MHC class II DRB variability in wild black howler monkeys (*Alouatta pigra*), an endangered New World primate

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Abstract

*MHC class II DRB variability in wild black howler monkey (*Alouatta pigra*), an endangered New World primate.* The genes of the major histocompatibility complex (MHC) are the most important genetic component of the immune system in vertebrates. Their variability is known to influence a species' ability to recognize and respond to pathogens. Here, we present the first data of the MHC class II DRB exon 2 for the endangered black howler monkey (*Alouatta pigra*), one of the most northerly distributed platyrrhines. Twenty–one DRB sequences corresponding to four new lineages were identified in 44 individuals through a combination of cloning and reference strand conformational analysis. The detection of up to eight sequences per individual suggests the existence of at least four loci in the species. A relatively low DRB sequence diversity, but similar lineage and loci numbers. were found in *A. pigra* when compared to other platyrrhines. The reduced DRB allelic diversity in the species appears to be a consequence of drift, reflecting the colonization by its ancestors from South to Central America. Finally, the allelic diversity in the species might be enabling an adequate immune response in wild populations to cope with current pathogens, but it might entail a risk for these populations in case of the emergence of new pathogens.

Key words: Major histocompatibility complex, Polymorphism, Howler monkey, Genetic bottleneck

Resumen

*Variabilidad del gen DRB del CMH de clase II en el mono aullador negro (*Alouatta pigra*), un primate del Nuevo Mundo en peligro.* Los genes del complejo mayor de histocompatibilidad (CMH) son el componente genético más importante del sistema inmunitario en vertebrados. Su variabilidad puede influir en la habilidad de una especie para detectar patógenos y reaccionar ante ellos. En este trabajo presentamos por primera vez datos relativos al exón 2 del gen DRB del MHC de clase II del mono aullador negro (*Alouatta pigra*), una de las especies de platirrinos con una distribución más septentrional. Se identificaron 21 secuencias de DRB, pertenecientes a cuatro nuevos linajes en 44 individuos de *A. pigra*. Se detectaron hasta ocho secuencias por individuo, lo que sugiere la existencia de al menos cuatro loci en la especie. En comparación con otros platirrinos, en *A. pigra* se observó una diversidad relativamente baja de secuencias de DRB, pero un número parecido de linajes y loci. La reducida diversidad alélica del gen DRB en la especie parece ser consecuencia de deriva génica, lo que refleja la colonización por parte de sus ancestros desde América del Sur hacia Centroamérica. Por último, la diversidad alélica de la especie está permitiendo que las poblaciones silvestres muestren una respuesta inmunitaria adecuada frente a los patógenos actuales, pero podría implicar un riesgo para esas poblaciones en caso de que surjan otros nuevos.

Palabras clave: Complejo mayor de histocompatibilidad, Polimorfismo, Mono aullador, Cuello de botella genético

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Introduction

The major histocompatibility complex (MHC) is the most functionally important genetic region involved in infectious disease resistance among jawed vertebrates (Hedrick, 1994). Classical class I and II genes encode for cell surface glycoproteins that bind antigens and present them to effector cells, triggering an adaptive immune response (Hedrick, 1994; Knapp, 2005b). Class I molecules are expressed on the surface of all nucleated cells, and present peptides of intracellular origin (such as those naturally produced by a cell as well as those originating from a virus) to cytotoxic T cell lymphocytes (CD8+ T cells), targeting infected cells for destruction. Class II molecules are only expressed on specialized antigen–presenting cells, such as macrophages, dendritic cells and B cells, and present antigens with an extracellular origin (such as those derived from cellular pathogens) to helper T lymphocytes (CD4+ T cells), initiating the antigen–specific immune response (Janeway et al., 2001). The MHC is characterized by a particularly high polymorphism, which is mainly located at residues involved in antigen binding (antigen binding sites, ABS) (Hedrick, 1994). Such diversity appears to arise from pathogen–driven balancing selection (Ujvari & Belov, 2011; van Oosterhout, 2009). This selective force is thought to operate through the heterozygote advantage (i.e. overdominance; Doherty and Zinkernagel, 1975) or through the emergence of rare alleles (i.e. negative frequency–dependent selection; Takahata & Nei, 1990). Nevertheless, other mechanisms like mating preferences, maternal–fetal interactions and olfactory–based markers for kin recognition and inbreeding avoidance, have also been proposed to play a significative role in MHC polymorphism (Ekblom et al., 2010; Ujvari and Belov, 2011; Penn and Potts, 1999).

Although most natural vertebrate populations that have been studied exhibit high levels of MHC diversity (Klein, 1986), some species show a limited variation (Sommer et al., 2002; Seddon and Baverstock, 1999). This indicates that balancing selection may not always play the most important role in shaping MHC diversity in natural populations, and that neutral forces, such as genetic drift and gene flow, might also influence variation in these functionally important genes (Miller and Lambert, 2004). It has been proposed that low MHC polymorphism in some cases can be explained by reduced selection pressure due to a solitary lifestyle, or low exposure to pathogens in high latitudes or in marine environments (Mainguy et al., 2007; Caballero et al., 2010; Ellegren et al., 1996). Also, low MHC polymorphism has been suggested as a consequence of a restrictive mating system, a small population size, or past population bottleneck (Sommer et al., 2002; Bollmer et al., 2011; Hedrick et al., 2000). In such cases, the strength of genetic drift may exceed that of balancing selection (Miller and Lambert, 2004).

There is growing evidence showing a correlation between MHC gene variability and disease resistance (Bernatchez and Landry, 2003; Froeschke, 2005; Westerdahl et al., 2005; Worley et al., 2010). However, the consequences of a low MHC variation for most wildlife are not clear (Radwan et al., 2010; Bollmer et al., 2011), and it has been hypothesized that low levels of variation might increase susceptibility to infectious diseases and limit the capacity of populations to respond to emerging pathogens. This possibility is of particular concern for endangered species (O'Brien et al., 1985; Lafferty and Gerber, 2002; Siddle et al., 2007; Zhang et al., 2015). Transmittable virus–associated cancers such as Tasmanian Devil Facial Tumour Disease and Canine Transmissible Venereal Tumour are examples of the relevance of MHC variation in conservation biology, since both diseases are assumed to have emerged and disseminated due to low MHC diversity in Tasmanian devil, wolf and dog populations (Siddle et al., 2007; Belov, 2011; McCallum, 2008); some populations of Tasmanian devils have been driven to a rapid decline (Belov, 2011). Assessing the degree of polymorphism of MHC can thus provide critical baseline information on a species' immune response and adaptive potential.

In the case of non–human primates, many of which are classified as endangered species, the development of standardized techniques for MHC studies using non–invasive samples (e.g., faeces, hair) has increased our possibility to target vulnerable wild populations (Lukas et al., 2004; Knapp, 2005a; Müller et al., 2014; Hans et al., 2015; Lukas and Vigilant, 2005). Nonetheless, current knowledge of MHC diversity from wild New World monkey (NWM) populations is restricted to isolated studies of the family Cebidae (Nino–Vasquez et al., 2000; Gyllensten et al., 1994; Suárez et al., 2006). In this study, non–invasive samples were used to characterize the polymorphism at MHC class II loci from wild *A. pigra*, a representative species of the Atelidae family and one of the most northerly distributed platyrrhines.

A. pigra is distributed in a relatively small area in the Yucatan Peninsula of Mexico, Belize and central Guatemala (Rylands et al., 2005). This species is classified as 'Endangered' (Marsh et al., 2008), mainly due to habitat destruction, illegal hunting and the pet trade. Furthermore, the fast and continuous loss of their habitat and human encroachment is increasing their exposure to both human and domestic animal pathogens (Vitazkova and Wade, 2006; Nunn and Gillespie, 2016; Martínez–Mota et al., 2015), raising the risk of emerging infectious diseases. This highlights the importance of the assessment of immune gene variability in the species for its inclusion in conservation strategies. Analyses of genetic variation on neutral markers (i.e., microsatellites and mtDNA) have detected low levels of variation in *A. pigra* compared to other *Alouatta* species distributed further south (Amendola, 2009; Van Belle et al., 2012; James et al., 1997). These low levels of variation have been mainly attributed to a founder effect due to the colonization of *A. pigra* ancestors from South to Central America (Ellsworth and Hoelzer, 2006; Cortés–Ortiz et al., 2003). Contrary to neutral markers, MHC genes are thought to be under balancing selection. It is therefore possible that variation in these genes is retained in species that have undergone a bottleneck like *A. pigra* (Aguilar et al., 2004). Nevertheless, in numerous cases, low variation at neutral loci has been

correlated with reduced *MHC* diversity, indicating that balancing selection had been overpowered by genetic drift (Bollmer et al., 2011; Miller and Lambert, 2004).

In this study, we assessed the level of variation on exon 2 of MHC–DRB loci of wild *A. pigra*, in terms of its allelic, lineage, and loci polymorphism. Our aim was to to generate baseline information on immune gene diversity for this endangered NWM species. Nonetheless, we also intended to determine whether the evolutionary forces shaping the neutral genetic variation in this species had also influenced the variation in genes linked to adaptive immunity like the *MHC*, or whether balancing selection had managed to counteract the effects of genetic drift. We chose to study the MHC–DRB since it is the most widely studied MHC loci in NWM and other non–human primates (De Groot et al., 2012), thus allowing a better comparison of MHC diversity. Furthermore, we focused on exon 2 of DRB loci, which encodes most of the ABS and has a primary immune function, making it relevant for conservation purposes (Ujvari and Belov, 2011; Hedrick, 1994; Zhang et al., 2015).

Table 1. Number of faecal samples of *Alouatta pigra* individuals (Ni); number of social groups (Nsg) collected from June to November 2014 at each locality; and number of successfully analyzed samples (Nsas): PNP, Palenque Nacional Park; CBR, Calakmul Biosphere Reserve; and CQR, Coba Quintana Roo.

Tabla 1. Número de muestras fecales de individuos (N); número de grupos sociales (Ngs) de Alouatta pigra *recogidas entre junio y noviembre de 2014 en cada localidad; y número de muestras analizadas (Nsas): PNP, Parque Nacional de Palenque; CBR, Reserva de la Biosfera de Calakmul; CQR, Cobá Quintana Roo.*

Population	Ni	Nsg	Nsas
CBR	7	23	23
PNP	6	20	17
CQR	2	6	4
Total	15	49	44

Material and methods

Sample collection

Considering the conservation status of the species and the possible health risks of a chemical immobilization procedure to obtain blood samples, we chose to use a non–invasive faecal DNA sampling approach for this study. A total of 49 faecal samples from adult animals belonging to 15 troops and two solitary individuals were collected from June to November 2014 (table 1). Sampling took place at three different sites: (1) Calakmul Biosphere Reserve (CBR) in the state of Campeche (18º 36' 43'' N, 89º 32' 53'' W), the largest Mexican tropical forest having a surface area in excess of 720,000 ha; (2) Palenque National Park (PNP) in the state of Chiapas (17º 29' 00'' N, 92º 03' 00'' W), a protected natural area of 1,772 ha; and (3) a seven hectare patch close to Coba, Quintana Roo (CQR) of low and medium subdeciduous forest (20º 28' 51.58'' N, 87º 44' 18.40'' W), see figure 1.

Monkey troops were visually tracked and closely monitored to allow expedite collection of fresh faecal samples from well–identified and sexed adult individuals. Faecal samples were collected using single–use gloves that were changed between samples to avoid cross–contamination. Approximately 2 g of stool sample were placed in sterile tubes with 3 ml of RNAlater solution (Ambion, Austin, TX, USA) and stored at ambient temperature for a maximum of 18 days. Once in the laboratory, they were stored at –20 ºC until DNA extraction.

Genotyping of MHC–DRB by cloning–RSCA and sequencing

DNA was extracted from approximately 15 mg of stool sample using the Fecal DNA Extraction Kit (Zymo Research, Irving, California, USA) following the manufacturer's instructions. To amplify a 269 bp of exon 2 we used the primers DRBP1 (5' CCGGATCCTTCGTGTCCCCACAGCACG 3') and DRBP2 (5' TCGCCGCTGCACTGTGAAG 3') (Tiercy et al., 1990). Primer selection was based on the alignment of DRB exon 2 sequences from all known NWM lineages, including species most closely related to *Alouatta* (i.e., families Atelidae and Pitheciidae). PCR amplification was carried out in a total volume of 25 µl with 20 pmol of each primer, 200 µM of dNTPs, 2 mM $MgCl_2$, 1 U of GoTaq (Promega, Madison, WI, USA) and 4 µl of DNA extract. In order to minimize the differences in the effectiveness of allele amplifications, a 'Touchdown' protocol for the PCR was implemented (Hans et al., 2015). Thermocycling conditions included an initial denaturalization step of 94 ºC for 5 min, followed by two cycles of denaturing 94 ºC for 30s, annealing at 68 ºC for 30s, extension at 72 ºC for 30s, two cycles of 94 ºC for 30s, 63 ºC for 30s, and 72 ºC for 30s, and 30 cycles of 94 ºC for 30s, 58 ºC for 30s, and 72 ºC for 30s, with a final extension step of 72 ºC for 10 min. Products were visualized and documented in 1.5 % agarose gels.

For DRB genotyping, a combination of two molecular methods was used: the classic cloning–sequencing approach, and a Reference Strand Conformational Analysis (RSCA) strategy (Argüello et al., 1998). Both techniques achieve high resolution for the genotyping of human and non–human MHC (Drake et al., 2004; Babik, 2010; Strand and Höglund, 2011; Oppelt and Behrmann–Godel, 2012). However, given the source of DNA samples, we found amplicon cloning yielded better results for detecting all DRB sequence variants within a sample (data not shown). In order to avoid sequencing a great number of identical clones,

we used RSCA as a screening tool to differentiate sequence variants among the obtained clones, thus reducing the set of clones subjected to nucleotide sequencing. To ensure reliability of genotyping result, two or three independent amplification products from each individual sample were cloned into a pGEM–T easy vector (Promega, Madison, WI, USA) and the ligation mix was transformed into Top10 cells (Invitrogen) using heat shock. Verification of the cloned fragment size relied on subsequent PCR amplification using primers DRBP1 and DRBP2; those having the expected size were then subjected to RSCA.

RSCA allows the differentiation of sequence variants present in amplicons that are hybridized to a fluorescently labelled pre–sequenced reference strand (FLR). The electrophoretic mobility of the heteroduplex is affected by mismatches, allowing the identification of different sequence variants. For this study, we used two FLRs of human origin (*HLA–DRB*1*10:01:01 and *HLA–DRB*3*01:16) so as to better ensure that no homoduplex would be formed when hybridizing with the *A. pigra* samples (Argüello et al., 1998). Lastly, we selected three to five clones (from different individuals) for each putative allelic variant identified and subjected them to bidirectional Sanger sequencing at LANBAMA, Mexico and Macrogen, South Korea using M13 universal primers.

Sequence analysis and artefact detection

The alignment and edition of sequences were accomplished in the sequencer 5.0.1 software (Gen Codes Corporation). Visual inspection of sequences was performed to detect possible base calling errors based on the consensus sequence. All sequences were translated into protein sequences to verify whether there was evidence of pseudogenes, such as the presence of premature stop codons or indels. Samples exhibiting information suggestive of chimeric sequence artefacts (resulting from template switching by the DNA polymerase) were detected using six simultaneous analyses in RDP 4.56 (Martin et al., 2015): RDP, MaxChi2, BootScan, Chimaera, Siscan and 3Seq (Martin and Rybicki, 2000; Smith, 1992; Posada and Crandall, 2001; Martin et al., 2005; Gibbs et al., 2000; Boni et al., 2007). Finally, we considered true alleles as those present in at least two different individuals or independent PCR products from the same individual (Cutrera and Lacey, 2006; Real–Monroy et al., 2014).

Statistical and phylogenetic analyses

To assess variation at the DRB gene, we considered the number of alleles, number of lineages, nucleotide diversity (π), and number of segregating sites (S). These calculations were made using DnaSP 5.1 software (Rozas, 2009).

Since the primers used may amplify multiple copies of DRB in the samples, a phylogenetic analysis was carried out to identify groups of sequences that potentially represent different loci, based on the assumption that alleles of a particular MHC locus have a tendency to form a phylogenetic cluster (Oppelt et al., 2010; Lukas et al., 2004). A Bayesian inference analysis was implemented using Mr. Bayes 3.2.6 (Ronquist et al., 2012). The best nucleotide substitution model (HKY+I) was selected using the Akaike information criterion in the jModelTest software (Posada, 2009). Markov chain Monte Carlo (MCMC) were run for 15 million generations, sampling trees and parameters every 1,000 generations. Analysis consisted of two independent runs with one cold and three hot chains. The majority–rule consensus tree was obtained, with their respective posterior probabilities, after discarding the initial 25% of the accumulated trees. Sequences representing the 35 DRB lineages from platyrrhine species were included in the analysis. In addition, sequences with the highest degree of homology available in the IPD–MHC NHP database (De Groot et al., 2012) from catarrhine species were also included. A human DRB sequence (*HLA–DRB*1*01:01:01) was used as the outgroup.

Lastly, to detect molecular level evidence of selection on DRB, the relative rates of synonymous substitutions (d_S) and non–synonymous substitutions (d_N) were calculated using the Nei and Gojobori with the Jukes and Cantor correction in DnaSP 5 (Rozas, 2009). Calculations were performed for the antigen binding sites (ABS) [extrapolated from those described in humans (Brown et al., 1993)] and non–ABS sequence regions for each cluster of *A. pigra* DRB sequences (putative loci) and for all sequences. Finally, a *Z*–test was carried out to evaluate departure from neutrality for the d_N/d_S ratios. Tests were executed in Mega 7.0.2 (Kumar et al., 2016).

Results

From a total of 49 samples of *A. pigra*, amplification and cloning of DRB exon 2 PCR products of 44 individuals was achieved (table 1). RSCA analysis of clones (20 to 42 clones per sample) identified 33 DRB sequence variants. Twelve of these variants were discarded from subsequent analyses as ten of them were only found in one or two clones from a single amplification of one individual. These discarded sequences showed a substitution of one or two nucleotides relative to true DRB sequences present in the same sample (eight variants), or they had a deletion of a single nucleotide at position 61 or 117 that shifted the reading frame (two variants). The other two sequence variants were found to be in vitro recombinants, having as parental sequences *Alpi–DRB**W107:04 and *Alpi–DRB**W105:05 (recombinant 1), *Alpi–DRB**W105:04 and *Alpi–DRB**W106:04 (recombinant 2). Finally, all sequence variants considered true alleles (N = 21) were identified in clones from two or more independent amplifications of one individual (*Alpi–DRB*3*06:09), or in clones of more than two individuals (table 2).

DRB variation

Based on the nucleotide sequences, 21 DRB alleles were detected in the analysed samples of *A. pigra*.

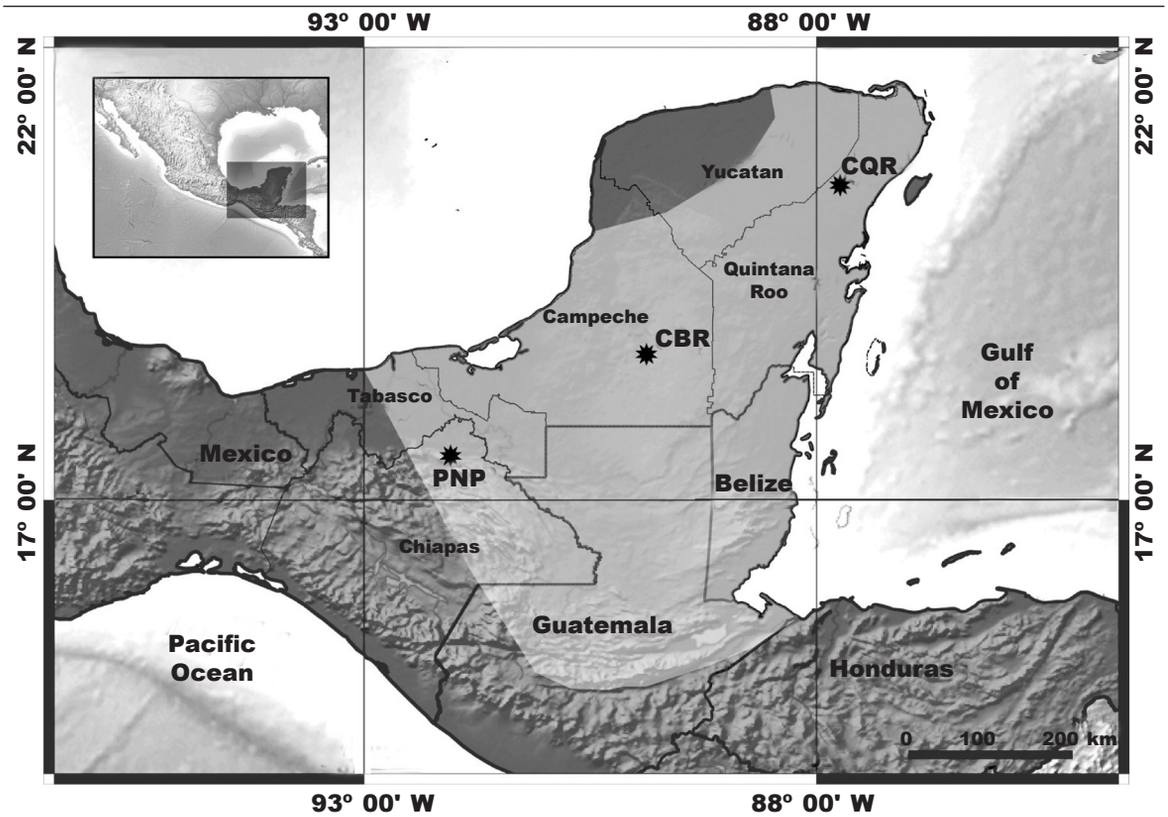

Fig. 1. Location of the three sites where fecal samples of *Alouatta pigra* were collected from June to November 2014. Sites are marked with stars: PNP, Palenque National Park; CBR, Calakmul Biosphere Reserve; and CQR, Coba Quintana Roo. (The light–shaded area indicates the putative distribution range of the species.)

Fig. 1. Ubicación de las tres localidades donde se recogieron las muestras fecales de Alouatta pigra *entre junio y noviembre de 2014. Las localidades están marcadas con estrellas: PNP, Parque Nacional de Palenque; CBR, Reserva de la Biosfera de Calakmul; CQR, Cobá Quintana Roo. (El área con sombreado claro indica el rango de distribución supuesto de la especie).*

These alleles had a 93 % to 95.9 % nucleotide similarity to DRB sequences from other primates registered in GenBank, mainly from platyrrhine species (e.g. *Aotus nancymaae, Callithrix jacchus,* and *Cebus apella*). A total of 45 segregating sites and a general nucleotide diversity of 0.057 were found (table 3). The pairwise difference among *A. pigra* alleles ranged from 1 to 33 bp, with a mean of 15.26 differences along the 269 bp fragment. The 21 alleles translated to 20 different amino acid sequences as the difference between two alleles is a single synonymous substitution (fig. 2). Within the 89 amino acid positions, 27 (30.34 %) were found to be variable. All alleles were submitted to GenBank/IPD–MHC databases (accession numbers: MF136723–MF136743) and were named by the IPD–MHC NHP nomenclature committee (De Groot et al., 2012). Since it was not clear whether alleles belonged to different lineages from a single or multiple loci, *A. pigra* alleles were designated as W (workshop): *Alpi–DRB**W105, *DRB**W106, *DRB**W107, and *DRB**W108 (table 4). No insertions or deletions were observed within the true allele set, and when translated, no premature stop codons were revealed. Therefore, no evidence suggesting the presence of pseudogenes was found.

In the Bayesian analysis, all *A. pigra* alleles were recovered into three clusters with moderate and high support values (fig. 3). The four alleles from cluster I were closely related to lineages *DRB*5*03 and *DRB**W37 from *Macaca* spp., as well as several other lineages from NWM species (e.g. *DRB*11*01, *DRB**W38, *DRB**W12 and *DRB**W19). Cluster II contained alleles from *A. pigra* from lineages *DRB**W105 and *DRB**W108, and was the sister group to *Aotus nancymaae* (*Aona–DRB**W89:01). The third cluster corresponded to alleles from lineage *DRB**W106. For this cluster, the relationship between the sequences was unclear.

The number of alleles detected per individual ranged from two to eight (table 2), with a mean of 4.75 alleles (table 3). Most of the individuals ana-

Table 2. MHC–DRB exon 2 sequences detected per *Alouatta pigra* individual analyzed. The last letter in an individual's code indicates gender (M, male; F, female). The names of the alleles correspond to those assigned by the IPD–MHC NHP nomenclature committee (De Groot et al., 2012): Indiv, individuals; N, number of alleles.

Tabla 2. Secuencias del exón 2 del gen DRB del CMH detectadas por individuo analizado de Alouatta pigra*. La ultima letra del código del individuo indica el sexo (M, macho; F, hembra). Los nombres de los alelos corresponden a los asignados por el Comité de Nomenclatura del IPD–MHC NHP (de Groot* et al.*, 2012): Indiv, individuos; N, número de alelos.*

	Cluster II							Cluster III										Cluster I				
Indiv	*Alpi–DRB*W105:01*	*Alpi–DRB*W105:02*	*Alpi–DRB*W105:03*	*Alpi–DRB*W105:04*	*Alpi–DRB*W105:05*	*Alpi–DRB*W105:06*	*Alpi–DRB*W108:01*	*Alpi–DRB*W106:01*	*Alpi–DRB*W106:02*	*Alpi–DRB*W106:03*	*Alpi–DRB*W106:04*	*Alpi–DRB*W106:05*	*Alpi–DRB*W106:06*	*Alpi–DRB*W106:07:01*	*Alpi–DRB*W106:07:02*	*Alpi–DRB*W106:08*	*Alpi–DRB*W106:09*	*Alpi–DRB*W107:01*	*Alpi–DRB*W107:02*	*Alpi–DRB*W107:03*	*Alpi–DRB*W107:04*	N
IS01M			+	+		+				+				+				+		+		7
IS02H								+		+			+	+				+		+		6
IS03H		*						+					+	+		+		+	+			7
IR01H		+	+																	+		3
IR02M	+		+	+						+	+		+									6
IR03H	+	+						+										+				4
IR04H		+		+							+		+					+				5
IR05H		+		+	+				+	+												5
IR06M					+				+	+							+		+		+	6
K261M		+		+		+				+		+						+		+		7
K262M				+												+					+	3
K263H		+																+				2
K264H	+	+			+		+			+								+	+			7
K265H										+				+				+				3
DR01H	+		+		+		+		+				+					+	+			8
DR02M			+							+		+						+				4
DR03H			+			+								+								3
ZA21M	+			+		+	+							+		+		+				7
ZA22H	+	+		+														+		+		5
ZA11H	+		+			+					+			+								5
PU01M	+	+		+														+		+		5
PU02H					+					+								+		+		4
PU03H		+	+								+							+		+		5
FM01M		+	+							+								+			+	5
FM02H	+			+		+				+	+							+			+	7
FM03H		+	+	+																		3
FM04H		+																+				2
CP01H			+	+						+		+				+	+					6
CP02H				+		+																2

Table 2. (Cont.)

	Cluster II							Cluster III										Cluster I				
Indiv	*Alpi–DRB**W105:01	*Alpi–DRB**W105:02	*Alpi–DRB**W105:03	*Alpi–DRB**W105:04	*Alpi–DRB**W105:05	*Alpi–DRB**W105:06	*Alpi–DRB**W108:01	*Alpi–DRB**W106:01	*Alpi–DRB**W106:02	*Alpi–DRB**W106:03	*Alpi–DRB**W106:04	*Alpi–DRB**W106:05	*Alpi–DRB**W106:06	*Alpi–DRB**W106:07:01	*Alpi–DRB**W106:07:02	*Alpi–DRB**W106:08	*Alpi–DRB**W106:09	*Alpi–DRB**W107:01	*Alpi–DRB**W107:02	*Alpi–DRB**W107:03	*Alpi–DRB**W107:04	N
AP01M				+								+								+		3
AP02H	+		+	+							+											4
AP03M		+	+					+		+												4
CN01M	+	+	+				+														+	5
CN02M		+		+		+							+									4
CN03H		+		+					+		+											4
CN04H	+	+			+		+													+	+	6
CN05H				+		+		+	+							+					+	6
PC01M			+	+				+			+		+				+	+				7
P01H		+	+	+				+			+											5
EM01H				+							+											2
CU01M		+								+			+	+				+				5
CU02H				+						+	+		+	+								5
CU03H		+		+				+		+								+				5
CU04H		+		+																		2

Table 3. MHC–DRB polymorphism detected in faecal samples of *Alouatta pigra* from: PNP, Palenque National Park; CBR, Calakmul Biosphere Reserv; CQR, Coba Quintana Roo; N, number of individuals; π, nucleotide diversity; S, segregating sites; MAI, mean number of alleles per individual.

Tabla 3. Polimorfismo del gen DRB del MHC detectado en muestras fecales de Alouatta pigra *de: PNP, Parque Nacional de Palenque; CBR, Reserva de la Biosfera de Calakmul; CQR, Cobá Quintana Roo; N, número de individuos; π, diversidad de nucleótidos; S, sitios segregados; MAI, número promedio de alelos por individuo.*

Locality	N	No. alleles	No. lineages	π	S	MAI
CBR	23	21	4	0.057	45	5.09
PNP	17	18	4	0.057	44	4.41
CQR	4	8	3	0.055	39	4.25
Total	44	21	4	0.057	45	4.75

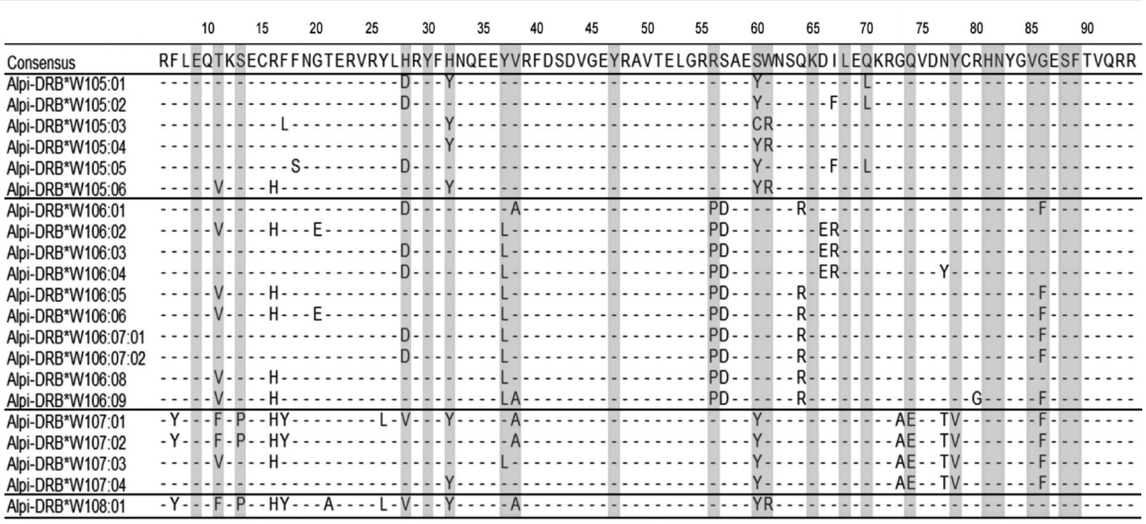

Fig. 2. Amino acid sequence alignment of the 21 DRB alleles of *Alouatta pigra*. The numbers at the top indicate the amino acid position based on *HLA–DRB*1*01:01:01 allele. The shaded columns correspond to antigen binding sites deduced from human positions.

Fig. 2. Alineamiento de secuencias de aminoácidos de los 21 alelos DRB de Alouatta pigra*. Los números en el margen superior indican la posición de los aminoácidos basada en el alelo* HLA–DRB*1*01:01:01. Las columnas sombreadas corresponden a los sitios de unión al antígeno deducidos a partir de las posiciones descritas en humanos.*

Table 4. *Alouatta pigra* MHC–DRB alleles and lineages detected per locality: PNP, Palenque Nacional Park; CBR, Calakmul Biosphere Reserve; CQR, Coba Quintana Roo.

Tabla 4. Alelos y linajes del gen DRB del MHC de Alouatta pigra *detectados por localidad: PNP, Parque Nacional de Palenque; CBR, Reserva de la Biosfera de Calakmul; CQR, Cobá Quintana Roo.*

	Locality		
Lineage	CBR	PNP	CQR
Allele	N = 23	N = 17	N = 4
*DRB**W105			
*Alpi–DRB**W105:01	+	+	
*Alpi–DRB**W105:02	+	+	+
*Alpi–DRB**W105:03	+	+	
*Alpi–DRB**W105:04	+	+	+
*Alpi–DRB**W105:05	+	+	
*Alpi–DRB**W105:06	+	+	
*DRB**W106			
*Alpi–DRB**W106:01	+	+	+
*Alpi–DRB**W106:02	+	+	
*Alpi–DRB**W106:03	+	+	+
*Alpi–DRB**W106:04	+	+	+
*Alpi–DRB**W106:05	+	+	
*Alpi–DRB**W106:06	+	+	+
*Alpi–DRB**W106:07:01	+		+
*Alpi–DRB**W106:07:02	+		
*Alpi–DRB**W106:08	+	+	
*Alpi–DRB**W106:09	+	+	
*DRB**W107			
*Alpi–DRB**W107:01	+	+	+
*Alpi–DRB**W107:02	+		
*Alpi–DRB**W107:03	+	+	
*Alpi–DRB**W107:04	+	+	
*DRB**W108			
*Alpi–DRB**W108:01	+	+	
Total	21	18	8

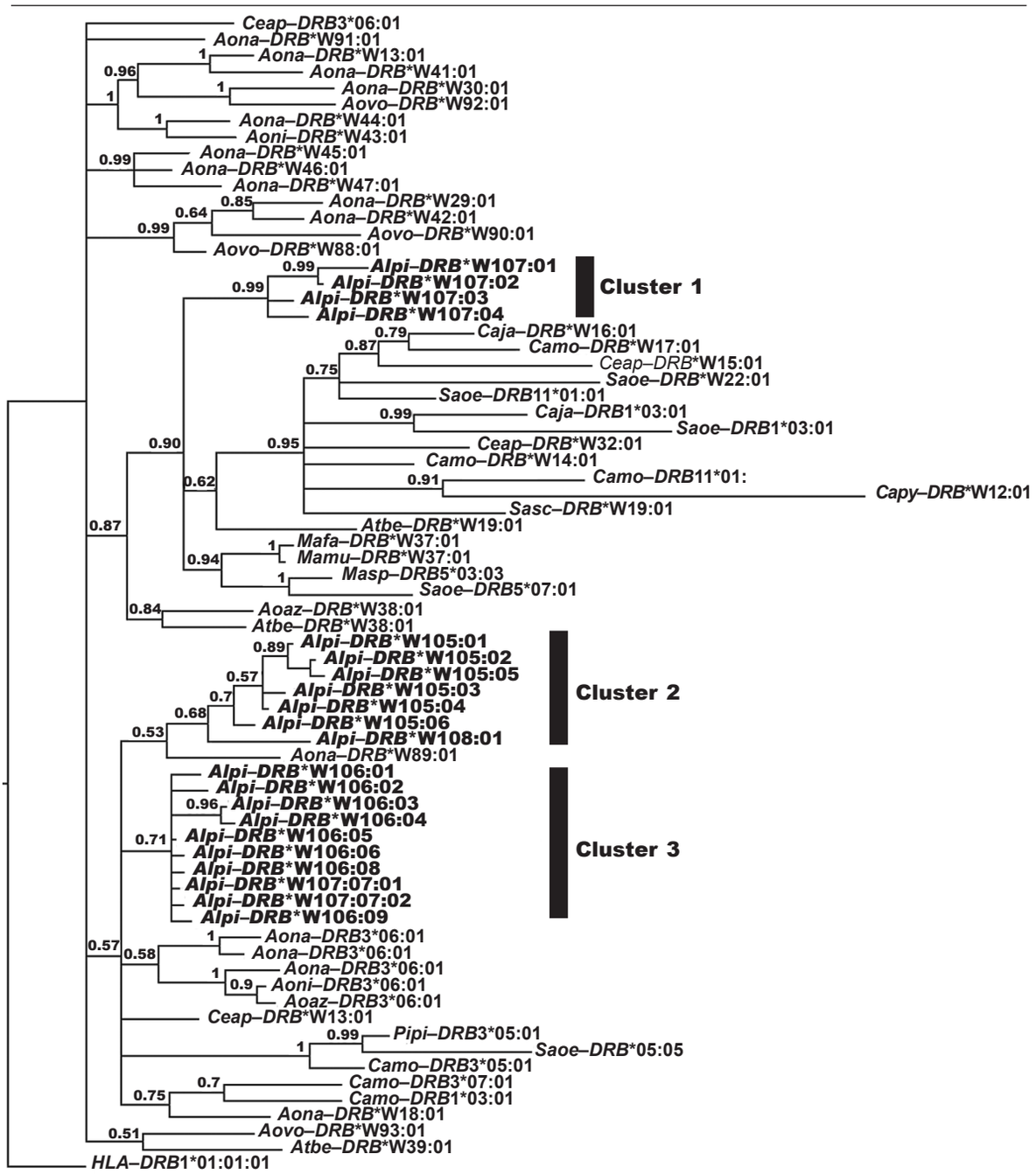

Fig. 3. Consensus Bayesian tree of DRB exon 2 of *Alouatta pigra* and selected sequences representing all known lineages of NWM. A human sequence was used as outgroup (*HLA–DRB*1*01:01:01): *A. pigra* (*Alpi–DRB*) sequences are shown in bold. Other sequences are: *Ceap–DRB*, *Cebus apella*; Saoe, *Saguinus oedipus*; *Camo–DRB*, *Callithrix moloch*; *Pipi–DRB*, *Pithecia pithecia*; *Aoaz–DRB*, *Aotus azarai*; *Aoni–DRB*, *Aotus nigriceps*; *Aona–DRB*, *Aotus nancymaae*; *Atbe–DRB*, *Ateles belzebuth*; *Aovo–DRB*, *Aotus vociferans*; *Capy–DRB*, *Callithrix pygmaea*; *Caja–DRB*, *Callithrix jacchus*; *Maca–DRB*, *Macaca mulatta*; *Mafa–DRB*, *Macaca fascicularis*; *Masp–DRB*, *Mandrillus sphinx*. The numbers above the lines correspond to Bayesian posterior probabilities.

Fig. 3. Árbol bayesiano consenso del exón 2 del gen DRB de Alouatta pigra *y secuencias que representan todos los linajes conocidos de platirrinos. Se usó una secuencia de humano como grupo externo (*HLA–DRB*1*01:01:01). Las secuencias de* A. pigra *(*Alpi–DRB*) están marcadas en negritas. Los números encima de las líneas corresponden a las probabilidades posteriores bayesianas. (Para las abreviaturas de las distintas secuencias, véase arriba.)*

Table 5. *Z*–test for positive selection at putative antigen binding sites (ABS) as described for humans by Brown et al. (1993) and the non–ABS positions of *DRB* exon 2 alleles of *Alouatta pigra:* d_S, synonymous substitutions; d_N, non–synonymous substitutions; d_N/d_S, ratio of nonsynonymous to synonymous substitutions; P, probability value. Sequences were grouped according to their position in the phylogenetic analysis.

Tabla 5. Prueba de Z para la selección positiva en sitios de unión al antígeno (ABS) descritos para humanos por Brown et al. *(1993) y en las posiciones de aminoácidos no pertenecientes a ABS (non–ABS) de los alelos del exón 2 del gen* DRB *detectados en* Alouatta pigra: *d_S, sustituciones sinónimas; d_N, sustituciones no sinónimas; d_N/d_S, tasa entre sustituciones no sinónimas a sinónimas; P, valor de probabilidad. Las secuencias se agruparon según la posición que ocupan en el análisis filogenético.*

	d_S	d_N	d_N/d_S	*P*	*Z*–value
ABS N = 24					
Clade I	0	0.114 ± 0.047		**0.009**	2.387
Clade II	0	0.097 ± 0.057		**0.004**	2.696
Clade III	0.014 ± 0.029	0.072 ± 0.042	5.14	0.081	1.407
All sequences	0.078 ± 0.071	0.181 ± 0.094	2.305	**0.045**	1.535
Non–ABS N = 65					
Clade I	0.021 ± 0.014	0.022 ± 0.012	1.04	0.468	0.081
Clade II	0.020 ± 0.022	0.023 ± 0.019	1.13	0.401	0.252
Clade III	0.033 ± 0.037	0.024 ± 0.027	0.73	1	–0.327
All sequences	0.028 ± 0.022	0.039 ± 0.022	1.39	0.282	0.578

lyzed (26 out of 44 individuals) had more than five DRB sequences. Some individuals presented more than two DRB alleles (up to four) that belonged to the same phylogenetic cluster. Seventeen individuals harboured three or four alleles from cluster II, and nine individuals had three or four alleles from cluster III (table 2).

Alouatta pigra from CBR showed a slightly higher allelic variation (N = 21) than *PNP* (N = 18). In CQR, where only four samples were successfully analysed, eight sequence variants were detected. Allele *Alpi–DRB**W106:07:01 was not detected in PNP and alleles *Alpi–DRB**W106:07:02 and *Alpi–DRB**W107:02 were unique for CBR (table 4). As for the nucleotide variation and the number of segregating sites, values were similar for CBR (π = 0.057; S = 45) and PNP (π = 0.057; S = 44) (table 3). CQR values were slightly lower than those for the other two best–represented localities (π = 0.055, S = 39, and an average number of alleles per individual of 4.25). The greatest difference in the mean number of alleles per individual was between CBR (5.09) and CQR (4.25) (table 3).

Positive selection

Within the 24 amino acid positions putatively belonging to the ABS, 13 (54.2%) were detected as variable. On the other hand, only 14 (20%) out of 65 positions of the non–ABS part were variable, and half of them were located next to an ABS. The values of the rates of non–synonymous (d_N) vs. synonymous (d_S) substitutions were higher for the ABS (d_N/d_S = 2.305) than for the non–ABS sequence region (d_N/d_S = 1.39), for all sequences and for each cluster. Even though both values of d_N/d_S were higher than 1, the *Z*–test for positive selection was significant (*P* = 0.045) only for the ABS (table 5). For the ABS of Cluster III, although the d_N/d_S rate showed a high value, *Z* test was not significant.

Discussion

Current knowledge on MHC–DRB polymorphism in platyrrhines is concentrated in a few species of the Cebidae family, which are commonly used in biomedical research (Nino–Vasquez et al., 2000; Suárez et al., 2006; Mee et al., 2011). This study is therefore, the first to investigate DRB gene nucleotide sequence polymorphism in a representative species of the Atelidae family. In *A. pigra*, we detected a relatively low level of allelic diversity (N = 21) and four different DRB lineages from those previously reported in other primate species.

DRB allelic diversity in *A. pigra*

MHC allelic diversity can vary considerably among primate species. Many catarrhine species have been

found to possess a high DRB allelic diversity (e.g. 2311 in humans, 211 crab–eating macaques), while in general, a more modest allelic diversity has been detected in *NWM* (e.g. 44 in common marmoset, 110 in owl monkeys, and 47 in cotton–top tamarins) (De Groot et al., 2012; Robinson et al., 2013). Thus, comparing the DRB allelic diversity found in *A. pigra* (i.e. 21 sequences in 44 individuals) to that reported in other studies from *NWM* (e.g. *Aotus nancymaae*, 34 sequences in 15 individual and 67 sequences in 71 individuals; *Saguinus oedipus,* 28 sequences in 13 individuals; *Callithrix jaccus,* 21 sequences in 30 individuals [Antunes et al., 1998; Suárez et al., 2006; Nino–Vasquez et al., 2000; Gyllensten et al., 1994]), it can be considered as low.

This difference in the level of DRB sequence diversity of *A. pigra* compared to other *NWM* could be related to several factors, such as differences in selection pressure (Mainguy et al., 2007; Hambuch and Lacey, 2002). Although *NWM* have mainly arboreal lifestyles, parasite exposure could vary between species given their differences in: (a) geographical distribution, as evidence suggests a decrease in parasite richness from the equator to poles (Guernier et al., 2004; Mainguy et al., 2007); (b) the size of the social groups (i.e. 2–8 individuals in *A. pigra;* 15–30 in *C. jaccus;* 2–5 in *A. nancymaae*; and 2–13 in *S. oedipus*), the population density and their contact with related species, which influence lateral transfer of parasites (Nunn et al., 2003); and (c) the species diet, as those that include insects in their diet (*A. nancymaae, S. oedipus,* and *C. jacchus*) are expected to have higher parasite diversity than herbivores (*A. pigra*), as insects can serve as intermediate hosts for parasites (Lafferty, 1999; Vitone et al., 2004). Alternatively, the reduced allelic variation of *A. pigra* could be explained by a process of past purifying selection against a parasite (Bollmer et al., 2011), favouring the fixation of only a limited number of DRB sequences.

Historical events such as founding events and bottlenecks, however, can be translated into low *MHC* sequence variation. In such events, the strength of balancing selection to maintain polymorphisms is exceeded by genetic drift. therefore, randomly losing genetic diversity (Sommer, 2005). This seems the most likely explanation for the sequence diversity found in *A. pigra*. First, due to its biogeographic history, as Central American *Alouatta* species (i.e., *A. pigra* and *A. palliata*) have their origin in populations of northern South America that invaded Central America and southern Mexico after the formation of the Panama isthmus (Fleagle, 1999). Microsatellite loci studies of both Central American *Alouatta* species show that this founder effect derived from the process of colonization, creating a pattern of gradual loss of genetic variation northward of their distribution (Ellsworth and Hoelzer, 2006). Contrary to neutral markers such as microsatellites, *MHC* loci are subjected to positive selection due to their role in the host immune response. Nevertheless, the strong genetic drift derived from this process of colonization could have affected *MHC* allelic diversity in this northern species. This hypothesis is strongly supported by the low neutral variability (i.e. microsatellites) previously detected in the species (Amendola, 2009; Van Belle et al., 2012). It is also in agreement with the almost homogenous geographical distribution of DRB sequences detected among the sampled localities (table 4). Thus, although neither of the two former explanations can be ruled out, they cannot necessarily account for the low variation in neutral markers reported for *A. pigra* in other studies. Therefore, the relatively low level of DRB allelic diversity in *A. pigra* is most likely a consequence of drift due to the biogeographical history of this species.

DRB loci and lineages

Some of the known DRB platyrrhine alleles show high similarity to catarrhine alleles from *DRB*1*03, *DRB*3*01, and *DRB*5 loci. However, evidence indicates that the similarity between DRB alleles of platyrrhines and catarrhines has arisen through convergent evolution, given the differences of the codons determining the shared amino acid motifs (Kriener et al., 2000). None of the sequences detected in *A. pigra* belong to this group of alleles, and the four lineages to which they were assigned (*DRB*W105, *DRB*W106, *DRB*W107, and *DRB*W108) are different from those previously reported in other primate species, although they show a high nucleotide similarity (93–95.9 %) to alleles of other NWM lineages.

The number of alleles detected per animal ranged from two to eight, indicating the existence of at least four DRB loci in *A. pigra*. This variation in the number of MHC–DRB loci between individuals of a species has been reported for several mammals, such as humans, other non–human primates, bats, tree shrews, and voles (Bontrop, 2006; Oppelt et al., 2010; Kloch et al., 2010; Salmier et al., 2016). These gene duplications contribute to MHC diversity and are known to play an important role in the adaptive evolution of organisms (Hughes and Yeager, 1998). Phylogenetic analysis revealed three *A. pigra* DRB allele clusters, which could be interpreted as hypothetical loci, on the premise that alleles from a particular DRB locus tend to cluster together. However, if a relatively recent gene duplication has occurred, the alleles of both genes will be highly related, difficulting the distinction of the two loci by phylogenetic clustering of sequences (Oppelt et al., 2010). This is probably the case in *A. pigra* since data indicate the existence of at least four loci, but only three clusters were recovered. Furthermore, the allelic composition of the screened animals suggests that two of the phylogenetic clusters likely represent more than a single loci. This is revealed by a high number of individuals harbouring more than two alleles from cluster II or III (table 2). In contrast, no more than two alleles from cluster I were found in a single individual. Nonetheless, the evolution and mechanisms generating polymorphisms in DRB exon 2 are complex and do not follow specific patterns (Takahata and Satta, 1998). Consequently, the exclusive use of exon 2 sequences to evaluate allele phylogenetic relationships may lead in some cases to erroneous conclusions. Moreover, it has been shown that the relationship between alleles is more accurately inferred by using information from

introns or exons that are not under selective pressure (Kriener et al., 2000). The inclusion of intron sequence data could thus help clarify the relationships between the alleles of *A. pigra*, providing a more precise identification of loci.

In addition, the observed variation in the composition of lineages present in each animal could indicate the existence of haplotype (i.e. combinations of sequences that are inherited as blocks) polymorphism in the species. Althoughwe cannot rule out the possibility that mutations in the hybridization sites of the primers and allelic drop out may have led to a failure to detect some of the allelic variants , the methodological approach used here minimizes this possibility, and a real haplotype variation is likely occurring. However, due to the lack of data on mother–infant pairs or kinship information of the analysed animals, it was not possible to define DRB haplotypes.

Since genomic DNA was used, it cannot be ascertained whether the DRB alleles amplified for *A. pigra* are expressed. However, no evidence indicating the presence of pseudogenes was found, as none of the studied sequences have indels or premature stop codons. Also, an excess of non–synonymous substitution at the ABS reveals that selection has acted on these genes, but such a process is not necessarily on–going (Garrigan and Hedrick, 2003). Therefore, transcription studies are needed to confirm their functionality, particularly because genomic and expression analyses in other NWM species have uncovered the presence of non–functional alleles from different lineages (Doxiadis et al., 2006; Trtková et al., 1993).

Finally, although *A. pigra* has a reduced set of alleles, sequence level divergence between some of the alleles from the different lineages is marked (up to 33 p). Thus, they may likely recognize antigens from very different pathogens (Hedrick et al., 2000). Furthermore, polymorphism is observed in the number of lineages and loci, which is comparable to that found in other NWM species, except *Aotus nancymaae* (Trtková et al., 1993; De Groot et al., 2012; Gyllensten et al., 1994). It is also observed in allelic configurations of individuals, which suggests haplotype variation. This observed polymorphism, taken as a whole, appears to have been sufficient for wild *A. pigra* to cope with parasites under current ecological conditions, given that there is no evidence that the species suffers from increased susceptibility to parasites when compared with other *Alouatta* species (Trejo–Macías and Estrada, 2012; Vitazkova and Wade, 2006; Stoner and Gonzalez Di Pierro, 2006). However, this relatively low variation in adaptive immune response genes may not be enough to give populations the necessary protection in case of the emergence of novel pathogens. This could be a cause of concern for the conservation of the species if a greater loss of this diversity occurs derived from the ongoing population declines.

Nevertheless, the relatively low level of polymorphism detected in these functionally important loci (MHC–DRB), together with previous low variation reported in neutral markers (microsatellites and mtDNA), suggests that genetic drift due to the biogeographical history of the species had an impact on the general genetic diversity of the species. As a result, conservation programs should consider the maintenance of the maximal genetic diversity (including *MHC* and other immune genes). Future research should also consider spatio–temporal variations of MHC–DRB diversity in *A. pigra* populations so as to monitor and detect possible increases in the risk of infectious diseases. This applies particularly to those at higher risk of pathogen introduction,and those suffering from steep population declines or isolation derived from human encroachment to their habitat (Ujvari and Belov, 2011; Grogan et al., 2017).

Another point of relevance is the need to incorporate in future studies other functionally important immune genes, such as *MHC* class I genes, particularly *MHC–B* and *MHC–G* like genes that have shown to be highly polymorphic and that are expressed in several NWM, including some from the Atelidae family (van der Wiel et al., 2013; Lugo and Cadavid, 2015; Cao et al., 2015). Non–*MHC* immune genes, especially those related to viral resistance like the OAS1 (oligoadenylate synthetase) gene (Acevedo–Whitehouse and Cunningham, 2006; Rios et al., 2007), should also be considered since there have been reports of yellow fever outbreaks in populations of other *Alouatta* species (De Almeida et al., 2012; Crockett, 1998; Holzmann et al., 2010), that could represent a possible threat to the conservation of *A. pigra* populations. All this would allow a better understanding of the immunogenetic status of the species and gauge its response to novel pathogen threats.

In summary, a relatively low MHC–DRB exon 2 variation was detected at the allelic level in *A. pigra* compared to that reported for other NWM species. The consistency of these results with those from previous studies on neutral marker variation, and the nearly homogeneous allele and lineage distribution observed across localities of this study, suggest that this reduced allelic diversity in free–ranging *A. pigra* is most likely associated with a historical founder effect during the northward expansion of their ancestors from South America. However, the excess of nonsynonymous substitutions at the antigen binding sites indicates that sequence evolution was driven at some point by positive selection. Finally, despite the relatively low DRB allelic diversity in the species, polymorphism in lineage and loci, as well as the level of differentiation between some of the alleles, might be enabling wild populations to initiate an adequate immune response to cope with current pathogens, but it might entail a risk for these populations in case of the emergence of new ones.

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