHC allele calling were conducted as described in detail by Sommer et al. (2013). We applied the quality check implemented in the Roche 454 Genome Sequencer FLX Software (package 2.0.00.22), which is based on the sequencing results of DNA control beads and only keeps sequences with a Phred quality score < 20. After these filtering steps, 134,352 reads in the first 454 run and 182,165 reads in the second 454 run remained and passed this key quality control step. Next, we removed all reads shorter than 200 bp and assigned them to individual PCR replicates. We removed all unique sequences (singletons), as they are probably a result of sequencing errors (see Sommer et al. 2013). The remaining 111,020 and 88,763 reads (454 runs 1 and 2, respectively) were assembled into clusters with 100 % identity of the reads. As our reads appeared very similar, we adjusted the pipeline by Sommer et al. (2013) by dividing clusters into 'clusters with 1 nucleotide difference from most frequent cluster' and into '>1 nucleotide differences' (step I in Fig. 2, Sommer et al. 2013). In total, 661 different variants occurring more than once were identified. These were applied to our strict and very conservative pipeline (steps II, III) to identify true MHC-DRB alleles, which were used for subsequent analyses. We analysed all reads of each individual replicate and run (up to four independent PCRs per individual) separately to compare the performance and check for consistency. Animals were excluded from further analysis when conflicts between PCRs appeared.

Data analysis and statistics

In order to test whether the amplified MHC DRB exon 2 region has been under selection over long periods of time, the rates of non-synonymous (d_N) and synonymous (d_S) base pair substitutions were calculated separately for non-antigen (non-ABS) and antigen-binding sites (ABS, i.e. residues with side chains in the binding groove plus the residues, which are involved in hydrogen bonding) as defined for house mice by Bondinas et al. (2007) using *MEGA 6.06* (Tamura et al. 2013). To be able to compare our results with previous studies, we repeated the analyses using the human ABS annotated by Brown et al. (1988, 1993). We utilised the method according to Nei and Gojobori (1986) and applied the Jukes-Cantor correction for multiple hits (Jukes and Cantor 1969). The d_N/d_S rates were tested for significant differences from neutrality ($d_N = d_S$) using the implemented Z-test. MEGA 6 was also used to calculate pairwise allelic distances between the

MHC alleles of each individual based on the number of differences between the nucleotide and amino acid sequences, respectively, applying the pairwise deletion option. The extent of population differentiation and changes in allele frequency across years was examined by pairwise F_{ST} (10,000 permutations (Wright 1965) and the Global test (Markov chain length: 10,000 steps) using ARLEQUIN 3.5 (Excoffier et al. 2005). Allele frequencies were calculated on the amino acid level; i.e. nucleotide allele numbers translating into the same amino acid allele were summed up. Furthermore, MEGA 6 was applied to perform model selection to construct a phylogenetic tree of MHC class II DRB exon 2 alleles based on maximum likelihood. Phylogenetic relationships of MHC alleles were estimated based on 1000 bootstraps using the Whelan and Goldman (WAG) model with partial deletion and uniform rates among sites. Each terminal (nucleotide allele) was labelled by its occurrence in each year and location.

Results

MHC characteristics of common vole

In total, we analysed 156 individuals using 454 pyrosequencing (Roche Diagnostics GmbH, Mannheim, Germany). One hundred forty-three animals remained after applying the strict criteria of our bioinformatics pipeline (Sommer et al. 2013). On average, 677 reads were obtained per individual (min 173, max 2207 reads). Table 1 shows the distribution of individuals across study sites and years.

We detected 25 alleles at the nucleotide level translating into 14 unique amino acid MHC class II DRB exon 2 alleles (Supplementary Fig. 1). No stop codons were detected. Two nucleotide alleles (*Miar*-DRB*01f, *Miar*-DRB*03c) revealed a codon deletion leading to the loss of an amino acid but not to a frame shift of the subsequent amino acid sequence (Supplementary Fig. 1). MHC alleles were named *Miar*-DRB*01 to *Miar*-DRB*14 based on the standard nomenclature given by Klein et al. (1990) (Supplementary Fig. 1). Nucleotide alleles translating into the same amino acid allele were indicated by additional letters (a–f). A BLAST search confirmed the identity of *Miar*-DRB alleles to other rodent and microtine DRB sequences with >97 % for the top 10 hits. The allele frequencies at the amino acid level ranged from 0.004 to 0.550 (Table 1).

The number of variable nucleotide position was 51 out of 149 (24 out of 49 amino acids) (Supplementary Fig. 1). The mean number of pairwise nucleotide differences was 18.91 ± 2.38 (0 to 34). The mean number of pairwise amino acid differences was 9.18 ± 1.58 (0 to 17). The number of non-synonymous substitutions was 4.4 and 5.1 times higher in the ABS (according to Bondinas et al. (2007) and Brown et al. (1988);

Table 1 MHC class II allele frequencies across study sites and year

	Overall	Guelpe			Potsdam		
		2010	2011	2012	2010	2011	2012
N	143	28	6	29	24	26	30
No. of copies	278	51	11	57	52	52	55
m	14	7	3	7	9	5	7
AAdist	3.24 ± 0.48	3.29 ± 1.09	5.67 ± 2.99	4.62 ± 1.24	3.33 ± 1.18	2.62 ± 1.06	1.87 ± 0.72
Gene Diversity	0.610	0.697	0.691	0.686	0.567	0.441	0.559
Miar-DRB*01	0.554	0.451	0.364	0.404	0.615	0.731	0.618
Miar-DRB*02	0.004	0.000	0.000	0.000	0.000	0.000	0.018
Miar-DRB*03	0.284	0.314	0.455	0.386	0.250	0.173	0.255
Miar-DRB*04	0.004	0.000	0.000	0.018	0.000	0.000	0.000
Miar-DRB*05	0.014	0.078	0.000	0.000	0.000	0.000	0.000
Miar-DRB*06	0.018	0.020	0.000	0.000	0.019	0.000	0.055
Miar-DRB*07	0.011	0.000	0.000	0.000	0.019	0.038	0.000
Miar-DRB*08	0.007	0.000	0.000	0.000	0.000	0.019	0.018
Miar-DRB*09	0.014	0.020	0.000	0.018	0.019	0.000	0.018
Miar-DRB*10	0.032	0.039	0.000	0.053	0.019	0.038	0.018
Miar-DRB*11	0.004	0.000	0.000	0.018	0.000	0.000	0.000
Miar-DRB*12	0.004	0.000	0.000	0.000	0.019	0.000	0.000
Miar-DRB*13	0.047	0.078	0.182	0.105	0.019	0.000	0.000
Miar-DRB*14	0.004	0.000	0.000	0.000	0.019	0.000	0.000

Individuals harboured one to four different MHC alleles

N number of individuals, *No. of copies* number of allele copies, *m* number of alleles detected, AA_{dist} mean amino acid divergence ± standard error within an individual

Brown et al. (1993), respectively) than in the non-ABS sites, a sign of strong positive selection. Accordingly, ABS showed a higher mutation rate than non-ABS, but non-synonymous and synonymous substitutions were not significantly different. In non-ABS, synonymous substitutions were 2.4 to 3.3 times more frequent than non-synonymous substitutions ($p < 0.05$, Table 2).

The phylogenetic relationships of amino acid MHC alleles suggested that the *Miar* alleles form at least three distinguished clusters with high bootstrap support (Fig. 1). All three clustered well with DRB alleles from closely related microtine species (*Microtus oeconomus* and *Microtus montanus*, see Fig. 1 for accession numbers). The expression of these alleles was partly proven by cDNA-based sequencing, which supports the functionality of our detected alleles. Individuals exhibited one to four alleles both at the nucleotide as well as at the amino acid level per individual (mean number of alleles ± standard deviation: nucleotide = 1.94 ± 0.84, amino acid = 1.69 ± 0.74). Thirty-six out of 143 individuals (25.2 %) harboured two nucleotide alleles, which translated into the same amino acid allele. Each but one individual (99.3 %, 142 out of 143) harboured an allele
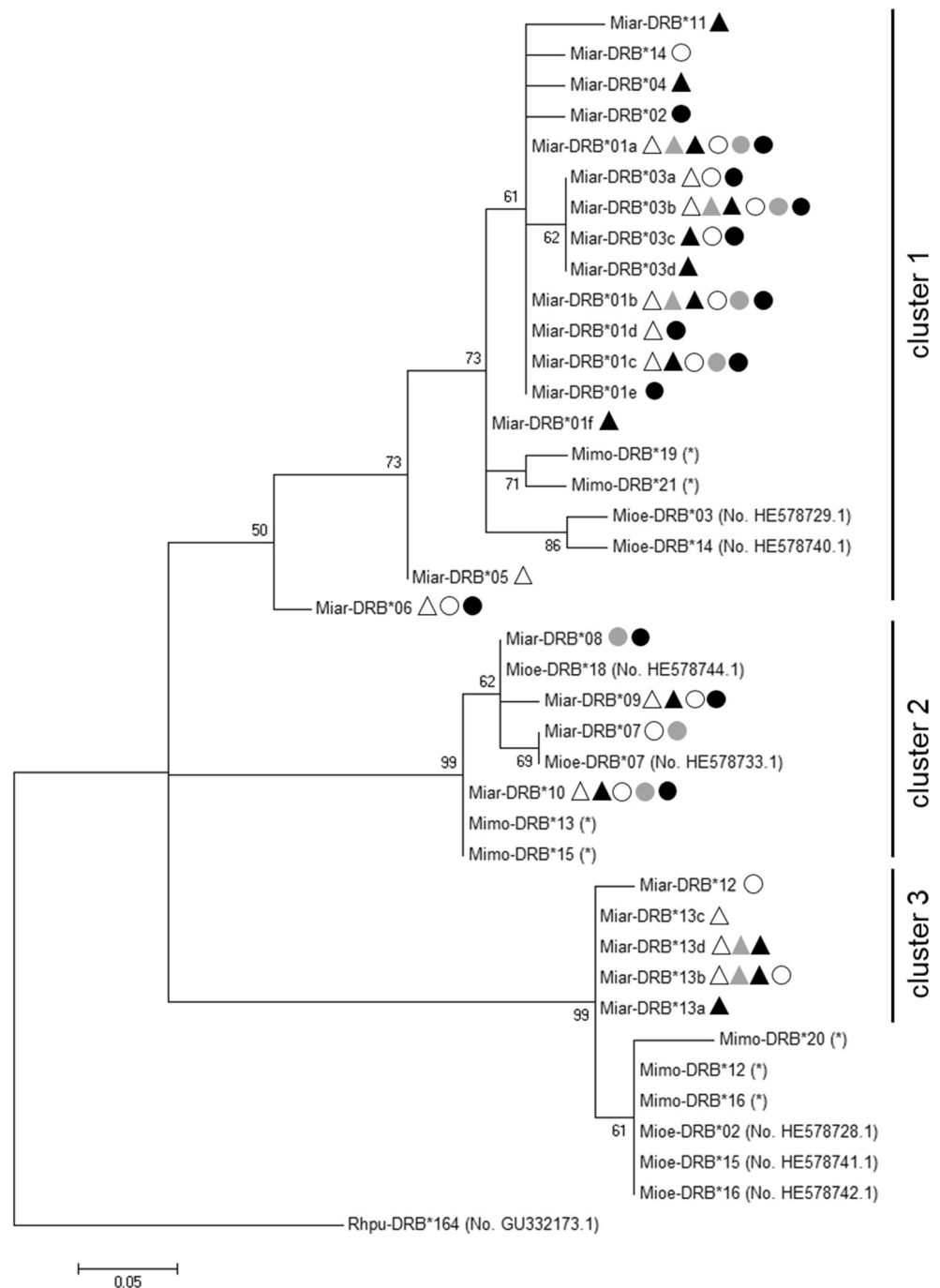
from cluster 1, and most individuals (86.0 %, 123 out of 143) carried *Miar*-DRB*01. Twelve individuals harboured alleles from cluster 1 and cluster 2 (8.4 %), ten individuals from clusters 1 and 3 (7.0 %), and four individuals (2.8 %) from all three clusters.

Population differentiation in space and time

The overall genetic diversity, i.e. expected heterozygosity, was higher in the Guelpe (0.69 ± 0.03) than in the Potsdam population (0.52 ± 0.04), as well as the mean amino acid divergence ± standard error within an individual (Guelpe: $AA_{dist} = 4.13 \pm 0.81$, Potsdam $AA_{dist} = 2.55 \pm 0.57$, MWU test: n.s.). However, the mean number of alleles per individual did not differ between the two populations (Guelpe: nucleotide = 1.89 ± 0.88, amino acid = 1.76 ± 0.78, Potsdam: nucleotide = 1.99 ± 0.82, amino acid = 1.64 ± 0.72, MWU test: n.s.).

The most frequent MHC alleles (>10 % Miar-DRB*01 and 03) were found in both populations and within all years (Table 1). Guelpe voles harboured nine and Potsdam 11 different MHC class II DRB amino acid alleles. Six alleles occurred at both study sites. Both study populations did not show significant population differentiation between years

(all pairwise F_{ST} *P* values > 0.29). However, they showed a significant population differentiation between sites ($F_{ST} = 0.06$, $P < 0.001$).

Discussion

In this study, we analysed for the first time the MHC class II DRB exon 2 allelic variation in common voles, a rodent well known for its frequent population cycles. *Microtus arvalis* exhibited a moderate variability of MHC alleles in our two populations studied. In 143 individuals, we detected 25 alleles on the nucleotide level translating into 14 unique amino acid alleles but distributed across at least three MHC class II DRB loci. Only two alleles (Miar-DRB*01, Miar-DRB*03) occurred in high frequencies (> 25 %), whereas the 12 others occurred in very low frequencies (< 5 %, Table 1). The number of detected alleles in common voles is considered remarkably

Table 2 Estimated rates (± SE) of non-synonymous (d$_N$) and synonymous (d$_S$) substitutions for antigen-binding sites (ABS) and non-antigen-binding sites (non-ABS) assuming concordance with ABS inferred for mice and polymorphic sites inferred from the human sequence and their ratio in *Microtus arvalis* MHC class II sequences

DRB	Sites	N	d$_N$	d$_S$	d$_N$/d$_S$	P value
Bondinas et al. (2007)	ABS	9	0.412 ± 0.206	0.450 ± 0.161	0.92	ns
	Non-ABS	40	0.094 ± 0.025	0.230 ± 0.074	0.41	(*)
	All	49	0.138 ± 0.032	0.296 ± 0.081	0.47	*
Brown et al. (1988, 1993)	ABS	13	0.331 ± 0.142	0.431 ± 0.160	0.77	ns
	Non-ABS	36	0.065 ± 0.017	0.213 ± 0.071	0.31	*
	All	49	0.121 ± 0.029	0.265 ± 0.069	0.46	*

N number of codons in each category, *P* is the probability that d$_N$ and d$_S$ are different using a two-sided Z-test, *ns* not significant

*$P < 0.05$, (*)$0.05 < P < 0.1$

high for a species with very regular fluctuations in population size. Our results are in agreement with a previous study in another cyclic population of a *Microtus* vole species: Winternitz et al. (2014) reported moderate levels of diversity with 21 MHC DRB alleles belonging to at least two loci in 123 individuals of *Microtus montanus*, considered as remarkably high for a species with very regular density fluctuations. Furthermore, Bryja et al. (2006) found eight MHC alleles in 16 investigated individuals of common voles at the duplicated DQA locus. The number of DQA alleles was within the same range in other vole species (water vole *Arvicola terrestris*: *N* = 96, seven MHC-DQA alleles, two loci; bank vole *Myodes glareolus*: *N* = 16, seven MHC-DQA, two loci, see Bryja et al. 2006). This was interpreted as a generally high DQA diversity in voles (Bryja et al. 2006). In comparison, in non-cyclic rodent species such as wood mice (*Apodemus sylvaticus*), 38 unique DRB alleles (*N* = 119) and in yellow-necked mice (*Apodemus flavicollis*) 15 DRB alleles (*N* = 60) were detected (Musolf et al. 2004), respectively. In 36 Eastern woodchucks (*Marmota monax*), Moreno-Cugnon et al. (2015) identified 15 MHC alleles, which were expressed in two independent loci. In Alpine marmots (*Marmota marmota*), 11 MHC DRB alleles were detected at two loci in 38 individuals (Kuduk et al. 2012).

In our vole populations, not all loci were present in all individuals though all loci seem to be functional. Individuals exhibited one to four alleles (mean number of alleles = 1.94) indicating the presence of at least two loci at the individual level. Since many MHC alleles at the nucleotide level (up to six) translated into the same amino acid allele, most gene duplications seemed to have occurred recently. Almost all individuals carried at least one allele from cluster 1 (99.3 %), 12 individuals (8.4 %) carried at least one allele from clusters 1 and 2, and 10 individuals (7.0 %) harboured an allele from clusters 1 and 3, but in only 4 individuals (2.8 %), an allele from each cluster was detected. We consider a potential loci amplification bias as unlikely since we used optimal fitting species- and target-specific primers during intensive preliminary analyses though this can never be fully

excluded without sequencing whole genes. Our primers have been used in three separate next-generation sequencing runs including independent individual replicates. As we obtained equal results and allele sequences, variations in number of alleles per individual seem to reflect the individual copy number variation. Moreover, copy number variations within populations are common in mammals, and especially within *Microtus* species, gene duplications are a common phenomenon (Winternitz and Wares 2013). For instance, *Microtus oeconomus* (root vole) and *Microtus montanus* (montane vole) inhabit at least two DRB loci (Winternitz and Wares 2013). Furthermore, the MHC class II DQA locus also seems to appear duplicated within *Arvicolinae* (e.g. *Microtus arvalis*, Bryja et al. 2006; *Myodes glareolus*, Axtner and Sommer 2007).

The mean number of pairwise amino acid differences was 9.18 ± 1.58 (0 to 17), which is considered high for a species subject to high amplitude fluctuations and in comparison to other rodent species. For example, in montane voles (*Microtus montanus*), on average, 6.5 amino acid differences were detected among 21 MHC-DRB alleles (Winternitz and Wares 2013). In wood mice (*Apodemus sylvaticus*), on average, 5.8 amino acid differences were observed between 13 MHC DQA alleles (de Bellocq et al. 2008). Further, in striped mice (*Rhabdomys pumilio*), a rodent with a large geographic range ranging from South Africa, across Namibia to the border of Angola, Froeschke and Sommer (2005) revealed a mean number of 7.8 amino acid differences between 20 DRB alleles (*N* = 58 individuals). In accordance with the observed elevated substitution rate in ABS, this suggests strong positive selection maintaining functional important MHC diversity.

A recent meta-analysis revealed that MHC and neutral genetic diversity are lost during bottleneck events in most vertebrate populations (Sutton et al. 2011). A study in voles (*Microtus arvalis*) using neutral markers detected, however, that vole migration increased with population density, and the authors assumed that this counteracts the local loss of genetic variety during the low-density phases (Gauffre et al. 2014). Also, in water voles (*Arvicola terrestris*), high variance in

population size did not negatively affect genetic diversity in both neutral (Berthier et al. 2006) and adaptive MHC markers (Bryja et al. 2007).

In our study populations, we did not observe a strong impact of genetic drift on MHC diversity. We found no differences in MHC allele composition and frequencies in both populations between the years. Though the study sites differ in the rhythm and main causes of population crashes, the population dynamics at both study sites seem to be similar. At the Potsdam site, populations may crash due to internal or external biotic reasons (such as diseases, parasites, food shortage, predation pressure, e.g. Andreassen et al. 2013), while in the Guelpe population, declines might primarily be due to flooding as an external, abiotic factor. Interestingly, the mean number of alleles per individual did not differ between the two populations and the overall genetic diversity, and the mean amino acid divergence within an individual was even higher in Guelpe than in Potsdam. This suggests that occasionally occurring wipeouts of the Guelpe population by flooding are not complete. Some local voles might survive a population crash and serve as founders of a new population, sufficient to maintain the observed MHC diversity. There are refugia during flooding, such as a dwelling mount of ca. 1 ha around the research station that was not flooded since the house was build some 150 years ago and a sand dune ridge. Contrarily to the Potsdam population, however, which is connected to a larger continuous population, it is unlikely that the Guelpe population is colonised from neighbouring populations, since the study site is situated on an island surrounded by arms of the river *Havel*.

Though the two study populations did not differ to a large extent in their MHC diversity, we observed a strong overall differentiation between the populations. The significant F_{ST} value between the two populations could be due to many different factors, including divergent selection, neutral drift with reduced migration or bottlenecks after previous recolonisation, and can thus not be interpreted without comparing with similar analyses using a neutral marker. Evidence for parasite-driven selection and the association of specific MHC alleles, MHC heterozygosity and MHC expression with parasite load have been stated for many other rodent species (Axtner and Sommer 2007; Meyer-Lucht et al. 2008). Vole individuals in our study populations did not show any visible health problems. As already detected in other bottlenecked species like cheetahs and beavers (Babik et al. 2005; Castro-Prieto et al. 2011), a reduced number of MHC alleles do not necessarily lead to more susceptible populations in terms of infections and pathogens (Radwan et al. 2010). It might, however, involve a risk once new pathogens emerge since the adaptation ability might be reduced (Sommer 2005). In *Apodemus flavicollis*, it was shown that a lower number of MHC-DRB alleles per population were associated with an elevated overall helminth infection and vice versa (Meyer-Lucht and Sommer 2009).

To conclude, our results highlight the key role of selection in maintaining MHC diversity even in species with high population size fluctuations. Detailed studies of how pathogens vary across space and time will be crucial to further elucidate the counteracting forces of pathogen-driven selection versus genetic drift affecting MHC and neutral marker diversity in cyclic populations.

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