Patterns of MHC Polymorphism in Endangered San Joaquin Kit Foxes Living in Urban and Non-urban Environments

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Abstract Genes of the major histocompatibility complex (MHC) play a primary role in resistance to infectious disease and have been implicated in mate choice and recognition of close relatives. We describe genetic diversity at three MHC class II genes in urban and non-urban populations of San Joaquin kit fox (*Vulpes macrotis mutica*), an endangered canid endemic to California's San Joaquin Valley. We used multi-tagged pyrosequencing synthesis to characterize genetic diversity in 96 kit foxes at three MHC class II genes, DQA1, DQB1, and DRB1. Using rigorous bioinformatics methods to limit sequencing artifacts, we identified 11 DQA alleles, 13 DQB alleles, and 29 DRB alleles, which translated into 1, 7, and 28 unique antigen-binding sites for the respective genes. The high polymorphism did not correlate with sequencing depth or sample location but was found across individuals and indicated polygenic loci for DQB (two gene copies) and DRB (three copies). A phylogeny based on all known carnivore MHC alleles resolved two major clades,

Electronic supplementary material The online version of this chapter (https://doi.org/10.1007/978-3-030-33334-8_13) contains supplementary material, which is available to authorized users.

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J. Ortega and J. E. Maldonado (eds.), *Conservation Genetics in Mammals*,
https://doi.org/10.1007/978-3-030-33334-8_13

one with alleles from DQA and one with alleles from DQB and DRB, regardless of the taxa. Based on Tajima's D, we found evidence of negative selection in DQA ($D = -1.90$) and positive selection in DRB ($D = 2.36$). We analyzed 48 urban kit foxes that were living in the city of Bakersfield and 48 non-urban foxes living in the nearby LoKern and Carrizo plain areas. We found similar levels of MHC allelic diversity in the urban and non-urban populations. However, many of the common DRB alleles found in the non-urban population were absent in the urban population. This could result from a founder effect or different selective pressures on the two populations. The levels of MHC diversity in the SJKF are similar to or higher than MHC diversity of more wide-spread canid species, although this subspecies has been isolated in the San Joaquin Valley for millennia and has low mtDNA haplotype diversity. Our study provides baseline data on three MHC genes that can contribute to future studies of disease resistance and mate choice in the endangered San Joaquin kit fox.

Keyword Kit fox · Major histocompatibility complex · DQA1 · DQB1 · DRB1 · Balancing selection · Tests of neutrality · Immunogenetics

1 Introduction

The major histocompatibility complex (MHC) is a highly variable part of the genome that codes for immune system genes. MHC genes code for proteins that bind to antigens, molecules from a foreign substance or pathogen, and initiate an immune response. MHC genes are grouped into three classes (I, II, and III) depending on their structure, which also determines the type of immune system pathway. Class II molecules have two subunits, an alpha and a beta strand (e.g., DQA and DQB), which form a groove that binds the antigen, i.e., the antigen-binding site (ABS). Variation in the ABS can be generated from codominant, highly polymorphic, and/or polygenic (multiple copies of the same gene) MHC genes. MHC polymorphism may be derived from multiple evolutionary mechanisms, including pathogen mediation, over-dominance selection (i.e., heterozygote advantage; Hughes and Nei 1988), negative frequency-dependent selection (Tollenaere et al. 2008), or disassortative mating (Milinski 2006).

Genetic variation in the immune system has been shown to affect the survival of individuals. Furthermore, it can result in parasite resistance or inbreeding avoidance which may have direct and/or indirect effects on the fitness of progeny (Landry et al. 2001). Selection on the MHC can arise when pathogen evolution is occurring rapidly or in areas with high pathogen loads, such as urban environments (Hedrick et al. 2003). A limited number of endangered mammal species are able to use the urban landscape (Randa and Yunger 2006), and often little is known about how these species behave in the urban habitat and whether they are losing genetic variation over time. Carnivores that inhabit urban ecosystems, such as raccoons and skunks, have increased disease risks from interactions with domestic dogs and cats. Pathogens spread by domestic carnivores have resulted in increased disease outbreaks in wild

carnivores worldwide (Woodroffe et al. 2004); for example, domestic dogs spread rabies to highly endangered Ethiopian wolves (*Canis simensis*) and African wild dogs (*Lycaon pictus*, Laurenson et al. 2004).

Urban areas may become an alternative refuge for species that are rapidly losing wild habitats (Parris and Hazell 2005). Such is the case for the endangered San Joaquin kit fox (SJKF; *Vulpes macrotis mutica*) (Fig. 1), which has declined to approximately 3,000 individuals with the contraction of populations attributed to loss of 90% of its habitat (US Fish and Wildlife Service 2010). A sizeable population of SJKFs inhabits the city of Bakersfield, California. Kit foxes are opportunistic foragers with preferences for small mammals like rodents and rabbits but also benefit from increased resources in an urban setting such as water and anthropogenic food sources (Cypher 2010; Bjurlin et al. 2005; Newsome et al. 2010). Urban kit foxes live in monogamous pairs and occasionally larger social groups within small territories and have smaller, more overlapping home ranges (Ralls et al. 2001, 2007; Westall et al. 2019). Overall, the urban environment can support a higher population density of kit foxes than their natural environment. While this adaptation to the urban environment appears beneficial, the increased density of kit foxes may also increase disease transmission (Cypher 2010). Urban SJKFs live in close proximity to domestic and urban carnivores and may be vulnerable to canine diseases such as rabies, canine distemper virus (CDV), canine parvovirus (CPV), and sarcoptic mange, all of which have been documented in SJKF (Cypher et al. 2017; Miller et al. 2000; Standley and McCue 1992; White et al. 2000). By studying the MHC genes of SJKF, we can better understand the amount of MHC diversity that can assist in the immune response of this endangered species and determine whether there are differences between foxes in urban and non-urban habitats.

Next-generation sequencing methods have made MHC studies of non-model organisms much more feasible (Babik et al. 2009; Wegner 2009). To date, MHC

Fig. 1 San Joaquin kit fox (*Vulpes macrotis mutica*) shown in urban (right) and non-urban (left) environments. The San Joaquin kit foxes live primarily in arid grasslands and shrublands in the southern portions of the San Joaquin Desert. With a loss of over 90% of this habitat in the last century, a population of kit foxes has inhabited the city of Bakersfield, California. Photographs taken by California State University, Stanislaus, Endangered Species Recovery Program

genes have been characterized in the following canids: *Urocyon*, *Lycalopex*, *Lyacon, Canis*, and *Vulpes* (Aguilar et al. 2004; Hedrick et al. 2000, 2002; Kennedy et al. 2007b; Marshall et al. 2016; Seddon and Ellegren 2002, 2004; Ploshnitsa et al. 2012).

This study is the first to characterize patterns of MHC genetic diversity and natural selection in SJKF (*Vulpes macrotis mutica*), and to span an urban-to-rural gradient. We first developed methods to amplify and sequence three class II MHC genes (DQA1, DQB1, and DRB1) for a few kit foxes with Sanger sequencing, followed by multi-tagged pyrosequencing of all individuals (MTPS; Gillevet et al. 2010). We studied the genetic variation of these genes in the urban kit fox population in Bakersfield and two non-urban reference populations in LoKern and Carrizo Plains, California. These urban and non-urban foxes have different demographic histories, different ecological characteristics (such as diet, mortality, and territory size; Cypher 2010; Cypher and Frost 1999), and likely experience different selection pressures with respect to disease due to the differences between their environments. We compared the levels of MHC diversity in kit foxes between the two environment types, compared MHC variation in kit foxes to variation in other carnivores, and tested for evidence of selection.

2 Methods

Samples—We selected 96 ear tissue samples collected during previously published studies: 48 collected in Bakersfield from 2001 to 2009 (Cypher 2010), 24 in LoKern from 2005 to 2008 (Bremner-Harrison and Cypher 2011), and 24 in the Carrizo Plains National Monument in 1998 (Bean 2002). We extracted DNA from approximately ½ of a 3 mm diameter circle of ear using a DNeasy blood and tissue kit (QIAGEN). All extractions were carried out in a separate room under quasi-clean conditions to prevent contamination. Negative controls (no DNA material added to the extraction) accompanied each set of extractions and were used to check for contamination. We also used high amplification success of microsatellites from the same samples as an indicator of high-quality DNA (unpublished data).

Amplification—We focused on class II MHC genes in exon 2 because it is a hypervariable region that translates into functional differences in pathogen recognition. DQA1 in SJKF amplified using a primer set that had previously been used in gray wolves (Kennedy et al. 2007b) and was developed for dogs (Wagner et al. 1996; DQAin1: 5′-TAAGGTTCTTTTCTCCCTCT-3′; DQAin2: 5′-GGACAGATTCAGTGAAGAGA-3′). For DQB1, we used the forward primer designed for seals and subsequently used for island foxes (Aguilar et al. 2004; Hoelzel et al. 1999; DQB1: 5′-TCGTGTACCAGTTTAAGGGC-3′) and the reverse primer designed for wolves by Kennedy et al. (2002; DQBR2: 5′-CACCTCGCCGCTGCAACGTG-3′). The DRB1 gene from SJKF is successfully amplified using primers designed for Mexican wolves (Hedrick

et al. 2000: DM-1: 5′-AAGTCCGAGTGCTATTTCACC-3′ and DM-2: 5′-TCGCCGCTGCACCGTGAAGCT-3′). These primer sets amplified DNA fragments of 288 bp for DQA1, 227 bp for DQB1, and 207 bp for DRB1.

454 Next-generation sequencing—Next-generation sequencing (NGS) has increasingly been used in the last decade to genotype the MHC in a diversity of organisms (Gonzalez-Quevedo et al. 2015 and references therein). With the introduction of NGS technologies, such as the Roche 454 pyrosequencing platform, it has been possible to obtain sequences from individual DNA strands, allowing fast and efficient sequencing in parallel of co-amplified alleles (Margulies et al. 2005). Another advantage of using an NGS platform is the potential for obtaining sequences from a large number of identified individuals in a single run by using 'barcoded fusion' primers. This allows for the subsequent assignment of sequences to individuals during the sequence filtering stage. We therefore used a Roche 454 NGS platform to screen MHC variation in the three MHC genes (DQA, DQB, and DRB) for all 96 samples in one-pooled NGS run (Babik et al. 2009; Wegner 2009). NGS methods are prone to sequencing errors primarily due to chimeric sequence, which can be an important artifact in a gene complex such as the MHC. Since fusion primers reduce the number of PCRs needed to uniquely barcode samples, they minimize the chance of creating chimeric sequences (Smyth et al. 2010). Thus, we modified the three sets of primers to include a 5′ addition of a 19-mer adaptor and a 4 bp key sequence (TCAG) required for emulsion PCR and 454 sequencing. In addition, we created 96 forward primers that included a unique 8 bp barcode (individual sample tag) between the key sequence and the primer that differed by at least 2 base pairs and did not create any homopolymer sites (Supp. Table 1–3). Fusion primers were synthesized by Operon (Louisville, KY) with the barcoded forward primer on a plate format at 10 nmol concentration to avoid contamination that may be introduced in manipulating primers.

We amplified each gene in 20 μL volumes as follows: 2.0 μL of 10 × PCR buffer, 2.0 μL of 10 μM dNTPs (2.5 μM each; Invitrogen, Carlsbad, CA, USA), 1.75 μL of MgCl2 (25 mM), 2.0 μL of BSA (25 mM), 0.25 μL of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 3.0 μL of template DNA, 0.75 μL of 10 μM fusion forward primers, 0.75 μL of 10 μM reverse primers, and 7.5 μL of sterile water. A touchdown PCR protocol was used for DRB and DQB amplifications, which consisted of an initial 10 min at 95 °C, 16 touchdown cycles of 95 °C for 30 s, 45 s touchdown annealing step (starting at 63 °C and reducing by 1 °C each cycle), and then 72 °C for 45 s. This was followed by 29 cycles of 95 °C for 40 s, 47 °C for 55 s, and 72 °C for 55 s plus a final extension at 72 °C for 10 min.

Amplification of DQA required a modified protocol because the PCR for DQA did not amplify with the fusion primer. Therefore, we amplified DQA with the original primer set (non-fusion primers DQAin1 and DQAin2) using the above touchdown protocol with only ten cycles at the constant annealing temperature of 47 °C. Next, we cleaned PCR reagents from each reaction using 2.5 μL of 1:10 diluted ExoSAP-IT (Fisher Scientific). We used 3 μL of these cleaned products in new PCR with the fusion primers in the same 20 μL volume recipe as above with the following conditions: an initial 10 min at 95 °C, 20 cycles of 95 °C for 40 s, 47 °C for 55 s, and

72 °C for 55 s, followed by a final extension at 72 °C for 10 min. Using this modified protocol, we investigated the presence of DQA amplicons, both with and without the 5′ modifications (adaptor, etc.), using a dissociation curve on a qPCR machine (Mx300P, Agilent Technologies). We confirmed our amplicons of DRB and DQB with fusion primers in the same dissociation curve.

We cleaned the PCR products two times with Sera-Mag SpeedBeads (Thermo-Scientific, Waltham, MA) in a PEG/NaCl buffer (i.e., MagNA beads; Rohland and Reich 2012) and visualized pre- and post-cleanup PCR products on agarose gels using GelRed (Phenix Research Products, Candler, NC). Samples (96) from each gene were pooled using gel visualization, such that 10 μL of most amplicons were added to the pool except when they were very strong or very weak. Only 5 μL were added when amplicons were very strong, and 20 μL when amplicons were weak. Each gene pool was cleaned again using MagNA beads. The pre- and post-cleanups of gene pools were run on an agarose gel to confirm the presence and size of amplicons and the lack of PCR primers and truncated products. Gene pools were then quantified using the fluorescent PicoGreen DNA assay and pooled in equimolar concentrations for emulsion PCR. The pool was sequenced in two separate runs on the 454 GS Junior (Roche) located in the Microbiome Analysis Center at George Mason University (MBAC). Each run consisted of clonal amplification in two separate emPCR kits, each with independent sequencing according to manufacturer's instructions.

Bioinformatic pipelines—The data generated from 454 sequencing were analyzed in several ways that have been suggested in the literature (BioPerl script and MUSCLE alignment—Neiman et al. 2011; SESAME—Meglécz et al. 2011; Oomen et al. 2013), with a final analysis based on the stepwise procedure in Galan et al. (2010). This process was used to filter out as many non-target genes and sequencing errors as possible. Briefly, the steps are as follows:

- Step 1: We used custom PERL scripts on Galaxy (Goecks et al. 2010) in the MBAC Metabiome Portal to remove sequences shorter than 150 bp and to trim bases with a quality score less than 9 from the 3′ end. Sequences from each gene were then separated into three files using the forward primer sequence with no base pair mismatches allowed. We used jMHC to detect variants (unique sequences that are putative alleles) within each gene pool and index the number of sequences for each variant in each sample (Stuglik et al. 2011). In this process, jMHC uses forward and reverse primer sequences and sample barcodes to create a cross-referenced SQL database. We discarded variants with three or fewer reads in the entire gene pool due to the chances for sequencing errors.
- Step 2: The $T1$ threshold developed by Galan et al. (2010) uses probability calculations to determine the number of sequences required to determine the complete genotype for a sample. This threshold depends upon the number of copies of the gene, which was not known for SJKF. However, with such deep sequencing, each sample had hundreds of reads for each variant or did not amplify at all. To have a 99.9% probability of genotyping an individual, we selected $T1 = 18$ for $m = 2$, $T1 = 46$ for $m = 4$, and $T1 = 74$ for $m = 6$, where m is the maximal number of

variants for the gene within a sample (Galan et al. 2010). Thus, all samples with reads were kept and would pass the $T1$ threshold despite the potential number of variants (m).

- Step 3: Each variant detected by jMHC was aligned in Geneious (http://www.geneious.com, Kearse et al. 2012) and grouped based on high similarity and disregarding homopolymer errors. Random G-nucleotide SNPs were prevalent in the dataset, so these were ignored unless a larger number of reads or variants identified by jMHC confirmed the SNP. We chose this conservative approach in refining the dataset because of the known error rates in 454 sequencing (Babik et al. 2009; Metzker 2009). Reads for all variants within a new group were summed for each individual using Microsoft Excel.
- Step 4: We calculated summary statistics according to Galan et al. (2010), including: the total number of reads (N), the total number of variants (A), and the number of sequences for each variant j(Nj). We also calculated the following for each individual: number of sequences per individual i(Ni), the number of variants per individual (Ai), the number of sequences for each variant (Nij), and the frequency of each variant per individual (Fij) which equal Nij/Ni. To assess whether gene duplication exists in SJKF, we calculated the most common ratio of the number of sequences (N) for each variant frequency per individual (Fij) and compared the number of modes in the histogram to patterns found in gene duplications in rodents (Galan et al. 2010). We also used the histograms to look for the appropriate $T2$ threshold, which is used to eliminate artifactual variants and is expected to vary based on the number of gene copies and the complexity of the MHC system (Galan et al. 2010). A large number of low-frequency variants can be assumed to be artifacts due to SNP and indel errors from 454 sequencing (Babik et al. 2009; Harismendy et al. 2009). After determining the $T2$ threshold for each gene, all variants with $Fij < T2$ were removed.
- Step 5: The remaining variants were aligned in MEGA version 7 (Kumar al. 2016) as protein-coding nucleotide sequences and examined for stop codons. Variants were then ordered from the most to least abundant alleles and named using the conventional MHC naming protocol (Klein et al. 1990).
- Step 6: To test if our bioinformatics pipeline included enough quality checks to ensure that we eliminated individuals without enough sequences for complete genotyping ($T1$ threshold), we measured the correlation between the number of reads per individual (Ni) and the number of alleles per individual (Ai). We used the test statistic of a linear regression to check for a correlation between the number of reads of an individual and the number of alleles identified for that individual.

3 Genetic Diversity and Tests for Selection

We constructed a minimum spanning network between alleles of each gene using TCS 1.21 (Clement et al. 2000). Circle sizes are roughly scaled to represent the

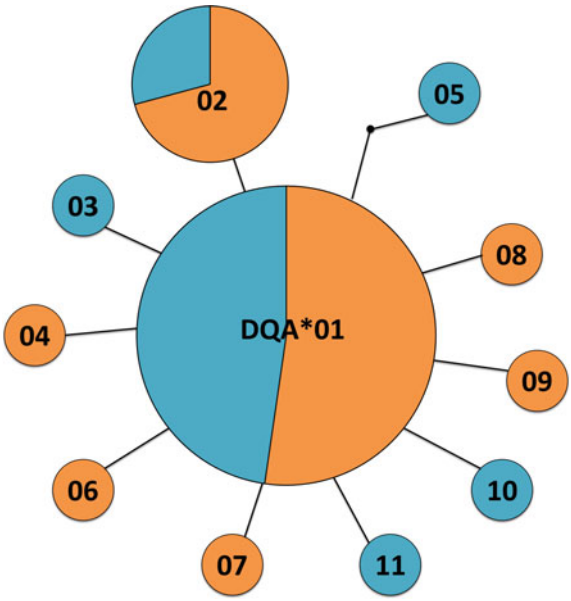

Fig. 2 DQA Haplotype Network. Circle sizes are roughly representative of the number of individuals carrying the allele with orange representing urban foxes and blue representing non-urban foxes

number of individuals that carried each allele with color-coding according to the presence of that allele in individuals in urban (Bakersfield) and non-urban (Carrizo Plain and LoKern) environments (Figs. 2, 3, and 4). Because allelic dosage (the number of copies of an allele at a particular locus; Dufresne et al. 2013) varied among individuals, we could not assign genotypes to individuals or use conventional metrics of population genetics that rely on the premise of frequency statistics for one orthologous locus. To better understand the allelic diversity found in natural and urban environments, we calculated two statistical measures of community diversity—the Jaccard similarity coefficient (Jaccard 1901) and the Bray–Curtis dissimilarity index (Bray and Curtis 1957). The Jaccard coefficient compares presence/absence of types in two communities, whereby 0 means no overlap and 1 means they contain the same diversity. On the other hand, the Bray–Curtis dissimilarity index compares the abundance of types in two communities, whereby 0 means the same composition and 1 means completely different compositions. We counted each allele detected in an individual as one occurrence, even though the individual could have more than one copy of the allele because it was not possible to distinguish between a complete or partial homozygous genotype. This would not affect the Jaccard coefficient but may affect the Bray–Curtis dissimilarity index. These statistics were calculated using Microsoft Excel.

Although there are no studies on the crystalline structures of MHC loci for foxes, amino acid positions involved in antigen binding for canids have been assigned from human HLA studies. The antigen-binding sites (ABS) varied slightly between those identified by Brown et al. (1993; used in studies for wolves, dogs, and arctic fox) and those sites more recently defined by Reche and Reinherz (2003; used in

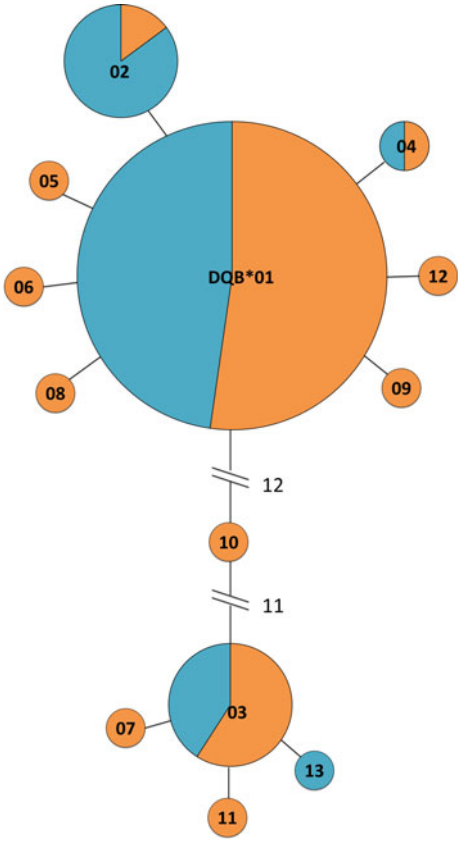

Fig. 3 DQB Haplotype Network. Circle sizes are roughly representative of the number of individuals carrying the allele with orange representing urban foxes and blue representing non-urban foxes

studies for badgers and bears). However, the differences are largely contained within the hypervariable regions assigned to MHC loci of dogs (Kennedy et al. 1999). Therefore, we included all variable amino acid positions used in studies of arctic foxes (Ploshnitsa et al. 2012) and recently identified by Reche and Reinherz (2003). We compared the ABS within SJKF as well as across *Vulpes* species to identify identical ABS.

We used MEGA version 7 (Kumar et al. 2016) to calculate the following statistics: (a) estimated frequencies of non-synonymous (d_N) and synonymous (dS) substitutions in the ABS and non-ABS sites using the joint maximum likelihood reconstructions of ancestral states under a Muse–Gaut model of codon substitution (Muse and Gaut 1994) and Felsenstein's model of nucleotide substitution (Felsenstein 1981), (b) a Z-test to test rejection of two null hypotheses, neutrality ($d_N = d_S$) and positive selection ($d_N > d_S$), using the modified Nei and Gojobori (1986) method with Jukes–Cantor correction (1969; transition/transversion bias = 2, Zhang et al. 1998) and significance computed with 1000 bootstraps, and (c) Tajima's D statistic for ABS and non-ABS sites to test for neutrality or evidence of selection (Tajima 1989). We

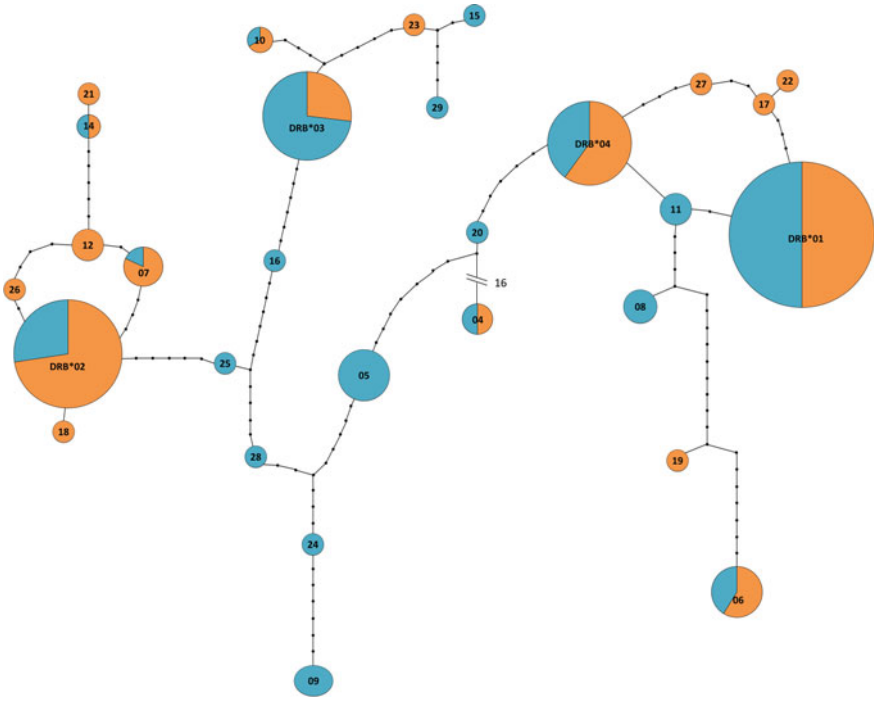

Fig. 4 DRB Haplotype Network. Circle sizes are roughly representative of the number of individuals carrying the allele with orange representing urban foxes and blue representing non-urban foxes

also calculated the same community diversity metrics—the Jaccard similarity coefficient and the Bray–Curtis dissimilarity index—for unique ABS, which represents the functional diversity upon which selection would act.

4 Phylogenetic Reconstruction

We also compared SJKF sequences for exon 2 of DQA1, DQB1, and DRB1 to other carnivores using sequences downloaded from GenBank (Table 1; Supplemental Tables 4–6). We included all alleles from the genus *Vulpes* and up to four sequences per species from other genera within the suborder Caniformia (order Carnivora), using as many different studies for each species as possible. We added two alleles for each of two outgroup species from the suborder Feliformia (order Carnivora): the spotted hyena (*Crocuta crocuta*) and the striped hyena (*Hyaena hyaena*). We only used sequences translated from mRNA when a taxon was otherwise underrepresented. We used the MAFFT algorithm implemented in Geneious Prime 2019.0.4 (https://www.geneious.com) to align sequences for each gene and performed model

Table 1 Number of sequences for each species included in phylogenetic analysis to compare the relationships of DQA1, DQB1, and DRB1 (exon 2). Accession numbers and citations for each sequence are listed in the Appendix Tables 12, 13, and 14

Family	Species	Common name	DQA	DQB	DRB
Canidae	*Canis aureus*	Golden jackal	1	2	2
Canidae	*Canis latrans*	Coyote	2	0	2
Canidae	*Canis lupus*	Wolf	2	2	2
Canidae	*Canis lupus baileyi*	Mexican wolf	0	0	2
Canidae	*Canis lupus dingo*	Dingo	2	1	1
Canidae	*Canis lupus familiaris*	Domestic dog	3	4	4
Canidae	*Canis rufus*	Red wolf	1	0	2
Canidae	*Canis simensis*	Ethiopian wolf	1	2	2
Canidae	*Lycalopex fulvipes*	Darwin's fox	0	1	2
Canidae	*Lycaon pictus*	African wild dog	1	2	2
Canidae	*Urocyon littoralis*	Island fox	0	2	2
Canidae	*Vulpes lagopus*	Arctic fox	1	12	13
Canidae	***Vulpes macrotis mutica***	**San Joaquin kit fox**	**11**	**13**	**29**
Canidae	*Vulpes vulpes*	Red fox	1	2	26
Hyaenidae	*Crocuta crocuta*	Spotted hyena	2	2	2
Hyaenidae	*Hyaena hyaena*	Striped hyena	2	2	2
Mustelidae	*Enhydra lutris*	Sea otter	2	2	2
Mustelidae	*Gulo gulo*	Wolverine	0	0	2
Mustelidae	*Meles meles*	European badger	2	2	2
Mustelidae	*Mustela itatsi*	Japanese weasel	0	0	2
Mustelidae	*Mustela lutreola*	European mink	0	0	2
Mustelidae	*Mustela nivalis*	Least weasel	0	0	2
Mustelidae	*Mustela putorius furo*	Ferret	2	2	2
Mustelidae	*Mustela sibirica*	Siberian weasel	0	0	2
Mustelidae	*Taxidea taxus*	American badgers	1	1	2
Ursidae	*Ailuropoda melanoleuca*	Giant panda	3	4	5
Ursidae	*Tremarctos ornatus*	Spectacled bear	0	0	2
Ursidae	*Ursus arctos*	Brown bear	0	2	2
Ursidae	*Ursus maritimus*	Polar bear	2	1	2
Ursidae	*Ursus thibethanus*	Asian black bear	0	4	2
Total			42	65	126

selection using jModelTest 2 (Guindon and Gascuel 2003; Darriba et al. 2012) with the default parameters. Phylogenetic trees were reconstructed using both maximum likelihood and Bayesian inferences. The ML inferences were carried out using the RAxML 8.2.11 plugin (Stamatakis 2014) implemented in Geneious Prime using the rapid bootstrap and search for best-scoring ML tree method (−f a − × 1), 1000 bootstrap repeats, and the GTR + G model. Bayesian inference was carried out using the CIPRES version of MrBayes v3.2.6 (Miller et al. 2010; Ronquist and Huelsenbeck 2003), with two runs (nruns = 2) of four chains each (nchains = 4), 10 million MCMC generations, sampling frequency every 1000 generations and discarding the first 25% as burn-in. Trees were visualized and summarized using FigTree v1.4.4 (Rambaut 2018).

5 Results

Successful amplification of exon 2 of each gene showed distinct bands on agarose gels, which lacked primer dimer bands after cleaning with MagNA beads. Gel imaging and dissociation curves confirmed the modified protocol for DQA contained the larger-sized molecule with the adaptor on the 5′ end for pyrosequencing. We obtained a total of 236,567 sequences from both of the 454 GS Junior runs using regular processing protocols. After filtering reads shorter than 150 bp and separating them based on the forward primer, we obtained 22,546 reads of DQA, 76,398 reads of DQB, and 93,590 reads of DRB, which equaled a loss of 19% due to short or non-specific reads. Separation of the gene reads into individuals was successful for DQB and DRB but difficult for DQA. Briefly, we removed the first two bases from the reverse sequence used by jMHC and were then able to demultiplex reads to individuals (see supplemental materials for more details). For each gene, 70–80% of sequences were assigned to 75–95 samples (Table 2). The number of variants identified by jMHC that included a barcode was enormous (374 for DQA, 529 for DQB, and 935 for DRB), with the overall total of unique sequences being 3,847, 6,883, and 13,130, respectively. We plotted the number of sequences based on the frequency of each variant in a sample (*Fij*) in 10% intervals as well as in smaller increments for variants with *Fij* less than 10% (Supp. Figures 1–3). By visualizing the histograms and the number of high-frequency alleles within a sample (see Supplementary Figs. 6, 7, and 8), we identified $T1$ thresholds corresponding to a duplicated gene in a diploid species for DQA and DQB (46 reads) and a triploid gene in a diploid species for DRB (74 reads; Galan et al. 2010). Therefore, we removed ten samples from DQA, one from DQB, and zero from DRB, which resulted in a minor loss in reads overall (Table 2). Alignment of the barcoded variants revealed large numbers of random SNPs, and homopolymer mistakes identified as sequencing errors. Variant alignment and grouping in Geneious reduced the number of variants greatly and removed a large amount of sequencing noise. From the modal distributions shown in the frequency histograms, we chose a $T2$ threshold of 5% for DQA, 3% for DQB, and 6% for DRB (Supp. Figures 1–3). We did not find stop codons in any of the alleles after translation

Table 2 Bioinformatics Pipeline and Data. Bioinformatics procedures numbered here from 1 to 5 match steps explained more fully in the methods. Percentages indicate the number of reads retained from that step as compared to the previous step, not the original total

	Run 1 reads:	Run 2 reads:	Total:
454 GS Jr. regular processing (raw data)	1,28,064	1,08,503	236,567 reads
(1) Galaxy filtering: >150 bp	104,964 (82%)	101,885 (94%)	**206,849 reads** (87%)

		DQA	DQB	DRB
(1) Galaxy gene separation	MTPS33 reads:	13,952	35,088	46,684
	MTPS34 reads:	8,594	41,310	46,906
	Total:	22,546	76,398	93,590
(1) jMHC Individual Separation	Reads	17,361	61,610	66,164
	% Reads	77.00%	80.60%	70.70%
	Variants (total)	374 (3,847)	529 (6,883)	935 (13,130)
	Individuals	75	94	95
(2) T1 Threshold	Reads	17,173	61,610	66,163
	% Reads	98.90%	100%	100%
	Variants	374	529	935
	Individuals	65	93	94
(3) Variant grouping in Geneious	Reads	16,351	59,368	62,150
	% Reads	95.20%	96.40%	93.90%
	Variants	214	43	56
	Individuals	65	93	94
(4) T2 threshold	Reads	13,925	57,650	50,067
(5) Translation check	% Reads	85.20%	97.10%	80.60%
	Alleles	*11*	*13*	*29*
	Individuals	*65*	*93*	*94*

into amino acid sequences, and thus characterized 11 alleles of DQA from 13,925 reads, 13 alleles of DQB from 57,650 reads, and 29 alleles of DRB from 50,067 reads (Table 2). The test statistic showed no correlation between the number of reads and the number of alleles (data not shown).

Low read number of DQA made determination of true and artifactual variants difficult. While random SNPs could be generally ignored with DQB and DRB during variant grouping, with DQA the low number of reads per individual meant that a SNP could have a high frequency per individual (*Fij*) with a low actual number (*Nij*). In the end, we reviewed the variants that passed the $T2$ threshold and removed ones that appeared to be sequencing errors in homopolymer regions. While this seems to be the best strategy thus far, other SNPs that passed as alleles could be sequencing error that were clonally amplified in emulsion PCR rather than true alleles. This same issue could exist with alleles that passed the $T2$ threshold for DQB and DRB and were

only found in one individual. However, we were conservative in ignoring random SNPs that did not appear in more than one variant detected by jMHC or did not have a high number of reads overall. Therefore, we likely avoided the error of confusing sequencing artifacts and true alleles. Our bioinformatic methods, particularly the $T1$ and $T2$ thresholds, appear to have been stringent enough to avoid these errors because the test statistic found no correlation between the number of reads and the number of alleles identified for an individual. If we had used lower thresholds, we would expect more artifactual alleles to be identified and may have found a greater correlation between read and allele numbers. Our thresholds and error checking methods are similar, if not identical, to previously suggested approaches (Babik et al. 2009; Galan et al. 2010; Oomen et al. 2012), but clonal amplification errors from emulsion PCR are still hard to detect. We translated all alleles into amino acid sequences and confirmed that no alleles included stop codons and are therefore functional. However, it is very difficult to verify alleles even with cloning or mRNA studies, so we must rely on bioinformatics standards to assess the validity of MHC alleles at this time.

We labeled the alleles *Vuma*-DQA*01-11, *Vuma*-DQB*01-13, and *Vuma*-DRB*01-29 (Tables 3, 4, and 5; see supplemental materials for GenBank accession numbers). Minimum spanning networks of DQA and DQB show star-shaped formations with a few common alleles shared by individuals from both urban and natural populations, as well as a few alleles unique to individuals from either urban or natural populations (Figs. 2 and 3). DQB has four alleles that are quite distant from the rest of the network, with 11 bp substitutions connecting them to *Vuma*-DQB*10 and another 12 bp substitutions to the major allele (Fig. 3). For both DQA and DQB,

Table 3 DQA Alleles. Numbers of individuals sequenced in each population are shown in parentheses. Alleles above the line were found in greater than 1 individual. Alleles found only in urban foxes are highlighted in orange, and alleles found only in wild foxes are highlighted in blue

MHC allele	Urban (n=37)	Non-urban (n=32)	Overall (n=69)
Vuma-DQA*01	34	31	65
Vuma-DQA*02	17	7	24
Vuma-DQA*03	0	1	1
Vuma-DQA*04	1	0	1
Vuma-DQA*05	0	1	1
Vuma-DQA*06	1	0	1
Vuma-DQA*07	1	0	1
Vuma-DQA*08	1	0	1
Vuma-DQA*09	1	0	1
Vuma-DQA*10	0	1	1
Vuma-DQA*11	0	1	1

Note The number of individuals is smaller than the sum of individuals with each allele because the number of alleles per individual ranged from 1 to 3

Table 4 DQB Alleles. Numbers of individuals sequenced in each population are shown in parentheses. Alleles above the line were found in more than 1 individual. Alleles found only in urban foxes are highlighted in orange, and alleles found only in wild foxes are highlighted in blue

MHC allele	Urban (n=46)	Non-urban (n=47)	Overall (n=93)
Vuma-DQB*01	46	42	88
Vuma-DQB*02	4	23	27
Vuma-DQB*03	13	9	22
Vuma-DQB*04	1	1	2
Vuma-DQB*05	1	0	1
Vuma-DQB*06	1	0	1
Vuma-DQB*07	1	0	1
Vuma-DQB*08	1	0	1
Vuma-DQB*09	1	0	1
Vuma-DQB*10	1	0	1
Vuma-DQB*11	1	0	1
Vuma-DQB*12	0	1	1
Vuma-DQB*13	0	1	1

Note The number of individuals is smaller than the sum of individuals with each allele because the number of alleles per individual ranged from 1 to 3

most individuals carried either one or two alleles, with a few individuals having three alleles for DQB (data not presented).

The network for DRB is much more complex with 1–16 bp changes connecting alleles and no star formations (Fig. 4). While individuals in urban and natural environments had many unique alleles (9 and 11, respectively; Table 5), four alleles (*Vuma*-DRB*01-04) were common in both populations. Most individuals carried 3–4 DRB alleles, with a few carrying one allele and one individual carrying six alleles (data not presented here). Even if we removed all of the rare alleles, the number of DRB alleles per individual would still range from 1 to 6, which suggests that variation in gene copy number may exist among individuals.

The community diversity statistics summarize the patterns of allelic diversity between the urban and natural environments. The Jaccard similarity coefficient ranged from 0.182 to 0.310 (Table 5), indicating that the presence of alleles is more dissimilar than similar (closer to 0). However, the Bray–Curtis dissimilarity index ranged from 0.224 to 0.396 (Table 6), indicating that the composition of common alleles is more similar between the two environments (closer to 0). In other words, common alleles are shared by both environments while rare alleles are unique to each environment.

The number of variable nucleotide positions was 11 for *Vuma*-DQA (out of 288), 36 for *Vuma*-DQB (out of 225), and 47 for *Vuma*-DRB (out of 207). The mean number of pairwise nucleotide differences between alleles was 2.00 (range: 1–3) for *Vuma*-DQA, 14.00 (range: 1–28) for *Vuma*-DQB, and 17.09 (range: 1–35) for *Vuma*-DRB. The number of variable amino acids was 7/95 for *Vuma*-DQA, 21/75 for *Vuma*-DQB, and 23/69 for *Vuma*-DRB (Table 7). However, the diversity in the amino

Table 5 DRB Alleles. Numbers of individuals sequenced in each population are shown in parentheses. Alleles above the line were found in more than 1 individual. Alleles found only in urban foxes are highlighted in orange, and alleles found only in wild foxes are highlighted in blue

MHC allele	Urban (n=47)	Non-urban (n=47)	Overall (n=94)
Vuma-DRB*01	35	35	70
Vuma-DRB*02	45	17	62
Vuma-DRB*03	14	38	52
Vuma-DRB*04	27	18	45
Vuma-DRB*05	0	21	21
Vuma-DRB*06	10	7	17
Vuma-DRB*07	9	2	11
Vuma-DRB*08	0	5	5
Vuma-DRB*09	0	5	5
Vuma-DRB*10	2	1	3
Vuma-DRB*11	0	3	3
Vuma-DRB*12	3	0	3
Vuma-DRB*13	1	1	2
Vuma-DRB*14	1	1	2
Vuma-DRB*15	0	2	2
Vuma-DRB*16	0	2	2
Vuma-DRB*17	1	0	1
Vuma-DRB*18	1	0	1
Vuma-DRB*19	1	0	1
Vuma-DRB*20	0	1	1
Vuma-DRB*21	1	0	1
Vuma-DRB*22	1	0	1
Vuma-DRB*23	1	0	1
Vuma-DRB*24	0	1	1
Vuma-DRB*25	0	1	1
Vuma-DRB*26	1	0	1
Vuma-DRB*27	1	0	1
Vuma-DRB*28	0	1	1
Vuma-DRB*29	0	1	1

Note The number of individuals is smaller than the sum of individuals with each allele because the number of alleles per individual ranged from 1 to 6

acid sequences only translates to 1 functionally different group of amino acids in the ABS for DQA, 7 for DQB, and 28 for DRB, which is most relevant for immunity.

The overall d_N/d_S ratio was 0.578 for *Vuma*-DQA, 1.121 for *Vuma*-DQB, and 0.919 for *Vuma*-DRB, with d_N/d_S ratios four and five times higher in the ABS as compared to non-ABS for DQB and DRB, respectively (Table 7). The Z-test for positive selection was only significant for positive selection in the ABS of the DRB gene (Table 7). We found a notably low negative value of Tajima's D for DQA and notably high positive values for DRB (Table 7). While Tajima's D was elevated for DQB, it was not greater than 2 and neutrality could not be rejected (Schmidt and Pool 2002).

Table 6 Comparison of diversity in urban and rural foxes. The number of alleles in a population refers to presence/absence of an allele in the population, while the number of allele copies includes the abundance of each allele in the population. Functional diversity is compared using antigen-binding site (ABS) haplotypes

		Urban	Rural	Total (shared)	Jaccard	Bray–Curtis
DQA	# Individuals	37	32	69		
	# Allele copies	56	42	98		
	# Alleles	7	6	11 (2)	0.182	0.224
	# ABS haplotypes	1	1	1 (1)	1.000	0.143
DQB	# Individuals	46	47	93		
	# Allele copies	71	77	148		
	# Alleles	11	6	13 (4)	0.308	0.243
	# ABS haplotypes	6	4	7 (3)	0.429	0.149
DRB	# Individuals	47	47	94		
	# Allele copies	155	163	318		
	# Alleles	18	20	29 (9)	0.310	0.396
	# ABS haplotypes	18	19	28 (9)	0.321	0.378

Amino acid diversity varied between genes and across *Vulpes* species (Table 8; amino acids numbered according to Reche and Reinherz 2003). Only one amino acid in the ABS of DQA was the same for SJKF (*Vulpes macrotis mutica*) and *Vulpes lagopus* but different from *Vulpes vulpes* (Supplemental Figure 4). For DQB, 18 amino acids within the ABS varied between *Vulpes* species, which translates into 19 ABS haplotypes across *Vulpes* (7 for SJKF; Supplemental Figure 5). For DRB, 19 amino acids within the ABS translated into a total of 61 ABS haplotypes for all *Vulpes* species (28 for SJKF; Supplemental Figure 6). Although DQB and DRB were both highly variable, some amino acids were fixed in one or two of the species, and none of the ABS haplotypes were shared between species.

To get a measure of functional differences between urban and non-urban foxes, we also used community diversity statistics to compare the ABS haplotypes. For all genes, the Jaccard index increased and the Bray–Curtis index decreased. When translating the nucleotide sequences to the amino acid ABS, the urban and non-urban foxes became more similar in composition. The Jaccard index rose to 1 for DQA since there was only one ABS haplotype in urban and non-urban foxes, but the Bray–Curtis did not fall completely to zero because the total number of allele copies differed between groups, which is somewhat problematic because it highlights a difference between urban and non-urban populations that may have been due to the number of individuals sampled, the number of gene copies, or both. Future use of these statistics to compare polygenic loci with different sample sizes may require standardization.

Alignment of all taxa including the three genes was poor because DQA was very different from DQB and DRB. For exploratory purposes, we created a phylogenetic tree of the three loci, and it resolved two major clades, one with alleles from DQA

Table 7 Rates of substitution and test of selection

	S_T	d_N	d_S	d_N/d_S	H_O: $d_N = d_S$		H_O: $d_N > d_S$		S_V	D
					Z	p	Z	p		
DQA ($n = 11$)										
Overall	95	0.034	0.059	0.578	−0.495	0.622	−0.508	1.000	7	−1.897
ABS	21	0.000	0.231	0.000	−1.752	0.082	−1.865	1.000	0	n/c
non-ABS	74	0.044	0.010	4.369	1.380	0.170	1.350	0.090	7	−1.897
DQB ($n = 13$)										
Overall	75	0.168	0.150	1.121	0.543	0.588	0.541	0.295	21	1.067
ABS	29	0.309	0.141	2.190	1.475	0.143	1.499	0.068	13	1.616
non-ABS	46	0.079	0.155	0.510	−0.625	0.533	−0.605	1.000	8	0.056
DRB ($n = 29$)										
Overall	69	0.771	0.839	0.919	1.197	0.234	1.183	0.120	23	1.735
ABS	25	1.878	1.335	1.407	1.961	0.052	2.029	**0.022**	17	2.361
non-ABS	44	0.143	0.557	0.256	−0.816	0.416	−0.797	1.000	6	−0.206

S_T total number of amino acid sites
d_N rate of non-synonymous substitutions
d_S rate of synonymous substitutions
Z Z-statistic
S_V number of variable amino acid sites
D Tajima's D

Table 8 Summary of variable amino acids in the antigen-binding sites (ABS) for DQA, DQB, and DRB across the genus *Vulpes*, shown with the standard one-letter amino acid code. First two rows highlight which positions were indicated in antigen binding by Brown et al. (1993) and Reche and Reinherz (2003; *F* indicates flanking region of ABS and ? indicates not likely to be functionally important). Fixed amino acids within a species are indicated in bold. See Appendix for complete haplotypes for all individuals sequenced

(a) DQA - This variable amino acid creates 2 ABS haplotypes.

	66
Vulpes lagopus	**A**
Vulpes macrotis mutica (SJKF)	**A**
Vulpes vulpes	**G**

(b) DQB - combinations of these 18 variable amino acids translates to 19 ABS haplotypes (n=10 for *Vulpes lagopus*, n=7 for *Vulpes macrotis mutica*, n=2 for *Vulpes Vulpes*).

Brown et al ABS																		
Reche Reinhart ABS																		
Amino Acid Position	14	26	28	37	38	47	57	60	61	67	68	70	71	74	77	82	85	86
Vulpes lagopus	?	A/F	**A**	F/H/L/Y	V/M	F/Y	D/I/W	S/Y	L/W	E/I	L/V	E/R	K/R/V	E/V	R/T	**N**	**V**	**E**
Vulpes macrotis mutica (SJKF)	**E**	A/L	A/R/V	H/Y	V/L	**Y**	D/E	S/Y	**W**	I/T/V	L/V	**R**	R/V	E/K	**T**	D/N	G/V	E/G
Vulpes vulpes	E/Q	A/F	A/T	I/Y	V/L	**F**	D/E	S/Y	**W**	I/V	L/V	E/R	K/V	E/V	R/T	**N**	T/V	E/G

(c) DRB- combinations of these 19 variable amino acids creates 61 ABS haplotypes (n=13 for *Vulpes lagopus*, n=28 for *Vulpes macrotis mutica*, n=20 for *Vulpes vulpes*).

Brown et al ABS																		
Reche Reinhart ABS																		
Amino Acid Position	14	26	28	37	38	47	57	60	61	67	68	70	71	74	77	82	85	86
Vulpes lagopus	?	A/F	**A**	F/H/L/Y	V/M	F/Y	D/I/W	S/Y	L/W	E/I	L/V	E/R	K/R/V	E/V	R/T	**N**	**V**	**E**
Vulpes macrotis mutica (SJKF)	**E**	A/L	A/R/V	H/Y	V/L	**Y**	D/E	S/Y	**W**	I/T/V	L/V	**R**	R/V	E/K	**T**	D/N	G/V	E/G
Vulpes vulpes	E/Q	A/F	A/T	I/Y	V/L	**F**	D/E	S/Y	**W**	I/V	L/V	E/R	K/V	E/V	R/T	**N**	T/V	E/G

and one with alleles from DQB and DRB, regardless of the taxa (data not shown). In general, the gene trees for DQA, DQB, and DRB showed each carnivore family forming reciprocally monophyletic groups (Fig. 5a–c). The only exception was within the DRB tree where two polar bear sequences clustered within Canidae. Within Canidae, the three gene trees showed a similar pattern; species were largely unresolved (see Supplementary Figures 4–6 for comprehensive phylogenetic trees). In DQA, SJKF was paraphyletic to the two other *Vulpes* sequences. In DQB, SJKF clustered into two groups that were paraphyletic to other species. In DRB, SJKF sequences were found across the Canidae clade.

6 Discussion

6.1 Patterns of MHC Allelic Diversity in Urban and Non-urban Kit Foxes

DQA. The most common alleles were shared between the urban and non-urban populations, but the rare alleles tended to be found in only one population. Despite the presence of unique DQA alleles, all of the DQA alleles found in both populations translated into the same functional antigen-binding site (ABS) and were, therefore, functionally equivalent.

DQB. Four alleles were common in both urban and non-urban foxes (DQB*01-04). The 13 DQB alleles produced 7 ABS haplotypes. The urban and non-urban foxes shared three functionally different ABS haplotypes, while three types were only found in urban foxes and one type was only in non-urban foxes.

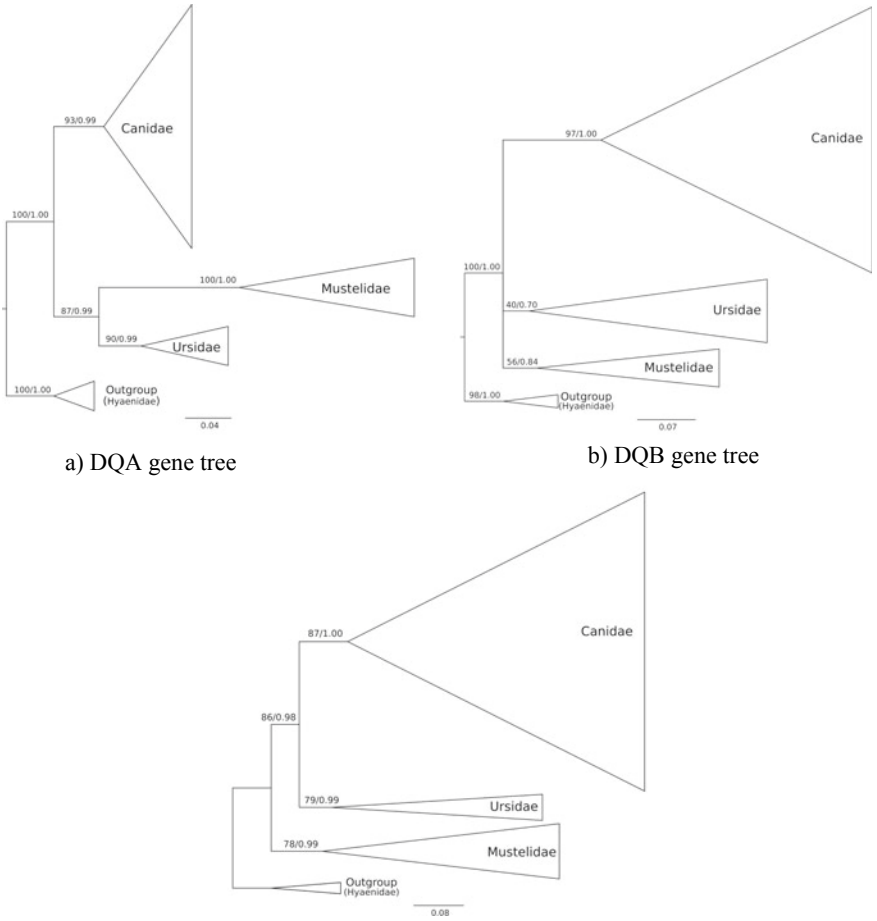

Fig. 5 Phylogenetic tree of DQA, DQB, and DRB exon 2 alleles for the San Joaquin kit fox (Vuma) in relation to other species of Carnivora—(**a**) Bayesian tree for DQA, (**b**) Bayesian tree for DQB, (**c**) Bayesian clade for DRB. Maximum likelihood and Bayesian support are shown on branches of each gene trees and Hyaenidae as an outgroup. Branches are collapsed to illustrate separation between families. Full trees available in Appendix (**a**) DQA gene tree, (**b**) DQB gene tree, (**c**) DRB gene tree

DRB. Seventeen of 25 amino acid positions in the ABS were variable, with 29 alleles resulting in 28 unique ABS for pathogen recognition. Most of the high-frequency DRB alleles were present in both populations, except for *Vuma*-DRB*05, which was absent in the urban population but present in 45% of the non-urban foxes. In addition, no urban foxes carried *Vuma*-DRB*08 or *Vuma*-DRB*09, whereas each of these alleles were found in 11% of the non-urban populations. These three DRB alleles (*05, *08, and *09) translated into functionally unique ABS. The allelic differences

between the urban and non-urban populations may have been the result of different selective pressures in the urban and non-urban environments. Additional sampling might reveal one or more of these three alleles in the urban population. However, this is unlikely because our sample sizes were robust ($n = 96$) and contained approximately equal numbers of urban and non-urban foxes. Our results suggest that if these alleles are not completely absent, they must be extremely rare in the urban population. Other less frequent alleles translated into unique proteins that were only present in individuals from one of the two populations.

We found gene duplication and moderate levels of allelic diversity in DQA and DQB in SJKF. We also detected three gene copies and high levels of allelic diversity in DRB of SJKF compared to other carnivores (Aguilar et al. 2004; Marsden et al. 2012; Oomen et al. 2013; Ploshnitsa et al. 2012; Seddon and Ellegren 2002, 2004). Nucleotide diversity within exon 2 of MHC genes in SJKF results in substitutions in the ABS that influence the pathogen-binding ability of the immune system. Additionally, the gene duplication we observed in these MHC genes increases the number of alleles that can be expressed by an individual at one time (Janeway et al. 2001). Therefore, our results suggest that gene duplication and allelic diversity may be important processes contributing to the functional diversity of the MHC in SJKF.

6.2 Comparisons of Allelic and ABS Diversity to Other Canids

Our results revealed that SJKF has similar numbers of MHC alleles as other wild canids.

DQA. Except for two putative mRNA sequences for DQA (one from the arctic fox and one from the red fox), no previous studies have described patterns of polymorphism of this locus in other species of foxes. However, DQA has been characterized in other wild and endangered canids such as the European wolves (Seddon and Ellegren 2002). In this study, wolves evinced moderate levels of DQA allelic diversity (9 alleles); a slightly lower number of alleles than what we found in SJKF (11 alleles). Notably, all of the nucleotide substitutions within the ABS of the DQA alleles were synonymous mutations (Table 7), implying that all alleles are functionally equivalent.

DQB. We also found moderate levels of DQB diversity in SJKF (13 alleles), which is comparable to European wolves (10 alleles; Seddon and Ellegren 2002) and arctic foxes (11 alleles; Ploshnitsa et al. 2012). The d_N/d_S ratio within the ABS of SJKF DQB alleles was greater than 2 and more than four times the ratio in non-ABS sites. These results strongly suggest that balancing selection is acting on this locus, even though these values did not statistically deviate from neutrality using Z statistics or Tajima's D. The lack of statistical significance may be due to the low number of alleles and the comparison of substitution rates for only a short DNA sequence. This might also reflect that balancing selection on DQB has not been strong.

DRB. We found higher DRB allelic diversity for SJKF (29 alleles) than found in other wild canids—13 in Arctic fox (Ploshnitsa et al. 2012), 26 in red fox (Marshall et al. 2016), 19 in African wild dog (Marsden et al. 2012), and 17 in European wolves (Seddon and Ellegren 2002). The Z-test for positive selection was significant in SJKF ($p = 0.022$) and Tajima's D indicated a deviation from neutrality ($D = 2.361$). D statistics higher than two are correlated with an excess of common alleles produced by positive selection (Schmidt and Pool 2002). We also have evidence of balancing selection from an increased rate of non-synonymous to synonymous substitutions within ABS (d_N/d_S ratio = 1.41 in ABS and 0.26 outside of ABS).

6.3 Evolution of MHC Diversity

Phylogenetic comparison of alleles from all three MHC loci for SJKF and other carnivores only separated DQA alleles from DQB and DRB alleles, with alleles from different species completely intertwined. DQB and DRB have similar structure composition (Klein 1986) and have been shown to have similar nucleotide motifs that suggest recombination between these two genes (Seddon and Ellegren 2002). MHC genes in canids show varying degrees of diversity that may reflect the different functions of these genes and/or varying selection pressures in the past. Lower levels of diversity at DQA or DQB could signify more recent disease-related selective pressure (Hedrick et al. 2003), but these patterns are also observed in other canids (see Table 3 in Marsden et al. 2009). The general patterns of diversity (i.e., lower diversity in DQA and DQB, and higher diversity in DRB) are somewhat consistent across carnivores in general, and thus may represent the genic diversity of the Order Carnivora resulting from millions of years of MHC evolution with gene conversion or recombination creating different numbers of gene copies in different species. Based on MHC data on European wolves, the diversification within DQA (6.4 ± 3.6 mya) was estimated to have occurred more recently than that of DQB (27.2 ± 10.1 mya) and that of DRB (37.0 ± 11.3 mya; Seddon and Ellegren 2002). Thus, these ancient patterns in carnivores, together with more recent species history along with other processes of evolution—drift, mutation, migration, and selection—would dictate the patterns of polymorphism we found within SJKF.

Similar to MHC genes in other species, the high diversity of SJKF alleles is spread across the phylogenetic trees and displays trans-species evolution (see supplemental figures; Klein 1980; Hedrick et al. 2000; Seddon and Ellegren 2002; Garrigan and Hedrick 2003; Archie et al. 2010). Trans-specific conservation of MHC alleles is typically thought to reflect balancing selection over long time periods (potentially over millions of years including speciation within genera or families; Garrigan and Hedrick 2003), or positive selection of MHC variants that are beneficial in responding to pathogens. Garrigan and Hedrick (2003) showed that it takes hundreds of generations to create significant differences between the rates of synonymous and non-synonymous substitutions. Although it is hard to definitively say if these trees represent shared ancient lineages, indicate some level of convergent evolution, or are

phylogenetically unresolved due to the short sequence length, most canid research suggests ancestral MHC lineages that have been conserved over time. Extensive behavioral, pedigree, and population data are needed to tease apart the contributions from demographic and evolutionary processes (Piertney and Oliver 2006). Because these other types of information have already been collected for the SJKF, future studies may be able to explore these evolutionary processes and assess whether these MHC differences are important for antigen binding or kin recognition.

Interestingly, SJKF maintains a high level of diversity although restricted to a smaller geographic distribution (the southern part of the San Joaquin Valley in California) than red foxes, European wolves, and African wild dogs. This high allelic diversity suggests that SJKF did not lose MHC diversity after they became isolated in the San Joaquin Valley, separated from other kit foxes over the last millennium by mountain ranges including the Sierra Nevada and California's transverse ranges (Mercure et al. 1993). Much of the original diversity may have been maintained by balancing selection throughout the last millennium of changing environments within the San Joaquin Valley (Matocq et al. 2012). It would be interesting to see if kit foxes from other areas in the western USA or Mexico also maintained a high level of MHC diversity.

Additionally, when we compare urban-to-non-urban kit foxes, we find similar levels of allelic diversity. The population of urban kit foxes in Bakersfield is smaller and more isolated than the non-urban foxes from LoKern and the Carrizo Plains, and likely to have less genetic exchange with other populations. However, this isolation goes back only a few decades. Until recently, disease has rarely been an issue for kit fox when compared to other canids (Cypher 2003). Mange was first documented in urban SJKF in Bakersfield in March 2013 (Cypher et al. 2017) and has since been documented in over 400 cases, including almost 100 fatalities, potentially resulting in a population decline of more than 50% (Cypher *personal communication*). There is no evidence that kit foxes can recover from mange without treatment, and they do not appear to develop any immunity to the disease (Cypher *personal communication*). Not only does mange present a significant threat to the survival of the Bakersfield kit fox population, but if uncontrolled, it may have very drastic consequences in the genetic diversity at both neutral and adaptive loci like MHC.

6.4 Conservation Implications

This study presents the first characterization of MHC genes in the San Joaquin kit fox and provides the first opportunity to compare MHC diversity between populations of a canid species in natural and urban environments. The conservation of wild species and habitat is challenged by human population growth, urbanization, habitat fragmentation, and ever-increasing demands on natural resources. The overall chance for disease exposure is likely to increase, but the disease vectors may be different between urban and non-urban populations. In any future events, it will be helpful to

have our data to compare MHC alleles to survival. Although SJKF has low mitochondrial diversity, they have high levels of MHC diversity, which increases their chances of surviving an epizootic event.

Acknowledgements We wish to thank Stacey Lance, Kenneth Jones, Maria Ruiz-Lopez, and Jennifer Bollmer for discussion and suggestions on improvement of MHC data analysis during and after the 2011 NSF-AGA workshop on Evolutionary Genomics of Non-Model Species: Next-Gen Sequencing, Data Management, and Hypothesis Testing. We would like to thank also Nancy McInerney and the many other CCG laboratory members working on NGS analysis and their suggestions throughout this project. Funding was provided by the Smithsonian Conservation Biology Institute, the Smithsonian Next-Generation Sequencing Small Grant Program, and the NSF GK-12 SUNRISE program at George Mason University. Sequencing costs were provided by the Microbiome Analysis Center.

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Multitask Genetic Applicability to Preserve Species in the Future

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