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Parasite load and MHC diversity in undisturbed and agriculturally modified habitats of the ornate dragon lizard

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Abstract

Major histocompatibility complex (MHC) gene polymorphism is thought to be driven by host–parasite co-evolution, but the evidence for an association between the selective pressure from parasites and the number of MHC alleles segregating in a population is scarce and inconsistent. Here, we characterized MHC class I polymorphism in a lizard whose habitat preferences (rock outcrops) lead to the formation of well-defined and stable populations. We investigated the association between the load of ticks, which were used as a proxy for the load of pathogens they transmit, and MHC class I polymorphism across populations in two types of habitat: undisturbed reserves and agricultural land. We hypothesized that the association would be positive across undisturbed reserve populations, but across fragmented agricultural land populations, the relationship would be distorted by the loss of MHC variation due to drift. After controlling for habitat, MHC diversity was not associated with tick number, and the habitats did not differ in this respect. Neither did we detect a difference between habitats in the relationship between MHC and neutral diversity, which was positive across all populations. However, there was extensive variation in the number of MHC alleles per individual, and we found that tick number was positively associated with the average number of alleles carried by lizards across reserve populations, but not across populations from disturbed agricultural land. Our results thus indicate that local differences in selection from parasites may contribute to MHC copy number variation within species, but habitat degradation can distort this relationship.

Keywords: copy number variation, land clearing, MHC diversity, ornate dragon lizard, parasite load

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Introduction

Major histocompatibility complex (MHC) proteins are involved in the recognition of nonself-antigens, derived primarily form parasites (broadly interpreted and including pathogenic microorgansims). Genes coding for classical MHC proteins (class I and class II) are highly polymorphic, with populations typically hosting dozens or even hundreds of allelic variants. At the individual level, some vertebrates carry a few MHC loci (e.g. humans, mice, chicken; reviewed in Kelley *et al.* 2005), but in other species, the numbers of copies of functionally similar MHC loci can be higher, with copy number variation between haplotypes, and consequently between individuals (Westerdahl *et al.* 2000; Reusch *et al.* 2001; Zagalska-Neubauer *et al.* 2010).

The high polymorphism of MHC genes is thought to be primarily driven by selection imposed by parasites (reviewed by Piertney & Oliver 2006; Spurgin &

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Richardson 2010), although mate choice for MHC-dissimilar mates may also play a role (Penn 2002; Hedrick 2004). According to the heterozygote advantage hypothesis, polymorphism is maintained because heterozygotes can recognize a wider array of pathogen-derived antigens (Doherty & Zinkernagel 1975; Hughes & Nei 1988). Another mechanism, rare allele advantage, assumes that parasites are most likely to adapt to the most frequent host genotypes, and thus, rare alleles are more often associated with parasite resistance, which prevents their loss from populations (Bodmer 1972; Potts & Wakeland 1990; Borghans *et al.* 2004). Additionally, fluctuating selection may also contribute to a high polymorphism (Hedrick 2003), for example if the selective pressure associated with given parasite changes temporally (Hill *et al.* 1991) or spatially (Kloch *et al.* 2010; Loiseau *et al.* 2011).

Association between MHC variants and parasite load have been demonstrated in numerous cases (reviewed by Spurgin & Richardson 2010). The link between MHC variants and fitness via parasite load has also been demonstrated in some systems (Eizaguirre *et al.* 2012; Kloch *et al.* 2013). While these studies show that MHC alleles are under selection associated with their role in parasite recognition, they provide only indirect evidence that MHC polymorphism is driven by pressure from parasites. The evidence is indirect because it is based on the strong assumption that this selection is balancing. It is, however, possible that other mechanisms, such as ongoing selective sweeps, may lead to associations between resistance to parasites and MHC alleles, while leading ultimately to the loss of variation. More direct support can be obtained by looking at the link between parasite diversity and MHC diversity across populations or species, but such studies are scarce. Across taxa, richness of helminth species have been shown to be associated with increased MHC diversity in rodents (de Bellocq *et al.* 2008). However, a recent meta-analysis (Winternitz *et al.* 2013) has found that within mammals the patterns may differ: MHC diversity increased with parasite richness for bats and ungulates, but decreased with parasite richness for carnivores.

Within species, parasite diversity was shown to be positively correlated with MHC diversity across populations of sticklebacks (Wegner *et al.* 2003) and humans (Prugnolle *et al.* 2005). However, Meyer-Lucht & Sommer (2009) found that in yellow-necked mice populations, a larger number of MHC alleles were associated with lower parasite loads. The authors argue that this result stems from the fact that the loss of MHC diversity in yellow-necked mice, sampled in suburban areas, is recent and reflects population fragmentation due to habitat degradation. Therefore, the MHC-parasite load association does not reflect the state of evolutionary equilibrium between hosts and pathogens. When there has been a recent bottleneck, it can be expected that populations with depleted MHC diversity may be more susceptible to parasites due to the loss of resistant alleles and because parasites may adapt more easily to a few common MHC alleles (Radwan *et al.* 2010). An alternative explanation is offered by a theoretical model showing that selection from parasites tends to deplete, rather than increase, MHC diversity in small populations (Ejsmond & Radwan 2011). Irrespective of the causality, a negative association between MHC diversity and parasite load can be predicted for populations that have experienced recent bottlenecks, but in stable populations at host–parasite equilibrium, the opposite trend can be expected. Here, we were able to test these contrasting predictions using a population system of the ornate dragon lizard, *Ctenophorus ornatus*.

The ornate dragon, a diurnal, agamid lizard with an average lifespan of 3 years (Bradshaw 1965), is highly adapted to life on granite outcrops (Bradshaw 1965; Whithers 2000), which allow unambiguous delineation of populations. Like most reptiles, *C. ornatus* is infected by several ectoparasites, the most common of which is a small (approximately 0.5 mm) red trombiculid mite. As ticks act as vectors for many haemoparasites such as haemogregarines (e.g. Lewis & Wagner 1964; Allison & Desser 1981; Smallridge & Paperna 2000), other protists (e.g. Bonorris & Ball 1955) and bacteria (Camin 1948), their abundance can be used as a general index of parasite pressure. Indeed, infection with vector-borne microorganisms have been shown to vary with vector abundance, both temporarily (Bennett & Cameron 1974; Godfrey *et al.* 2011) and spatially (Sol *et al.* 2000). Tick-transmitted haemoparasites are likely to impose substantial selection on their reptile hosts; for example, haemogregarines have been shown to cause reduced haemoglobin concentration, locomotor speed, tail regeneration and endurance in common lizards (Oppliger *et al.* 1996; Oppliger & Clobert 1997; Clobert *et al.* 2000) and decreased home ranges in Australian sleepy lizards (*Tiliqua rugosa*) (Bouma *et al.* 2007). In addition, ectoparasite infections themselves can have negative impacts on metabolism (Klukowski & Nelson 2001), growth rates, condition, stress (Clobert *et al.* 2000; Godfrey *et al.* 2010), activeness and home range size (Main & Bull 2000), as well as causing skin lesions, inflammation and blood loss (Goldberg & Bursey 1991; Goldberg & Holshuh 1992). Other significant selection pressures on this species are likely to be bird and snake predation (Bradshaw 1965), but these are likely to interact with parasite load, which should make individuals more susceptible to predation.

The ornate dragon provides an opportunity to assess the genetic effects of habitat fragmentation because it is found both in remnants of native woodlands, and in highly disturbed areas. The majority of the ornate dragon's range is located within the agriculturally-intensive region (wheat belt) of southwest Western Australia. The wheat belt has undergone extensive clearing over the past 150 years (Saunders 1989) and is now made up of approximately 140 000 km^2 of exotic grasses and cereal crops with only small isolated pockets of native woodlands, scrub, heath and thicket persisting throughout the region (Saunders 1989). Because granite outcrops inhabited by the ornate dragon are unsuitable for agriculture, and therefore have been spared direct clearing and size reductions (Yates *et al.* 2007), the effect of fragmentation is not confounded by the effect of habitat loss. Levy *et al* (2010) found that even though land clearing around the outcrop leaves outcrop size unchanged, it restricts gene flow, reducing genetic variation and increasing population structure. Furthermore, simulations showed that the reduction in genetic diversity is very recent and that gene flow must have been negligible since fragmentation (Levy *et al.* 2010).

Thus, we predicted that within the agricultural land, a negative association between MHC diversity and parasite load would be observed, similar to the yellow-necked mice study. In reserves, however, the number of alleles is more likely to reflect long-term host–parasite evolutionary dynamics rather than recent fragmentation. Furthermore, higher connectivity should facilitate inflow of genes from other populations. Such inflow can be selected for by a negative-frequency-dependent selection (Nadachowska-Brzyska *et al.* 2012), which should be stronger in populations experiencing higher pressure from parasites. Consequently, in native woodlands, a positive association between parasite load and MHC variation would be expected.

Additionally, based on theoretical results (Ejsmond & Radwan 2011) suggesting that in small, fragmented populations MHC diversity is depleted faster than neutral variation, we predicted that the slope of regression of MHC diversity on microsatellite allelic richness (AR) would be steeper in the agricultural land than in the reserve.

Finally, we were able to investigate whether our proxy for pathogen pressure predicts the average number of alleles carried by individuals in a given population. Individuals that possess more alleles should be able to recognize a wider range of parasites, and this advantage should be more pronounced in populations with higher parasite load. While the advantage of possessing multiple alleles may be counterbalanced by the higher loss of lymphocytes in the process of negative selection (Nowak *et al.* 1992), Woelfing *et al.* (2009) speculated that under such a trade-off, individuals 'with an MHC diversity just high enough to present peptides of locally abundant parasites and pathogens efficiently will be selected'. Consequently, populations under lower pressure from parasites can be expected to have the optimum at lower individual MHC diversity. Consistent with this idea is the finding that river sticklebacks, exposed to lower parasite diversity than the lake fish, have a relatively lower number of MHC IIB alleles (Wegner *et al.* 2003). Based on these considerations, we expected to observe a positive association between the number of ticks and average number of MHC alleles per individual across reserve populations, but not across the agricultural land populations, where composition of haplotypes bearing various numbers of MHC genes would be shaped by drift rather than by host–parasite co-evolution.

Materials and methods

Samples

This study took place at Tutanning nature reserve (32.541°S, 117.325°E) and in an area in the adjacent agricultural land (32.626°S, 117.486°E), located approximately 180 km southeast of Perth, Western Australia. Lizards from 24 outcrops were sampled: 12 outcrops from within Tutanning reserve (henceforth: reserve) and 12 from outcrops in the adjacent agricultural land. In the agricultural land, only outcrops that were fenced off from the surrounding paddocks or showed minimal degradation were sampled. To control for the potential effects of outcrop size and distance between outcrops on genetic structure, only outcrops as similar as possible with respect to size and distance apart were selected for sampling (average outcrop size: reserve = 26 034 $\pm$ 9264 m^2, agricultural land = 37 032 $\pm$ 5592 m^2, average distance apart: reserve = 3.68 $\pm$ 0.48 km, agricultural land = 3.09 $\pm$ 0.27 km (see Levy *et al.* 2010 for details). In total, 391 lizards from 24 outcrops ranging in size from 0.10 to 149.94 ha (mean = 14.02 ha), with samples from 10 or more adult lizards (mean sample size = 20, range = 10–41), were examined. For all the analyses below, however, we used only populations for which we were able to obtain MHC and microsatellite genotypes of at least eight individuals. Therefore, the final data set included nine populations from the agricultural land and eight from the reserve (Table 2).

Lizards were captured by hand at first light, when they were still relatively inactive, by lifting exfoliated granite slabs. If fewer than 10 adult lizards on a single outcrop were captured by this method, the outcrop was resampled during the afternoon, using nets to catch additional lizards. The sex of each lizard was recorded.

As juveniles are likely to have lower levels of parasitism compared with adults (Reardon & Norbury 2004), only adults classed as individuals with a SVL greater than 69 mm (Baverstock 1972) were used in parasite load analyses.

To estimate red trombiculid mite abundance, each lizard was examined thoroughly and the number of mites present was counted, paying close attention to the tail and the skin folds around the neck. Finally, each lizard was toe-clipped to provide a tissue sample for genetic analysis and then returned to its outcrop. Toe-clips were stored in 100% ethanol prior to DNA extraction. In lizards, toe and tail clipping is commonly used for identification and DNA analysis (Olsson 1994; Lebas 2001; Berry *et al.* 2005) and *Ctenophorus ornatus* are known to lose toes as a result of predation and rock-associated injuries (N. LeBas, personal observation).

Development of primers

We developed primer amplifying highly polymorphic 2nd exon coding for α1 domain, which forms a part of the peptide-binding groove of MHC I molecule. Infection with haemoparasites in birds, which similar to reptiles possess nucleated blood cells, has been shown to be associated with MHC class I variants (e.g. Westerdahl *et al.* 2005; Loiseau *et al.* 2008). Moreover, MHC I variants can be associated with resistance to ticks themselves (Stear *et al.* 1990; Olsson *et al.* 2005). First sequences were obtained from 12 individuals using degenerate primers (RepMHC1F1: 5′-GTTSCAGYDGATGTDYGGCTGYGA-3′ and RepMHC1R1: 5′-CTC GABRCASIYBYSCTCCAGRTA-3′) designed by Miller *et al.* (2006) from aligned reptile (including birds), amphibian, fish and mammalian MHC class I sequences. On the basis of these sequences, we designed internal primers (drawF1: 5′-TGACAAGGAG ACCCTCACCTG-3′; drawF2: 5′-GGAGACCCTCAC CTGGACGGC-3′; drawR1: 5′-GCCGTCCAGGtGAGG GTCTCC-3′ and drawR2: 5′-CAGGtGAGGGTCTCC TTGTCA-5′) for vectorette PCR approach (Ko *et al.* 2003). This allowed us to obtain sequences flanking exon 2. We adopted a modified vectorette PCR protocol used by Ko *et al.* (2003), as described in Babik *et al.* (2008). Based on flanking sequences obtained from vectorette PCR, we designed new primers specific to *C. ornatus*: DRA_I_F: 5′-ATGTAYGGYTGTGAGMTGA-3′ and DRA_I_R: 5′-TSTCCTTCCSGTASTCCAGG-3′.

Amplification and sequencing

Amplification was performed using fusion primers, which contained the 454 titanium adaptor (A in forward and B in reverse primer), 6-bp barcode and MHC class I specific primer. Details of the amplification strategy are described in Kuduk *et al.* (2012). PCR products were pooled in approximately equimolar quantities; pools were purified with the MinElute PCR Purification Kit (Qiagen) and sequenced at the Plant-Microbe Genomics Facility (The Ohio State University). A subsample of 20 randomly selected individuals were amplified and sequenced in duplicates to estimate the genotyping error. Extraction of reads from multifasta files, assignment of reads to individuals and generation of alignments of variants present in each amplicon were performed with JMHC (Stuglik *et al.* 2011). The output from JMHC was analysed using BLAST, EXCEL and BIOEDIT (Hall 1999).

Validation of alleles, filtering artefacts and genotyping

Preliminary assessment of the data indicated that due to a large number (>10) of variants coamplifying within individuals, considerable coverage was required for reliable genotyping. Only individuals with coverage of at least 300× were considered for validation of alleles, filtering out artefacts and genotyping; 300× coverage was sufficient for reliable genotyping (see Results).

The procedure for the validation of true alleles (TA) and filtering out artefacts (AA) followed Radwan *et al.* (2012) with modifications as detailed below. We started with calculating the maximum per amplicon frequency (MPAF) for each variant; MPAF is the maximum fraction of the individual's reads attributed to a given variant across all individuals in which the variant was present. Then, for all variants with MPAF ≥2%, we constructed a neighbour-joining tree (Fig. S1, Supporting information) to visually assess the diversity of variants and check for the presence of well-supported clusters of similar variants. This data set of 360 variants obviously contained an unknown proportion of artefacts and was used only for exploratory purposes. One motivation behind this exploration was the presence of common variants of the lengths 200, 201, 202 and 205 bp, implying the occurrence of frameshift mutations in high frequencies, signatures of pseudogenization. We wanted to check whether such potential pseudogene variants could be identified as separate cluster(s) and excluded from further analyses. Indeed, 96 variants 200, 201 and 202 bp long formed a cluster with 82% bootstrap support. These putative pseudogene variants are not considered further.

For the remaining 264 variants, we applied criteria outlined in Radwan *et al.* (2012) to distinguish TA from AA at the level of the entire data set, that is to distinguish variants which were TA in at least some individuals from variants which could in all cases be explained as AA. Because AA with sequences identical to TA may

appear as a result of PCR/sequencing errors or in vitro recombination during PCR, the same variant may in some individuals be an AA and in other individuals a TA. In brief, starting from the lowest MPAF values, the method first compares variants to more common variants within the same amplicon to check whether they can be explained as PCR/sequencing errors. Two thresholds are than set: the lower, below which most sequences can be considered artefacts, and the higher one, above which the sequence cannot be explained as an artefact (chimera, indel or substitution) derived from more common variants. For individual genotyping, all sequences below the first threshold are discarded as AA, and all above the threshold are accepted as TA. For sequences in the 'grey zone' between the two thresholds, comparisons to TAs within the same amplicon are made on a case-by-case basis. This method is very reliable, typically resulting in 90–100% congruence in independent replicates (e.g. Radwan *et al.* 2012; Sepil *et al.* 2012; Kloch *et al.* 2013).

The lowest MPAF of the variant classified as TA was 2.3%, but TAs were generally rare in the MPAF range 2–3% (85.7% variants in this range were AA). True alleles dominated the MPAF ranges 3–4% (33.3% AA) and 4–5% (only 12.5% AA). The overwhelming majority of variants classified as TA were 202 or 205 bp long. The total of four variants, three 204 bp and one 201 bp could not be explained as AA, were thus classified as pseudogene alleles and removed from further analyses. Additionally, two variants (202 and 205 bp) classified as TA had premature stop codons; these were also excluded from further analyses as nonfunctional. We defined the 'grey zone' (Radwan *et al.* 2012), in which both TA and AA co-occur, as the MPAF range of 2.3–4.3%.

For individual genotyping, only TA were considered. All TA which occurred in a given individual in more than 4.3% reads were accepted as alleles indeed present in the individual. The TA in the grey zone (2.3–4.3% reads) were accepted as the individual's alleles only if they could not be explained as artefacts (PCR chimaera or one bp substitution) derived from other TA present in this individual in a larger number of reads. A custom Python script was used to perform this check. To confirm that our minimum coverage was sufficient for reliable genotyping, we performed a posteriori check using the approach and R scripts of Sommer *et al.* (2013), which allow unequal allele amplification efficiencies. Amplification efficiencies were estimated using maximum likelihood and used to calculate the number of reads required to achieve a given genotyping confidence, that is the probability of 0.95 that all alleles detected in an amplicon would be present in at least seven reads (which is based on our minimum coverage of 300 and minimum TA threshold of 2.3%). Because the optimization algorithm did not reach convergence with the entire data set, such calculations were performed for a random sample of 100 individuals.

Genotyping error was calculated from samples sequenced in duplicates by dividing the number of alleles found only in one replicate by the total number of alleles detected in all samples analysed in duplicates (conservatively, if two duplicates of a sample differed in the number of alleles, the lower was accepted as the true number).

Expression analysis

To check which MHC class I alleles are transcribed, we extracted RNA from tail tips of five individuals using RNeasy Mini Kit (Qiagen). Extracted RNA was DNase treated to remove residual DNA and reverse transcribed using Omniscript RT Kit (Qiagen) following the manufacturer's protocols, and resulting cDNA was used for PCR amplification of MHC class I. Seqeuencing was performed as described above. For four of these individuals, also genomic DNA was analysed in parallel.

MHC class I sequence diversity and tests of selection

The location of putative antigen-binding sites (ABS) was inferred from the structure of human HLA genes (Reche & Reinherz 2003). We conservatively considered only ABS common for HLA-A, HLA-B and HLA-C proteins. For all alleles, the average pairwise nucleotide distances (Kimura 2-parameter model – K2P), Poisson-corrected amino acid distances, as well as the average rates of synonymous (dS) and nonsynonymous (dN) substitutions, using the Nei–Gojobori method (Nei & Gojobori 1986) with the Jukes–Cantor correction for multiple substitutions, were computed in MEGA5 (Tamura *et al.* 2011) for all, ABS and non-ABS codons. Standard errors were obtained through 1000 bootstrap replicates.

Two standard approaches were used to test for the signatures of positive selection in MHC class I alleles. First, we used the one-sided Z test implemented in MEGA5 to compare dN and dS for all, ABS and non-ABS sites. Second, codon-based method implemented in PAML 4.7 (Yang 2007) was used to compare the fit of the following models of codon evolution to data: M0 (assumes a single dN/dS ratio for all codons), M7 (assumes that all codons evolve under purifying selection or neutrally, and the rate variation among codons is modelled using beta distribution) and M8 (as M7 but allows for positive selection). Model selection was based on the Akaike information criterion (AIC). Positively selected codons were identified through the

Bayes empirical Bayes procedure (Zhang *et al.* 2005). A neighbour-joining tree was constructed from the matrix of the Jukes–Cantor distances between TA to visualize diversity of TA and reveal any clusters of similar sequences.

Microsatellite data and comparison of variation and differentiation in two classes of markers

Data on genotypes in 22 microsatellite loci are from Levy *et al.* (2010). In this study, we excluded any individuals which had missing genotypes in more than one microsatellite locus. Because we could not assign MHC alleles to loci, they were analysed as dominant markers with each allele encoded as a dominant biallelic locus. AR for each population was calculated as the mean number of alleles present in 1000 randomly drawn samples of eight individuals.

To check whether the relationship between microsatellite and MHC AR differs between the agricultural land and reserve, we tested for differences of the slope and intercept using lm function in R package (R-core development team). Relationships between geographic distance and F_{ST} for reserve and the agricultural land were calculated as in Levy *et al.* (2010) using IBDWS (Jensen *et al.* 2005).

Analysis of associations between MHC variation, parasite load and habitat type

The effect of the type of habitat (agricultural land vs. reserve) on tick number was analysed using a generalized mixed model with Poisson error distribution, population as a random effect, and sex and habitat as fixed effects. The model was implemented in MCMCGLMM R package (Hadfield 2010).

To analyse the association between MHC AR and parasite load across populations, we calculated median number of ticks for each population. These data were analysed with general linear models implemented in lm function in R, with MHC AR as a dependent variable and median tick number, habitat and microsatellite AR as predictors. To test the hypothesis that the slope of the relationship between MHC diversity and parasite load differs between habitats, the habitat × parasite load interaction was entered into the model. Equivalent models were run with average number of alleles per individual in a population (henceforth MHC AN) as a dependent variable except that we entered MHC AR rather than microsatellite AR as a predictor. This was done to account for the fact that the number of alleles per individual may not only depend on the number of MHC loci, but also depend on their heterozygosity. Heterozygosity will in turn depend on the number of alleles segregating in a population.

Results

MHC diversity, signatures of selection and expression

This section, describing the overall MHC class I variation in the ornate dragon, is based on a broader sample from a larger geographical range (Levy *et al.* 2010; 2012, 2013). A total of 547 lizards were genotyped; the mean coverage ± (SD) was 705 (±404) reads. Extensive variation between individuals was detected in the number of alleles which ranged from 3 to 14, with the mean of 8.0 ± (SD) 1.79 (Fig. 1). The minimum number of reads required for 95% genotyping confidence when allowing for unequal allele amplification did not exceed 300 for any of the 100 tested individuals (max = 294, mean 155 ± 52 reads). This and the lack of significant correlation between the number of alleles and coverage ($r = 0.06$, $P = 0.14$) indicate that the coverage was sufficient for reliable genotyping. The minimum coverage of 300 required for genotyping was attained for 19 of 20 duplicates, and genotyping error was estimated as 3.7%. Overall, we identified 226 putatively functional alleles; 46 of them were found in single individuals only (Fig. 2). Alleles showed considerable sequence divergence (Table 1), with both synonymous and nonsynonymous variation higher in ABS. Nonsynonymous divergence in ABS tended to be higher than synonymous ($P = 0.09$, one-sided Z test of selection), whereas the opposite trend was observed in non-ABS codons (Table 1). The M8 model of sequence evolution fitted the data much better than the M7 (ΔAIC = 187) or M0 (ΔAIC = 890) models, indicating the operation of positive selection in the evolutionary history of the ornate

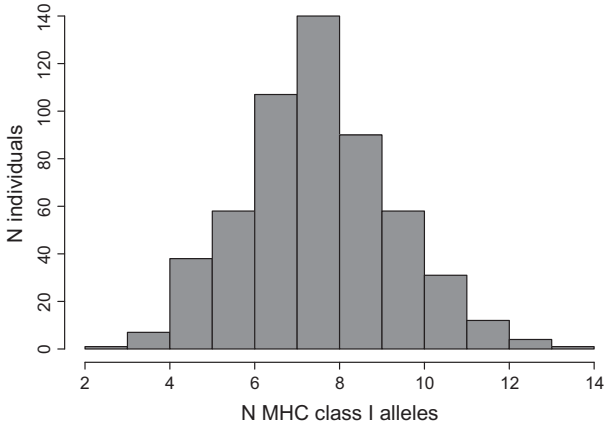

Fig. 1 The distribution of the number of MHC class I alleles per individual among 547 genotyped ornate dragons.

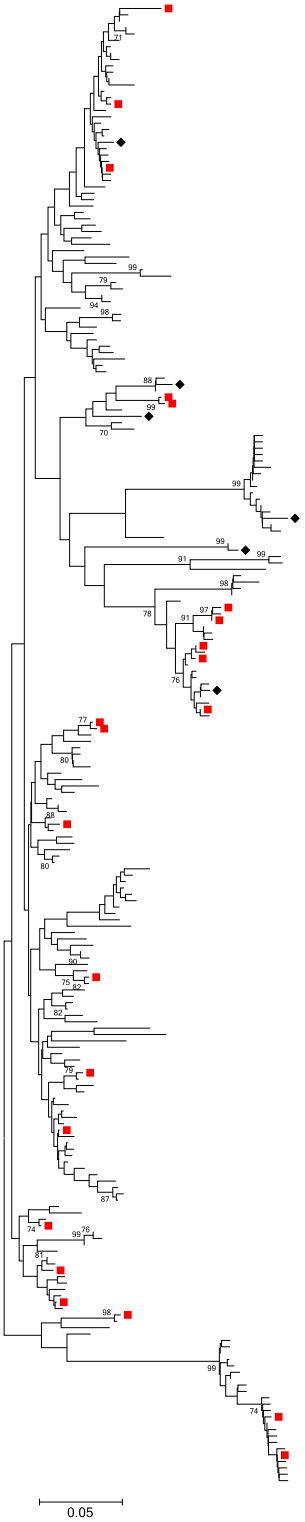

Fig. 2 A Neighbour-joining tree showing the diversity of putatively functional ornate dragon's MHC class I alleles. Alleles which have been confirmed as transcribed are marked with squares. The tree does not contain pseudogene clusters (see main text), but includes several putative pseudogene alleles (variants with stop codons/fameshift mutations, marked with diamonds).

dragon MHC class I, although only two codons (positions 49 and 53 in the alignment) were identified as evolving under positive selection (both with PP > 0.99). One of these codons is at a putative ABS, and the other is an insertion with respect to the human MHC class I protein sequence.

We collected expression information for five individuals, including four cDNA–gDNA pairs. Unfortunately, a rigorous comparison between genotypes obtained from genomic DNA and cDNA was not possible because in no pair did both cDNA and gDNA reach the threshold of 300 reads required for reliable genotyping. We were nevertheless able to confirm the transcription of 22 alleles. The distribution of those alleles on the tree (Fig. 2) indicates that expressed alleles do not form separate groups/clusters; thus, all alleles were classified as putatively expressed. On the other hand, the presence of a few obviously nonfunctional (pseudogene) sequences scattered across the tree (see Materials and methods) suggests that in some cases pseudogene and functional alleles may have similar sequences. Consequently, in all analyses below, we take into account all alleles detected in an individual except for those carrying clear signatures of pseudogenisation (stop codons or frameshifts). However, comprehensive classification of alleles as potentially functional and pseudogene would require collection of high-quality RNA from many individuals which is not currently feasible in the ornate dragon due to conservation concerns.

MHC variation within and between populations

The comparison of variation between MHC and microsatellites was performed only in the subset of populations assayed for MHC variation. These were the seventeen populations located in the area where strong differences in genetic variation within outcrops and differentiation among outcrops were detected by Levy *et al.* (2010) (Table 2). MHC genotypes were available for 304 and microsatellite genotypes for 478 individuals (Table 2).

Major histocompatibility complex AR differed between habitats (mean ± SD = 23.1 ± 2.5 in reserve and 19.0 ± 4.1 in the agricultural land, $t_{15} = 2.47$, $P = 0.020$). However, MHC AR was significantly correlated with that of microsatellites (Fig. 3; $t_{15} = 3.34$, $P = 0.004$), so the difference between habitats likely reflected a general difference in genetic variation between habitats. Indeed, in a model in which microsatellite AR was entered as a covariate, neither the effect of habitat nor that of the microsatellite AR × habitat interaction was significant (Table 3).

Mean F_{ST} between populations was much higher in the agricultural land (0.32 for MHC, 0.31 for microsatellites

Table 1 The overall mean genetic distances between putatively functional MHC class I alleles

	nt dist	aa dist	d*S*	d*N*	*P*
All	0.128 (0.014)	0.217 (0.035)	0.156 (0.031)	0.119 (0.022)	1.0
ABS	0.278 (0.074)	0.451 (0.163)	0.212 (0.094)	0.313 (0.122)	0.09
Non-ABS	0.106 (0.014)	0.183 (0.033)	0.149 (0.035)	0.091 (0.019)	1.0

All distance measures were calculated for all codons (All), codons located in putative ABS (ABS) and those outside ABS (non-ABS). Distances are as follows: nt dist, nucleotide Kimura 2 parameter distance; aa dist, amino acid distance, Poisson corrected for multiple substitutions; d*S* and d*N*, synonymous and nonsynonymous distances calculated according to the Nei & Gojobori (1986) method with the Jukes–Cantor correction for multiple substitutions. *P*, *P* value of the *Z* test of the null hypothesis $dN/dS \leq 1$.

Table 2 Sample sizes and allelic richness (AR) for MHC and microsatellites

Habitat	Population	*N* MHC	*N* ticks	*N* msat	MHC AR	Microsatellite AR
R	Eagle	28	27	25	23.1	6.79
R	N	20	39	62	24.8	7.69
R	O	29	24	42	25.1	7.67
R	P	9	8	21	21.8	7.68
R	R	13	12	10	21.2	6.28
R	S	22	12	45	18.5	6.65
R	Tammar	11	17	22	24.7	6.85
R	Y	11	5	15	25.7	6.62
R	Mean ± SE				23.1 ± 0.9	7.0 ± 0.2
C	BBQ	8	11	19	20.0	6.29
C	Dryandra	15	10	17	19.4	4.81
C	Fresh Fields	14	4	12	16.8	2.38
C	Lilleystone (LS)	17	18	19	15.6	5.79
C	RNR	17	15	19	18.6	5.16
C	Sheep Flock Rock	31	30	30	20.9	3.76
C	Simpson	18	21	17	16.2	5.03
C	Windmill	19	14	14	15.2	3.86
C	Z	22	17	89	28.4	6.54
C	Mean ± SE				19.0 ± 1.4	4.8 ± 0.4

R, reserve; C, cleared agricultural land.
See Levy *et al.* (2012) for the map of locations.

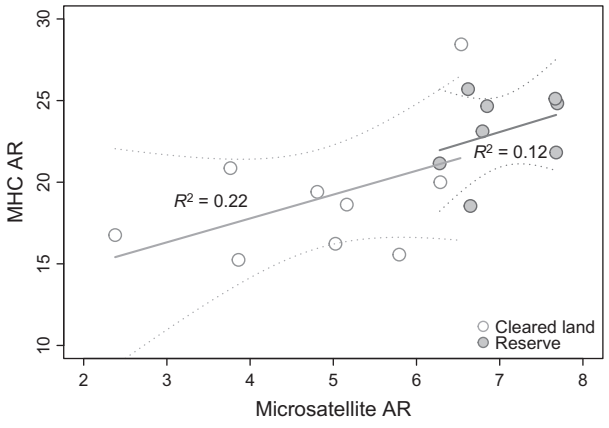

Fig. 3 Relationships between MHC and microsatellite allelic richness (AR).

coded as dominant markers and 0.22 for microsatellites coded as codominant markers) than in reserve (0.11, 0.09 and 0.06, respectively); thus, it appears that genetic differentiation in MHC mirrors closely that in microsatellites.

MHC diversity and parasite load across populations

In a simple model with MHC AR as a dependent variable and the median number of ticks as a predictor, we have found a significant positive association (Fig. 4, $r = 0.492$; 95% CI: 0.013–0.971; $t_{15} = 2.19$, $P = 0.045$). However, habitats differed in parasite load (Table 4), so this relationship may simply reflect habitat differences. Indeed, after accounting for habitat, the association between MHC AR and parasite load became nonsignificant

(Table 5). Furthermore, the interaction between habitat and parasite load in their effect on MHC AR was not significant, indicating that the slope of the relationship is similar in the agricultural land and in the reserve (Table 5); removing this nonsignificant interaction did not affect the conclusion that MHC AR is not significantly associated with parasite load after habitat type is accounted for ($t_{14} = 1.05$, $P = 0.311$). Qualitatively identical results were obtained when microsatellite AR was not entered as a predictor.

Major histocompatibility complex AN showed habitat-specific association with parasite load after controlling for MHC AR (Table 6). Analysis without MHC AR as a covariate yielded the same conclusion (not shown).

Table 3 Results of a general linear model with MHC allelic richness (AR) as a dependent variable, and habitat type and microsatellite AR as predictors (error d.f. = 13)

	t	*P*
Habitat	0.021	0.984
Microsatellite AR	1.681	0.116
Interaction	0.035	0.972

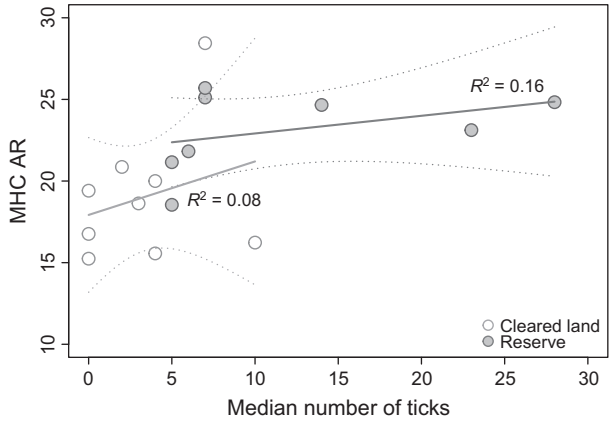

Fig. 4 Relationship between parasite load (median number of ticks per population) and MHC allelic richness across two habitat types: reserve and the agricultural land.

In the reserve, higher parasite load was associated with increased MHC AN ($r = 0.717$, 95% CI: 0.021–1.413, $t_6 = 2.52$, $P = 0.045$), but in the agricultural land, the correlation was not significant ($r = -0.479$, 95% CI: −1.264 to 0.305, $t_7 = -1.446$, $P = 0.191$; Fig. 5).

Discussion

Major histocompatibility complex genes are thought to evolve under selection imposed by parasites, and indeed, since the study of the MHC of nonmodel organisms has become popular, many authors have demonstrated associations between the MHC and resistance (or susceptibility) to parasites (reviewed in Spurgin & Richardson 2010). Likewise, molecular signatures consistent with models of host–parasite co-evolution are also apparent in most of the species investigated so far. The sequences coding for the peptide-binding region of the MHC class I molecule of the ornate dragon, which were characterized in this study, provide another example of positive selection favouring novel MHC peptides, which could be due to either heterozygote advantage or negative frequency dependence mechanisms (Takahata & Nei 1990). Both mechanisms can maintain MHC polymorphism (Takahata & Nei 1990; Borghans *et al.* 2004).

However, the empirical evidence for the association between selective pressure from parasites and MHC diversity has been limited and contradictory. While most of the few studies which have tested such an association report positive relationships (Wegner *et al.* 2003; Prugnolle *et al.* 2005), a negative relationship has recently been reported (Meyer-Lucht & Sommer 2009), and at the interspecific level among mammals, the sign of the relationship seems to vary according to taxonomic group (de Bellocq *et al.* 2008; Winternitz *et al.* 2013).

Although we did find a positive relationship between tick number and MHC diversity across populations, this relationship could be explained by the differences in the load of ticks between habitats. Tick number was higher in the reserve population, which also had higher genetic variation, both at natural markers (Levy *et al.* 2010) and at MHC genes (this study).

Table 4 The effect of habitat (R, reserve vs. C, cleared agricultural land) and sex on the load of ticks in the ornate dragon estimated using general mixed model implemented in MCMCglmm (see Materials and methods for details)

	Posterior mean	Lower 95%CI	Upper 95% CI	Effective sample	P_{MCMC}
Intercept	–0.25	−1.14	0.57	358.2	0.582
Habitat (R)	2.90	1.70	4.17	402.6	<0.001
Sex (male)	0.03	−0.49	0.55	617.3	0.936
Habitat × sex	0.41	−0.22	1.04	780.9	0.226

Table 5 Results of a general linear model with MHC allelic richness (AR) as a dependent variable, and habitat type, median number of ticks per population (ticks) and microsatellite AR as predictors (error d.f. = 12)

	t	*P*
Microsatellite AR	1.416	0.182
Ticks	−0.015	0.988
Habitat	−0.001	0.998
Habitat × ticks	0.203	0.842

Table 6 Results of a general linear model with per population average number of MHC alleles per individual (MHC AN) as a dependent variable, and habitat type and median number of ticks per population (ticks) as predictors (error d.f. = 13)

	t	*P*
MHC AR	3.688	0.003
Ticks	−3.303	0.006
Habitat	−4.486	0.001
Habitat × ticks	3.700	0.003

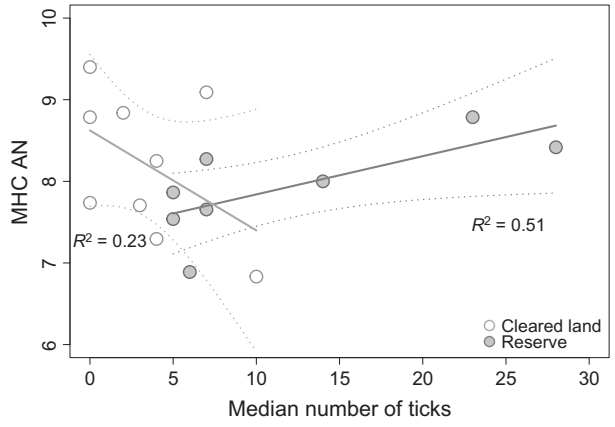

Fig. 5 Relationship between parasite load (median number of ticks per population) and the average number of MHC alleleles per individual in populations inhabiting two habitat types: reserve and the agricultural land.

Differences in the sign of the relationship between parasite pressure and MHC variation were hypothesized to result from differences in the histories of the populations investigated. In populations in which the number of MHC alleles reflects the history of co-evolution with parasites, a positive correlation could be expected, whereas in bottlenecked ones, in which reduced genetic diversity is a result of a recent disturbance, the relationship can be reversed (Meyer-Lucht & Sommer 2009; Ejsmond & Radwan 2011). Here, we were able to test this hypothesis by comparing the slopes of the relationships between parasite pressure and MHC diversity in two habitat types that differed in their recent history: undisturbed populations from the reserve and fragmented populations from agriculturally disturbed land. Tick abundance was used as an index for the abundance and diversity of parasites that they transmit. Based on this index, our results provided no support for the hypothesis: we found that the slopes of the relationship between average per population tick number and MHC AR did not differ significantly between the habitats.

Instead, our results suggest that the number of MHC alleles carried can respond to population-specific pressure from parasites. The extent of intraindividual variation (3–14 alleles per individual) clearly demonstrates that the variation is due to variation between haplotypes in the number of carried copies of MHC class I genes rather than variation in heterozygosity (in which case the lowest number should be no less than the half of the highest number of alleles). Nevertheless, heterozygosity (and hence number of alleles per individual) is expected to be higher in populations with more MHC alleles; therefore, we controlled for MHC AR in our analyses (see methods). Irrespective of whether we controlled for MHC AR or not, we found that the two habitats differed in the slope of the relationship between tick load and the average number of alleles carried by individuals in a population (MHC AN). The association was not significant among populations from cleared land, but in the reserve, parasite load predicted MHC AN. This result is consistent with the verbal argument put forward by Woelfing *et al.* (2009) that the optimal individual MHC diversity should be higher in populations exposed to higher pressure from parasites. More studies investigating how parasite pressure shapes the average number of copies carried by individuals should contribute to our understanding of why we observe such an extensive variation both within and between species (Kelley *et al.* 2005). We are aware of only one system where such an association was suggested: river sticklebacks, exposed to lower parasite diversity than the lake fish, have relatively lower number of MHC IIB alleles (Wegner *et al.* 2003).

In isolated populations in cleared land, however, we observed no significant relationship. The load of ticks in the cleared land was generally lower, possibly due to the weaker connectivity of populations, and the lower number of alternative hosts, preventing their spread. Weaker selection from parasites coupled with low gene flow between the agricultural land populations likely caused the frequency of MHC haplotypes to be driven by drift rather than by selection.

In the context of our finding that parasite load affects MHC AN across reserve populations, it is

intriguing as to why we have not observed the concurrent increase in MHC AR, especially that the two measures were correlated. We cannot exclude that our power to detect a significant relationships was compromised by the fact that we studied genomic DNA, and we did not know the expression status of all MHC alleles. However, we only included in our analyses those sequences which did not have signatures of pseudogenisation. Furthermore, we showed that expressed alleles are scattered on all branches of the MHC gene tree, which suggests that most of these potentially functional alleles are expressed.

Theory for MHC polymorphisms has so far only considered single loci, and the dynamics of multilocus systems may differ (e.g. when individuals in a population carry many MHC variants, the advantage of rare/novel alleles may be lower than in single-locus systems). Our results thus indicate that there is a need for explicit modelling of populations with haplotypes bearing multiple, functionally equivalent loci.

Our results also highlight the need to model explicitly the verbal argument put forward by Woelfing *et al.* (2009) that the optimal number of alleles per individual may vary depending on selective pressure of parasites. Under the optimality hypothesis, and with variable pathogen pressure, there could be negative frequency-dependent selection at the level of entire haplotypes, facilitating rapid response to selection when the optimum changes. This could also maintain extensive copy number variation among populations. Furthermore, given copy number variation between populations, migration may contribute to the variation within populations. Indeed, given that there should be an optimal individual MHC diversity, it is intriguing why an extensive variation in the number of MHC copies exists within populations (e.g. Radwan *et al.* 2012). Our results suggest that this may be due to gene flow between populations differing in optimal MHC copy number.

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J.R. desinged the study, carried out statistical analyses and drafted the manuscript; K.K. designed primers and analysed expression data; N.L. and E.R. analysed tick load and microsatellite diversity and helped to draft the manuscript; W.B. carried out genotyping, population-genetic analyses and helped to draft the manuscript.

Data accessibility

sequences of MHC alleles, microsatellite and MHC genotypes, expression analysis data, tick abundance data: DRYAD entry doi: 10.5061/dryad.19264.

Supporting information

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

Fig. S1 A neighbor-joining tree showing relationships between all variants with the maximum per amplicon frequency ≥2%.