

# MHC class I genes of birds of prey: isolation, polymorphism and diversifying selection

Miguel Alcaide · Scott V. Edwards ·  
Luis Cadahía · Juan J. Negro

Received: 14 May 2008 / Accepted: 4 August 2008  
© Springer Science+Business Media B.V. 2008

**Abstract** The threat of emerging infectious diseases encourages the investigation of functional loci related to host resilience, such as those belonging to the major histocompatibility complex (MHC). Through careful primer design targeting to conserved regions of MHC class I sequences in birds, we successfully amplified a genomic fragment spanning exons 2–4 in three birds of prey. The identification of a highly conserved region within intron 2 allowed cross-amplifying complete exon 3 sequences in diurnal raptors, owls and New World vultures. We found evidence through PCR and cloning for 1–2 polymorphic class I loci, although this is almost certainly an underestimate. Inferences of diversifying selection in the kestrel MHC revealed that the two major regions of exon 3 exhibiting positive selection mostly agree with those described for the human HLA-A2 molecule. In contrast to passerines, where a high incidence of gene duplications and pseudogenes has been commonly documented, birds of prey emerge as nice model species for the investigation of the evolutionary significance and conservation implications of MHC diversity in vertebrates.

**Keywords** Adaptive variation · Conservation genetics · Immunogenetics · Genetic diversity · Raptors · Infectious diseases

## Introduction

During the last two decades, the major histocompatibility complex (MHC) has been the focus in studies of evolutionary ecology and conservation because of its implication in many relevant biological processes (reviewed by Sommer 2005; Piertney and Oliver 2006, but see Acevedo-Whitehouse and Cunningham 2006). Thus, several studies have emphasized the potential of MHC genes as valuable molecular markers to assess the evolutionary and adaptive potential of endangered populations and species in relation to the menace of changed and emerging diseases (e.g. Yuhki and O'Brien 1990; Hedrick and Parker 1998; Garrigan and Hedrick 2004; Wan et al. 2006; Bollmer et al. 2007). MHC genes encode cell surface glycoproteins that play an essential role in the immune response by presenting short peptides derived from the processing of pathogens and subsequent initiation of an adaptive immune response (e.g. antibody production or destruction of the antigen presenting cell). MHC class I glycoproteins are expressed in all nucleated cells and are related to immune defence against intracellular pathogens such as viruses and some protozoa. Critically for studies of MHC variation in non-model species, single class I  $\alpha$  genes possess two polymorphic exons ( $\alpha_1$  and  $\alpha_2$ ) encoding the polymorphic peptide binding region (PBR) (Bjorkman et al. 1987).

As far as we know, MHC class I genes have only been investigated in detail in a handful of avian species, namely the chicken *Gallus gallus* (Kaufman et al. 1999), quail *Coturnix japonica* (Shiina et al. 1995), great reed warbler *Acrocephalus arundinaceus* (Westerdahl et al. 1999), Seychelles warbler *Acrocephalus sechellensis* (Richardson and Westerdahl 2003), Florida sandhill crane *Grus canadensis* (Jarvi et al. 1999), domestic goose *Anser anser* (Xia et al. 2005) and the mallard *Anas platyrhynchos*

---

M. Alcaide (✉) · L. Cadahía · J. J. Negro  
Estación Biológica de Doñana (CSIC), Pabellón de Perú. Avda.  
M<sup>a</sup> Luisa s/n, 41013 Sevilla, Spain  
e-mail: malcaide@ebd.csic.es

S. V. Edwards  
Department of Organismic and Evolutionary Biology, Harvard  
University, 26 Oxford St., Cambridge, MA 02138, USA

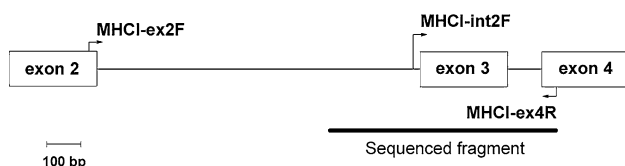
(Moon et al. 2005). Importantly, nearly all the above studies investigating class I diversity in non-model avian species have done so at the cDNA level. Thus, our knowledge of class I intron sequences in birds is limited. In the present study, we aimed at developing molecular methods for the isolation of genomic MHC class I sequences in birds of prey, focusing on the  $\alpha_2$  domain encoded by exon 3.

## Materials and methods

### Amplification, sequencing and alignment of MHC class I fragments

Amplification strategies relying on the polymerase chain reaction (PCR) were performed on genomic DNA, extracted following the protocol described by Gemmell and Akiyama (1996), using a PTC-100 Programmable Thermal Controller (MJ Research Inc.). We designed degenerate primers (MHCI-ex2F: CGCTACAACCAGASCRRSG and MHCI-ex4R: GGGTAGAAGCCGTGAGCRC; see Fig. 1) across conserved regions of exons 2 and 4 emerging from an alignment of mRNA sequences of a few bird species deposited in GenBank (species names and GenBank accession numbers: Chicken *Gallus gallus* L28958, Domestic goose *Anser anser* AM114924, Mallard *Anas platyrhynchos* AB115246 and Sandhill Crane *Grus canadensis* AF033106). Our aim at this stage was obtaining intronic sequences flanking exon 3 in order to design specific primers for the amplification of this polymorphic region in raptors. After sequencing the target MHC fragment in at least three raptor species, we created an alignment including genomic class I sequences of the chicken AM279340, domestic goose AY387655 and mallard AY854375.

The PCR profile consisted of 4 min at 94°C following 35 cycles of 40 s at 94°C, 40 s at 56°C, 40 s at 72°C and finally, 4 min at 72°C. Each 25  $\mu$ l reaction contained 0.2 units of Taq polymerase (Bioline), 1 $\times$  kit-supplied PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.02% gelatine, 5% DMSO, 0.12 mM of each dNTP, 10 pmol of each primer and, approximately, 25 ng of genomic DNA. Sequencing reactions were carried out using the Big Dye 1.1 Terminator



**Fig. 1** Schematic illustration of part of an MHC class I gene of hawks and allies. The position of the primers used in this study is indicated by arrows

technology and labelled fragments were resolved in a 3100 automated sequencer (Applied Biosystems). DNA sequences were aligned and edited using the software BioEdit (Hall 1999). Primer design was tested using Oligo 6.0 (Molecular Biology Insights).

### Molecular cloning and sequencing analyses

Investigation of variation at MHC loci requires separating the different PCR amplification products, either because of the possibility of amplifying more than one locus, or because individuals are likely to be heterozygous for these loci. Cloning and sequencing protocols followed those described for raptor MHC class II genes (see Alcaide et al. 2007; Alcaide et al. 2008 for details). Polymorphism statistics within species were generated using the software DNAsp (Rozas et al. 2003). Putative amino acid sequences were obtained after alignment to the chicken BF1 gene (Shaw et al. 2007). The phylogenetic relationships of class I sequences were visualized through Neighbour Net networks built in the software Splitstree 4 (Huson and Bryant 2006).

### Inference of positive selection in the presence of recombination

Genetic hallmarks of positive selection at functionally important amino acid sites are identified from an excess of non-synonymous substitutions ( $d_N$ ) over synonymous substitutions ( $d_S$ ), where  $\omega = d_N/d_S > 1$ . The use of phylogenetic methods, such as those implemented in the PAML package (Yang 2000), are believed to cause high numbers of false positives when high levels of recombination are operating (Anisimova et al. 2003). The recently developed software OmegaMap (Wilson and McVean 2006) permits to infer positive selection in the presence of recombination. Previous analyses of positive selection at MHC class II sequences in lesser kestrels have already demonstrated that OmegaMap is less prone to overestimate the number of amino acid sites experiencing positive selection (see Alcaide et al. 2008 for details). Thus, we followed the same analytical protocol used for the kestrel class II data set to investigate diversifying selection at MHC class I sequences.

## Results

### Amplification of MHC class I fragments and complete exon 3 sequences

We successfully sequenced part of a genomic MHC class I fragment spanning exons 2–4 (~1.4 kb as estimated in

1.5% agarose gels) in one Spanish Imperial Eagle *Aquila adalberti* (EU120724), one Bearded Vulture *Gypaetus barbatus* (EU120725) and one White-backed Vulture *Gyps africanus* (EU120723). The length of introns 2 and 3 was estimated at about 1 kb and 75 bp, respectively (see Fig. 1). The length of intron 2 appears much longer in birds of prey than in the chicken BF1 and BF2 genes (228 bp, Shaw et al. 2007). However, the alignment with genomic class I sequences of the chicken, mallard and goose (see above) revealed that part of the intron 2 sequence flanking exon 3 is quite well conserved across different avian orders. Nevertheless, intron 3 sequences were quite divergent and exhibited an extremely high GC content (>70%), precluding optimal primer design and walking. We therefore designed a new degenerate primer (MHCI-int2F: CATTCCCTYGTGTTTCAGG) sitting in the conserved flanking region of intron 2, which we used in conjunction with the reverse primer MHCI-ex4R (see above). We predicted this approach would be successful given the small size of intron 3 (Fig. 1). Subsequently, we amplified a fragment of about 450 bp in all the species summarized in Table 1 using primers MHCI-int2F and MHCI-ex4R, following the same PCR protocol described above.

MHC class I polymorphism and gene duplications

The number of MHC sequences isolated per individual ranged from 1 to 4 as measured by cloning and sequencing of PCR amplicons. Consequently, the number of putative MHC class I loci amplified per species was estimated to range from 1 to 2. Genetic variation at several species of raptors is summarized in Table 1. The amino acid sequences of 23 class I alleles isolated from the Eurasian kestrel *Falco tinnunculus* are shown in Fig. 2. None of the DNA sequences reported here showed any signs of non-functionality, such as stop codons or frameshift mutations. On the other hand, the phylogenetic network constructed from the genomic class I sequences of birds of prey failed to identify any kind of orthologous relationships among putative class I loci, regardless whether we analyzed exon 3, the flanking sequence composed of intron 3 and part of exon 4, or the entire sequence spanning exons 3–4 (see Fig. 3).

Test of selection

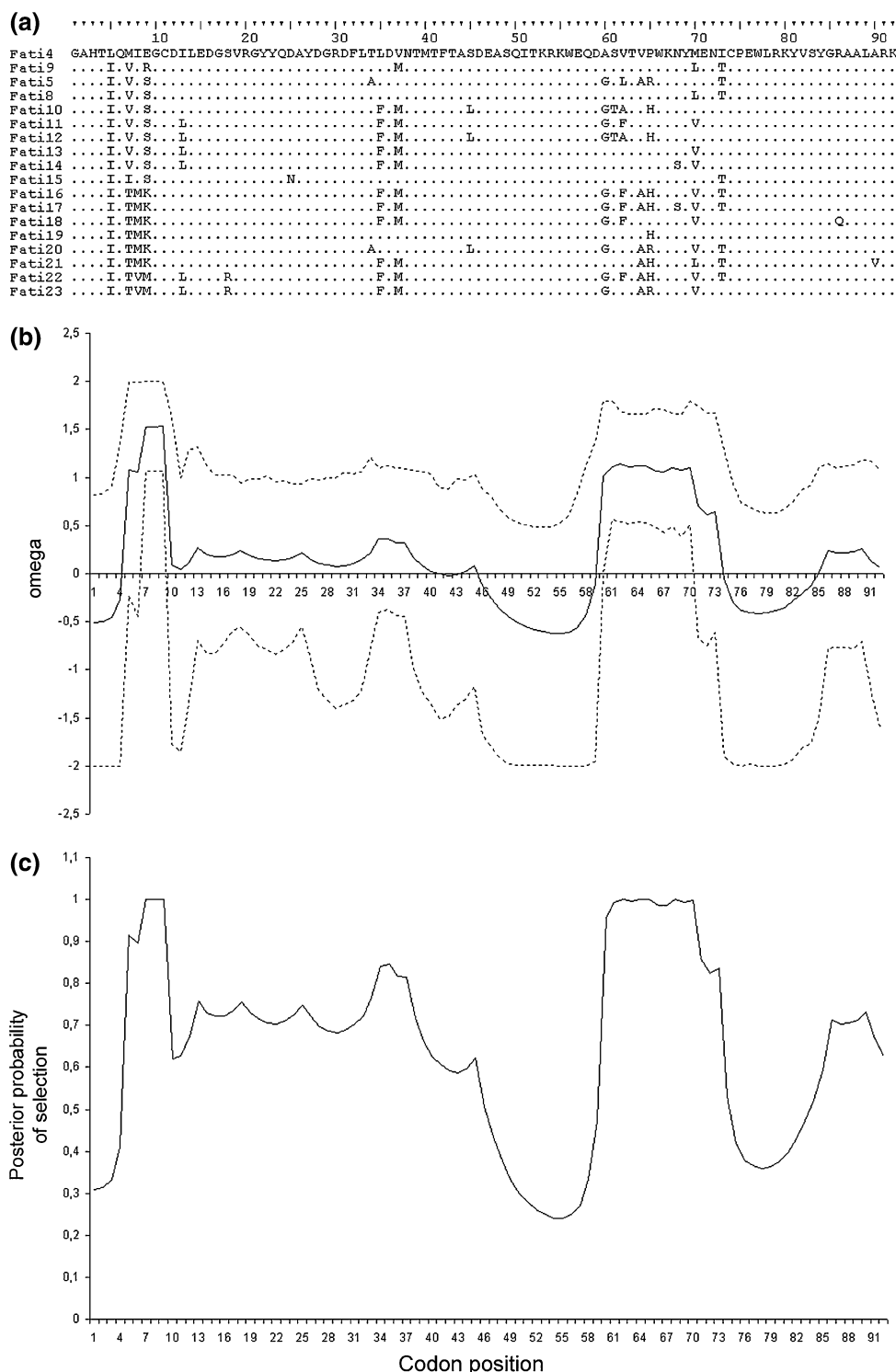
The mean value per codon of the selection parameter across the entire exon 3 was set at  $\omega = 3.82$ . The mean

**Table 1** Birds of prey in which exon 3 sequences from MHC class I loci have been isolated

Species Family	No. of clones analysed per individual	Exon 3 seqs (No. of individuals)	GenBank accession no	Species code	Country of origin
Eurasian Kestrel <i>Falco tinnunculus</i> Falconidae	6	23 (25)	EU120698-722	Fati	Spain
Lesser Kestrel <i>Falco naumanni</i> Falconidae	6	18 (25)	EU120664-79	Fana	Spain
Black-shouldered Kite <i>Elanus caeruleus</i> Accipitridae	8	4 (1)	EU120680-83	Elca	Spain
Spanish Imperial Eagle <i>Aquila adalberti</i> Accipitridae	8	9 (8)	EU120684-87	Aqad	Spain
Eurasian Black Vulture <i>Aegypius monachus</i> Accipitridae	8	4 (1)	EU120688-91	Aemo	Spain
Andean Condor <i>Vultur gryphus</i> Cathartidae	8	6 (8)	EU120692-94	Vugr	Argentina
Eagle Owl <i>Bubo bubo</i> Strigidae	1	1 (1)	EU120697	Bubub	Spain

The taxonomy follows Brooke and Birkhead (1991). The number of MHC haplotypes isolated and the number of individuals analysed per species are indicated. The codes here proposed will be employed for the naming of MHC sequences following the nomenclature recommended by Klein et al. (1990)

**Fig. 2** (a) Alignment of putative amino acid sequences of 23 class I (exon 3) alleles of the Eurasian Kestrel. *Dots* indicate identity with the top sequence. (b) Spatial variation in the logarithm of the selection parameter  $\omega$ . Parameter estimates were carried out in the software package OmegaMap using an objective set of prior distributions (Wilson and McVean 2006). The sitewise mean (solid line) and 95% HPD intervals (dotted lines) are shown. (c) Spatial variation in the posterior probability of positive selection

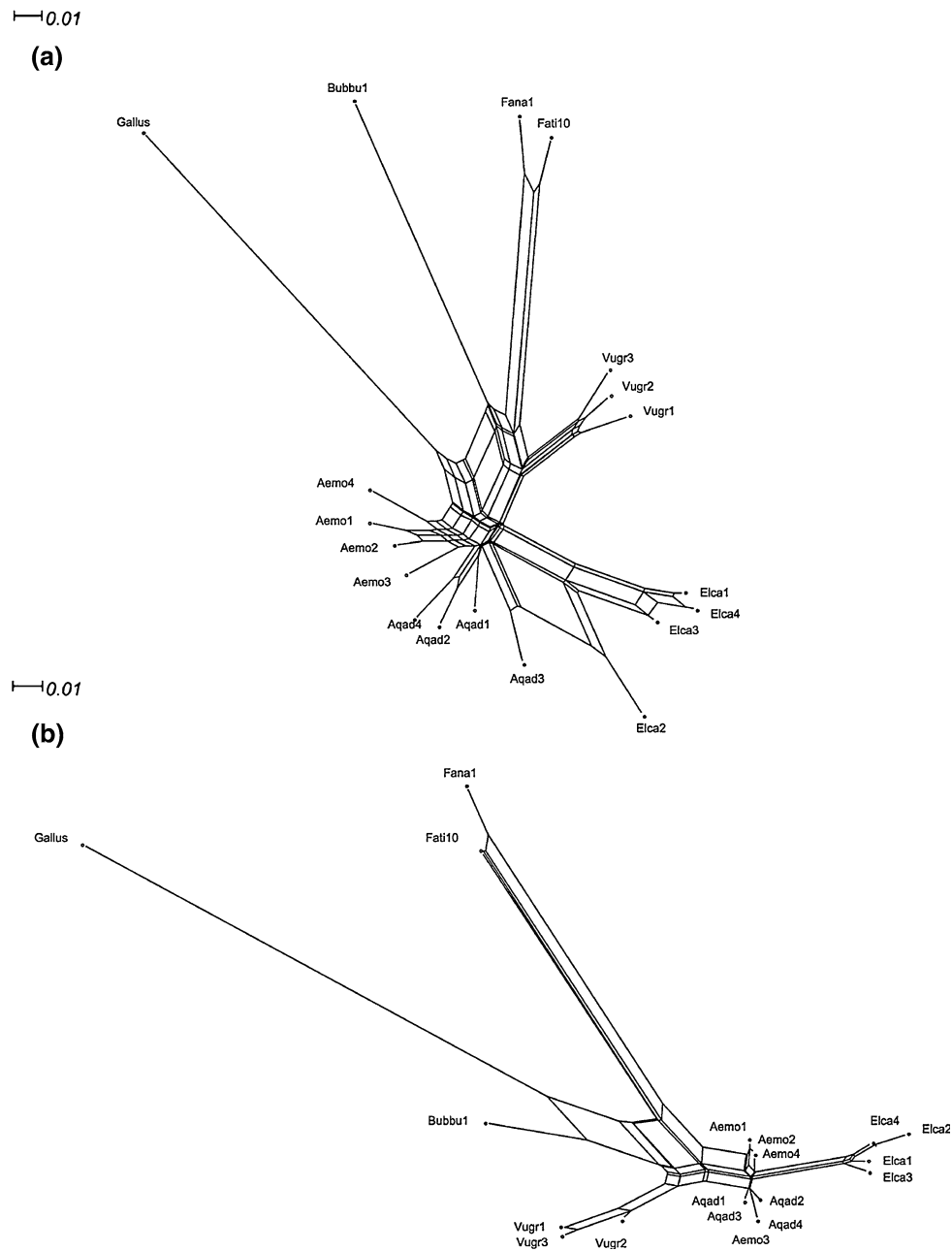


amount of population recombination per codon ( $\rho = 0.44$ ) showed to greatly exceed the mean amount of population mutation ( $\theta = 0.010$ ). Thus, this evidence for a predominant role of intragenic recombination and/or gene conversion during the evolutionary history of exon 3 suggests that inferring selection at this locus without also assuming recombination may be inappropriate.

**Discussion**

To our knowledge, this is the first study isolating and reporting polymorphism patterns in classical MHC class I genes of birds of prey and one of the very few studies of class I gene structure in non-model avian species. Based on our PCR approach, the genomic architecture and

**Fig. 3** A neighbor-net constructed from genomic class I sequences of the entire exon 3 (a) and intron 3 plus part of exon 4 (b) that were isolated in different species of birds of prey. The same sequence from the chicken *Gallus gallus* is used as outgroup



complexity of raptor MHC class I genes support previous data collected for class II genes (see Alcaide et al. 2007). Hence, the MHC of birds of prey seems closer to the relatively simple and compact MHC of the chicken (Kaufman et al. 1999) than to that of passerines. In this respect, we have found evidence for a low number of class I loci (1-2) in comparison to passerine species where genetic evidence for up to seven class I loci has been reported within the same individual (e.g. Witzell et al. 1999b; Westerdahl et al. 2004). However, our estimate is likely biased downward since PCR approaches might selectively amplify particular genes in multigene families (Wagner et al. 1994), and Southern blots would help resolve this issue

further (Westerdahl et al. 1999; Witzell et al. 1999a, b; Edwards et al. 2000). On the other hand, the lack of stop codons or frameshift mutations in any coding region suggests a low incidence of pseudogenes. Pseudogenes, as indicated by stop codons or disrupted open reading frames, have been commonly documented in passerines for both classes I and II (Westerdahl et al. 1999; Hess et al. 2000; Edwards et al. 2000) but appear almost absent in other avian groups (e.g. Kaufman et al. 1999; Ekblom et al. 2003; Alcaide et al. 2007).

Our survey of MHC polymorphism has revealed high genetic diversity in several raptor species. Ongoing research in other birds of prey such as the Egyptian vulture

*Neophron percnopterus* is currently reporting more than 10 class I alleles (Agudo and Alcaide, unpublished data). Positive selection at the same amino acid sites that are known to comprise the PBR of the human HLA-A2 molecule (Bjorkman et al. 1987) indicates that balancing selection is operating. Although we have not performed gene expression analyses in this study, other studies have generally observed a correlation between signatures for balancing selection and level of expression of MHC genes (Zoorob et al. 1990; Jacob et al. 2000). Finally, the lack of orthologous relationships among putative different class I loci support the concerted evolution hypothesis proposed for the avian MHC (see also Edwards et al. 1995; Witzell et al. 1999a; Alcaide et al. 2007), or possibly post-speciation gene duplication (Edwards et al. 1999). This latter possibility should be considered, since our taxon sampling was low, with large periods of time between speciation events in the tree.

In conclusion, the molecular methods and sequence data collected in this paper should contribute to a better understanding of the evolutionary significance and conservation implications of the MHC in birds of prey. Moreover, the primers designed for this study are targeting highly conserved regions across class I genes, and therefore, similar fragments in other avian groups are likely to be cross-amplified successfully. Given that MHC genes may decisively determine pathogen and parasite resistance (Edwards and Potts 1996; Hedrick 2001), this study may also aid in the preservation of genetic diversity in raptors.

**Acknowledgements** This study was supported by the MCyT (project CGL2004-04120) and the CSIC, which also provided research grants to M. Alcaide.

## References

- Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife immunogenetics? *Trends Ecol Evol* 21:433–438. doi:10.1016/j.tree.2006.05.010
- Alcaide M, Edwards SV, Negro JJ (2007) Characterization, polymorphism and evolution of MHC class II B genes in birds of prey. *J Mol Evol* 65:541–554. doi:10.1007/s00239-007-9033-9
- Alcaide M, Edwards SV, Negro JJ, Serrano D, Tella JL (2008) Extensive polymorphism and geographical variation at a positively selected MHC class II B gene of the lesser kestrel (*Falco naumanni*). *Mol Ecol* 17:2652–2665. doi:10.1111/j.1365-294X.2008.03791.x
- Anisimova M, Nielsen R, Yang Z (2003) Effect of recombination on the accuracy of the likelihood method for detecting positive selection at amino acid sites. *Genetics* 164:1229–1236
- Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC (1987) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* 329:512–518. doi:10.1038/329512a0
- Bollmer JL, Hernán Vargas F, Parker PG (2007) Low MHC variation in the endangered Galápagos penguin (*Spheniscus mendiculus*). *Immunogenetics* 59:593–602. doi:10.1007/s00251-007-0221-y
- Brooke M, Birkhead M (1991) *The Cambridge encyclopedia of ornithology*. Cambridge University Press, Cambridge
- Edwards SV, Potts WK (1996) Polymorphism of genes in the major histocompatibility complex: implications for conservation genetics of vertebrates. In: Smith TB, Wayne RK (eds) *Molecular genetic approaches in conservation*. Oxford University Press, Oxford, pp 214–237
- Edwards SV, Grahn M, Potts WK (1995) Dynamics of Mhc evolution in birds and crocodylians: amplification of class II genes with degenerate primers. *Mol Ecol* 4:719–729. doi:10.1111/j.1365-294X.1995.tb00272.x
- Edwards SV, Nusser J, Gasper J (2000) Characterization and evolution of major histocompatibility complex (MHC) genes in non-model organisms, with examples from birds. In: Baker AJ (ed) *Molecular methods in ecology*. Blackwell, Oxford, pp 168–207
- Edwards SV, Hess CM, Gaspar J, Garrigan D (1999) Toward and evolutionary genomics of the avian MHC. *Immunol Rev* 167:119–132
- Eklblom R, Grahn M, Höglund J (2003) Patterns of polymorphism in the MHC class II of a non-passerine bird, the great snipe (*Gallinago media*). *Immunogenetics* 54:734–741
- Garrigan D, Hedrick PW (2004) Class I MHC polymorphism and evolution in endangered California Chinook and other Pacific Salmon. *Immunogenetics* 53:483–489. doi:10.1007/s002510100352
- Gemmell NJ, Akiyama S (1996) An efficient method for the extraction of DNA from vertebrate tissues. *Trends Genet* 12:338–339. doi:10.1016/S0168-9525(96)80005-9
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
- Hedrick PW (2001) Conservation genetics: where are we now? *Trends Ecol Evol* 16:629–636. doi:10.1016/S0169-5347(01)02282-0
- Hedrick PW, Parker KM (1998) MHC variation in the endangered Gila Topminnow. *Evol Int J Org Evol* 52:194–199. doi:10.2307/2410934
- Hess CM, Gasper J, Hoekstra HE, Hill CE, Edwards SV (2000) MHC class II pseudogene and genomic signature of a 32-kb cosmid in the house finch (*Carpodacus mexicanus*). *Genome Res* 10:613–623. doi:10.1101/gr.10.5.613
- Huson H, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* 23:254–267. doi:10.1093/molbev/msj030
- Jacob JP, Milne S, Beck S, Kaufman J (2000) The major and a minor class II beta-chain (B-LB) gene flank the Tapasin gene in the B-F/B-L region of the chicken major histocompatibility complex. *Immunogenetics* 51:138–147. doi:10.1007/s002510050022
- Jarvi SI, Goto RM, Gee GF, Briles WE, Miller MM (1999) Identification, inheritance, and linkage of B-G-like and MHC class I genes in cranes. *J Hered* 90:152–159. doi:10.1093/jhered/90.1.152
- Kaufman J, Milne S, Göbel T, Walker BA, Jacob JP, Auffrey C et al (1999) The chicken B locus is a minimal-essential major histocompatibility complex. *Nature* 401:923–925. doi:10.1038/44856
- Klein J, Bontrop RE, Dawkins RL, Erlich HA, Gyllensten UB, Heise ER et al (1990) Nomenclature for the major histocompatibility complexes of different species: a proposal. *Immunogenetics* 31:217–219
- Moon DA, Veniamin SM, Parks-Dely JA, Magor KE (2005) The MHC of the duck (*Anas platyrhynchos*) contains five differentially expressed class I genes. *J Immunol* 175:6702–6712
- Piertney SB, Oliver MK (2006) The evolutionary ecology of the major histocompatibility complex. *Heredity* 96:7–21

- Richardson DS, Westerdahl H (2003) MHC diversity in two *Acrocephalus* species: the outbred Great reed Warbler and the inbred Seychelles warbler. *Mol Ecol* 12:3253–3259. doi:[10.1046/j.1365-294X.2003.02005.x](https://doi.org/10.1046/j.1365-294X.2003.02005.x)
- Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19:2496–2497. doi:[10.1093/bioinformatics/btg359](https://doi.org/10.1093/bioinformatics/btg359)
- Shiina T, Ando A, Imanishi T, Kawata H, Hanzawa K, Gojobori T et al (1995) Isolation and characterization of cDNA clones for Japanese quail (*Coturnix japonica*) major histocompatibility complex (*MhcCoja*) class I molecules. *Immunogenetics* 42:213. doi:[10.1007/BF00191227](https://doi.org/10.1007/BF00191227)
- Shaw I, Powell TJ, Marston DA, Baker K, van Hateren A, Riegert P et al (2007) Different evolutionary histories of the two classical class I genes BF1 and BF2 illustrate drift and selection within the stable MHC haplotypes of chickens. *J Immunol* 178:5744–5752
- Sommer S (2005) The importance of immune gene variability in evolutionary ecology and evolution. *Front Zool* 2:16. doi:[10.1186/1742-9994-2-16](https://doi.org/10.1186/1742-9994-2-16)
- Wagner A, Blackstone N, Cartwright P, Dick M, Misof B, Snow P et al (1994) Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. *Syst Biol* 43:250–261. doi:[10.2307/2413465](https://doi.org/10.2307/2413465)
- Wan QH, Zhu L, Wu H, Fang SG (2006) Major histocompatibility complex class II variation in the giant panda (*Ailuropoda melanoleuca*). *Mol Ecol* 15:2441–2450. doi:[10.1111/j.1365-294X.2006.02966.x](https://doi.org/10.1111/j.1365-294X.2006.02966.x)
- Westerdahl H, Wittzell H, von Schantz T (1999) Polymorphism and transcription of *Mhc* class I genes in a passerine bird, the great reed warbler. *Immunogenetics* 49:158–170. doi:[10.1007/s002510050477](https://doi.org/10.1007/s002510050477)
- Westerdahl H, Wittzell H, von Schantz T, Bensch S (2004) MHC class I typing in a songbird with numerous loci and high polymorphism using motif-specific PCR and DGGE. *Heredity* 92:534–542. doi:[10.1038/sj.hdy.6800450](https://doi.org/10.1038/sj.hdy.6800450)
- Wilson DJ, McVean G (2006) Estimating diversifying selection and functional constraint in the presence of recombination. *Genetics* 172:1411–1425. doi:[10.1534/genetics.105.044917](https://doi.org/10.1534/genetics.105.044917)
- Witzell H, Bernot A, Auffrey C, Zoorob R (1999a) Concerted evolution of two *Mhc* class II B loci in pheasants and domestic chickens. *Mol Biol Evol* 16:479–490
- Witzell H, Madsen T, Westerdahl H, Shine R, von Schantz T (1999b) MHC variation in birds and reptiles. *Genetica* 104:301–309. doi:[10.1023/A:1026421607089](https://doi.org/10.1023/A:1026421607089)
- Xia C, Hu T, Yang T, Wang L, Xu G, Lin C (2005) cDNA cloning, genomic structure and expression analysis of the goose (*Anser cygnoides*) MHC class I gene. *Vet Immunol Immunopathol* 107:291–302. doi:[10.1016/j.vetimm.2005.05.005](https://doi.org/10.1016/j.vetimm.2005.05.005)
- Yang Z (2000) Phylogenetic analysis by maximum likelihood (PAML), version 3.0. University College London, London
- Yuhki N, O'Brien SJ (1990) DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. *Proc Natl Acad Sci* 87:836–840
- Zoorob R, Behar G, Kroemer G, Auffrey C (1990) Organization of a functional chicken class II B gene. *Immunogenetics* 31:179–187