Maintaining functional major histocompatibility complex diversity under inbreeding: the case of a selfing vertebrate

A. Ellison¹, J. Allainguillaume², S. Girdwood¹, J. Pachebat¹, K. M. Peat¹, P. Wright³ and S. Consuegra¹,*

¹IBERS, Aberystwyth University, Penglais Campus, Aberystwyth SY23 3DA, UK
²Department of Applied Sciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY, UK
³Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Major histocompatibility complex (MHC) genes encode proteins that present pathogen-derived antigens to T-cells, initiating the adaptive immune response in vertebrates. Although populations with low MHC diversity tend to be more susceptible to pathogens, some bottlenecked populations persist and even increase in numbers despite low MHC diversity. Thus, the relative importance of MHC diversity versus genome-wide variability for the long-term viability of populations after bottlenecks and/or under high inbreeding is controversial. We tested the hypothesis that genome-wide inbreeding (estimated using microsatellites) should be more critical than MHC diversity alone in determining pathogen resistance in the self-fertilizing fish *Kryptolebias marmoratus* by analysing MHC diversity and parasite loads in natural and laboratory populations with different degrees of inbreeding. Both MHC and neutral diversities were lost after several generations of selfing, but we also found evidence of parasite selection acting on MHC diversity and of non-random loss of alleles, suggesting a possible selective advantage of those individuals with functionally divergent MHC, in accordance with the hypothesis of divergent allele advantage. Moreover, we found that parasite loads were better explained by including MHC diversity in the model than by genome-wide (microsatellites) heterozygosity alone. Our results suggest that immune-related overdominance could be the key in maintaining variables rates of selfing and outcrossing in *K. marmoratus* and other mixed-mating species.

**Keywords:** *Kryptolebias marmoratus; MHC; mixed-mating; outcrossing; parasites; inbreeding*

1. **INTRODUCTION**

Elucidating the mechanisms that maintain genetic diversity in natural populations is a central issue in evolutionary biology. Genetic variation is needed to maintain the adaptive potential of species and has been correlated with both population [1] and individual fitness [2]. A reduction in genetic diversity within populations may render them more vulnerable to pathogen infections [3,4], particularly if it is accompanied by the loss of variation at immune-related genes [5,6]. Inbreeding can further increase susceptibility to infections [7], and loss of immunocompetence is one of the most common consequences attributed to inbreeding [8,9]. Inbreeding depression arises from mating between close relatives as a consequence of increased homozygosity of deleterious recessive alleles and loss of heterosis [10]. Although inbreeding avoidance is considered the norm, it can vary depending on the relative costs of inbreeding in relation to, for example, outbreeding [11] or dispersal [12]. In fact, inbreeding tolerance depends on the strength of inbreeding depression but also on the mating system and mate availability [13]. Thus, mating with close relatives could be beneficial, for example, in species with low inbreeding depression and limited mate availability [13] or when there is a conflict over parental care and related parents display increased cooperation in comparison with unrelated parents [14].

Among the immune-related genes involved in the defence against pathogens in vertebrates, the highly polymorphic genes of the major histocompatibility complex (MHC) are probably the most studied and better understood in terms of function and evolution [15]. MHC genes encode proteins that present pathogen-derived antigens to T-cells, initiating the adaptive immune response [16]. MHC diversity seems critical for the effectiveness of the immune response and most variation in individual MHC genes is concentrated in the region that binds antigens from pathogens [17]. Polymorphism within the antigen-binding region is thought to be maintained by balancing selection driven by pathogens (either through overdominance or via frequency-dependent selection) [18,19], but also by mate choice [20,21]. Although MHC diversity seems to be positively correlated with parasite resistance—particularly in the case of multiple pathogens [22], in many cases, an optimal rather than a maximum number of alleles is assumed to maximize pathogen resistance [20,23,24]. This is because too much allelic diversity could result in a reduction in T-cell receptor diversity driven by negative selection, ultimately reducing the ability to fight infection [25]. Evidence of MHC heterozygote advantage [26,27],
rare-allele advantage [28], association of individual MHC genotypes or alleles with susceptibility to infection [29,30] and shifts in allele frequencies after exposure [31] suggest that parasites are the primary source of selection on MHC.

MHC genes in most natural populations display high heterozygosity, number of alleles and genotypic diversity, as they can be present in multiple copies. MHC copy numbers can vary between species and also between individuals from the same species [32]. Inbreeding and population reductions can greatly impact on MHC diversity at both individual and population levels, although low MHC diversity is not necessarily associated with inbreeding. Inbred populations tend to have a lower number of unique MHC genotypes and fewer alleles compared with their outbred counterparts [6,33], but it is assumed that MHC diversity can be maintained even under high levels of inbreeding owing to the intensity of selection acting upon MHC genes [34,35]. Although high MHC diversity does not necessarily guarantee low susceptibility to infectious diseases [36], populations with low MHC diversity tend to be more prone to be affected by pathogens [5,37]. However, there are cases of bottlenecked populations that persist and even increase in numbers despite low MHC diversity [38–40]. In clonal fishes, such as the Amazon molly (Poecilia fromosa), number of MHC alleles seems to be less important for pathogen resistance than genotypic diversity [41]. Thus, the relationship between MHC diversity, the ability to respond to pathogens and the long-term viability of bottlenecked and/or inbred populations remains unclear [5,42]. As a consequence, recommended conservation measures driven by the maintenance of MHC diversity [43] contrast with the view that avoidance of genome-wide inbreeding should be of greater concern than focusing solely upon a single family of genes [5].

Self-fertilization (selfing) represents a particularly extreme case of inbreeding, and as such, selfing organisms may provide ideal models to study the maintenance of immunocompetence under high rates of inbreeding. Selection for reproductive assurance under conditions of low mate availability can favour selfing. However, selfing could also render organisms more susceptible to parasites if disease resistance was lowered as a consequence of inbreeding and loss of heterozygosity. In these conditions, small amounts of outcrossing would break up linkage disequilibrium and increase heterozygosity across the genome, potentially counteracting not only the negative implications of accumulation of deleterious mutations [44], but also the loss of variability at immune-related genes.

The mangrove rivulus (Kryptolebias marmoratus) is a self-fertilizing fish with mixed-mating reproduction (variable rates of selfing and outcrossing) [45]. Its mode of reproduction [46,47], unique among vertebrates, makes it an ideal model to study the evolution of MHC genes under high inbreeding rates. Natural populations of K. marmoratus are composed of highly homozygous (based on microsatellite markers) hermaphrodites that can reproduce by internal self-fertilization [46] or by outcrossing with males, present at low proportions varying between 1 and 25 per cent [48]. Despite the near total homozygosity at neutral loci displayed by K. marmoratus, MHC genes seem to retain some variation in the species even in individuals completely homozygous for microsatellites [49]. The distribution of MHC diversity in this species is assumed to be the result of a complex pattern of interactions among the rate of selfing, the intensity of balancing selection and the persistence of ancestral lineages [49].

We have investigated the role of selection in maintaining MHC diversity under high inbreeding conditions by analysing the diversity of the exon 3 of class I MHC in a natural population of K. marmoratus. A negative correlation between genetic diversity at neutral markers (microsatellites) and parasite loads had been previously observed in this species [50], and here we tested the hypothesis that genome-wide microsatellite heterozygosity may be more critical than MHC diversity alone in determining pathogen resistance. We also compared the natural MHC diversity with that in two laboratory lines that had undergone more than 10 generations of selfing. Given the predominance of highly homozygous selfing hermaphrodites and low levels of outcrossing in wild K. marmoratus populations, we expected low individual MHC diversity but higher diversity of genotypic combinations in the natural population. By contrast, low allele and genotypic diversity was expected in the laboratory lines.

2. METHODS

(a) Sample collection

A total of 183 K. marmoratus were analysed. Of them, 72 were collected at 4 locations in Calabash Caye, Turneffe Atoll, Belize (17°16′N, 87°48′W) by using cup traps and wire minnow traps [50]. Fish were euthanized and stored in 95 per cent ethanol. A subset of 50 fish had been screened for gill and gastro-intestinal parasites as in Ellison et al. [50]. Briefly, the gills and gastro-intestinal tract were screened for parasite infections under a dissecting microscope. We found that bacterial gill cysts, trichodinids and acanthocephalans were the most common infections. Counts of these 3 infections were added to produce an estimate of total parasite load. The remaining 111 fish belonged to 2 different selfing lines maintained in the laboratory for at least 10 generations (R and DAN, [51]).

(b) MHC and microsatellite genotyping

Total genomic DNA was extracted from muscle tissue using the Wizard SV 96 DNA Purification Kit (Promega Corp.). Fish were genotyped for MHC class I using the degenerate forward primer KM-MHCF (5′acagctgacctagGGCTGTCARTTGGRAYGAWG3′) in combination with the modified reverse primer KFR1 (5′acagctgacctagCTCCCATAGTTCACATCCTTC3′, [49]) in order to amplify a 185–192 bp fragment of the K. marmoratus MHC class I 3rd exon. The degenerate forward primer was designed to amplify all loci previously published [49]. The adaptor tail (acagctgacctag) complementing each primers at the 5′ terminus, allowed for the DNA tagging of each individual PCR template for 454 analysis following the procedure described below. Samples were divided into two groups (1 and 2). Group 1 was amplified with the above primer combinations where n is A, and group 2 where n is T. Polymerase chain reactions (PCR) were performed in 10 μl and contained 1 μl (approx. 20 ng) of genomic DNA, 1 μl (2 μM) equimolar mixture of both primers and 5 μl BioMix (Bioline). PCR conditions were 95°C/5 min, 5 × (94°C/30 s,
55°C/30 s, 72°C/45 s), 20 × (94°C/30 s, 50°C/30 s, 72°C/45 s), 72°C/10 min. The 10 μl product was then diluted 1:20 for the second round of PCR. Samples in groups 1 and 2 were split again into two: group 1a, 1b; 2a and 2b. Each sample in each group was then amplified with a single primer complementary to the adaptor tail each bearing a unique 6bp DNA tag indicated by Ns at the 5’ (5’-ANNNNNGG AAACAGCTATGACCATG3’) (see the electronic supplementary material, appendix 1). 2nd PCRs were performed in 25 μl and contained 2.5 μl diluted product from 1st PCR, 2.5 μl (2 μM) primers and 12.5 μl BioMix (Bioline). Products from the 2nd PCR were then pooled in approximately equimolar quantities into two pools: pool A (group 1a and 2b) and pool B (group 1b and 2a). The two pools were purified using AMPure PCR purification kit (Agencourt). The purified pools were sequenced in a single 454 FLX run, according to the 454 AmpliSeq sequencing protocols provided by the manufacturer (Roche 454). Prior to sequencing, each pool was uniquely tagged with Roche MID adaptors. This allowed both pools to be run together on a single part of the Pico-Titer plate and ensured a minimal number of unique sequences to be run together on a single part of the 454 Genome Sequencer FLX Software Package v. 2.0.00.22.

Following initial quality assessment using standard settings of the 454 software, the following procedure was used to increase stringency of quality control. Only sequences showing a perfect match to the original primers (KMMHCf and KMFR1) and with no N-calls were extracted from the multi-fasta file produced by 454 using jMHC MHCf and KMFR1) and with no N-calls were extracted to increase stringency of quality control. Only sequences determining the clustering trees to create supertypes [64].

c) Data analyses

For MHC alleles, the presence of potential recombination sites was determined using SSBP and GARD within the HyPhy software package at http://www.datamonkey.org [57]. The occurrence of gene conversion was assessed using the program GENECONV v. 1.81 [58], using the option that allows only synonymous sites of coding sequences to be included in the analysis, thus avoiding the possible effects of selection [59]. In order to analyse signatures of selection on specific codons of the peptide-binding region (PBR) of MHC alleles, the number of non-synonymous versus synonymous substitutions was analysed using both the random effects likelihood (REL, Bayes factor = 50) and the fixed effects likelihood (FEL, posterior probability = 0.05) approaches. The analyses were implemented using the HyPhy software package at http://www.datamonkey.org [57]. Only positively selected sites (PSS) identified by both methods were used for subsequent analyses.

For studies on MHC variation, the method for defining and quantifying MHC diversity requires careful consideration, as differences in nucleotide sequences may not necessarily translate in MHC protein binding differences. Therefore, an extra allele does not necessarily confer the ability to respond to greater range of pathogen peptides. In contrast, changes in the amino acid sequence that alter the binding properties of the MHC binding pocket can affect the range of pathogen peptides an individual can respond to. In addition, the complex nature of these closely linked duplicated genes means it is often very difficult to identify specific loci and calculate MHC heterozygosity. For these reasons, grouping MHC alleles by ‘supertypes’ based on their binding properties is often considered biologically more significant than allelic diversity [28,60,61]. We defined MHC-supertypes by applying amino-acid-sequence-based clustering, as proposed by Doytchinova and Flower [60]. The amino-acid sequences of all PSS were aligned. All amino acids outside PSS were excluded. Each amino acid was described by five z-descriptors: z1 (hydrophobicity), z2 (steric bulk), z3 (polarity), z4 and z5 (electronic effects) [62] and translated accordingly into a mathematical matrix. This matrix was imported into PAST v. 2.04 [63] and cluster analysis was applied using both Ward’s and paired linkage algorithms with three similarity measures (Euclidean distance, Pearson correlation coefficient and cosine correlation efficient). Bootstrapping (n = 1000) was applied to all trees generated. In the absence of established criterion to define supertypes from a clustering tree (when antigen-binding affinities cannot be assessed via immunoassays), those sequences that were consistently grouped together in all trees were considered part of the same supertype. This approach (rather than tracing an arbitrary line in one of the trees) was considered conservative because it minimized the risk of grouping sequences with distinct binding affinities within a same supertype by taking into account only robust nodes of the clustering trees to create supertypes [64].

Global individual genetic diversity was estimated from microsatellite genotypes using the homozygosity by locus index (HL), a measure that weighs contribution of each locus to overall homozygosity, depending on its allelic variability and correlates better than uncorrected homozygosity (Ho, measured as proportion of homozygous loci) with genome-wide homozygosity and inbreeding coefficients in open populations [63]. Data were checked for normality and homogeneity of variances. Parasite counts and number
of MHC supertypes were square-root-transformed. HL was arcsine-transformed, and fish length was log_{10}-transformed. The correlation between the number of MHC supertypes and microsatellite homozygosity (HL) was assessed using Pearson’s method. For each individual, the number of generations of selfing since an outcrossing event was estimated using INSTRUCT [66] and then correlated with MHC diversity (no. of MHC supertypes). INSTRUCT was run ten times for each value of K (1–10) with a burn-in period of 10,000 replicates followed by 50,000 replicates to collect estimated parameters and likelihoods, as detailed in Ellison et al. [67]. Fully homozygous individuals were excluded from this analysis as INSTRUCT is unable to estimate the number of selfing generations under total homozygosity.

(d) Evidence of pathogen-mediated acting on MHC loci
The relationship between parasite load and MHC diversity (number of MHC supertypes) was assessed using general linear models (GLMs). The full model included number of MHC supertypes (number of MHC supertypes)^2, heterozygosity (HL), host length and sampling site as factors plus all two- and three-term interactions. The square of the number of supertypes was used in order to test whether the relationship was polynomial, to test for the existence of an optimal rather than maximal number of supertypes [68,69]. HL was included as has it has been previously shown to be significantly correlated with parasite loads in *K. marmoratus* [50]. Host length was included as it had been previously shown to be influential on parasite loads in fish [70]. Sampling site was also included as parasite loads significantly differed between locations [67]. Model selection was then carried out based on the AICc (AIC corrected for small sample size) criterion [71]. GLMs and model selection were also carried out upon each infection category in order to assess their individual effects.

To test whether any of the MHC supertypes were significantly associated with individual parasite loads, t-tests were used to compare counts between fish carrying different supertypes. For each supertype, the relative risk of being infected was estimated by odds ratio tests. Significant p values were corrected for multiple tests, using a Bonferroni procedure [72].

In order to assess the divergence of alleles across the range of individual MHC diversities, a correlation was estimated between number of alleles and mean and minimum dissimilarity of MHC alleles, estimated as the total number of amino acid differences (across entire sequence) calculated using MEGA v. 4 [55].

All data are available through Dryad doi:10.5061/dryad.8k608.

3. RESULTS

(a) MHC diversity
In total, 165 of the 183 fish were successfully genotyped. The median number of reads per individual was 351 (minimum = 50, maximum = 2238, s.d. = 389.1). Following sequencing artefact removal (see the electronic supplementary material, appendix 2), 51 putative alleles were identified (EMBL accession numbers HE616687 to HE616733). Four of these alleles (A1-02, H1-01, H1-02 and H1-03) had been previously described in *Kryptolebias marmoratus* populations [49]. All the sequences identified by cloning in 6 individuals were present in the 454 sequences and only in one case, one of the sequences with the lowest number of 454 reads was not represented in the cloning data. Analysis of the sequences using GENECONV found no evidence for gene conversion events. A single recombination event (base pair 124 of aligned nucleotide sequences) was identified and taken into account for analysis of positive selection.

Of the 63 codons, 11 were found to be under positive selection (see the electronic supplementary material, figure S2), using both the random effects likelihood (REL) and fixed effects likelihood (FEL) approaches. The physicochemical properties of the amino acids at the 11 positively selected sites (PSS) were used to determine MHC supertypes. Cluster analysis obtained 16 consensus supertypes, each possessing between 2 and 6 alleles (figure 1). Clusters (supertypes) and their bootstrapping values were consistent among all methods used (see the electronic supplementary material, figure S3). No fish possessed more than two alleles in each supertype. Given the complex nature of closely-linked duplicated MHC genes [73], no attempt was made to estimate the actual number of loci. The number of supertypes possessed by wild individual ranged between 1 and 8, whereas the number of alleles ranged between 1 and 11. Only two wild fish possessed the same MHC genotype and the average number of supertypes in the wild fish was 3.9. In contrast to wild fish, captive-bred fish (resulting from self-fertilization over 10 to 20 generations) possessed between 3 and 4 supertypes and alleles. All fish from strain R possessed the same genotype, and only two distinct genotypes were found in the DAN strain.

The number of MHC supertypes in wild fish was highly correlated with microsatellite homozygosity (r = −0.440, p < 0.001), indicating that fish with high levels of global genetic heterozygosity (low HL) had also higher numbers of supertypes. There was a significant negative correlation between number of selfing generations and MHC diversity (r = −0.464, p < 0.001), i.e. fish that had gone through more selfing generations had fewer MHC supertypes (figure 2). Both mean and minimal amino acid dissimilarity among alleles per fish increased with a decreasing number of alleles (mean; r = −0.298, p = 0.020, figure 3a, minimum; r = −0.681, p = <0.001, figure 3b).

(b) Evidence of pathogen-mediated selection
In total, 43 of the 66 fish successfully genotyped were screened for bacterial gill cysts, trichodinids and acanthocephalans. The model that best fitted the data according to the AICc criterion [71] excluded HL, site and host length as predictors (plus all interactions) and indicated that parasite load increased with decreasing number of supertypes (p = 0.007, AICc = 165.1) (see the electronic supplementary material, table S1a). For bacterial gill cysts, the most probable model included number of supertypes (p = 0.010) and sampling site (p < 0.001) (see the electronic supplementary material, table S1b). In the trichodinid model, only number of MHC supertypes was included (p = 0.044) (see the electronic supplementary material, table S1c). The model that best fitted the acanthocephalans data included only sampling site (p ≤ 0.001) (see the electronic supplementary material, table S1d). All models excluded the square
of MHC supertypes, indicating that, within our data range, there was no optimal number of MHC supertypes (table 1). As MHC supertype number and HL were correlated, GLMs were re-run with the residuals of the correlation instead of separate variables. For total parasite load, the strongest model was MHC-HL residuals ($p = 0.022$). For bacterial gill cysts, the most probable model included residuals ($p = 0.032$) and sampling site ($p < 0.001$). In the trichodinid model, only residuals were included ($p = 0.052$). The model that best fitted the acanthocephalans data included only sampling site ($p < 0.001$). There was no effect of predictor order on the significance of any GLM.

After Bonferroni correction, two significantly negative associations were found of specific MHC supertypes with individual parasites infections (table 2, electronic supplementary material, figure S4). In particular, fish that possessed supertype S16 had significantly fewer bacterial gill cysts ($t = 4.60$, $p < 0.001$) and fish with the supertype S1 had significantly fewer trichodinids. Supertypes S14 and S15 were also found to be negatively associated with bacterial gill cysts (S14; $p = 0.010$, S15; $p = 0.021$), but these relationships were not significant after applying Bonferroni corrections. Two positive associations (increased load with possession of supertype) were also found between supertype S4 and bacterial gill...
cysts (p = 0.008) and supertype S8 and acanthocephalans (p = 0.008), but again these were not significant after correction for multiple tests.

Odds-ratios tests could only be conducted with bacterial gill cysts and trichodinids infections as only one fish was found to be uninfected with acanthocephalans. Although non-significant after correction for multiple tests, supertypes S1 and S2 showed reduced risk of infection with trichodinids (S1; OR = 0.16, p = 0.007, S2; OR = 0.13, p = 0.005) and supertype S13 reduced risk of infection with bacterial gill cysts (OR = 0.00, p = 0.017).

4. DISCUSSION
The mode of reproduction of K. marmoratus, characterized by selfing and occasional outcrossing, provides a unique case for investigating the role of pathogen-mediated selection in shaping MHC diversity under high inbreeding conditions. Selfing allows the preservation of advantageous allele combinations and enhances local adaptation, but also increases homozygosity as a consequence of continued inbreeding, whereas a low level of outcrossing maintains the potential for generating new genotypes. We found that K. marmoratus MHC diversity is lost after successive generations of selfing (i.e. individuals with higher MHC diversity resulted from fewer selfing generations) and also a positive correlation between heterozygosity at microsatellites and MHC diversity (measured as number of supertypes), suggesting that MHC might be evolving in a similar way to neutral markers (MHC allele loss over generations: y = −0.6785x + 6.4532, microsatellite allelic richness loss: y = −0.6852x + 6.9832), although not necessarily. Despite the low levels of MHC observed in the highly inbred laboratory lines and in the most inbred of the wild individuals, the distribution of MHC alleles in K. marmoratus seems to be influenced by selection. Thus, we found evidence of positive selection acting on a number of sites within the peptide-binding region of the MHC class I. Moreover, our results suggest that the retention of supertypes in individuals with low diversity is not random but that in cases where only few alleles were preserved, those were highly divergent. As supertypes were defined on the basis of their molecule binding properties, they provide a particularly good approximation of their functional differences [74]. The preservation of few and very divergent functional MHC supertypes in individuals with low microsatellite heterozygosity could be interpreted as the consequence of balancing selection shaping MHC diversity, as in other species that have undergone severe bottlenecks [75,76]. This pattern suggests that individuals with divergent MHC alleles could have a selective advantage as a consequence of being able to fight a broader range of pathogens, possibly under a mechanism of divergent allele advantage [77–79]. Our results also indicate that diversity of MHC supertypes, and not only microsatellite heterozygosity, is critical for parasite resistance. In this sense, the best models for predicting total and individual parasite loads included MHC diversity, but not microsatellite heterozygosity, suggesting that MHC can be a better predictor of parasite resistance than overall microsatellite heterozygosity. Further evidence of pathogen-mediated selection is provided by the association of particular supertypes with low parasite loads. Three of these supertypes associated with particular pathogens (S1, S14 and S16)
eral generations of inbreeding. In fact, MHC diversity where MHC diversity is lost, as in neutral loci, after sev-
is related to parasite loads even in selfing conditions and not only genome-wide microsatellite heterozygosity, balancing the transmission advantage of selfing [84].

would produce enough variability to evade parasites, of outcrossing in mixed-mating organisms [83], such as parasites can magnify the effects of inbreeding depression owing to genome-wide homozgyosity of deleter-
ious recessive alleles and/or homozgyosity at overdominant loci; our study supports the hypothesis that immune-
related overdominance could be the key to maintaining variables rates of selfing and outcrossing in K. marmoratus and other mixed-mating species.

The average diversity of MHC (number of supertypes) per individual in the natural population of K. marmoratus was 3.9, similar to the diversity found in the laboratory lines after more than 10 generations of selfing. But, in contrast to the laboratory lines, the natural population displayed a high diversity of genotypes, and only two individuals were identical in their MHC genotype, despite the high levels of homozgyosity and low MHC diversity observed in some of them. This is similar to the distribution of MHC variability in the clonal Amazon molly, where the genotypic variation of the population seems to be more critical than the individual number of MHC copies [41]. However, the ability of K. marmoratus to self-fertilize and outcross means that it can produce new gene combinations through recombination that ensure genotypic diversity. In theory, overdominance for fitness (e.g. immunocompetence) at one or more loci could maintain mixed mating by selection on selfing alleles [81,82], as selfing alleles would become rapidly associated with homozgyous individuals with lower fitness (e.g. higher infection susceptibility). Our results indicating that higher MHC diversity was related to lower parasite loads would fit the hypothesis that overdominance in loci related to parasite resistance could play a role in the maintenance of outcrossing in mixed-mating organisms [83], such as K. marmoratus, as even a low amount of outcrossing would produce enough variability to evade parasites, balancing the transmission advantage of selfing [84].

In summary, our study revealed that MHC diversity, and not only genome-wide microsatellite heterozygosity, is related to parasite loads even in selfing conditions where MHC diversity is lost, as in neutral loci, after several generations of inbreeding. In fact, MHC diversity predicted parasite loads better than heterozygosity alone. We also found evidence of parasite selection acting on MHC diversity and of non-random loss of alleles, suggesting a possible selective advantage of those individuals with functionally divergent MHC supertypes, in accordance to the hypothesis of divergent allele advantage. Parasites can magnify the effects of inbreeding depression owing to genome-wide homozgyosity of deleter-
ious recessive alleles and/or homozgyosity at overdominant loci; our study supports the hypothesis that immune-
related overdominance could be the key to maintaining variables rates of selfing and outcrossing in K. marmoratus and other mixed-mating species.

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