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Original Investigation

Spatial distribution of microsatellite and MHC-DRB exon 2 gene variability in the Jamaican fruit bat (*Artibeus jamaicensis*) in Mexico

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ABSTRACT

Genetic diversity is essential to the evolutionary and adaptive potential of a species. Several empirical genetic studies have highlighted the importance of considering both neutral and adaptive genetic variation when characterizing microevolutionary dynamics. Genes at the major histocompatibility complex (MHC) have become excellent models for researching adaptive variation and natural selection, because of the crucial role they play against pathogens. The Jamaican fruit bat, *Artibeus jamaicensis*, is one of the most common and well-studied Neotropical mammals and is characterized by generalist feeding habits, high dispersal capability, and abundant populations. Fifteen localities of *Artibeus jamaicensis* were genetically assessed using ten neutral microsatellites and one expressed MHC class II locus (DRB) in order to detect footprints of balancing selection. Extensive polymorphism was found at both markers. Overall, 161 alleles were identified at DRB exon 2 gene, and 315 at microsatellites. The observed and expected heterozygosity averaged over all localities (±SD) was 0.756 ± 0.15 and 0.885 ± 0.11, respectively, with all localities in Hardy–Weinberg equilibrium. Pairwise genetic differentiation estimates were generally significant, but the overall differentiation was lower at DRB gene ($F_{st} = 0.039$) than at microsatellites ($F_{st} = 0.154$). We detected significant isolation by distance at microsatellite loci, but not at DRB exon 2 gene. STRUCTURE and BAPS analyses detected a population genetic structure made up of five defined clusters. Our results suggest that balancing selection has maintained the allele frequencies of DRB exon 2 gene across the distribution of *A. jamaicensis* in Mexico.

Introduction

Genetic diversity influences the evolutionary and adaptive potential of populations and species (Alcaide et al., 2008). Populations are exposed to a dynamic balance between local selective forces and genetic drift, and these contrasting mechanisms produce different signatures in distinctive parts of the genome (Coscia et al., 2012; Excoffier et al., 2009). Genetic studies often use different markers to estimate the amount of genetic variation present in individuals and populations. This information has allowed to determine the dispersal pattern of individuals, the genetic structure of species, and the distribution of reproduction and relatedness among individuals within populations (Lukas et al., 2004). Such markers, however, are not really suitable for detecting adaptive variation and fitness-related loci. As local adaptations often require restricted gene flow, exploring variation at genes under selection may be useful not only for this, but also for inferring population subdivision (Alcaide et al., 2008). In some cases, indeed, the separation of populations may be too recent to have left a signal at neutral loci, so that differences between them may only be detectable in genes under selection, such as those of the major histocompatibility complex (MHC; Lukas et al., 2004).

MHC is a family of highly polymorphic genes that encode cell surface glycoproteins that bind and present short peptides to specialized cells of the immune system in order to trigger the appropriate immune response (Bernatchez and Landry, 2003). Most MHC variation is concentrated in the antigen-binding site (ABS)—the part of the molecule that recognizes and binds antigens. An individual can only possess two alleles at any one locus; gene duplication is frequent; and many species express multiple types of MHC molecules to increase the range of pathogens recognized by their immune system (Cammen et al., 2011). Variation at MHC genes

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largely determines what foreign peptides an individual can respond to. This variation thus influences individual fitness, making it a good model to study adaptive variation (Bernatchez and Landry, 2003).

The level and spatial distribution of adaptive genetic variation will differ according to the strength and spatial scale of selection (Muirhead, 2001). Positive selection occurs when a new selectively advantageous mutation is segregating in a population. This type of selection may provide evidence for adaptation at the molecular level (Nielsen, 2001). Population genetics theory predicts that when selection increases the frequency of beneficial alleles in a population, the patterns of differentiation and diversity at linked sites change in a predictable way (Maynard-Smith and Haigh, 1974). Particular subsets of alleles may be favored locally (through local adaptation) but have limited success in invading other populations, thus resulting in higher levels of population differentiation than expected under neutrality (Muirhead, 2001; Nielsen, 2001).

Three main mechanisms may result in balancing selection, making them important in maintaining high levels of MHC variability. The first mechanism is the so-called 'heterozygote advantage', where individuals that are heterozygous at MHC loci are able to respond to a greater range of pathogen peptides than homozygotes. This hypothesis explains the persistence of highly divergent MHC alleles over millions of years (Wakeland et al., 1990). The second mechanism or hypothesis is frequency-dependent selection, which occurs when an allele or genotype is favored at one frequency, but disadvantaged at another frequency. For example, pathogens adapt to infect the most common host genotypes, leaving rare genotypes least infected. If alleles are favored when they are rare, but selected against when they are common, a balanced polymorphism results (Takahata and Nei, 1990; Penn and Potts, 1999). This could lead to continual cycling of host and parasite genotype frequencies within populations, hence maintaining high levels of MHC variability (Westerdahl et al., 2004). Finally, the hypothesis of fluctuating selection proposes that spatial and temporal heterogeneity in the type and abundance of pathogens may maintain diversity at the MHC (Hill, 1991). Host–parasite interactions are shaped by environmental conditions, which play an important regulating role in the distribution, transmission and developmental success of parasites and pathogens. Hence, co-evolutionary selection processes should vary not only in time but also in space, and different specific MHC alleles should have an advantage in different environments (Consuegra et al., 2011; Schwensow et al., 2007).

The difficulties in detecting selection in the MHC are compounded by multiple selective forces that act simultaneously at different geographical and evolutionary scales, neutral processes such as genetic drift, as well as the presence of multiple copies of particular MHC genes (Aguilar and Garza, 2006). One way of detecting selection is to contrast genetic structure at MHC with that at neutral loci across multiple populations (Aguilar and Garza, 2006; Spurgin and Richardson, 2010). In populations subject to heterozygote advantage or frequency-dependent selection favoring the immigration of rare alleles, MHC diversity should be high compared with total diversity, resulting in a lower population genetic structure at MHC than at neutral loci (Schierup et al., 2000). In populations subject to fluctuating selection, however, different subsets of MHC alleles will arise, and higher genetic structure will be observed at MHC than at neutral loci (Spurgin and Richardson, 2010).

Variation at the MHC class II has been under-investigated in the order Chiroptera. Diversity of the MHC-DRB exon 2 gene was first evaluated in the sac-winged bat (*Saccopteryx bilineata*). Evidence for two gene copies was found in 2% of the individuals, and allelic diversity generated by recombination was detected (Mayer and Brunner, 2007). The number of non-synonymous substitutions was higher than that of synonymous ones, this phenomenon being evidence for long-term balancing selection (Mayer and Brunner, 2007).

Evidence for positive selection and extensive polymorphism were found in other bat species. In *Noctilio albiventris*, the analysis of DRB gene variability revealed a single expressed polymorphic locus, with heterozygosity differences between males and females most likely due to MHC-mediated post-copulatory mechanisms or sex-specific survival differences (Schad et al., 2011). In *Myotis velifer*, a widespread continental species, DRB polymorphism was found to be similar to that commonly reported in other mammals. The geographically restricted *M. vivesi*, by contrast, showed very limited polymorphism (Richman et al., 2010).

Artibeus jamaicensis is one of the most common and well-studied Neotropical bats, inhabiting a wide variety of vegetation types. The species is essentially frugivorous but, if resources become scarce, it can consume insects, nectar and pollen. Potential roosting sites are very diverse and include caves, hollow trees and buildings (Ortega and Castro-Arellano, 2001). The species is abundant in highly-fragmented areas (Henry et al., 2007). The flexibility of these bats, capable of using various strata of vegetation, may have allowed them to take advantage of modified landscapes (Estrada and Coates-Estrada, 2002; Henry et al., 2007). A previous analysis of DRB exon 2 gene in *A. jamaicensis* (Del Real-Monroy et al., 2014) showed high levels of MHC polymorphism. Analyzed sequences from 193 bats resulted in 161 alleles likely to be representative functional alleles, as no deletions, insertions or stop codons were detected. The presence of three to five alleles in 40% of the individuals indicated duplication of the DRB exon 2 gene. The data were consistent with the hypothesis that recombination was involved in generating DRB diversity and maintaining variation in this population through positive selection (Del Real-Monroy et al., 2014). Here, we present the first study of an abundant bat that covers the whole species distribution, with a view to investigate the spatial patterns of divergence as obtained with two types of markers: adaptive (DRB exon 2 gene) and neutral (microsatellites). The specific aims of this study were: (i) to assess the genetic variation of *A. jamaicensis* in 15 localities using ten microsatellites and the DRB gene, and (ii) to obtain evidence for positive and balancing selection by characterizing and comparing population genetic structure as revealed by these two types of markers.

Material and methods

Sample collection and nucleic acid extraction

Fifteen sampling localities of *A. jamaicensis* were established across the species distribution in Mexico, from the slopes of the Pacific Ocean and the Gulf of Mexico to the Yucatán Peninsula (Fig. 1).

Ten to 22 *A. jamaicensis* individuals were captured at each location in February–August 2012. The bats were captured using mist nets placed near building roost sites as well as in agricultural fields known to be frequently visited by the species. Individuals were identified to the species level based on morphological characters (Medellin et al., 2008). Standard morphological measurements were taken on each individual using an electronic caliper with a precision of 0.1 mm. Then, a small (3-mm) piece of wing tissue was removed and the bat was released. All animal handling methods followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and The Animal Care Use Committee of the American Society of Mammalogists, 2016).

Tissue samples were stored in 96% ethanol and kept refrigerated at −20 °C. After digesting the samples in proteinase K (280 g/mL), total genomic DNA was extracted using a salt extraction protocol (Aljanabi and Martinez, 1997).

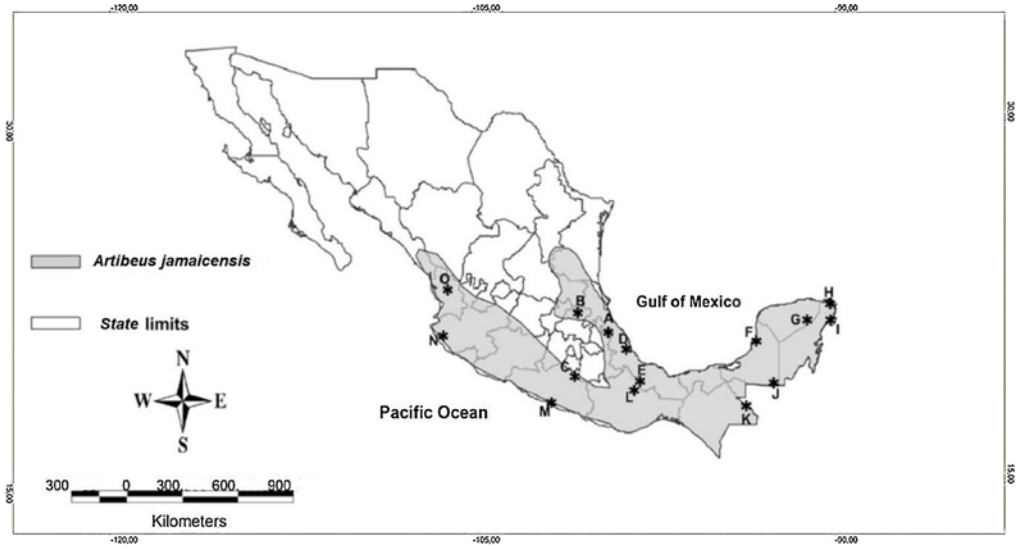

Fig. 1. Map of the studied localities of *Artibeus jamaicensis* in Mexico. A) Cave 'Las Vegas', Puebla (20°08′54″N, 97°24′39″W); B) Tepehuacán de Guerrero, Hidalgo (21°0′49.12″N, 98°50′25.96″W); C) Quebrantadero–Contla, Morelos (18°30′58.86″N, 98°48′4.72″W); D) Buena Vista, Veracruz (19°24′23.0″N, 96°34′20.5″W); E) Villahermosa, Tabasco (17°59′15.21″N, 92°55′44.93″W); F) Chiná, Campeche (19°45′37.00″N, 90°29′45.99″W); G) Cenote Suytún, Yucatán (20°41′54.8″N, 88°07′34.0″W); H) Cenote Salsipuedes, Quintana Roo (21°25′00.1″N, 87°02′60.0″W); I) Hotel Mayakoba, Quintana Roo (20°41′30.1″N, 87°02′00.3″W); J) Calakmul, Campeche (17°54′55″N, 89°41′10″W); K) Yaxchilán, Chiapas (16°53′44.31″N, 90°57′57.69″W); L) San Gaspar Yagalaxi, Oaxaca (17°35′2.28″N, 96°12′28.94″W); M) Aguas Blancas, Guerrero (17°02′34.6″N, 100°03′41.0″W); N) Cajón de Peña, Jalisco (19°59′36.5″N, 105°07′45.9″W) and O) Punta Raza, Nayarit (22° 23′59″N, 104° 55′88″W). The gray area shows the distribution of *Artibeus jamaicensis* in Mexico (IUCN, 2016).

Genotyping and molecular cloning of major histocompatibility complex

The DRB exon 2 locus was chosen for analysis because this region is known to code for the ABS of the MHC molecule (Alcaide et al., 2008; Schad et al., 2011). A 216-bp fragment of exon 2 was amplified using JS2Cape-DRB, a specific exon 2 primer, and JSi2Cape-DRB, a specific intron primer, both developed for *Carollia perspicillata* (Schad et al., 2012). Each PCR reaction consisted of a 20-μL volume, as described by Del Real-Monroy et al. (2014). PCR products were visualized by electrophoresis on 1.2% agarose gels, followed by staining with ethidium bromide.

All amplicons of the correct size were cloned and sequenced to characterize allelic variation at the DRB gene. Cloning was performed using the TA Cloning® Kit with TOP10 competent cells (Invitrogen ™). To verify that cloning products matched the expected insert size, DNA from positive bacterial colonies was reamplified using M13 forward and reverse primers specific to the TA cloning vector (Cutrera and Lacey, 2006). For each reaction, DNA template was obtained by touching the edge of a white colony with an autoclaved toothpick, dipping the toothpick into the master mix, and then mixing the reaction components. Amplicons with inserts of the correct size were cycle-sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems ™). An average of eight clones per individual were screened. For some individuals, up to 25 clones had to be screened to obtain all possible variants. To avoid analyzing PCR artifacts, DRB exon variants were considered true alleles only if they occurred in at least two individuals or two independent PCRs of the same individual (Cutrera and Lacey, 2006).

Genotyping of microsatellites

Ten microsatellite primers developed for *A. jamaicensis* (Ortega et al., 2002) were used: AjA47, AjA74, AjA84, AjA107, AjA123, AjA151, AjA180, AjA185, AjA199 and AjA464. Each PCR reaction consisted of a 20-μL volume, as described by the same authors. PCR products were visualized by electrophoresis on 1.2% agarose gels, followed by staining with ethidium bromide. After amplification, microsatellite products were multiplexed and allele size was determined with GeneMapper® Software v3.7 (Applied Biosystems ™).

Genetic diversity

The DRB gene sequences obtained were aligned and edited using Geneious 6.1.2 (Biomatters Ltd.). Primers were trimmed and the open reading frame was determined by downstream analysis. The similarity of DRB sequences was confirmed by NCBI BLAST searches. The number of allelic variants and allele frequencies were obtained using ARLEQUIN 3.5 (Excoffier et al., 2005). Allele frequencies per locality were computed as the number of individuals carrying a specific allele divided by the total allele count observed in the population (Ekblom et al., 2007).

For microsatellites, Micro-Checker 2.2.3 (van Oosterhout et al., 2004) was used to check for the presence of null alleles, large allele drop-out, and scoring errors, using a 95% confidence interval and 10 000 repetitions, and applying the Bonferroni correction. Possible departures from Hardy–Weinberg equilibrium (HWE) were examined by an exact test, and unbiased *P*-values were calculated using a Markov chain algorithm (Guo and Thomposon, 1992) with 10 000 dememorizations, 1000 batches and 10 000 iterations per batch. Linkage disequilibrium was also assessed with a log-likelihood ratio statistic (*G*-test) in GENEPOP 4.0 (Raymond and Rousset, 1995). Genetic variability was estimated at each locality by computing allele frequencies and the number of observed alleles (number of alleles sampled per locus and individual, A_o) with FSTAT 2.9.3.2 software (Goudet, 1995). The observed and expected heterozygosity (H_o and H_e, respectively) were evaluated with ARLEQUIN 3.5 (Excoffier et al., 2005) for each locus and across loci for each locality.

Departures from neutrality

Both a molecular- and a population-level approach were taken to detect departures from neutrality at DRB exon 2 gene (Cutrera and Lacey, 2006). The molecular approach used included three

complementary Maximum Likelihood (ML) methods for detecting sites under selection, which were performed using Datamonkey (http://www.datamonkey.org), a web-based gateway to a suite of algorithms, executed by HyPhy, a molecular evolution analysis platform (Pond and Frost, 2005). Datamonkey uses an integrative approach, combining the strengths of the likelihood-based approach described by Nielsen and Yang (1998) and the parsimony-based counting method of Suzuki and Gojobori (1999), implemented in the ADAPTSITE program written by Suzuki et al. (2001).

The three complementary methods used were: 1) single likelihood ancestor counting (SLAC), a heavily modified and improved derivative of the Suzuki–Gojobori counting approach (Pond and Frost, 2005); 2) fixed effects likelihood (FEL), a new likelihood-based and statistically rigorous method to fit an independent d_N (non-synonymous substitution rate) and d_S (synonymous substitution rate) to every site in the context of codon substitution models, and test whether $d_N = d_S$ (Pond and Frost, 2005); and 3) random effects likelihood (REL), an improved variant of the Nielsen–Yang approach, which uses flexible but not overly parameter-rich rate distributions and allows both d_S and d_N to vary across sites independently. It has been suggested that accounting for nucleotide substitution biases and synonymous site-to-site variation helps reduce Type I errors (Pond and Frost, 2005). These analyses were carried out both for the whole set of sequences and for each locality separately. FEL and REL analyses estimate selection in a phylogenetic context and take recombination into account (Seifertová and Simková, 2011).

For microsatellite loci, departures from neutrality were evaluated with Ewens–Waterson test using the algorithm given in Manly (1985) and implemented in POPGENE 1.31 (Yeh et al., 1999).

Analysis of population structure

Several approaches were taken to assess genetic structure, all based on F_{st}. Due to duplication in exon 2 of the DRB gene, genetic structure was estimated from binary-encoded data, with each allele considered a separate dominant locus and its presence/absence coded 1/0 (Nadachowska-Brzyska et al., 2012).

An analysis of isolation by distance was performed for both markers with the ISOLDE subroutine of GENEPOP (Rousset, 2008), which allowed to estimate the correlation coefficient between $F_{st}/(1–F_{st})$ and the natural logarithm of the geographical distance between sampling localities (in kilometers). The significance of the relationship was evaluated by a Mantel test with 100 000 permutations. F_{st} was calculated between all pairs of localities, and its statistical significance was tested using 10 000 permutations and applying the Bonferroni correction. Computations were performed in ARLEQUIN 3.5 (Excoffier et al., 2005). To determine the most likely number of microsatellite clusters present in the sampled localities, the individual-based Bayesian clustering method implemented in STRUCTURE 2.3.4 was used (Falush et al., 2007; Hubisz et al., 2009; Pritchard et al., 2000). This model takes into account the presence of Hardy–Weinberg or linkage disequilibrium by introducing population structure, and attempts to find population groupings that (as far as possible) are not in disequilibrium. The program uses multilocus genotype data to group individuals into K genetic clusters, each one with a characteristic set of allele frequencies with respect to the geographic origin of the populations. K values from 1 to 15 were examined, and for each K value, five runs of 4×10^5 burn-in steps were performed, followed by 4×10^6 post-burn MCMC iterations (Evanno et al., 2005).

BAPS 6.0 was used to reduce the probability of overestimating the number of underlying populations (Corander et al., 2003; Corander and Marttinen, 2006). This software performs an analysis that clusters individuals based on genotype instead of the place of capture. This analysis is not affected by sample size and allows to estimate the number of populations in the study area and maximize genetic differentiation among them. This Bayesian model is estimated by implementing a Markov chain Monte Carlo scheme that considers both genetic data and geographic coordinates, and spatially infers genetic discontinuities between populations from individual multilocus genotypes (Guillot et al., 2009). It enables analytical integration of the parameters in both the spatial prior and the likelihood of genetic data, as well as in both individual and group analysis modes. BAPS uses a stochastic optimization to infer the posterior mode of genetic structure, runs much faster than STRUCTURE—which uses Markov chain Monte Carlo algorithms—, and does not require multiple trials for each possible value of K. This software better handles large and complex data sets. It uses both genotype and, in group mode, sample group information to infer population clusters. The maximum number of populations (prior information) was set to 15. Although BAPS gives the most probable cluster arrangement, probabilities of the best clusters were also estimated using the rule of Bayes by comparison with less probable clusters (Corander and Marttinen, 2006).

Once genetic clusters were inferred, two analyses of molecular variance (AMOVAs)—one on microsatellite data and the other on DRB exon 2 gene data—were performed considering the five clusters obtained from microsatellites. AMOVA was used to compare the population subdivision obtained from microsatellites, using BAPS and STRUCTURE, with variation in exon 2 of the DRB gene. Statistical significance of the AMOVA was calculated using a non-parametric test based on 20 000 permutations of genotypes among populations. All non-parametric tests were performed under the same conditions as described above.

For DRB exon 2 gene, the relationships among populations were analyzed based on a genetic distance matrix using principal coordinates analysis (PCoA), a multivariate analysis and type of multidimensional scaling that allows clusters of individuals to be identified (Evanno et al., 2005). Multivariate analysis has several advantages over other classical approaches used in population genetics. For example, this exploratory method does not require assumptions about an underlying genetic model, such as Hardy–Weinberg equilibrium or the absence of linkage disequilibrium. As it simply aims at summarizing the genetic variability, any kind of genetic structuring can be revealed (Jumbart et al., 2009). The analysis was carried out using PAST 3.0 software (Hammer et al., 2001) and an algorithm from Davis (1986), where the PCoA routine finds the eigenvalues and eigenvectors of a matrix containing the distances or similarities between all data points (Hammer et al., 2001).

Results

MHC genotyping

A total of 196 individuals were sequenced for DRB exon 2 gene, allowing a total of 161 alleles to be scored. Due to gene duplication, it was not possible to assign the sequences to specific loci based on phylogenetic analysis; DRB exon 2 variants were thus called ‘alleles’. All DNA sequences were deposited in GenBank (accession numbers: KJ010959–KJ011119) as variants of MhcArjaDRB locus. Two types of alleles were defined: shared alleles ($n = 39$) and private alleles, occurring in only one locality ($n = 122$; Fig. 2). Allele frequencies across localities ranged from 0.028 to 0.192 (see Supporting information S1).

Microsatellite analysis

A total of 257 individuals were analyzed at ten microsatellite loci (mean number of analyzed individuals per locality ± SD:

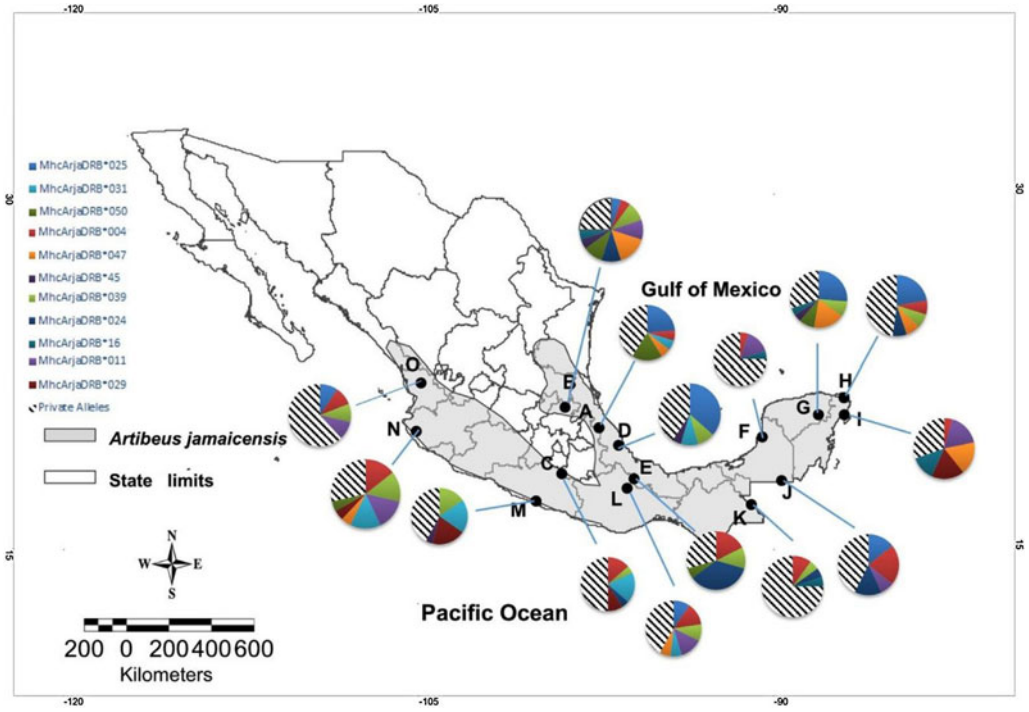

Fig. 2. Spatial variation in *Artibeus jamaicensis* DRB exon 2 gene. Pie charts show the frequency of the different alleles present in the 15 localities. The colored sections of the charts show the most frequent alleles, shared among localities; while striped sections show private alleles. The letters (A–O) correspond to the localities described in Figure.

17.13 ± 4.7). All microsatellite loci were in Hardy–Weinberg equilibrium in all localities. All ten loci were in linkage equilibrium, and there was no evidence of null alleles.

High genetic diversity values were found, with a total of 315 alleles detected at all ten loci and a range of 6–11 alleles per locality. Expected and observed heterozygosities had similar values for all 15 localities, ranging from 0.62 to 0.75 and 0.75 to 0.85, respectively. The number of observed alleles ranged from 5.3 to 9, and genetic diversity, from 0.753 to 0.857, both averaged across all loci (Table 1).

Departures from neutrality

For DRB exon 2 gene, evidence for positive selection was found in all localities when considering all 161 different DNA sequences. Averaged ω (d_N/d_S) ranged from 1.6 to 7.17. For all localities, the difference between the null model M1 (neutrality, which assumes that codons are either conserved [$\omega = 0$] or neutral [$\omega = 1$]) and the alternative model M2 (selection, where $\omega > 1$) was statistically significant (Table 2).

The positively selected sites (PSS) inferred by the analysis differed among localities; in locality L, for example, 28 sites were found to be under positive selection, while no such site was found in localities A, B and F. The most common codons detected were 5, 36 and 57, located at the beginning, middle and end of the gene sequence, respectively (Table 2).

The Ewens–Watterson test compares the observed to the expected Hardy–Weinberg homozygosity in a sample of *n* different alleles. The allele frequency distribution can be too even (suggestive of balancing selection) or too uneven (suggestive of a recent selective sweep) compared with the neutral expectation. For all ten microsatellite loci and 15 localities analyzed here, no evidence was found to reject the null hypothesis of neutrality.

Population genetic structure

Different levels of genetic diversity and structure were detected for the two types of genetic markers. The results of AMOVA showed that for both microsatellite loci and DRB exon 2 gene, over 80% of the genetic variation was partitioned within localities (Table 3). For microsatellites, about 3% of the genetic variation was partitioned among localities within clusters, and among-cluster differentiation was about 12%. For DRB exon 2 gene, slightly more than 4% of the genetic variation was partitioned among localities within clusters, and among-group differentiation was only 0.6%. Genetic variation among clusters was statistically significant in microsatellites, but not in DRB exon 2 gene.

A positive correlation was found between geographical distance and $F_{ST}/(1 - F_{ST})$ calculated from microsatellite loci (Fig. 3A; $r = 0.62$). This relationship was highly significant ($P < 0.001$), indicating isolation by distance. This correlation was not significant for DRB exon 2 gene (Fig. 3B; $r = -0.20$, $P = 0.096$), indicating a lack of isolation by distance at this marker.

As for pairwise genetic differentiation among localities, the F_{st} values estimated for DRB exon 2 gene were mostly significant and ranged from 0.0 to 0.117. Pairwise F_{st} values estimated for the ten microsatellite loci were almost all significant and ranged from 0.0004 to 0.206 (Table 4). Mean pairwise F_{st} values were significantly different for the two types of markers ($U = 1306.5$, $P = 0.0001$), being higher for microsatellite loci ($F_{st} = 0.154$) than for DRB exon 2 gene ($F_{st} = 0.039$). For this gene, inter-locality F_{st} estimates were generally low, indeed, indicating little genetic structure among localities.

A signature of population genetic structure was detected using the Bayesian clustering approach with microsatellite data. In the STRUCTURE analysis, the probability of the data $\Pr(X|K)$ was highest for $K = 5$. The individuals from localities N and O (north Pacific slope) were assigned to one group; and localities E, K and L (south Pacific and Gulf of Mexico slopes), to another. The individuals captured

Table 1
Levels of genetic diversity in *Artibeus jamaicensis* estimated from ten microsatellite markers. Values are averaged across loci for each locality. *N*, sample size; A_o, number of observed alleles (±SD); H_o, observed heterozygosity (±SD); H_e, expected heterozygosity (±SD). The letters (A–O) correspond to the localities described in Fig. 1.

Locality	*N*	A_o	H_o	H_e	Genetic diversity (±SD)
A	22	8.4 ± 1.1	0.840 ± 0.03	0.709 ± 0.04	0.8452 ± 0.44
B	12	6.5 ± 1.8	0.810 ± 0.06	0.733 ± 0.11	0.8105 ± 0.43
C	16	5.3 ± 1.7	0.753 ± 0.06	0.653 ± 0.13	0.7537 ± 0.40
D	22	7 ± 1.2	0.799 ± 0.03	0.627 ± 0.15	0.7993 ± 0.42
E	10	5.8 ± 1.4	0.802 ± 0.05	0.75 ± 0.15	0.8021 ± 0.43
F	12	7.4 ± 2.3	0.828 ± 0.07	0.725 ± 0.15	0.8286 ± 0.44
G	21	9 ± 1.8	0.854 ± 0.04	0.747 ± 0.1	0.8548 ± 0.44
H	18	8 ± 1.2	0.857 ± 0.02	0.688 ± 0.13	0.8573 ± 0.45
I	20	8.3 ± 1.7	0.849 ± 0.03	0.77 ± 0.13	0.8492 ± 0.44
J	15	8.7 ± 1.5	0.858 ± 0.03	0.766 ± 0.11	0.8583 ± 0.45
K	12	6 ± 1	0.803 ± 0.04	0.7 ± 0.05	0.8039 ± 0.43
L	13	6.8 ± 1.3	0.826 ± 0.04	0.746 ± 0.13	0.8267± 0.44
M	22	7.9 ± 0.7	0.819 ± 0.02	0.668 ± 0.1	0.8195 ± 0.43
N	20	8.1 ± 1.2	0.833 ± 0.04	0.695 ± 0.04	0.8334 ± 0.43
O	22	8.5 ± 2.1	0.828 ± 0.05	0.718 ± 0.11	0.8287 ± 0.43
Total	257				

Table 2
Estimates of selection pressures acting on DRB exon 2 gene in 15 localities of *Artibeus jamaicensis*. The letters (A–O) correspond to the localities described in Fig. 1. *N*, number of DNA sequences per locality; *P*-value ($\alpha \leq 0.05$); PSS, positively selected sites; ω, d_N/d_S; M1, neutrality; M2, positive selection.

Locality	*N*	Number of PSS	PSS	Mean ω	*P*-value	Null model (M1) Log(L)	Alternative model (M2) Log(L)
A	18	0	–	1.6	0.0478	−1008.85	−1006.3
B	18	0	–	2.77	0.00004	−1163.9	−1153.81
C	19	6	7,43, 49, 50, 53, 57	2.72	0.002	−1253.19	−1247.25
D	16	4	9, 36, 39, 65	2.51	0.0103	−822.41	−817.83
E	13	4	7, 39, 43, 57	2.91	0.0046	−925.46	−920.1
F	17	0	–	2.34	0.029	−936.07	−932.54
G	22	21	4, 5, 7, 9, 10, 13, 16, 17, 35, 36, 37, 39, 43, 46, 49, 50, 53, 57, 63, 65, 66	2.8	0.00005	−1086.65	−1077.4
H	17	2	59, 67	7.17	0.00012	−1050	−1041
I	16	14	4, 5, 13, 17, 29, 32, 35, 49, 50, 53, 56, 63, 65, 66,	4.13	0.0001	−982.33	−973.29
J	14	20	4, 7, 9, 13, 16, 17, 26, 29, 32, 36, 39, 42, 43, 46, 49, 50, 53, 57, 65, 71	4.6	0.001	−843.11	−827.26
K	26	11	7, 9, 17, 36, 39, 46, 50, 53, 63, 65, 66	3.28	1.296×10^{-8}	−1559.32	−1541.16
L	17	28	4, 5, 7, 9, 10, 12, 16, 17, 19, 26, 32, 36, 37, 39, 41, 42, 43, 46, 47, 49, 50, 53, 56, 57, 63, 65, 66, 71	2.7	0.0083	−893.69	−888.91
M	18	18	4, 5, 7, 9, 13, 16, 29, 32, 39, 42, 43, 46, 49, 50, 53, 57, 63, 65	1.54	0.0183	1017.84	1016.15
N	19	11	5, 7, 9, 16, 36, 39, 43, 46, 50, 53, 57	1.23	0.022	−1116.5	−1112.71
O	22	11	7, 9, 16, 36, 39, 43, 46, 50, 53, 57, 65	1.85	0.009	−1252.81	−1248.11
All localities	161	20	4, 7, 9,16, 17,29, 32, 35, 39, 42, 43, 46, 49, 50, 53, 54, 63, 65, 66, 67	4.16	0.00022	−1005	−999.85

Table 3
Results of the analysis of molecular variance (AMOVA) based on 15 localities of *Artibeus jamaicensis*. Genetic variation at ten microsatellite loci and DRB exon 2 gene is partitioned within clusters, among clusters and within clusters (see Materials and Methods).

Locus	Source of Variation	d.f.	Sum of squares	Variance component	Percentage of variation	Fixation index	*P*-value
Microsatellites	Among clusters	4	277.95	0.6039	12.35	0.123	0
	Among polulations within clusters	10	91.340	0.15062	3.08	0.035	0
	Within clusters	497	2055.63	4.1361	84.57	0.154	0
DRB exon 2 gene	Among clusters	4	83.27	0.0722	0.62	0.006	0.80
	Among populations within clusters	10	263.58	0.5198	4.44	0.038	0
	Within clusters	427	4806.07	11.7031	96.17	0.044	0

in the Yucatán Peninsula (localities F–J) were clustered together into another well-differentiated group, while those captured in central Mexico were clustered into two groups (A–C; and D and M; Fig. 4A). In the BAPS analysis, five genetic clusters ($K = 5$) were inferred, with 100% of the individuals assigned. There were five well-defined groups (north Pacific slope: N and O; south Pacific and Gulf of Mexico slopes: E, K and L; and Yucatán Peninsula: F–J).

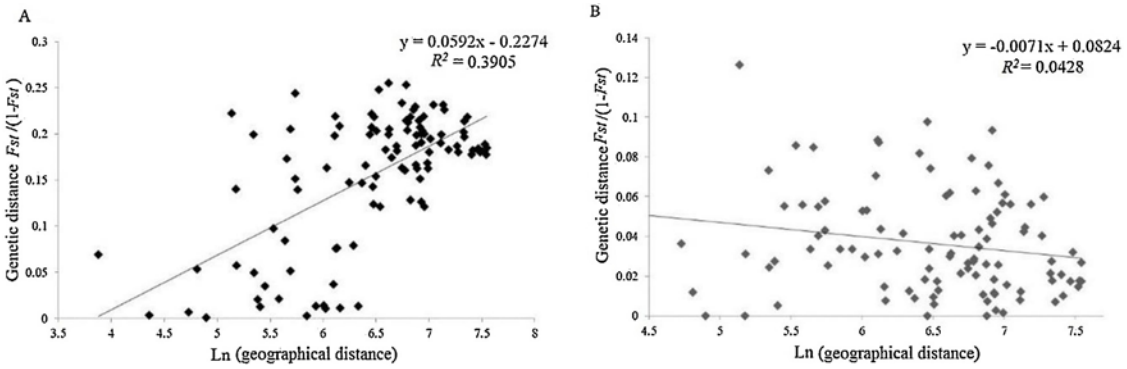

Fig. 3. Correlation between geographical distance and pairwise $F_{ST}/(1-F_{ST})$ in 15 localities of *Artibeus jamaicensis* in Mexico. (A) F_{st} values calculated from 10 microsatellite loci. (B) F_{st} values calculated from DRB exon 2 gene. The significance of the correlations was determined by Mantel tests with 100 000 permutations.

Table 4
Genetic differentiation at two markers among 15 localities of *Artibeus jamaicensis*. Pairwise F_{st} values at DRB exon 2 gene are shown above the diagonal, and those at ten microsatellite loci are shown below the diagonal. *, significant F_{st} estimates ($P \leq \alpha = 0.05$). The letters (A–O) correspond to the localities described.

Locality	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A	0	−0.0317	0.04236*	−0.006	0.06628*	0.05639*	−0.00241	0.00618	0.03191*	0.02628	0.01166	0.00932	0.05819*	0.01731	0.02573
B	0.05875*	0	0.06814*	0.11073*	0.04254*	0.03576*	0.05592*	0.04704*	0.0347*	0.11752*	0.02877*	0.06047*	0.04255*	0.00267	0.01086
C	0.03812*	0.08175*	0	0.05615*	0.05216*	0.00971	0.04438*	0.04984*	0.03079*	0.04034*	0.02727*	0.05323*	0.04829*	0.03958*	0.03833*
D	0.0547*	0.05472*	0.09355*	0	0.08968*	0.06992*	0.00008	0.01994	0.06133*	−0.00259	0.04614*	0.01227	0.10754*	0.06748*	0.06328*
E	0.15151*	0.18287*	0.19992*	0.18683*	0	0.04508*	0.05681*	0.05253*	0.07381*	0.07095*	0.04107*	0.0749*	0.08624*	0.06915*	0.0488*
F	0.15887*	0.18043*	0.20596*	0.18644*	0.14667*	0	0.03892*	0.04416*	0.02795*	0.06236*	0.02901*	0.03513*	0.03561	0.01461	0.0057
G	0.14564*	0.16597*	0.19088*	0.17251*	0.14188*	0.02495	0	−0.01709	0.02809*	0.01985	0.02299*	0.00028	0.0725*	0.03542*	0.02447
H	0.14387*	0.16464*	0.18909*	0.17125*	0.13662*	0.01972	0.00511	0	0.02768	0.03222	0.01929	0.0158	0.07091*	0.03687*	0.0279
I	0.14667*	0.16867*	0.19062*	0.17474*	0.14265*	0.01663	0.00941	0.00042	0	0.06464*	0.02062	0.0338*	0.04029*	0.02464	0.01507
J	0.1435*	0.16354*	0.18986*	0.17041*	0.13674*	0.0245*	0.00635*	0.01607*	0.01366*	0	0.02447	0.02495	0.11499*	0.08382*	0.06742*
K	0.15259*	0.18853*	0.19339*	0.18435*	0.07805*	0.12766*	0.13223*	0.13015*	0.13102*	0.12699*	0	0.03132*	0.05653*	0.03017*	0.01168
L	0.13542*	0.17587*	0.17414*	0.17077*	0.06852*	0.11441*	0.11706*	0.11279*	0.11521*	0.1113*	0.01809*	0	0.07562*	0.02286	0.0273*
M	0.03933*	0.07553*	0.052*	0.07501*	0.16971*	0.17112*	0.15751*	0.15706*	0.16026*	0.15655*	0.1625*	0.14449*	0	0.03017*	0.00611
N	0.15695*	0.17286*	0.20226*	0.17896*	0.18091*	0.16882*	0.15581*	0.15475*	0.15865*	0.15445*	0.18039*	0.16972*	0.16955*	0	0.01049
O	0.16044*	0.17498*	0.20648*	0.18248*	0.18332*	0.17131*	0.15827*	0.15726*	0.16111*	0.15702*	0.18275*	0.17218*	0.17311*	0.01625*	0

The fourth and fifth groups correspond to central Mexico localities (Fig. 4B).

Regarding DRB exon 2 gene sequences, genetic data could not be grouped, and the PCoA analysis showed a pattern without well-defined clusters (Fig. 5).

Discussion

This study integrated neutral and adaptive (DRB exon 2) genetic variation to estimate population genetic structure and demonstrated, by several complementary analyses, that balancing selection is maintaining the spatial distribution of adaptive genetic variation in *A. jamaicensis*, a bat species that inhabits a wide variety of vegetation types. It thus adds evidence for balancing selection as a mechanism maintaining variation at DRB exon 2 gene.

Evidence of natural selection at the molecular level

The region of the MHC molecule responsible for binding antigens, the so-called antigen-binding site (ABS), is coded by DRB exon 2 gene, which showed high levels of variation. In particular, the genes coding for these sites have been shown to display more non-synonymous than synonymous substitutions, hence changing the amino acid sequence and allowing binding of a diverse array of antigens. This indicates that selection processes have acted in the past in the functionally important regions of MHC (Oliver et al., 2009; Schwensow et al., 2007). Since synonymous substitution is selectively neutral or nearly so, the comparatively high level of non-synonymous substitution within the ABS shows that natural selection favors ABS diversity (Yeager and Hughes, 1999).

Genes within the MHC most often show extensive polymorphism at a variety of specific sites, especially the ABS. Alternative approaches based on maximum-likelihood models provide accurate estimates of the d_N/d_S ratio and allow the identification of species-specific positively selected sites (PSS). These methods are appropriate for detecting selection in MHC genes, where positive selection could be acting simultaneously in groups of codons (Schwensow et al., 2007). The d_N/d_S test is more powerful in detecting recurrent positive selection than population genetic tests. The main reason is that population genetic tests rely on capturing a selective sweep in action. If selective sweeps are common, the d_N/d_S ratio will be very large, making the d_N/d_S test even more powerful than population genetic tests; but if they are rare, population genetic tests will have very little power because they are unlikely to capture an ongoing selective sweep (Zhai et al., 2008). Here, the d_N/d_S approach allowed positive selection to be detected in all localities and, in most of them, several codons could be identified as PSS. The d_N/d_S ratios estimated here were quite low, similar to the ones reported for *Noctilio albiventris* (Schad et al., 2011). This approach was thus effective in detecting positive selection, even if no population genetic test of positive selection could be done due to duplication.

Evidence for positive selection was found in all localities, the highest number of PSS (28) occurring in locality L. Several PSS were shared among localities. The most common PSS were 7, 9, 29, 32 and 50, none of these corresponding to the human ABS of the HLA-DR1 or the ABS of the NoalDRB*01 locus in *Noctilio albiventris* (Schad et al., 2011), which suggests that these five PSS are unique to *A. jamaicensis* (Del Real-Monroy et al., 2014).

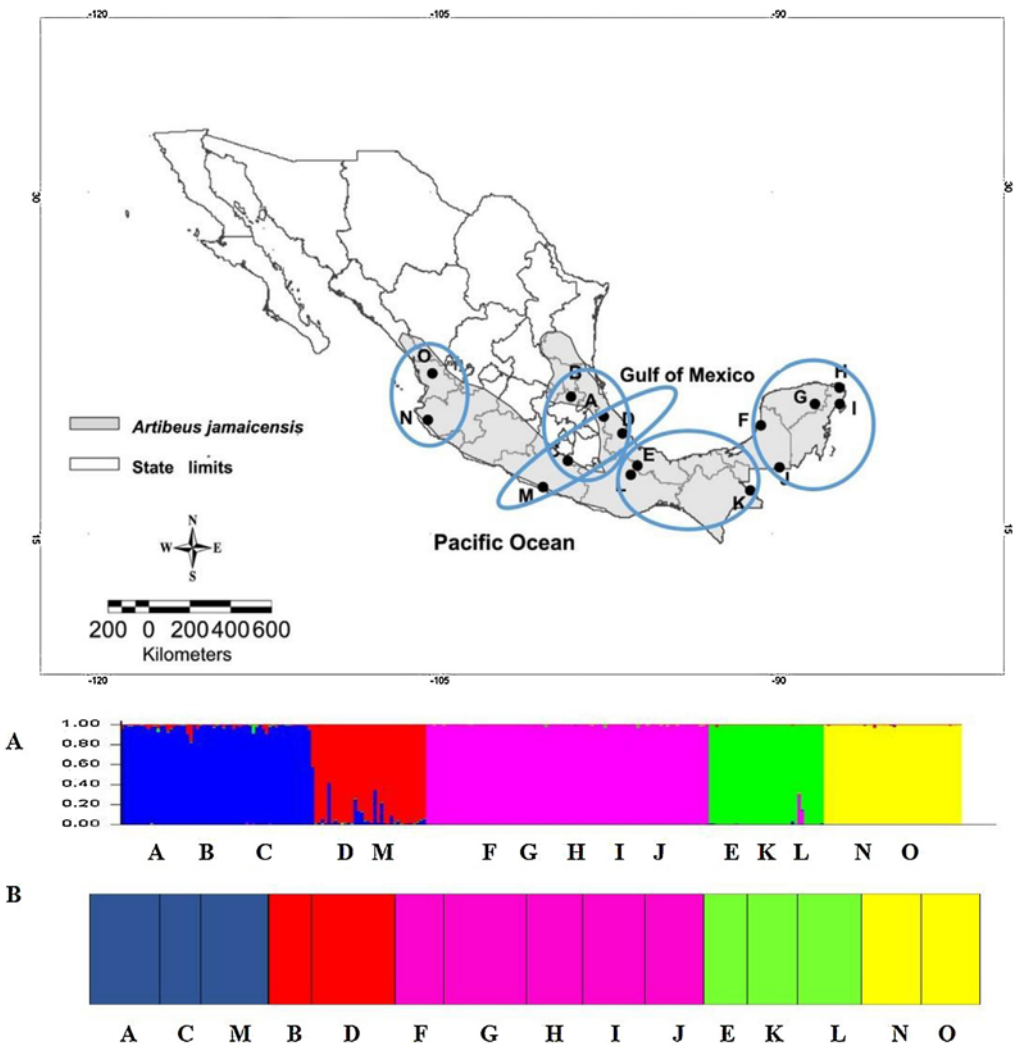

Fig. 4. Genetic structure detected from ten microsatellites in 15 sampling localities of *Artibeus jamaicensis* in Mexico. (A) The five clusters generated by STRUCTURE analysis (represented by circles on the map). (B) The five clusters generated by BAPS analysis (represented by circles on the map). The letters (A–O) correspond to the 15 localities described in Fig. 1.

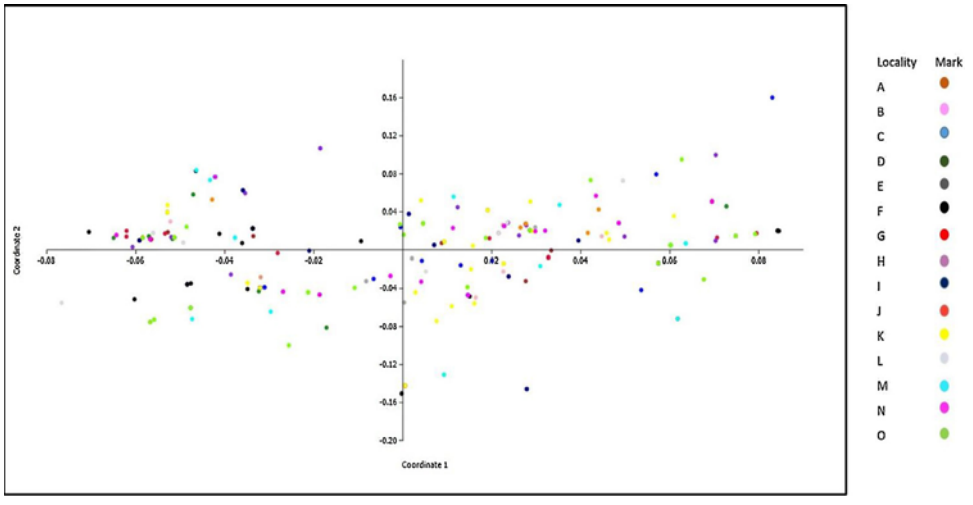

Fig. 5. Multidimensional scaling analysis of DRB exon 2 gene in 15 localities of *Artibeus jamaicensis*. The DNA sequences obtained for the 15 localities (A–O) are indicated as follows: A (brown circles), B (light pink circles), C (blue circles), D (dark green circles), E (dark gray circles), F (black circles), G (red circles), H (purple circles), I (dark blue circles), J (orange circles), K (yellow circles), L (light gray circles), M (turquoise circles), N (magenta circles) and O (light green circles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Evidence for selection at the population level

No previous studies of MHC genetic variation in bat populations have compared the genetic structure at DRB exon 2 gene and that at neutral loci to assess balancing selection across the whole species distribution. Many studies have found no difference between the genetic structure at MHC class II B loci (including DRB gene) and that at neutral loci, suggesting that little or no selection is operating across populations, or that multiple selective forces are operating and masking any overall effects (Spurgin and Richardson, 2010). In contrast, higher genetic differentiation at MHC class II B loci than at neutral loci has been reported among populations of fish species (Evans et al., 2010; Landry and Bernatchez, 2001) and great snipe (Ekblom et al., 2007). Other studies have reported high MHC differentiation among populations of lesser kestrel. In this case, the authors concluded that this pattern has arisen from geographically varying co-evolution, supporting the rare-allele advantage model of selection (Alcaide et al., 2008; Spurgin and Richardson, 2010). Few studies have however detected lower divergence levels at MHC than at microsatellites (e.g., in Trinidadian guppies, *Poecilia reticulate*), indicating that MHC diversity may be maintained by heterozygote or rare-allele advantage, by which old rare alleles are maintained or new rare immigrant alleles are favored (van Oosterhout, 2009). Our results provide support for such balancing selection.

As the DRB exon 2 gene of *A. jamaicensis* is duplicated (Del Real-Monroy et al., 2014), it was impossible to determine whether an individual was heterozygote or homozygote at this locus. An individual can only possess two alleles at any locus; gene duplication is frequent; and many species express multiple types of MHC molecules to increase the range of pathogens that their immune system can recognize (Cammen et al., 2011). Here, other approaches were taken to study balancing selection that involved comparing patterns of MHC variation with those expected for neutral loci, such as microsatellites (Piertney and Oliver, 2006).

The relative levels of neutral and MHC variation within and among populations can vary across related species, and even within a species depending on the spatial scale of analysis (Consuegra et al., 2011). If selection is operating constantly, it is expected that levels of genetic structure will be lower at MHC than at neutral microsatellites. Balancing selection will create a more even spatial distribution of alleles, or may favor new or rare alleles (Piertney and Oliver, 2006; Spurgin and Richardson, 2010). Conversely, under directional selection where selection pressures vary across space and time, one would expect to observe external biotic and abiotic forces driving spatiotemporal variation in pathogen abundance. This may lead to distinct subsets of alleles being selected for in different populations and/or at different time periods, and to a higher genetic structure when estimated from MHC loci than from microsatellites (Oliver et al., 2009).

Population genetic structure

The variation at neutral loci cannot provide direct information on selective processes involving the interaction of individuals with their environment. MHC variability, on the contrary, reflects evolutionary relevant and adaptive processes within and between populations (Schwensow et al., 2007). The main concern with direct comparisons of population structure revealed through MHC loci and microsatellites is that there is little consistent theory to derive predictions on the comparative patterns or between-population divergence for neutral loci and genes under selection in a subdivided population (Piertney and Oliver, 2006).

Functional clustering of MHC molecules is a highly challenging task due to the vast polymorphism of the MHC genomic region and the relationship between subtle amino acid substitutions and dramatic variations in binding specificity (Thomsen et al., 2013). Here, genetic structure was detected at microsatellites by all the above-mentioned analyses. Significant genetic variation at the geographical level was also obtained by AMOVA, indicating a possible genetic structure at the regional scale. For both types of markers, the highest genetic variation was among localities and, at the geographical level, the percentage of variation among clusters was higher for microsatellites than for DRB exon 2 gene. Thus, there appears to be a lack of structure among clusters at the expressed gene, but some kind of structure at microsatellite loci.

In some migratory species (e.g., *Tadarida brasiliensis*), populations are not genetically subdivided, reflecting extensive movements of individuals and random mating during migration (McCracken et al., 1994). Non-migratory species, such as the brown long-eared bat (*Plecotus auritus*) and Bechstein's bat (*Myotis bechsteinii*), are rather characterized by genetically isolated populations (Burland et al., 1999; Worthington-Wilmer et al., 1999). Key characteristics of the species studied here (*A. jamaicensis*), such as high movement capabilities, generalist habits—the use of different vegetation types, roosting structures and food resources (Muscarella et al., 2011)—, a polygynous mating system, female philopatry and differential dispersal of males, could have influenced population genetic structure and genetic diversity, resulting in some degree of genetic structure at microsatellites (Ortega and Arita, 1999). As both climatic factors and the topographic complexity of the region promote population fragmentation and isolation (Ruiz et al., 2013), the lack of genetic structure at positively selected markers may indicate that balancing selection have favored the retention of some alleles (Figueroa et al., 1988; McConnell et al., 1998).

Conclusion

This is the first report that compares the genetic structure revealed by an adaptive marker (DRB exon 2 gene) to that revealed by ten microsatellite loci, with a view to investigate balancing selection in a bat species with a wide geographical distribution. Our findings suggest a role for balancing selection in shaping the spectrum and frequencies of MHC alleles across several populations. This conclusion is drawn from AMOVA, pairwise estimates of F_{st} for the MHC gene and ten microsatellite loci, as well as the clear signature of genetic structure revealed by microsatellites, but not by the MHC gene. More analyses considering several loci of MHC class I and II are however required in order to explore the role of balancing selection in shaping genetic variability in bat populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mambio.2016.12.005.

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