M.Sc. in Conservation Biology

MHC Class I diversity influences haematozoon infection intensity in the polymorphic black sparrowhawk (*Accipiter melanoleucus*).

Liezl le Roux[1]

Supervisors:
Jacqueline M. Bishop[2] and Arjun Amar[1]

[1]*Department of Biological Sciences, Percy FitzPatrick Institute of African Ornithology, DST/NRF Centre of Excellence, University of Cape Town, Rondebosch, South Africa*
[2] *Department of Biological Sciences, University of Cape Town, Rondebosch, South Africa*

e-mail: liezl_leroux@hotmail.com

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Abstract:

The major histocompatibility complex (MHC) is a multigene family known to be the most variable gene group in vertebrates in terms of allelic diversity and gene number. Pathogen mediated selection is thought to be the major driving force behind the unusually high levels of MHC polymorphism in natural populations. In this study the relationship between MHC class I diversity and blood parasite infection intensity is explored in a population of black sparrowhawks (*Accipiter melanoleucus*). The species is of particular ecological and evolutionary interest as black sparrowhawks display a discrete polymorphism in plumage pattern across its range, occurring as a light and dark morph. It is well established in birds that carotenoïd and melanin-based pigmentation patterns are often associated with parasite resistance. In this study, functional variation at the peptide binding region (PBR) of MHC class I loci is investigated to explain individual variation in blood parasite infection in black sparrowhawks. Using DNA sequence data together with individual estimates of blood parasite load (for *Haemoproteus nisi* and *Leucocytozoon toddi);* the study tested the relationship between (i) allelic diversity and individual parasite load and (ii) specific alleles and individual parasite load using a Generalized Linear Model framework. Seven different, putatively functional, MHC class I alleles were identified. Number of alleles per individual ranged from one to three in individuals infected with *H. nisi*, whereas individuals infected with *L. toddi* had either two or three different alleles. A significant positive association was found between *H. nisi* infection intensity and MHC allele Acme_BF2*03. No significant association was found between *L. toddi* infection intensity and a specific allele. The results reported support the role of pathogen mediated selection of genetic variation at evolutionary relevant MHC genes through rare allele advantage. By characterizing the variation at MHC class I loci and testing for a relationship with parasitemia, it is now possible to elucidate the mechanisms and significance of MHC molecular adaptation in the black sparrowhawk.

Keywords: MHC class I diversity, black sparrowhawk, *Haemoproteus*, *Leucocytozoon*, plumage polymorphism, balancing selection.

Introduction

The importance of genetic adaptation in times of global change

Evolution is only possible in the presence of heritable genetic variation. Consequently, one central issue in conservation genetics is the amount of genetic variation present within an individual, population and species. The rate of adaptive evolution needs to reach the rate of environmental change if a population is to persist (Burger & Lynch 1995). Adaptive genetic variation is therefore critical for population adaptation and survival in response to a changing environment, e.g. climate change and habitat fragmentation (reviewed in Hoffmann & Sgrò 2011). The "central dogma of conservation genetics" is that genetic variability is advantageous, and thus worthy of primary conservation concern (Pertoldi et al. 2007). Generally, small fragmented populations are genetically depauperate (Palo et al. 2004), which threatens long-term adaptability in disturbed habitats (Lande & Shannon 1996) and increase the chance of inbreeding, which poses an immediate threat through the loss of genetic diversity (Lenormand 2002). Genetic diversity is typically characterized as polymorphism, heterozygosity, and allelic diversity. Polymorphism translates directly to "many forms", and refers to a gene or genetic locus (gene location). Speciation is possible when polymorphisms found at speciation loci are not shared between diverging populations (Wu 2001).

The adaptive immune response is unique to vertebrates. This response is initiated by generating receptors in the adaptive immune system and involves great variability and rearrangement of receptor gene segments. The adaptive immune system provide specific recognition of pathogen derived peptides (antigens) and immunological memory of infection (Janeway & Medzhitov 2002). The major histocompatibility complex (MHC) is a multigene family known to be the most variable gene group in vertebrates in terms of allelic diversity and gene number (Edwards & Hedrick 1998). MHC genes trigger the adaptive immune system by encoding peptide-binding glycoproteins which bind and present antigens to T -lymphocyte cells (Janeway et al. 2001). Two classes of MHC genes exist, where MHC class I (MHC-I) genes are monomeric proteins known to mostly present antigens derived from intracellular pathogens and MHC class II (MHC-II) molecules are dimeric proteins which bind antigens from extracellular pathogens (reviewed in Sommer 2005). Not only is the MHC polygenic (multiple genes) and polymorphic (multiples alleles), MHC alleles are co-dominant; as a result both maternal and paternal alleles are expressed equally in the cells. The high degree of population-level allelic richness, hereafter referred to as „diversity", of the MHC genes, together with their key role in the vertebrate

immune system has made this gene complex the ideal candidate for studying mechanisms and significance of molecular adaptation in vertebrates (Meyer & Thomson 2001).

Associations between MHC diversity and parasite load

Pathogen mediated selection is thought to be the major driving force behind the unusually high levels of MHC polymorphism in natural populations. Two models of balancing selection have been proposed to explain this: the „overdominance model" and the „negative frequency-dependent selection model". The overdominance model states that polymorphism is maintained through „heterozygote advantage", where heterozygous individuals are able to recognise a wider variety of parasites compared to homozygous individuals (Doherty & Zinkernagel 1975). The negative frequency-dependent selection model assumes that polymorphism is maintained by „rare-allele advantage" because selection favours parasites that can avoid recognition by the most common MHC variants and rare MHC alleles offer better parasite resistance (Clarke & Kirby 1966). The negative frequency-dependent selection model also incorporates the „optimality hypothesis" which states that individuals with an intermediate number of MHC alleles might have higher fitness due to lower chances of generating autoimmune disease as a result of self-reacting lymphocytes (Nowak et al. 1992; Wegner et al. 2003).

Evidence to support both the overdominance and negative frequency dependent model have been reported in wild avian populations (Westerdahl et al. 2005, 2013; Bonneaud et al. 2006b; Sepil et al. 2013; Dunn et al. 2013). The most prominent studies on the associations between MHC diversity and pathogens targeting blood cells of their avian hosts are summarized in Table 1.

Table 1 Evidence for pathogen-driven selection mechanisms in wild avian populations.

Host species	Population	Country	Infectious agent	Heterozygote advantage	Rare-allele advantage	Reference
Blue Tits (*Cyanistes caeruleus*)	Nest-box population	Sweden	*Haemoproteus majoris*	n/a	-ve association between infection intensity and MHC-I* allele 242	Westerdahl et al. 2013
Great reed warbler (*Acrocephalus arundinaceus*)	Migratory population	Sweden	*Haemoproteus payevskyi* and *Plasmodium* sp. (GRW2) and *Plasmodium* sp. (GRW4)	Positive association between numbers of MHC-I alleles and prevalence of the GRW2 parasite	+ve association between MHC-I allele (B4b), and prevalence of GRW2.	Westerdahl et al. 2005
Common Yellowthroat (*Geothlypis trichas*)	Migratory population	United States	*Plasmodium* sp.	Males with more MHC-II** alleles had greater apparent survival	+ve association between *Plasmodium* sp resistance and MHC-II allele 82	Dunn et al. 2013
House sparrow (*Passer domesticus*)	Nest-box and temporary aviary population	France	*Plasmodium* and *Haemoproteus* sp.	n/a	+ve association between resistance and MHC-I alleles a151 and a172, +ve association with increased susceptibility and allele a161	Bonneaud et al. 2006
Great tit (*Parus major*)	Nest-box population	United Kingdom	*Plasmodium* sp.	No significant relationship	-ve association between parasite prevalence and MHC-I supertype 17, +ve association between parasite intensity and MHC-I supertype 6	Sepil et al. 2013

*MHC-I: Major histocompatibility complex class I
**MHC-II: Major histocompatibility complex class II

These models of balancing selection are not mutually exclusive as they have been shown to act in concert with one another (Westerdahl et al. 2013; Dunn et al. 2013), which makes distinguishing the relative strength of each model challenging.

Adaptation in a changing pathogen environment

Recent studies suggest that many species are likely to experience range shifts in response to changing environmental conditions such as climate change and urbanization (McLaughlin et al. 2002; Hannah et

al. 2007; Klausmeyer & Shaw 2009; Evans et al. 2011; Perdinan & Winkler 2014). This in turn may result in a new set of threats as changing conditions may lead to locally modified pathogen communities (the subsets of pathogens that occur in each host population, Paulin 2007). Vector-borne parasites, such as intracellular avian haemosporidian parasites that target host red blood cells (protozoans of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) may be especially prone to range shifts due to climate change (Pérez-Rodríguez et al. 2014), as the life cycle of invertebrate vectors is strongly constrained by climate (Kovats et al., 2001; Gage et al., 2008). These pathogens vary in their relative pathogenicity and host spectrum (Remple 2004). In raptors *Plasmodium* is considered a host generalist and the most pathogenic of these three genera, whereas *Haemoproteus* and *Leucocytozoon* were believed to be relatively benign. However, *Haemoproteus syrnii* infection have been shown to cause severe anemia in snowy owls (Evans & Otter 1998) and Harris hawks (Mutlow & Forbes 2000), and *Leucocytozoon toddi* caused mortality in juvenile great horned owls (*Bubo virginianus*) during a year of food shortage (Hunter et al. 1997).

The black sparrowhawk

In this study the relationship between MHC class I diversity and blood parasite infection intensity is explored in an urban population of black sparrowhawks (*Accipiter melanoleucus*). This Western Cape population colonised and started breeding in the mid-1990s (Oettlé 1994) and has been monitored since 2000.

The black sparrowhawk is a widely distributed colour polymorphic raptor occurring throughout much of sub Saharan Africa (Ferguson-Lees & Christie 2001). The species is of particular ecological and evolutionary interest. Black sparrowhawks display a discrete polymorphism in plumage pattern across its range, occurring as a light and dark morph (Amar et al. 2013), which appears to confer some adaptive advantage (Lei et al. 2013). In most of its range the dark morph is regarded as the rarer morph. However, in the Cape Peninsula population where it is a recent colonist, a reversal of the morph frequencies is observed (Amar et al. 2013). Morph ratios in southern Africa appear to exhibit a cline which spans a distance of 1500 km, where the frequency of dark morphs fall from 80% to <20% with increasing distances from the Cape Peninsula (Amar et al. 2014). Rainfall and temperature have been shown to correlate with the proportion of dark morphs, where more dark morphs were found at lower elevations, with lower temperature and higher rainfall during the breeding period.

In black sparrowhawks the prevalence and infection intensity of *Haemoproteus nisi* and *Leucocytozoon toddi* have been investigated in the two morphotypes (Lei et al. 2013). While little evidence exist to support a direct effect of parasitemia by *Haemoproteus* and *Leucocytozoon* sp. on host fitness, both are found in increased numbers when the host experiences conditions of stress or immunosuppression. Therefore parasite intensity can act as an indicator of the presence of underlying disease or a predictor of a severe disease process (Bowman 1995).

The black sparrowhawk is an Austral winter breeding species (Roberts 2005) and thrives in the human modified landscape of the Cape Peninsula, where it is found nesting predominantly in exotic trees within pine and eucalyptus plantations (Malan & Robinson 2001). Understanding the mechanisms which contribute to adaptation and preservation of top predators in modified landscapes, such as the black sparrowhawk, is vital to inform policy, management and general strategies for biodiversity conservation (Sergio et al. 2006).

Associations between plumage polymorphism and parasite load

It is well established in birds that carotenoïd and melanin-based pigmentation patterns are often condition-dependent traits (Jacquin et al. 2013). The melanocortin-1 receptor (MC1R) which is involved in the production of eumelanic (black) and pheomelanic (yellow-reddish) pigments (Mundy 2005), also binds other melanocortins which regulate anti-inflammatory, antipyretic and anti-oxidative immune responses (Getting 2006) and may provide some fitness advantage to the species (Mundy 2005; Jacquin et al. 2011). Studies on barn owls (*Tyto alba*; Roulin et al. 2001), tawny owls (*Strix aluco*; Galeotti & Sacchi 2003) and feral pigeons (*Columba livia;* Jacquin et al. 2011) have shown that differently coloured individuals, within the same species, vary in their ability to cope with parasites. A similar trend has been observed in the black sparrowhawk, where light morphs have a higher intensity of *Haemoproteus nisi* parasites as compared to dark morphs (Lei et al. 2013). The only other study of its kind in birds of prey (Chakarov et al. 2008), showed that the intracellular parasite, *Leucocytozoon toddi*, was found at lower intensities in dark morphed common buzzard (*Buteo buteo*) nestlings as compared to their lighter conspecifics. Differences observed in parasite infection between avian morphotypes suggest that differential immune capacity may exist in polymorphic populations (Lei et al. 2013).

Adaptive immune diversity at MHC class I loci

Through exploring the relationship between parasite infection intensity, colour morph type and variability at genes of the MHC, one can gain insight into the role of selection in anthropogenic

environments. In this study, functional variation at the peptide binding region (PBR) of MHC class I loci is investigated as an alternative hypothesis to plumage pigmentation, to explain individual variation in blood parasite infection in black sparrowhawks. Using DNA sequence data together with individual estimates of blood parasite load the study tested the relationship between (i) allelic diversity and individual parasite load and (ii) specific alleles and individual parasite load using a Generalized Linear Model framework. The role of sex was also investigated, together with the possibility of any association between individual colour morph and specific alleles. The study presents the first characterization of adaptive immune diversity at MHC class I loci for an African raptor species.

Methods

Sample collection

Blood samples used in this study were collected as part of an on-going research program on black sparrowhawk population ecology in Cape Town, South Africa. Samples were collected during the period 2009 to 2012. Adult black sparrowhawks were trapped and sampled on their territories using a bal-chatri trap baited with live pigeons (*Columba livia;* Berger & Mueller 1959); the pigeons were not harmed in the process. Both dark and light morph individuals were sampled. The study area consists of heterogeneous habitats including urban gardens, alien pine (*Pinus* spp.) and eucalyptus (*Eucalyptus* spp.) plantations, indigenous Afromontane forest, and Fynbos. Altitude ranges from sea level to about 300 m, and the climate is temperate, with locally variable winter rainfall (Cowling & Simmons 1996). Blood was sampled from the cubital vein using a sterile 21 gauge needle (BD Micro-Fine Plus, Alpha Pharm East Cape, SA). Blood was then extracted from the small puncture hole using 80 µl x 75 mm heparinised capillary tubes (Lasec SA) and stored in 70% EtOH (Lei et al. 2013). Of the 23 samples used in this study, 20 were positive for either *H. nisi* or *L. toddi* infection; details are shown in Table 2.

Table 2 Details for adult black sparrowhawks used in this study for which exon 3 sequences from MHC class I loci were isolated. For each individual the sex, morph and the parasite infection intensity by *Leucocytozoon toddi* and *Haemoproteus nisi* is reported.

YEAR	SAFRING	Sex	Morph	*Haemoproteus nisi**	*Leucocytozoon toddi**
2009	798798	male	light	0	0
	7A00106	female	light	0	0
2010	687864	male	light	4	6
	798780	female	dark	2	1
	6H03206	male	dark	6	6
	6H03235	male	dark	4	1
	6H03237	male	dark	0	8
	6H03243	male	light	0	1
	6H03248	male	dark	2	2
	6H03256	male	dark	0	0
	7A00110	female	dark	1	2
	7A00126	female	dark	2	0
2011	681800	male	light	3	1
	784931	female	dark	1	1
	784932	female	dark	1	0
	784944	female	dark	1	0
	798763	female	dark	0	1
	798786	female	light	8	0
	798793	female	dark	2	0
	6H03270	male	dark	9	1
	6H03285	male	light	57	2
	6H03295	male	dark	3	0
2012	6H05477	male	dark	4	1

*parasite intensity = calculated as the number of parasites in 20 000 erythrocytes (adapted from Staats & Schall, 1996)

Parasite screening and quantification

Blood samples were collected to analyse infection by two common avian blood parasites documented in Accipitridae, *Leucocytozoon toddi* and *Haemoproteus nisi*. Blood smears for all individuals were analysed by Petra Sumasgutner using the following protocol: Standard thin blood smears were prepared in the field immediately after sampling, air dried and transferred for storage and transportation into a slotted slide carrier box. Slides were then fixed by immersion in methanol for 2 minutes prior to staining using Giemsa stain (106, diluted with Giemsa buffer) for 2 minutes to visualise blood cells and parasites (Gurr 1969). The stained blood slides were examined using a binocular compound microscope with oil immersion lenses (Nikon). First, the slide was scanned for parasites at 400x magnification for 15–20 minutes per slide covering most of the slide during this time. All slides received the same overall search

effort, and a good representation including both peripheries of the smear as well as the central areas with higher densities of erythrocytes. This was used to classify the sample as *L. toddi* and/or *H. nisi* positive or negative (Figure 1). Leucocytozoids and haematozoa were then quantified by counting the number of parasite infected erythrocytes within 20 000 erythrocytes (parasite intensity) at 1000× magnification under immersion oil (100–200 fields scanned). The fields were chosen in lines from one end of the slide to the other to compensate for differences in blood thickness across the smear.

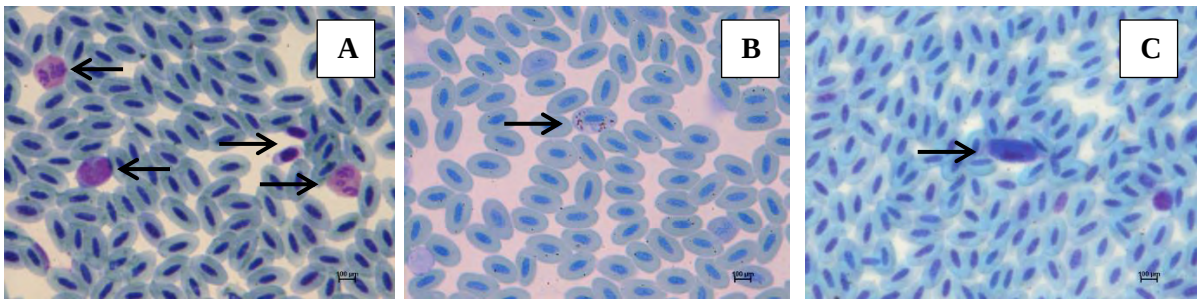


Figure 1 Giemsa stained black sparrowhawk blood slides indicating healthy erythrocytes and positive infection for haematozoan parasites (1000 x magnification); A. Healthy erythrocytes including immune cells indicated by black arrows (top left: eosinophil, bottom left: lymphocyte, top right: 2 thrombocytes, bottom right: eosinophil) , B. *Haemoproteus nisi* infected erythrocyte (black arrow), C. *Leucocytozoon toddi* infected erythrocyte (black arrow). Source: Petra Sumasgutner

PCR amplification, sequencing and alignment

A simple salt extraction protocol (adapted from Aljanabi & Martinez 1997 and Longmire et al. 1997; Appendix A) was used to obtain genomic DNA from full blood samples stored in 70% EtOH. Degenerate PCR primers, designed by Alcaide et al. (2008), were used to amplify the entire coding sequence of exon 3 of MHC class I (MHC-I) genes and a small section of the flanking intronic regions together with the 5‟region of exon 4 (Figure 2). The forward primer (MHCI-int2F: CAT TTC CCT YGT GTT TCA GG) falls within the intronic sequence upstream of exon 3 and the reverse primer (MHCI-ex4R: GGG TAG AAG CCG TGA GCR C) is located within the conserved 5‟‟region of exon 4, resulting in a 450 bp PCR product. PCR of MHC-I exon 3 was performed on a 2720 Thermo Cycler (Applied Biosystems) in a final volume of 20 µl containing 10 µl Dream Taq Green PCR Master Mix (2X) (Thermo Scientific), 10 pmol of each primer and~25 ng of genomic DNA.

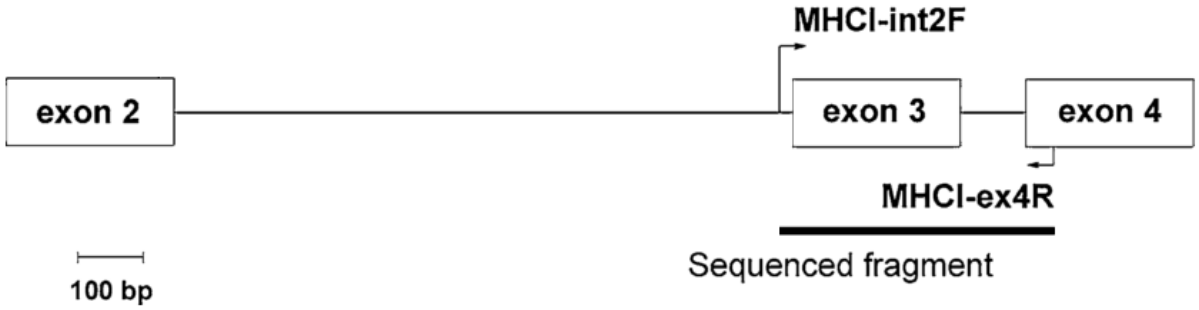


Figure 2 Schematic illustration of part of an MHC class I gene of hawks and allies. The position of the primers (MHCI-int2F and MHCI-ex4R) used in this study are indicated by arrows. (Adapted from Alcaide et al. 2008)

The PCR cycling profile consisted of an initial denaturation step of 2 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 56°C (annealing temperature), 40 s at 72°C and a final extension step of 5 min at 72°C. The PCR products were visualised under UV on a 1% agarose gel. GeneRuler 1kb DNA Ladder (Thermo Scientific) was run in parallel to size the PCR products. Bands corresponding to a size of 450 bp were excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Because of the possibility of amplifying more than one locus, and individuals are likely to be heterozygous for the target loci, PCR products were cloned using the pGEM®-T Easy Vector System I (Promega) and JM109 *E. coli* cells (Promega). Positive clones were identified using blue-white colour indication on IPTG/X-gal agar plates. Positive colonies were selected and used as template in colony PCRs, where inserts were re-amplified using universal M13 primers in a final volume of 20 µl containing 10 µl Dream Taq Green PCR Master Mix (2X) (Thermo Scientific), 10 pmol of each primer and a colony picked directly from the plate as DNA template. The PCR cycling profile consisted of an initial denaturation step of 2 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 62°C (annealing temperature), 40 s at 72°C and a final extension step of 5 min at 72°C using a 2720 Thermo Cycler (Applied Biosystems). The PCR products were again visualised under UV on a 1% agarose gel and GeneRuler 1kb DNA Ladder (Thermo Scientific) run in parallel to verify the size the PCR products. Twelve positive colonies were selected for each individual, based on the expected size (713 bp = 450 bp insert + 263 bp vector) of the colony PCR product and specificity of the product (no double banding). The selected samples were purified and sequenced through the EZ-seq plate purification service with M13 Forward primer (M13F: CGC CAG GGT TTT CCC AGT CAC GAC) by Macrogen Europe.

The identity of the sequences were verified using a nucleotide BLAST search on the NCBI database (Altschul et al. 1997). The Nucleotide collection (nr/nt) database was chosen as the „Searh Set" and the megablast program was selected to optimise for highly similar sequences. After verification, the MHC-I exon 3 sequences were edited in BioEdit v.7.2.5 (Hall 1999). The sequences were trimmed to contain only the coding DNA sequence (CDS) of MHC-I exon 3. These sequences were then screened to find different, putative alleles within each individual. Given the low number of clones screened (n=12) alleles were only considered as unique, presumably functional alleles, if the DNA sequences differed in at least two nucleotide positions and was found in more than one individual (following Alcaide et al. 2013). Alleles were considered pseudo genes if the same premature stop codon(s) or disrupted reading frame(s) occurred within a sequence of more than one individual. These criteria minimise the impact of PCR and sequencing artefacts in the allele repertoire. Sequences with premature stop codons, disrupted reading frames, truncations and low signal intensity for the chromatogram were excluded from downstream analyses (Figure 3). Sequences from non-infected individuals (n=3) were also excluded due to the small sample size, which is indicative of the high prevalence of adults infected with *Haemoproteus nisi* (72%) and *Leucocytozoon toddi* (59%) in the study population (Lei et al. 2013).

Quantifying MHC-I exon 3 polymorphism and tests for selection

A number of polymorphism statistics were calculated in DNAsp v5.10 (Librado & Rozas 2009) to investigate diversifying selection at MHC-I sequences. Positive selection can be inferred when an excess of non-synonymous substitutions (dN) is observed over synonymous substitutions (dS), at functionally important amino acid sites, i.e. $\omega = dN/dS > 1$.

Putative amino acid sites were identified by alignment with the chicken BF1 gene (Accession: HQ141385, Alcaide et al. 2014). The phylogenetic placement of the MHC-I sequences isolated here was determined using the Neighbour-joining method as implemented in MEGA v6.0 using the Tamura-Nei model (Tamura et al. 2011). Branch support was evaluated by 1,000 bootstrap replicates. Additional exon 3 sequences available for other raptor species were sourced from Genbank (Appendix B) and aligned using the Clustal W algorithm (Chenna et al. 2003) in BioEdit using default options.

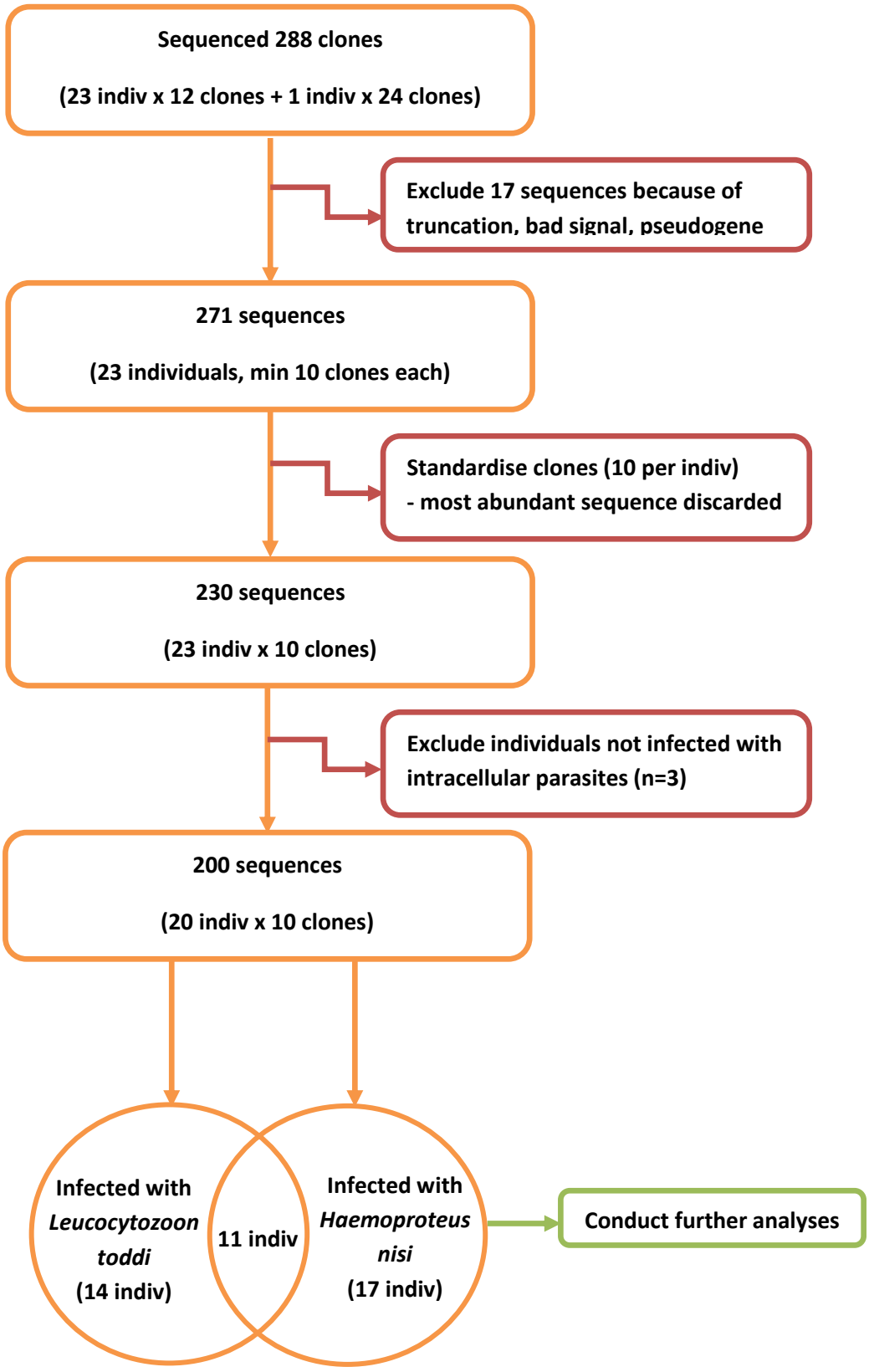


Figure 3 Sequence selection pipeline. A total of 200 sequences were included in the final dataset used to identify different MHC class I alleles of the Black sparrowhawk. (indiv = individual)

Evidence for positive selection on functionally important amino acid sites within exon 3 sequences was identified by testing for an excess of non-synonymous (dN) relative to synonymous (dS) substitutions in the final data set, where $\omega = dN/dS > 1$. Substitution rates were calculated in MEGA v6.0 (Tamura et al. 2011) according to the modified Nei-Gojorobi method with Jukes-Cantor correction and 1,000 bootstrap replicates. Three analyses were carried out, one including the whole MHC-I exon 3 sequence (full), one with only putative PBR codons (PBR only) and another including the remaining codons (non-PBR). Codons were labelled as PBR or non-PBR in accordance with previously documented patterns of positive selection across the avian MHC class I (Alcaide et al. 2008; Balakrishnan et al. 2010). The exon 3 codons classified as PBR-codons were 2, 4, 5, 6, 20, 22, 35, 56, 57, 58, 62, 63, 65 and 70 (Appendix C). Patterns of nucleotide substitution were also analysed using the online Selecton engine (Doron-Faigenboim et al. 2005, available at http://selecton.tau.ac.il/index.html). An alignment of the MHC-I alleles were uploaded to the Selecton engine where the default settings were used to test for positive selection using Yang"s M8 model (Yang 2003).

Statistical analysis

Generalized Linear Models (GLM) were used to explore differences in MHC-I diversity in relation to *L. toddi* and *H. nisi* infection intensity. Models were run in R v3.1.2 (R Development Core Team 2014). Individuals free of infection by either *H. nisi* or *L. toddi* were excluded from these analyses. The parasite intensity count data was found to be over-dispersed with a Poisson distribution. Thus, the *glm.nb* function from the *MASS* package was used to fit a negative binomial distribution using a log link function. GLMs (*glm.nb)* were also used to analyse the association between specific MHC alleles and infection intensity. For specific allele analysis, only MHC alleles that occurred in five or more individuals (n=4 MHC alleles) were included (following Westerdahl et al. 2013). Additional GLMs (*glm.nb)* were run to confirm the relationship, reported in Lei et al. (2013), between adult morph and parasite intensity, and sex and parasite intensity, for both *H. nisi* and *L. toddi*. This was necessary due to a methodological change in parasite quantification. No prior transformation was applied to the raw data (O"Hara & Kotze 2010).

Ethics

The protocol used for obtaining blood samples from black sparrowhawks was approved by the University of Cape Town"s Science Faculty Animal Ethics Committee (Permit number: 2012/V37/AA).

Results

Characterization of black sparrowhawk MHC class I exon 3

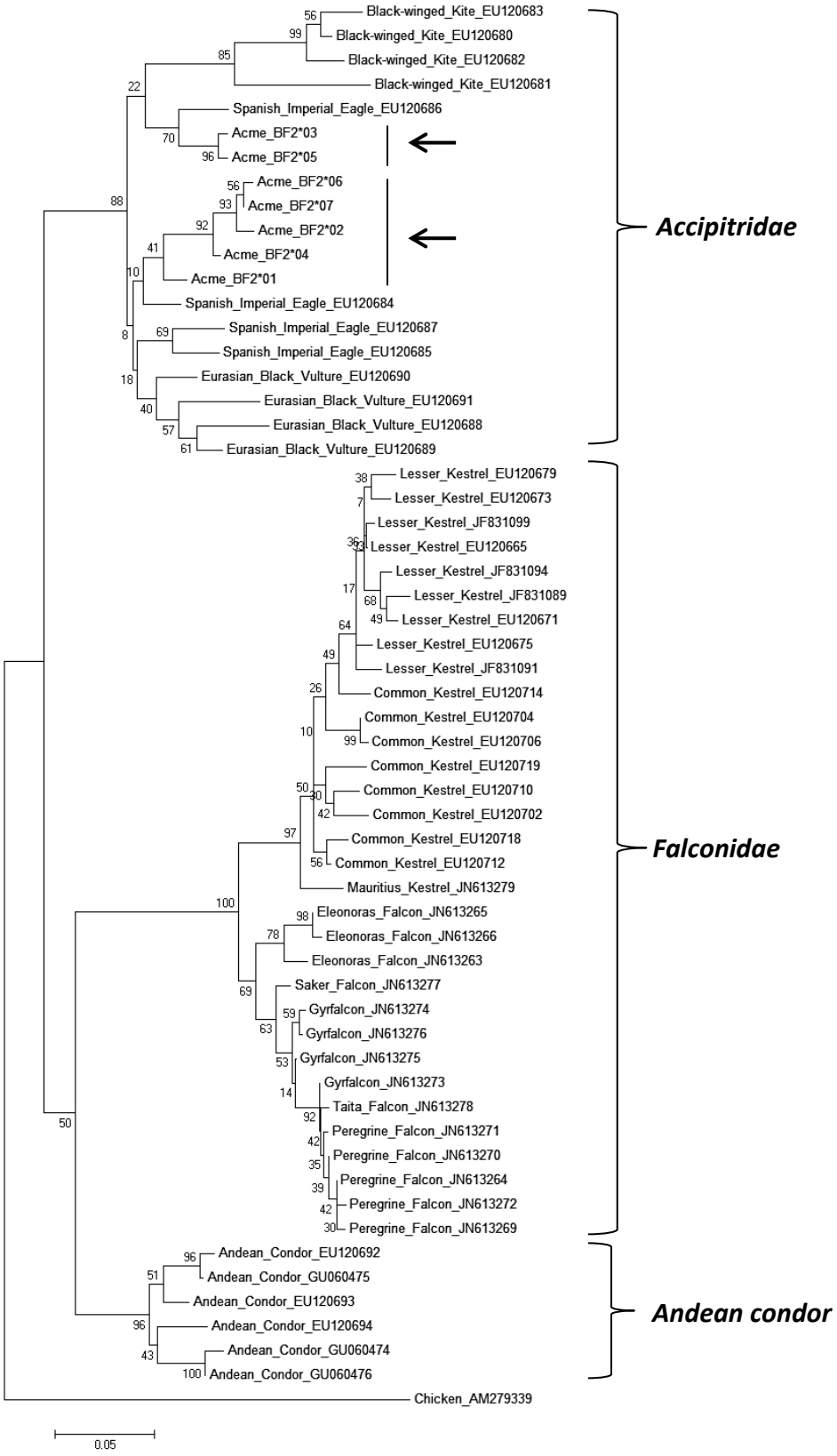
From a total of 200 clones seven different, putatively functional, MHC class I (MHC-I) alleles were identified. BLAST results revealed that all seven alleles produced the highest significant match with Spanish imperial eagle (*Aquila adalberti*) MHC class I partial cds sequences (Accession: EU120684, Alcaide et al. 2008). The most significant complete cds alignment was with the Red-billed gull (*Larus scopulinus*) Lasc-UAA*11 allele of the MHC class I antigen (Lasc-UAA) gene (Accession: HM008714, Cloutier et al. 2011). The seven alleles were also subjected to BLAT (Blast Like Alignment Tool) analysis (Kent 2002, available at: https://genome.ucsc.edu) against the most recent chicken genome (galGal4) and were found to align to the BF2 locus on Chromosome 16. The alleles were aligned to the BF2 locus of the chicken with an identity score of 83.7%.

Each individual bird had between one and three putatively functional MHC-I alleles (mean = 2.3 ± 0.15), suggesting the presence of at least two functional MHC-I loci in the black sparrowhawk. Measures of genetic diversity within the allele repertoire was relatively high with a nucleotide diversity of Pi = 0.039, and an average number of nucleotide differences between alleles (k) of 14.85, with 36 out of 381 nucleotide sites showing polymorphism. The translated amino acid sequence of the MHC-I alleles isolated here demonstrates a divergence of 4.33% (average number of codon differences/total number of codons) from another.

Phylogenetic relationship

A final alignment of the putatively functional MHC-I sequences isolated in this study together with published sequences isolated from a range of raptors (Alcaide et al. 2008) is included in Appendix B. This alignment (n=58 sequences) was used to explore the phylogenetic relationship among the MHC-I sequences through constructing a Neighbour Joining tree in MEGA (Figure 4). The resulting tree reveals two main clades, one including exon sequences from *Falconidae* and the other including the *Accipitridae* and the sequences from this study. The Andean condor sequences form a third clade branching from the *Falconidae* clade. All alleles isolated in this study are placed within the *Accipitridae* clade, but they do not cluster together. Rather they form two clear clades, one comprising alleles 3 and 5; the other comprising alleles 1, 2, 4, 6 and 7. Many of the nodes within the Neighbour Joining tree have low bootstrap support values (<50), however the most important node separating the two allele clades has a high bootstrap support value of 88 (Figure 4).

Figure 4 Phylogenetic placement of MHC-I exon 3 sequences from black sparrowhawk using neighbour joining tree construction and rooted with chicken MHC-1 BF1 gene. Bootstrap support values are indicated. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Arrows show the two allele clades, one comprising alleles 3 and 5; the other comprising alleles 1, 2, 4, 6 and 7.



Evidence for selection

As expected non-synonomous (dN) substitutions did not exceed synonomous (dS) substitutions when including the full coding sequence of the alleles (ω = dN/dS = 0.559) or the non-PBR codons (ω = 0.438) in the MEGA analysis. While non-synonomous substitutions (dN) did exceed synonomous substitutions (dS) at those codons identified as part of the PBR (ω = 1.23, Table 3) the difference was not significant (one sided z-test; Z-value = 0.290 p = 0.383, Table 3). The results from the Selecton Server analysis also identified a number of codons as evolving under positive selection, using a likelihood ratio test between Yang‟s M8 model, allowing for positive selection, and M8a null model, which does not allow positive selection (Yang 2003). The analysis identified 4 codons that have evolved under some degree of positive selection (codons 41, 57, 58 and 59; Figure 5); three of these codons correspond to codons identified as within the PBR (Figure 5).

Table 3 The overall mean genetic distances between putatively functional MHC class I alleles of the black sparrowhawk

	dS	dN	ω = dN/dS	P
All	0.059 ± 0.021	0.033 ± 0.012	0.559	1.0
PBR only	0.083 ± 0.080	0.102 ± 0.072	1.229	0.383
Non-PBR	0.057 ± 0.020	0.025 ± 0.010	0.438	1.0

All distance measures were calculated for all codons (All), codons located in putative PBR (PBR only) and those outside PBR (Non-PBR). Distances are as follows: dS and dN, synonymous and nonsynonymous distances calculated according to the modified Nei & Gojobori (1986) method with the Jukes–Cantor correction for multiple substitutions. P, P-value of the Z test of the null hypothesis dN/dS ≤ 1.

Figure 5 Amino acid alignment of the seven putatively functional MHC-I alleles isolated in this study. Dots indicate identity with the top sequence, + indicates codons labelled as PBR following Balakrishnan et al. (2010) and Alcaide et al. (2009), * indicates codons identified by Selecton as being under positive selection (Yang, 2003).

Individual MHC diversity and parasite infection intensity

To investigate the association between MHC diversity and parasite load in the black sparrowhawk, the distributions of MHC-I alleles isolated was expressed as allele frequencies (number of individuals that carry an allele/total number of individuals). Individuals were grouped on the basis of morph and sex respectively. Of those individuals infected with intracellular parasites (n=20), 85% were infected with *H. nisi*, 70% with *L. toddi* and 55% of individuals were infected with both *H. nisi* and *L. toddi.* All seven MHC-I alleles were found in both *H. nisi* and *L. toddi* infected individuals (male and female). The frequency distribution of the seven MHC-I alleles is shown in Table 4.

Table 4 Frequencies (number of individuals that carry an alleles/total number of individuals) of seven black sparrowhawk MHC-I alleles found in the 20 individuals that were infected with *H. nisi* and/or *L. toddi.*

Allele	Number of individuals	Frequency (indiv)	Dark morph (n = 15)	Light Morph (n = 5)	Male (n = 11)	Female (n = 9)
AcmeBF2*01	16	0.80	0.80	0.80	0.82	0.78
AcmeBF2*02	10	0.50	0.47	0.60	0.64	0.33
AcmeBF2*03	8	0.40	0.40	0.40	0.45	0.33
AcmeBF2*04	5	0.25	0.20	0.40	0.27	0.22
AcmeBF2*05	3	0.15	0.20	0	0.09	0.22
AcmeBF2*06	2	0.10	0.13	0	0.09	0.11
AcmeBF2*07	2	0.10	0.13	0	0.09	0.11

MHC-I alleles AcmeBF2*05, 6 and 7 were not found in light morphs, but given the low number of birds with these alleles (n=3, 2 & 2) and the low number of light morph birds (n=5), this could easily be due to random change. Light morphs had significantly higher infection intensities of *H. nisi* ($P = 0.002$) (Figure 6), but not *L. toddi* ($P = 0.936$), compared to dark morphs Males also experienced significantly higher infection intensities of *H. nisi* ($P = 0.003$) (Figure 6), but not *L. toddi* ($P = 0.140$), compared to females.

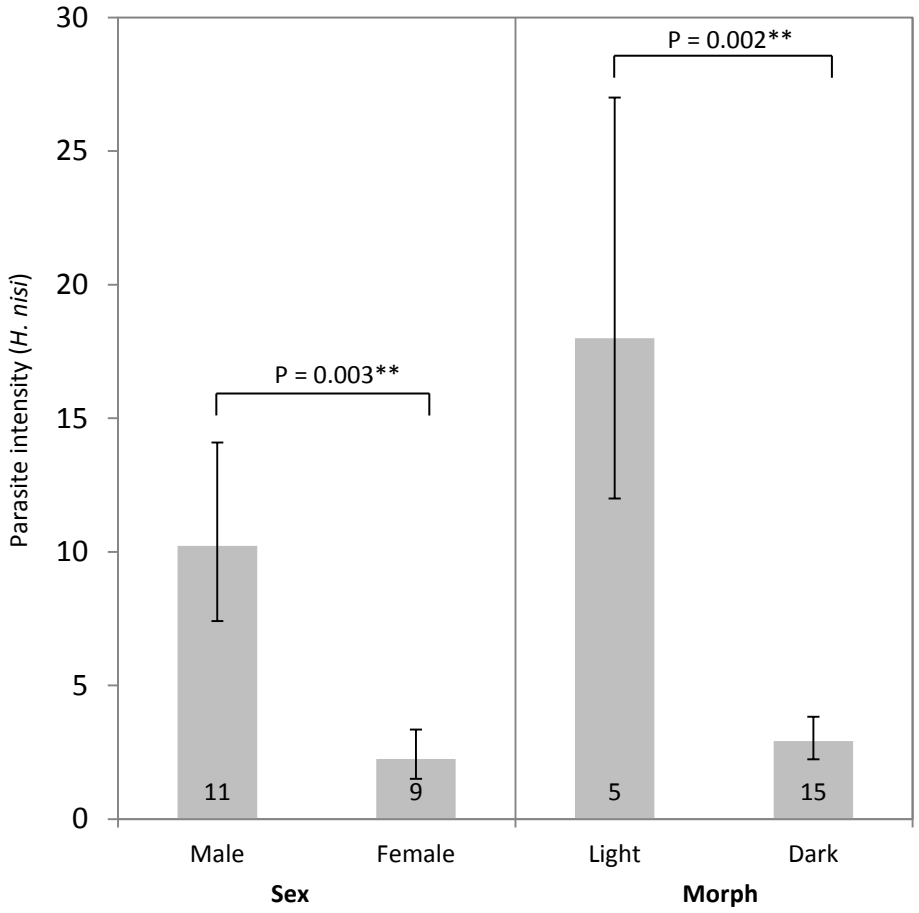


Figure 6 Infection intensity of *H.nisi* for the two sexes and morphs. The mean (± SE) infection intensity (number of intracellular parasites within 20 000 erythrocytes) is shown. Black numbers indicate sample size for each group (total n = 20). Significance code: ≤0.01 **.

Number of MHC-I alleles and parasite intensity

Number of alleles per individual ranged from one to three in individuals infected with *H. nisi*, whereas individuals infected with *L. toddi* had either two or three different alleles. Individuals with a total of three different alleles had significantly higher *H. nisi* parasite intensity (Z-value = -2.701, ndf = 16, ddf = 14, P = 0.019; Figure 7), compared to individuals with two different alleles. No significant difference was found between individuals with one and two alleles, or one and three alleles with respect to *H. nisi* parasite intensity (Z-value = 0.905, ndf = 16, ddf = 14, P = 0.637 and Z-value = -1.270, ndf = 16, ddf = 14, P =0.412 respectively; Figure 7). No significant relationship was found between *L. toddi* infection intensity and number of alleles per individual (P = 0.44).

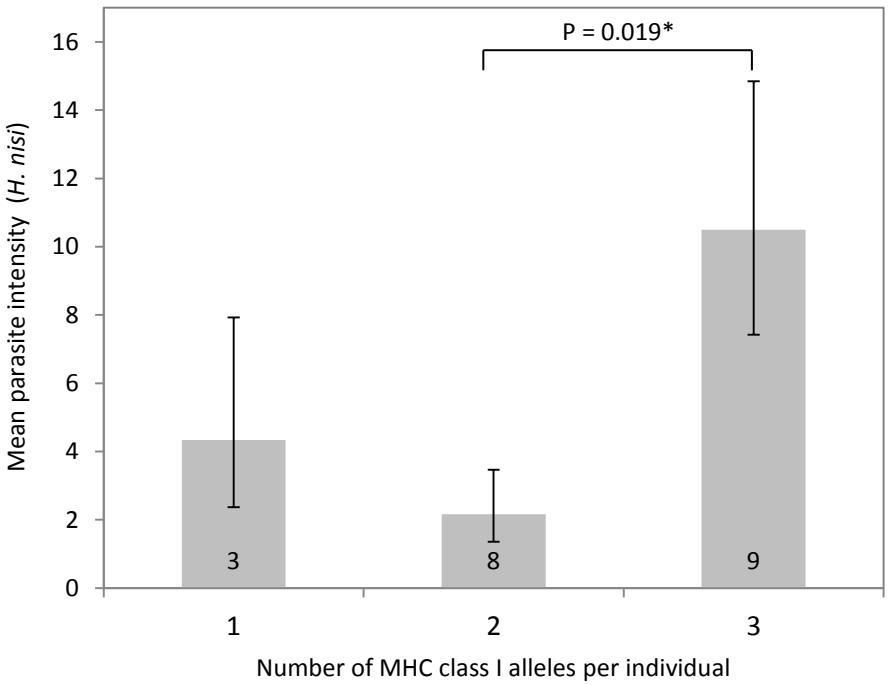


Figure 7 Mean *H.nisi* intensity in relation to number of MHC-I alleles per individual. Error bars indicate ± SE. Black numbers indicate sample size for each group (total n = 20). Siginificance code: ≤0.05*.

Specific MHC-I alleles and parasite intensity

Four out of the seven alleles isolated in this study occurred in five or more individuals and were therefore tested for association to parasite infection intensity. These alleles occurred in frequencies from 0.25 to 0.80 (Table 4). *H. nisi* infection intensity was significantly associated with one specific MHC allele (Acme_BF2*03) out of the four alleles tested (Z-value = 3.025, ndf = 15, ddf = 16, P=0.002; Figure 8). Individuals carrying Acme_BF2*03 had higher *H. nisi* intensities than individuals without this allele. A similar relationship was found for alleles Acme_BF2*01 and Acme_BF2*02, however, no significance was found for allele Acme_BF2*01 (Z-value = 1.151, ndf = 15, ddf = 16, P=0.25; Figure 8) and a near significant difference was found for allele Acme_BF2*02 (Z-value = 1.947, ndf = 15, ddf = 16, P=0.051; Figure 8). The opposite trend was observed for allele Acme_BF2*04, where individuals carrying this allele had lower *H. nisi* intensities than individuals without this allele, but this trend was also not quite statistically significant (Z-value = -1.834, ndf = 15, ddf = 16, P=0.067; Figure 8). No significant association was found between *L. toddi* infection intensity and a specific allele. The model outputs for *H. nisi* and specific MHC-I alleles are summarized in Table 5.

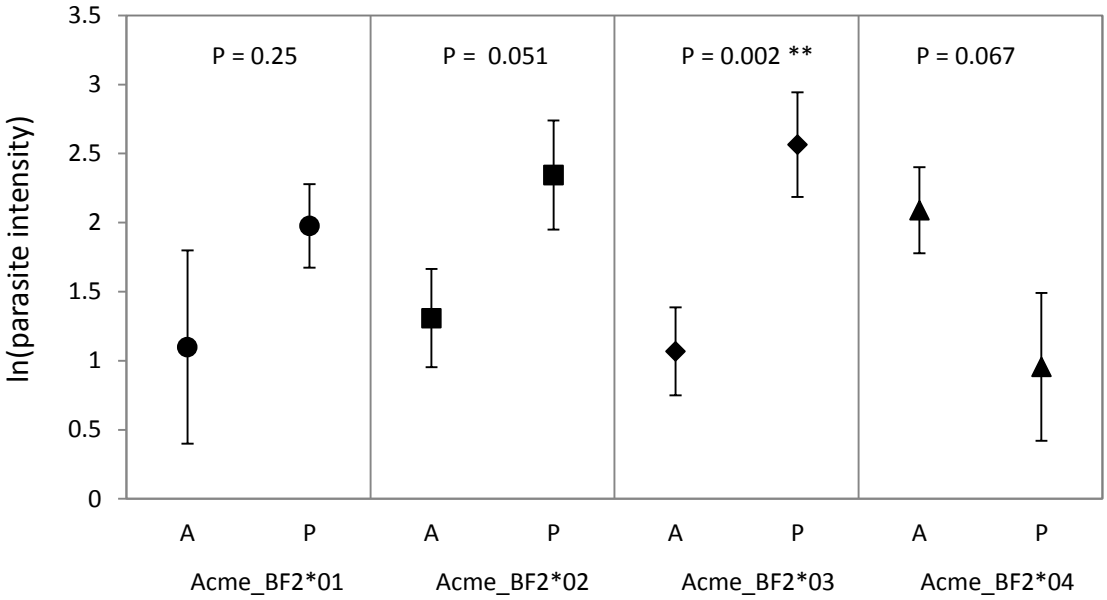


Figure 8 *H. nisi* infection intensity (ln transformed (parasite infection intensity)) of black sparrowhawk adults that carry (P - present) or do not carry (A - absent) the following alleles; Acme_BF2*01, Acme_BF2*02, Acme_BF2*03 or Acme_BF2*04 (mean±SE). Parasite intensity data was log link transformed using the glm.nb function. Significance code: ≤0.01 **

Table 5 Summary of models and outputs relating *H.nisi* infection intensity to the presence/absence of specific MHC-I alleles (1-4). Parasite data was log link transformed using the glm.nb function.

Model	Estimate	Std Error	Z-value	P-value	dev/df	Sample size (0:1)
H.nisi~ Acme_BF2*01	0.88	0.76	1.15	0.250	1.17	3:14
H.nisi~ Acme_BF2*02	1.04	0.53	1.95	0.051	1.14	7:7
H.nisi~ Acme_BF2*03	1.50	0.50	3.02	0.002**	1.07	8:6
H.nisi~ Acme_BF2*04	-1.13	0.62	-1.83	0.067	1.15	12:5

Siginificance code: ≤0.01 **

Discussion

Characterizing MHC class I variation in the black sparrowhawk

The diverse functions of the MHC make this gene complex a model candidate for studying the mechanisms and significance of molecular adaptation in vertebrates (Sommer 2005). Variation at the MHC has been linked to important fitness-related traits, including disease resistance and mate selection (Sommer 2005; Piertney & Oliver 2006; Bonneaud et al. 2006a; Spurgin & Richardson 2010; Sepil et al. 2013). With regard to parasite resistance, it is well known that birds infected with haemosporidians show a decrease in competitiveness (e.g. Johnson & Boyce 1991; Wiehn et al. 1997) and specific MHC alleles have been associated with resistance to parasites (e.g. Bonneaud et al. 2006b; Westerdahl et al. 2013a).

In this study, functional MHC diversity and its significance for individual parasitemia was investigated at class I loci in the black sparrowhawk. A relatively low number of class I loci (1-2) were identified compared to passerine species, where up to eight class I loci have been reported in the same individual (Dunn et al. 2013). From a total of 200 sequences isolated from 20 individuals a total of seven functional MHC-I alleles were identified, where the number of alleles per individual ranged from one to three. Alleles were only designated after processing the raw data through an established selection pipeline, thereby minimizing the chances of over-estimating diversity. Due to the relatively low number of clones screened per individual (n = 12) the total number of MHC-I alleles and loci may be higher than presented here. However, the number of putative loci found is comparable with the general trend observed in other raptors studied to date; Alcaide et al. (2008) reported the presence of 1-2 putative MHC class I loci across a broad range of species representing the Falconidae, Accipitridae, Carthatidae and Stringidae. The number of MHC class I, exon 3 sequences isolated for each species ranged from 23 for Eurasian kestrel *(Falco tinnunculus*) to one for eagle owl (*Bubo bubo*). The low allelic diversity found at the functionally important MHC-I exon 3 of the black sparrowhawk is analogous to the compact MHC of the chicken (*Gallus gallus*) which has two class I (BF1 and BF2) loci within the B-region and two class II (BLB1 and BLB2) loci within the Y-region on Chromosome 16 (Miller et al. 2004). The alleles isolated in this study aligned to the BF2 locus of the chicken with an identity score of 83.7%, using BLAT analysis (Kent 2002) .

In contrary to the low allelic diversity, measures of genetic diversity within the allele repertoire isolated here was relatively high with an average number of nucleotide differences between alleles (k) of 14.85, with 36 out of 381 nucleotide sites showing polymorphism. For example, Gangoso et al. (2012) report

average numbers of nucleotide differences between alleles ranging from 1 – 4.66, for six different species of falcons. This suggests that the low allelic diversity observed here is not an indication of low overall functional genetic variability in the study population of black sparrowhawks.

Evidence for selection in the Peptide Binding Region

The signatures of positive selection detected in the potential PBR suggest that the alleles isolated here are functional and expressed (Piertney & Oliver 2006). Genes which are under positive selection are good candidates for investigating adaptive genetic variation (Kohn et al. 2006).

Phylogenetic analysis placed all alleles isolated in this study within the *Accipitridae* clade. However, they form two clear clades, one comprising alleles 3 and 5; the other comprising alleles 1, 2, 4, 6 and 7. There are four common amino acid differences that separate alleles 3 and 5 from the rest, where three of these amino acids are under slight (but not significant) positive selection and one is under strong positive selection based on the Selecton analysis output. The phylogenetic separation of the alleles suggest that these alleles are specific to two distinct loci and positive selection of the amino acids that separate these alleles supports the hypothesis of differential functional roles for different loci (Cloutier et al. 2011; Walker et al. 2011).

MHC class I diversity influences haematozoon infection intensity

The results from this study suggest that MHC class I variation influences blood parasite infection by the avian malarial agent *Haemoproteus nisi* in black sparrowhawks. No significant associations were found between MHC class I variation and *Leucocytozoon toddi* infection. Analysis of MHC class I variation together with parasite quantification for individuals revealed a positive association between an intermediate number (two) of class I alleles and *H. nisi* parasitemia in the study population (Figure 7). Furthermore, a positive relationship between MHC-I allele BF2*03 and *H. nisi* infection intensity was found (Figure 8). These associations suggest that individuals carrying a total of two MHC-I alleles or individuals which do not carry BF2*03 cope better with parasite infection. Carrying two alleles only, may also decrease the chance of carrying allele BF2*03 as it is found at a lower frequency within the study population, compared to alleles BF2*02 and BF2*01 (Table 4). The low frequency of BF2*03 might reflect a cost to the individual when expressed together with BF2*01 and BF2*02 which support the negative frequency selection model. Therefore, allele BF2*03 might not confer the same level of fitness as the other alleles in the current pathogen environment.

An association was found between the presence of BF2*04 and reduced *H. nisi* infection ($p = 0.067$, Figure 8) which might indicate a role for this allele in conferring some degree of individual resistance to infection. These associations between specific alleles and parasite intensity suggest that the black sparrowhawk MHC-I alleles are subject to negative frequency-dependent selection through „rare-allele advantage". Moreover, the relationship between an intermediate number of MHC-I alleles and lower parasite load support the theoretical model of self-reactive T-cell elimination (Nowak et al. 1992; Mason 2001), also shown in sticklebacks (Wegner et al. 2003b). Although a high diversity of MHC class I genes allows presentation of more antigens, too many MHC variants will also result in presentation of more self-peptides, with subsequent elimination of corresponding self-reactive T-cell receptor lines which in turn limits the adaptive immune response (Mason 2001). Wegner et al. (2013) studied sticklebacks from eight study populations, sampled from lakes and rivers, and found that self-elimination effects only appear in fish sampled from lakes, where fish suffer from infestation by a wide array of parasite species. They suggest that the chance of mounting a functional immune response against a single pathogen does not depend on high levels of T-cell diversity, but rather on the effective presentation of the antigen by an MHC allele and recognition by a single T-cell receptor line. Thus, the presence of specific MHC-I alleles in the black sparrowhawk repertoire might be more informative than overall MHC-I diversity.

Another way of exploring the relationship between MHC diversity and parasite infection has been to characterize the presence of MHC supertypes in populations (e.g. Sepil et al. 2012, 2013; Dunn et al. 2013). MHC supertypes are allele clusters that are defined by their shared physiochemical properties of the amino acids at the peptide binding region (see Sandberg et al. 1998 and Doytchinova & Flower 2005 for details), rather than by their evolutionary relationships. For example Sepil et al. (2013) reported findings of a single MHC-I supertype which confers qualitative resistance to infection by *Plasmodium relictum* in a wild great tit (*Parus major*) population, while a different MHC-I supertype confers quantitative resistance to *Plasmodium circumflexum* infections in the same population. Grouping alleles into MHC "supertypes" was attempted in this study, however the number of alleles were too low to form reliably distinct clusters (7 alleles isolated here vs. 755 alleles isolated by Sepil el al. 2013).

Accordingly, MHC diversity of each individual was rather analysed in terms of number and identity of alleles carried. Interestingly, the rarer MHC-I alleles BF2*05, 6 and 7 (Table 4) were not found in light morphs. However, given the low number of birds with these alleles (n=3, 2 & 2 respectively) and the low number of light morph birds sampled (n=5), this could be due to random change. However, the ratio

of light to dark morphed individuals in the Western Cape (1:4) (Amar et al. 2014) closely resembles the ratio within this sample set (1:3) and a marked difference in the frequency of MHC-I allele BF2*02 between dark (0,47) and light (0.60) was observed. A positive association was observed for allele BF2*02 and *H. nisi* infection intensity suggesting that this allele might be a candidate to further explore the differences observed in parasite intensity between morphs (Lei et al. 2013 and this study). Repeating the analysis with an increased sample set will provide greater insight into the significance of this detected trend. The lowest pathogen burdens and the most diverse set of MHC alleles (n = 7) were found in dark morph birds. The differences observed in the MHC-I allele repertoire and parasite infection between the black sparrowhawk morphs, supports the theory that differential immune capacity may exist in polymorphic populations (Lei et al. 2013). The results presented in this study would therefore support the role of pathogen mediated selection of genetic variation at evolutionary relevant MHC genes through rare allele advantage.

Final comments

Characterizing the variation at MHC class I loci, and testing for a relationship with parasitemia provide the fundamental basis for further studies to elucidate the mechanisms and significance of MHC molecular adaptation in the black sparrowhawk. As the study is focused on a recently colonized population in a changing, urban environment, these data can be used to understand patterns of adaptive variation in a time of immense environmental change. Global change and urbanization will not only affect urban adaptors like the black sparrowhawk, but also the whole pathogenic environment. Relating the diversity observed in this population to populations in less urbanized environments will give insight into the effects of human impact on genetic variation and help inform policy, management and general strategies for biodiversity conservation.

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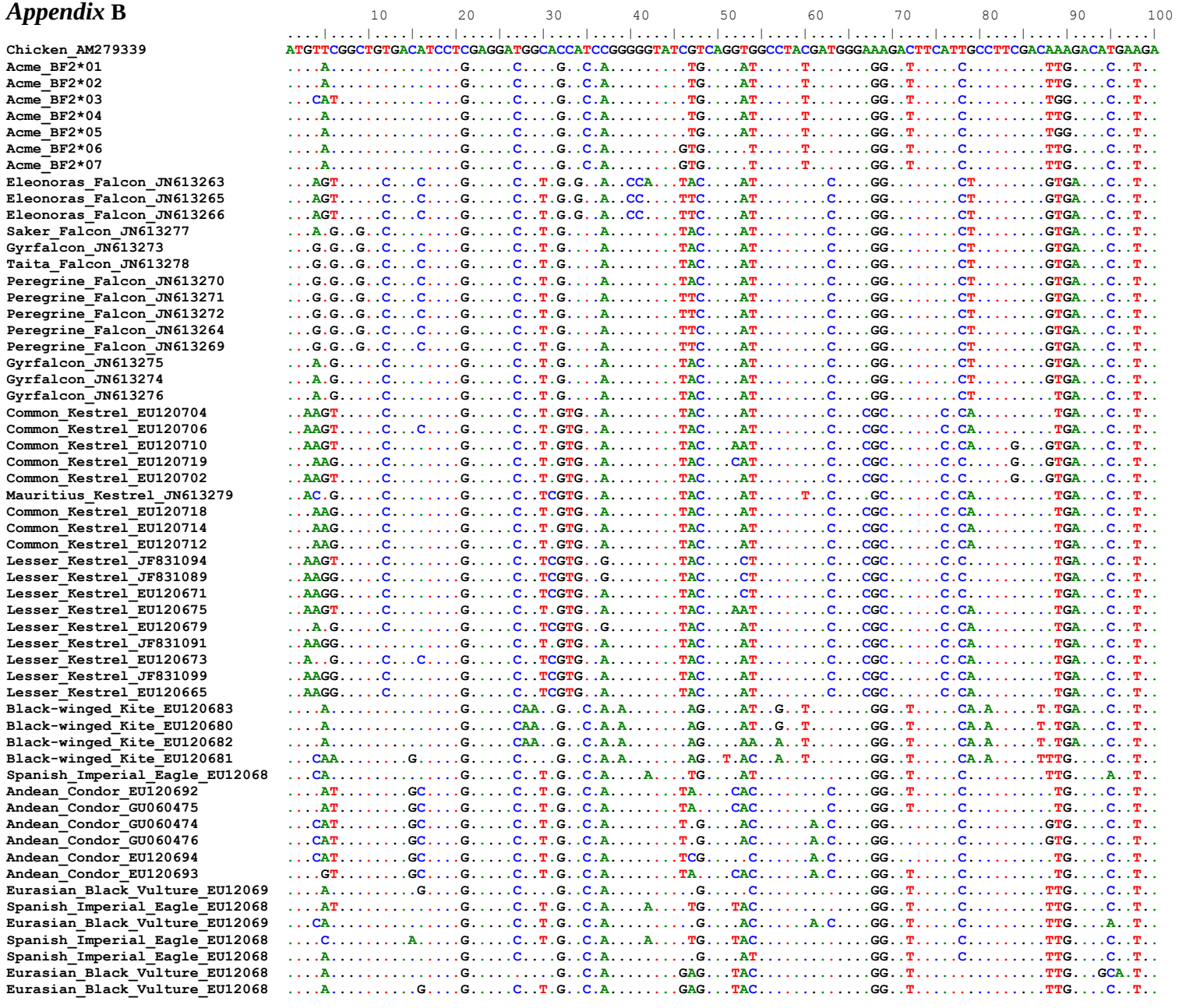
Appendix B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Acme_BF2*01 ....A.....G.....C....G..C.A.....TG....AT.....T.....GG..T.....C.....TTG....C..T..
Acme_BF2*02 ....A.....G.....C....G..C.A.....TG....AT.....T.....GG..T.....C.....TTG....C..T..
Acme_BF2*03 ...CAT.....G.....C....G..C.A.....TG....AT.....T.....GG..T.....C.....TGG....C..T..
Acme_BF2*04 ....A.....G.....C....G..C.A.....TG....AT.....T.....GG..T.....C.....TTG....C..T..
Acme_BF2*05 ....A.....G.....C....G..C.A.....TG....AT.....T.....GG..T.....C.....TGG....C..T..
Acme_BF2*06 ....A.....G.....C....G..C.A.....GTG.....T.....T.....GG..T.....C.....TTG....C..T..
Acme_BF2*07 ....A.....G.....C....G..C.A.....GTG.....T.....T.....GG..T.....C.....TTG....C..T..
Eleonoras_Falcon_JN613263 ...AGT.....C...C....G.....C..T.G.G..A..CCA...TAC....AT.....C....GG.....CT.....GTGA...C..T..
Eleonoras_Falcon_JN613265 ...AGT.....C...C....G.....C..T.G.G..A..CC....TTC....AT.....C....GG.....CT.....GTGA...C..T..
Eleonoras_Falcon_JN613266 ...AGT.....C...C....G.....C..T.G.G..A..CC....TTC....AT.....C....GG.....CT.....GTGA...C..T..
Saker_Falcon_JN613277 ...A.G..G..C.....G.....C..T.G....A.....TAC....AT.....C....GG.....CT.....GTGA...C..T..
Gyrfalcon_JN613273 ...G.G..G..C...C....G.....C..T.G....A.....TAC....AT.....C....GG.....CT.....GTGA...C..T..
Taita_Falcon_JN613278 ...G.G..G..C...C....G.....C..T.G....A.....TAC....AT.....C....GG.....CT.....GTGA...C..T..
Peregrine_Falcon_JN613270 ...G.G..G..C...C....G.....C..T.G....A.....TAC....AT.....C....GG.....CT.....GTGA...C..T..
Peregrine_Falcon_JN613271 ...G.G..G..C...C....G.....C..T.G....A.....TTC....AT.....C....GG.....CT.....GTGA...C..T..
Peregrine_Falcon_JN613272 ...G.G..G..C...C....G.....C..T.G....A.....TTC....AT.....C....GG.....CT.....GTGA...C..T..
Peregrine_Falcon_JN613264 ...G.G..G..C...C....G.....C..T.G....A.....TTC....AT.....C....GG.....CT.....GTGA...C..T..
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Gyrfalcon_JN613275 ...A.G.....C.....G.....C..T.G....A.....TAC....AT.....C....GG.....CT.....GTGA...C..T..
Gyrfalcon_JN613274 ...A.G.....C.....G.....C..T.G....A.....TAC....AT.....C....GG.....CT.....GTGA...C..T..
Gyrfalcon_JN613276 ...A.G.....C.....G.....C..T.G....A.....TAC....AT.....C....GG.....CT.....TGA...C..T..
Common_Kestrel_EU120704 ..AAGT.....C.....G.....C..T.GTG..A.....TAC....AT.....C....CGC.....C.CA.....TGA...C..T..
Common_Kestrel_EU120706 ..AAGT.....C...C....G.....C..T.GTG..A.....TAC....AT.....C....CGC.....C.CA.....TGA...C..T..
Common_Kestrel_EU120710 ..AAGT.....C.....G.....C..T.GTG..A.....TAC....AAT.....C....CGC.....C.CA....G...GTGA...C..T..
Common_Kestrel_EU120719 ...AAG.....C.....G.....C..T.GTG..A.....TAC....CAT.....C....CGC.....C.C.....G...GTGA...C..T..
Common_Kestrel_EU120702 ..AAGT.....C.....G.....C..T.GTG..A.....TAC....AT.....C....CGC.....C.C.....G...GTGA...C..T..
Mauritius_Kestrel_JN613279 ..AC.G.....C.....G.....C..TCGTG..A.....TAC....AT.....T..C....GC.....C.CA.....TGA...C..T..
Common_Kestrel_EU120718 ...AAG.....C.....G.....C..T.GTG..A.....TAC....AT.....C....CGC.....C.CA.....TGA...C..T..
Common_Kestrel_EU120714 ...AAG.....C.....G.....C..T.GTG..A.....TAC....AT.....C....CGC.....C.CA.....TGA...C..T..
Common_Kestrel_EU120712 ...AAG.....C.....G.....C..T.GTG..A.....TAC....AT.....C....CGC.....C.CA.....TGA...C..T..
Lesser_Kestrel_JF831094 ..AAGT.....C.....G.....C..TCGTG..G.....TAC....CT.....C....CGC.....C.C.....TGA...C..T..
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Lesser_Kestrel_EU120671 ..AAGG.....C.....G.....C..TCGTG..A.....TAC....CT.....C....CGC.....C.C.....TGA...C..T..
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Black-winged_Kite_EU120680 ....A.....G.....CAA..G..C.A.A.....AG....AT..G..T.....GG..T.....CA.A.....T.TGA...C..T..
Black-winged_Kite_EU120682 ....A.....G.....CAA..G..C.A.A.....AG....AA..A..T.....GG..T.....CA.A.....T.TGA...C..T..
Black-winged_Kite_EU120681 ...CAA.....G.....G.....C....G..C.A.A.....AG..T.AC..A..T.....GG..T.....CA.A.....TTTG....C..T..
Spanish_Imperial_Eagle_EU12068 ...CA.....G.....C..T.G..C.A....A....TG....AT.....GG..T.....C.....TTG....A..T..
Andean_Condor_EU120692 ....AT.....GC....G.....C..T.G..C.A.....TA....CAC.....C....GG..T.....C.....TG....C..T..
Andean_Condor_GU060475 ....AT.....GC....G.....C..T.G..C.A.....TA....CAC.....C....GG..T.....C.....TG....C..T..
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Andean_Condor_GU060476 ...CAT.....GC....G.....C..T.G..C.A.....T.G....AC.....A.C....GG.....C.....GTG....C..T..
Andean_Condor_EU120694 ...CAT.....GC....G.....C..T.G..C.A.....TCG.....C.....A.C....GG.....C.....TG....C..T..
Andean_Condor_EU120693 ....GT.....GC....G.....C..T.G..C.A.....TA....CAC.....A.C....GG..T.....C.....TG....C..T..
Eurasian_Black_Vulture_EU12069 ....A.....G....G.....C....G..C.A.....G.....C.....GG..T.....C.....TTG....C..T..
Spanish_Imperial_Eagle_EU12068 ....AT.....G.....C..T.G..C.A....A....TG...TAC.....GG..T.....C.....TTG....C..T..
Eurasian_Black_Vulture_EU12069 ...CA.....G.....C..T.G..C.A.....G....AC.....A.C....GG..T.....C.....TTG....A..T..
Spanish_Imperial_Eagle_EU12068 ....C.....A.....G.....C..T.G..C.A....A....TG...TAC.....GG..T.....C.....TTG....C..T..
Spanish_Imperial_Eagle_EU12068 ....A.....G.....C....G..C.A.....G....AT.....GG..T.....C.....TTG....C..T..
Eurasian_Black_Vulture_EU12068 ....A.....G.....G..C.A.....GAG...TAC.....GG..T.....TTG...GCA.T..
Eurasian_Black_Vulture_EU12068 ....A.....G....G.....C..T.G..C.A.....GAG...TAC.....GG..T.....TTG....C..T..


110 120 130 140 150 160 170 180 190 200
Chicken_AM279339 CGTTCACTGCGGCAGTTCCAGAGGCAGTTCCCACCAAGAGGAAATGGGAGGAAGGAGGT~~~GTTGCTGAGGGGTGGAAGAGTTACCTGGAGGAAACCTG
Acme_BF2*01
Acme_BF2*02
Acme_BF2*03
Acme_BF2*04
Acme_BF2*05
Acme_BF2*06
Acme_BF2*07
Eleonoras_Falcon_JN613263
Eleonoras_Falcon_JN613265
Eleonoras_Falcon_JN613266
Saker_Falcon_JN613277
Gyrfalcon_JN613273
Taita_Falcon_JN613278
Peregrine_Falcon_JN613270
Peregrine_Falcon_JN613271
Peregrine_Falcon_JN613272
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Peregrine_Falcon_JN613269
Gyrfalcon_JN613275
Gyrfalcon_JN613274
Gyrfalcon_JN613276
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Common_Kestrel_EU120702
Mauritius_Kestrel_JN613279
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Common_Kestrel_EU120712
Lesser_Kestrel_JF831094
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Lesser_Kestrel_EU120665
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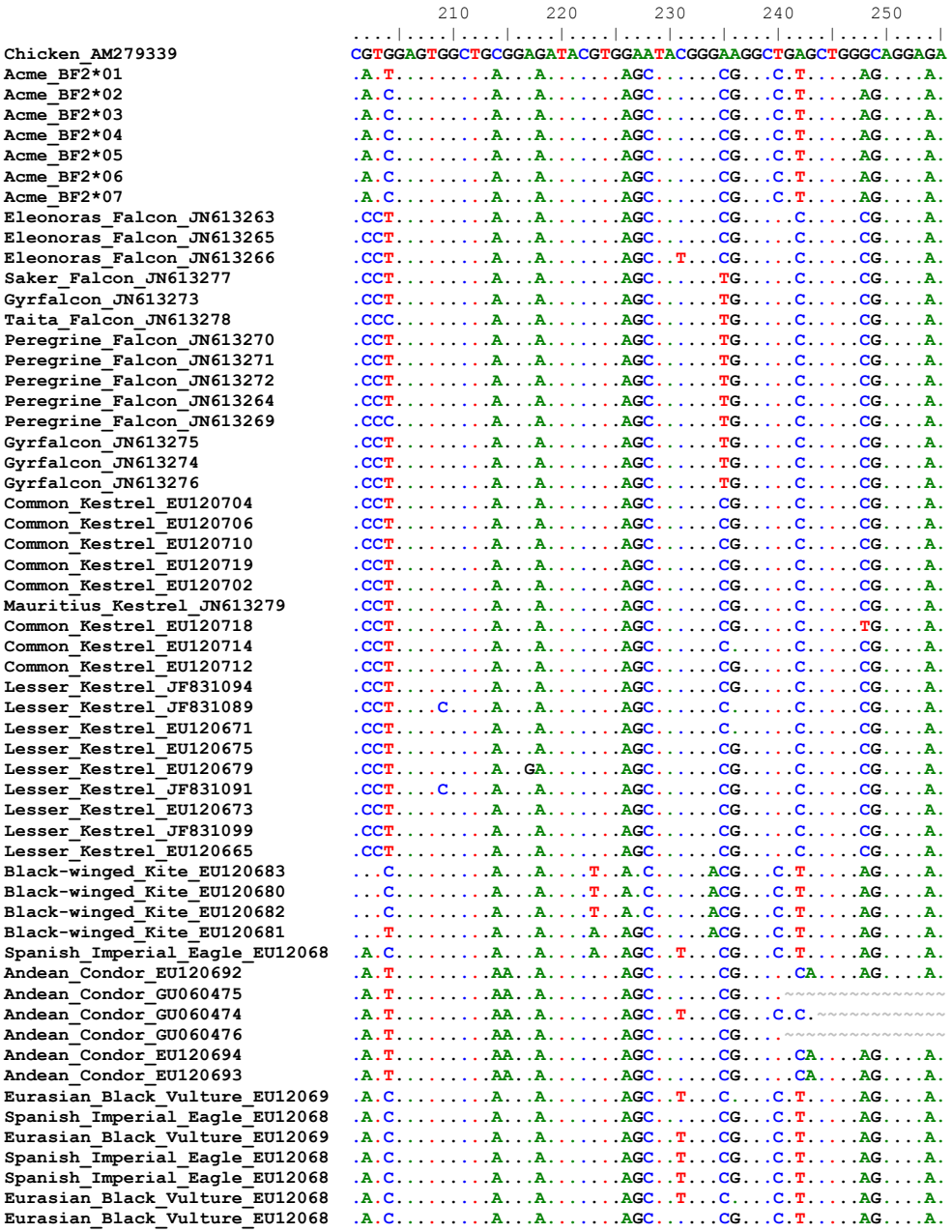

Figure 9 Multiple sequence alignment of raptor exon 3 sequences with the chicken BF1 gene (Accession: HQ141385, Alcaide et al. 2014)

Appendix C

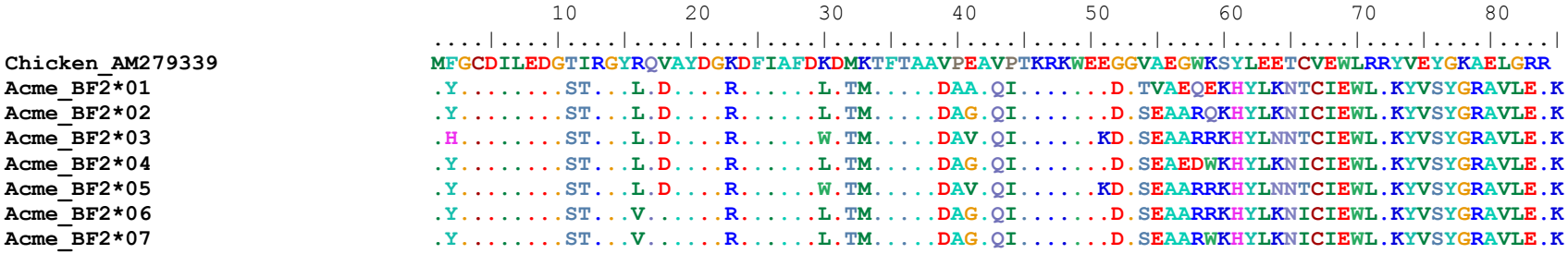

Figure 10 Alignment of the seven MHC-I alleles isolated here