MOLECULAR ECOLOGY
RESOURCES

Molecular Ecology Resources (2010) **10**, 237–251 doi: 10.1111/j.1755-0998.2009.02788.x

INVITED TECHNICAL REVIEW

Methods for MHC genotyping in non-model vertebrates

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Abstract

Genes of the major histocompatibility complex (MHC) are considered a paradigm of adaptive evolution at the molecular level and as such are frequently investigated by evolutionary biologists and ecologists. Accurate genotyping is essential for understanding of the role that MHC variation plays in natural populations, but may be extremely challenging. Here, I discuss the DNA-based methods currently used for genotyping MHC in non-model vertebrates, as well as techniques likely to find widespread use in the future. I also highlight the aspects of MHC structure that are relevant for genotyping, and detail the challenges posed by the complex genomic organization and high sequence variation of MHC loci. Special emphasis is placed on designing appropriate PCR primers, accounting for artefacts and the problem of genotyping alleles from multiple, co-amplifying loci, a strategy which is frequently necessary due to the structure of the MHC. The suitability of typing techniques is compared in various research situations, strategies for efficient genotyping are discussed and areas of likely progress in future are identified. This review addresses the well established typing methods such as the Single Strand Conformation Polymorphism (SSCP), Denaturing Gradient Gel Electrophoresis (DGGE), Reference Strand Conformational Analysis (RSCA) and cloning of PCR products. In addition, it includes the intriguing possibility of direct amplicon sequencing followed by the computational inference of alleles and also next generation sequencing (NGS) technologies; the latter technique may, in the future, find widespread use in typing complex multilocus MHC systems.

Keywords: genotyping, major histocompatibility complex, non-model organisms

Received 17 June 2009; revision received 20 August 2009; accepted 3 September 2009

Introduction

The Major Histocompatibility Complex (MHC) is a gene-dense genomic region present in all jawed vertebrates, encoding, among others, proteins involved in the immune response (Kelley *et al*. 2005). Classical molecules of MHC class I and II are specifically involved in the presentation of antigens derived from intra- and extracellular pathogens to the effector cells of the immune system, initiating the adaptive immune response (Klein 1986; Janeway *et al*. 2004). Although other genes of the MHC complex are also of great functional significance (Kulski *et al*. 2002; Kumanovics *et al*. 2003; Kelley *et al*. 2005; Acevedo-Whitehouse & Cunningham 2006), MHC I and II have been the subjects of the majority of research in the fields of ecology and evolution (reviewed in Sommer 2005; Milinski 2006; Piertney & Oliver 2006). This is because MHC includes the most polymorphic genes in vertebrate populations, with hundreds of alleles identified at some human loci (more than a thousand at *HLA-B* locus, http://www.ebi.ac.uk/imgt/hla/stats.html). Furthermore, fitness consequences of MHC genetic variation and evolutionary patterns observed in these genes make them the paradigm of adaptive evolution at the molecular level (Hughes & Nei 1989b; Bernatchez & Landry 2003; Garrigan & Hedrick 2003; Kumanovics *et al*. 2003).

The enormous polymorphism of MHC genes is believed to be the result of both positive selection for amino acid replacements in codons involved in antigen binding (Peptide Binding Region, PBR or Antigen Binding Sites, ABS), and long-term retention of allelic lineages (trans-species polymorphism model) due to the action of balancing selection (Klein 1987; Hughes & Nei 1988; Takahata & Nei 1990; Garrigan & Hedrick 2003; Klein *et al*. 2007; Wegner 2008). Pathogens are likely the most important selective factor maintaining MHC

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variation as there is ample evidence for the role of individual MHC genotypes or MHC heterozygosity in susceptibility to infection and resistance to pathogens in animals and humans (e.g. Briles *et al.* 1983; Thursz *et al.* 1997; Penn *et al.* 2002; Grimholt *et al.* 2003; Wegner *et al.* 2003; Wedekind *et al.* 2004; Froeschke & Sommer 2005; Bonneaud *et al.* 2006; Oliver *et al.* 2009). Two mechanisms of balancing selection are believed to be the most important for maintaining MHC polymorphism – overdominance (Doherty & Zinkernagel 1975), in which heterozygotes derive their superiority from the ability to present a wider spectrum of antigens, and negative frequency dependent selection, where rare alleles gain a selective advantage and follow the Red Queen prediction (Snell 1968; Borghans *et al.* 2004). The relative significance of these mechanisms as well as the role of demographic factors vs. natural selection in shaping MHC diversity are not sufficiently understood (Apanius *et al.* 1997; Hedrick 2002; Bernatchez & Landry 2003; Borghans *et al.* 2004; Piertney & Oliver 2006). Also, the role of MHC in conservation is a subject of controversy (O'Brien & Evermann 1988; Hughes 1991; Edwards & Potts 1996; Hedrick 2002), as it is still unclear whether reduced MHC variation indeed increases the risk of extinction or negatively affects population viability (Radwan *et al.* 2009). The role of MHC in reproduction and sexual selection may be an additional factor shaping high MHC variation (Milinski 2006). MHC has been invoked in maternal-foetal interactions (Thomas *et al.* 1985; Hedrick & Thomson 1988), in mate choice through inbreeding avoidance (Penn & Potts 1999; Penn 2002), search for genetic compatibility and in providing offspring with an optimal MHC repertoire (Milinski *et al.* 2005; Eizaguirre *et al.* 2009). An understanding of the role of MHC in these processes is far from complete, partly because information is restricted to humans and a few laboratory species, and the results from these are sometimes contradictory.

Thus, although much effort has been devoted to the study of MHC in the context of evolutionary ecology and population biology, many issues remain unresolved, leaving MHC the subject of active and growing research. Efficient and reliable genotyping is a prerequisite for understanding the role and significance of the MHC, but genotyping may be a surprisingly difficult task. Target loci are commonly present at multiple copies, and allele sequences even at a single locus may be very divergent, making the identification of all alleles carried by an individual and reconstructing its multilocus genotype challenging. The presence of both expressed loci and pseudogenes poses additional difficulties in identifying functional variants. Large scale genotyping is sometimes undertaken without sufficient understanding of the architecture of the system, due to the lack of background information, which could, in principle, be relatively easily obtained. Genotyping techniques themselves may introduce spurious variation (artefacts) which need to be identified and eliminated. The reader, particularly a novice in the field of MHC research should be aware of the pitfalls associated with MHC genotyping and I will try to emphasize the potential problems throughout this review. I will start with a brief discussion of the aspects of MHC structure relevant for genotyping. I will then present challenges that the idiosyncratic features of the MHC may pose in this respect, describe techniques currently used for genotyping, compare their usefulness in various situations, discuss strategies for efficient genotyping and finally try to outline what I consider the likely areas of progress in the future. While emphasizing the need for the background work on MHC genomic structure and expression as the basis for the more informed choice of the genotyping methods, I will not cover de novo characterization of full MHC sequences. This step, although clearly essential and necessary for a thorough understanding of the structure and function of MHC and forming the basis of the development of efficient genotyping methods, requires very different techniques and is beyond the scope of this review.

The emphasis will be put on PCR-based DNA genotyping techniques (both genomic and cDNA), as these are the only methods likely to be widely used for MHC typing in non-model vertebrates in the foreseeable future. For this reason most of the techniques which have been important in typing human MHC and crucial for transplantology, e.g. serological techniques, will not be covered here (Susskind 2007). Non-DNA based MHC typing methods have been used in a handful of non-model organisms (O'Brien *et al.* 1985; Jarvi & Briles 1992), however their resolution is not comparable to DNA-based typing and therefore these techniques will not be covered either. I will refer to methods used for Human Leukocyte Antigen (HLA) typing only in cases in which they can be potentially used for MHC typing in non-model vertebrates.

Structure of the MHC molecules determines genotyping targets

The parts of MHC proteins involved in peptide binding determine the functional differences between allelic variants and as such set the most frequent genotyping targets in non-model organisms. The MHC I molecule consists of a single transmembrane chain with three extracellular domains: α1, α2 and α3 which together with β2 microglobulin (monomorphic and coded outside MHC) form the complete MHC I molecule (Janeway *et al.* 2004). Domains α1 and α2 are coded by exons 2 and 3, form the antigen binding pocket, and contain the ABS sites (Bjorkman *et al.* 1987; Chelvanayagam 1996; Janeway *et al.* 2004). Consequently, exons 2 and 3 are the most

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variable and most commonly typed parts of the MHC I genes. The MHC II molecule is a heterodimer consisting of transmembrane chains α and β coded by distinct genes. The peptide binding pocket is formed by the α1/β1 domains encoded by second exons of these genes, which are thus the most variable and most often typed fragments of MHC II genes (Brown *et al.* 1993; Stern *et al.* 1994; Tong *et al.* 2006; Bondinas *et al.* 2007).

Variation in MHC genomic structure and modes of evolution complicate genotyping

Four factors pose significant challenges in MHC genotyping: (i) recent duplications, (ii) varying degree of concerted evolution, (iii) variation among haplotypes in the number of loci and (iv) the presence of MHC pseudogenes. In such situations the researcher is commonly forced to resort to multilocus genotyping as the design of locus-specific PCR primers is not possible; simultaneous analysis of genomic and cDNA is therefore frequently needed in order to distinguish pseudogenes from functional loci.

Major histocompatibility complex class I and II genes are present in all jawed vertebrates, but the structure of the complex may vary considerably (Kelley *et al.* 2005). Both classes are usually linked in a single chromosomal region but there are exceptions even to this rule – for example in teleosts MHC I and II genes are located on different chromosomes (Sato *et al.* 2000; Sambrook *et al.* 2005; Schaschl & Wegner 2007). Although certain structural features of MHC molecules are conserved across all vertebrates (Trowsdale 1995), orthology between genes is not conserved across groups. Mammal MHC I genes are characterized by a relatively short life span and retain orthologous relationships within orders, but not between them (Hughes & Nei 1989a). On the contrary, orthologous relationships of various MHC II genes are usually retained across mammals (Kumanovics *et al.* 2003), although deletions of some genes and expansions of others occur, due to recent duplications in various mammalian groups. Concerted evolution in multigene families and birth and death processes may contribute to this pattern (Hess & Edwards 2002; Nei & Rooney 2005). In non-mammalian vertebrates individual MHC genes appear to be generally short lived as manifested by the lack of orthology among loci within MHC I and MHC II (Nei *et al.* 1997; Kumanovics *et al.* 2003). In passerine birds for example, orthologous relationships of MHC II genes are not retained even on short evolutionary timescales, and sequences from various loci cluster in a species-specific manner (Westerdahl 2006). This indicates substantial concerted evolution, causing relative homogenisation of DNA sequences across loci in these birds (Hess & Edwards 2002). The effects of the extraordinary evolutionary dynamics of MHC, frequent recombination, duplications, etc., may cause differentiation in gene composition even within species – haplotypes differing in the number of loci have been described for a number of species (Ellis *et al.* 2005; Bontrop 2006). In the California sea lion and in the rhesus macaque extensive polymorphism results from the high number of loci characterized by low polymorphism, combined into a variety of haplotypes differing in locus composition (Bowen *et al.* 2004; Otting *et al.* 2005); a similar situation may occur in the axolotl MHC I (Sammut *et al.* 1999). Extreme, even hundredfold multiplication can occur, and profound differences between relatively closely related species have been described in mole rats and the African pygmy mouse (Vincek *et al.* 1987; Delarbre *et al.* 1992).

Another factor which may substantially complicate MHC genotyping is the presence of pseudogenes. Because most evolutionary and ecological studies place emphasis on the variation at functional MHC genes, the presence of pseudogenes may confound results. Pseudogenes themselves may provide important insights (Aguilar *et al.* 2005; Babik *et al.* 2009a), but their status as pseudogenes should first be unambiguously established. Sometimes the presence of frameshift or nonsense mutations in coding regions allows for their identification. However, a generally applicable approach to the pseudogene identification would be a comparison of genotypes obtained from RNA (cDNA) and genomic DNA. RNA should be extracted from tissues in which the MHC class of interest is expressed. Most tissues would be appropriate for the analysis of class I genes, expressed in all nucleated cells, but class II genes are expressed mainly in lymphocytes and other antigen presenting cell so their expression can be most easily detected in lymphoid organs (thymus, spleen, bone marrow) and blood (Janeway *et al.* 2004). Verification of the expression status of the detected alleles through typing of cDNA from a subset of individuals should be a standard in studies involving MHC genotyping. Genotyping techniques discussed below can be applied for this purpose.

RFLP – a non PCR DNA-based genotyping method

Historically, the first DNA-based technique used for MHC typing was hybridization of radioactively labelled probes obtained from cloned MHC genes to the total DNA digested by restriction enzymes and fractionated by gel electrophoresis (Restriction Fragment Length Polymorphism, RFLP). Developed in the eighties, this methodology has also been used with various modifications for non-model vertebrates, e.g. mole rat (Ben-Shlomo *et al.* 1988), beaver (Ellegren *et al.* 1993), salmon (Langefors *et al.* 2000) and particularly birds (Wittzell *et al.* 1994, 1999; Westerdahl *et al.* 1999; Freeman-Gallant *et al.* 2002; Miller

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& Lambert 2004; Kikkawa *et al.* 2009). Because the probes are typically hundreds of base pairs long, RFLP is immune to the problems of designing optimal PCR primers amplifying all alleles present in the sample. However the method has serious limitations: it is not suitable for typing of variation at high resolution due to the examination of only a fraction of nucleotide positions, it suffers from problems related to interlocus cross-hybridization of probes and requires large amounts of high quality DNA, which is difficult to obtain nondestructively. RFLP is also time consuming and low-throughput, which makes its use cumbersome and not generally suitable for larger scale genotyping (i.e. hundreds or thousands of samples). Several studies have actually used RFLP in combination with other techniques such as DGGE and cloning and sequencing of PCR products to characterize variation at the nucleotide level (Langefors *et al.* 2000; Bonneaud *et al.* 2004).

Whereas in actual genotyping RFLP has been mostly surpassed by PCR based methods, it remains extremely valuable at the initial stages of the MHC research in non-model species. RFLP, which may be combined with more quantitative dot-blot assays, provides approximate information about the number of loci (e.g. Delarbre *et al.* 1992; Edwards *et al.* 1999; Westerdahl *et al.* 1999), thus giving the researcher an idea about the complexity of the system, and may sometimes help in resolving haplotypes (Wittzell *et al.* 1994). Such background information is essential for choosing the appropriate genotyping strategy and should be encouraged whenever possible before leaping into large scale genotyping.

PCR-based methods

PCR-based methods are most widely used at present and are very likely to remain in widespread use in the foreseeable future. Irrespective of the MHC genomic features in a given species, determining whether: (1) single-locus typing, (2) multilocus typing with subsequent assignment of alleles to individual loci, or (3) multilocus typing without assigning alleles to loci would be adopted, there are two essential stages in PCR-based genotyping:

- Identification of primers reliably amplifying the entire variation the researcher is interested in,
- Accurate identification of variants amplified with these primers – detection of all true variants and elimination of artefacts, i.e. actual genotyping.

Primer design

For most non-model organisms used in ecological and evolutionary studies, extensive genomic resources and detailed information on the genomic organization of the MHC are lacking. In such cases, regardless of the method used for actual genotyping, the critical step required for the study of MHC polymorphism is identification of PCR primers amplifying all allelic variants of interest, usually all alleles present at a given locus or a well defined subset of alleles from single or multiple loci. This aspect is commonly underappreciated by investigators, who often use primers applied previously in more or less closely related species which may not amplify all variation present in the species of interests. In the case of single-locus amplification in diploids, basic population genetic signatures such as the excess of homozygotes and the presence of multiple types of homozygotes in population samples would help to identify and discard non-optimal primers. However, when multilocus amplification is necessary and/or haplotypes differ in the number of loci, then without rigorous assessment of the quality of primers, a substantial, or perhaps even more importantly, an unknown fraction of variation may be missed, resulting in unreliable genotyping and hence casting doubt the results of the study. Therefore, the importance of a careful selection of PCR primers in MHC studies cannot be overstated.

Despite very high polymorphism, MHC I and II molecules have certain conserved amino acid residues, even in the putative ABS (Kaufman *et al.* 1994; Hashimoto *et al.* 1999; Furlong & Yang 2008). For the initial characterization of MHC sequences these regions may be used to design 'universal' primers of broad specificity, often accommodating degenerate bases to allow for nucleotide polymorphisms sometimes encountered even in these conserved regions (Gyllensten *et al.* 1990; Hashimoto *et al.* 1990; Edwards *et al.* 1995; Mikko *et al.* 1999). Such primers are extremely useful for initial isolation of MHC sequences and may represent an attractive alternative for hybridization-based screening of cDNA libraries (Hashimoto *et al.* 1990). However, for studies of polymorphism, in which obtaining the complete spectrum of variation is critical, additional approaches to primer design should be considered. Initial successful amplification may encourage researchers to exclusively rely on primers which in fact amplify only a fraction of variants present in a given species, leading to unreliable genotyping. Therefore direct characterization of sequences of primer binding sites in many allelic lineages in a species of interest is always desirable (Edwards & Potts 1996). Genome walking techniques such as vectorette PCR may useful in this respect (Babik *et al.* 2008, 2009a; Biedrzycka & Radwan 2008). This technique enables the straightforward sequencing of genomic regions flanking a DNA segment of the known sequence (Arnold & Hodgson 1991; Ko *et al.* 2003).

Strategies for minimizing PCR artefacts

Various types of artefacts may be produced during a PCR reaction or in subsequent genotyping procedures.

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First, thermostable DNA polymerases are characterized by a relatively high error rate of the order 10^{-4}–10^{-6} per bp per replication (Cline *et al.* 1996). These kinds of errors result in point mutations (substitutions) which should appear more or less randomly throughout the sequence. Unless these occur at very early cycles of amplification and the starting amount of template is low, this type of error should not pose significant challenges for most genotyping methods, with the possible exception of cloning-based approaches; artefacts would differ only slightly from true alleles and should be relatively easy to eliminate (Acinas *et al.* 2005). Second, chimaeras are formed when incompletely elongated PCR products hybridize to templates and serve as PCR primers (reviewed in Kanagawa 2003). In PCR reactions containing multiple variants of template it is likely that an incompletely elongated 'primer' will in fact be derived from an allele differing from the template, and as a result a chimeric sequence will be formed. Third, heteroduplexes arise from the annealing of two strands derived from different alleles. These may result in the production of mosaics when cloned into bacteria due to the action of the bacterial mismatch repair systems (see below).

There is an extensive literature reporting PCR artefacts and presenting recommendations to minimize them (Zylstra *et al.* 1998; Qiu *et al.* 2001; Thompson *et al.* 2002; Kanagawa 2003; Acinas *et al.* 2005), also in the MHC genotyping context (Ennis *et al.* 1990; L'Abbe *et al.* 1992; Judo *et al.* 1998; Longeri *et al.* 2002; Jarvi *et al.* 2004; Lenz & Becker 2008). Recommendations, summarized by Lenz & Becker (2008), include: increased elongation time which minimizes the proportion of incompletely elongated products (these may serve as primers leading to the formation of chimeras), reconditioning PCR, which entails using an aliquot of the PCR reaction in a second PCR reaction with a low number of cycles and thus readjusting the primer to amplicon ratio, reducing the frequency of heteroduplexes, and avoiding the use of high fidelity polymerases which, paradoxically, seem to increase the frequency of artefacts (Judo *et al.* 1998; Zylstra *et al.* 1998). However, the most important factor reducing the incidence of PCR artefacts in multitemplate PCRs appears to be reducing the number of PCR cycles (Judo *et al.* 1998; Zylstra *et al.* 1998; Acinas *et al.* 2005; Lenz & Becker 2008), as the proportion of artefacts increases dramatically in later cycles. The general recommendation is that the number of PCR cycles should be the lowest possible which still enables analysis. In the case of amplification from vertebrate genomic DNA, 25–28 cycles seem reasonable and easily attainable (Lenz & Becker 2008).

Regardless of the precautions applied to reduce the frequency of artefacts, generally only alleles obtained in two independent PCR reactions are regarded as confirmed in MHC studies (e.g. Babik *et al.* 2005; Marsh *et al.* 2005), although even this approach might be too liberal (Lenz & Becker 2008).

Genotyping methods based on interrogation of known variation

In situations when all or almost all of the allelic variation in a given species or population is known, it is possible to design assays to distinguish all sequence variants and thus provide genotypes of samples. An enormous amount of work has been devoted to the typing of human MHC (HLA) using such methods, as it is crucial in transplantology (Susskind 2007). PCR with Sequence Specific Primers (PCR-SSP) is a very popular method based on the use of a panel of primers that amplify specific alleles or allele groups (Olerup & Zetterquist 1992; Bunce *et al.* 1995). Low to high resolution is possible. Typing is performed simply by multiple PCR amplifications with various primer combinations and checking for the presence of a PCR product on a gel. Internal amplification control is included in each reaction. This method requires a large number of primers for high resolution typing of highly polymorphic loci and may be time consuming. Another popular method based on interrogation of known variation is Sequence-Specific Oligonucleotide Probe (SSOP) hybridization, in which an amplicon obtained with generic primers is hybridized with allele-specific or allele-group specific oligonucleotide probes, afterwards a signal may be detected with a variety of methods (Leffell 2002).

Both PCR-SSP and SSOP methods have been commercialized and enable quick and cost-efficient genotyping of a large number of clinical samples. However, the development of such techniques capitalizes heavily on the unprecedented level of information on HLA available for model organisms. Consequently, these kinds of techniques have only rarely been used for the genotyping of non-model vertebrates. PCR-SSP has been applied to MHC I of the woodchuck (Zhou *et al.* 2003) and MHC II of the California sea lion (Bowen *et al.* 2004, 2006), in the latter case by means of real-time PCR instead of standard agarose gel electrophoresis detection. Outside the realm of HLA genotyping, SSOP has been used e.g. in typing of dog MHC (Kennedy *et al.* 1999, 2000). In non-model species this technique was applied for MHC II typing in the common marmoset (Antunes *et al.* 1998).

The logical extension of the SSOP approach is microarray analysis, which should enable high throughput parallel genotyping of multiple alleles and loci. However, the extreme polymorphism of the HLA led to considerable difficulties with establishing universally applicable microarrays and research is still ongoing (Zhang *et al.* 2005; Lee *et al.* 2008; Feng *et al.* 2009).

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A technique that may be useful for typing known variation as well as for *de novo* mutation detection is PCR-RFLP, where variation in MHC amplicons is detected by restriction enzyme digestion. As all PCR based methods, PCR-RFLP requires only a minute amount of DNA, and is fast and cheap. However its inherent limitation is the ability to interrogate only a fraction of sequence variation, and therefore PCR-RFLP has found only limited applications for MHC genotyping in non-model species (Wenink *et al.* 1998; Bak *et al.* 2006).

Conformation-based mutation detection methods

This family of techniques has been very popular in the studies of MHC variation. All methods from this category are able to detect previously unknown variation at the single base resolution, are relatively inexpensive and universally applicable. However, newly identified variants must be further characterized via sequencing.

Single Strand Conformation Polymorphism. Single Strand Conformation Polymorphism (SSCP) (Orita *et al.* 1989) is a technique frequently used for MHC genotyping in non-model vertebrates. It is widely applied for mutation screening and its use in molecular ecology was reviewed by Sunnucks *et al.* (2000). The method is based on the observation that in non-denaturing conditions the electrophoretic mobility of single stranded DNA fragments is conformation-dependent and the conformation is determined by the DNA sequence. The PCR product is denatured and then rapidly cooled; during this process single stranded molecules assume a sequence-specific conformation and then are electrophoresed under non-denaturing conditions. Electrophoresis is carried out at a low temperature in polyacrylamide or synthetic pre-cast gels (Hedrick *et al.* 1999; Sommer *et al.* 2002; Babik *et al.* 2008; Biedrzycka & Radwan 2008). The resulting bands are then visualized via autoradiography, silver or SYBR Gold staining (Oto *et al.* 1993). An obvious advantage of gel-based SSCP is the possibility of excising bands corresponding to individual alleles, reamplification and direct sequencing in order to obtain sequences of individual alleles (Sommer *et al.* 2002; Babik *et al.* 2008). Capillary electrophoresis of the fluorescently labelled amplicons in automated DNA sequencers (CE-SSCP) is also commonly employed (Binz *et al.* 2001; Lento *et al.* 2003; Babik *et al.* 2005, 2008, 2009a; Bryja *et al.* 2005; Schaschl *et al.* 2008). Each PCR primer is labelled with a different fluorescent dye, therefore strands can be distinguished and detection of the sequence differences which alter the mobility of only one strand is possible. Automated DNA sequencers provide a high level of control over temperature and electrophoretic conditions ensuring reproducibility and enable high throughput. The disadvantage of this approach is the lack of the possibility of excising bands. Therefore, sometimes gel-based and CE-SSCP are combined in studies of MHC polymorphism (Babik *et al.* 2008, 2009a; Biedrzycka & Radwan 2008).

The SSCP method is most suitable for fragments of 100–300 bp, in which it is able to detect the overwhelming majority of single-base substitutions, however its sensitivity drops markedly above 300 bp (Sunnucks *et al.* 2000). Multiple factors such as sieving matrix composition and concentration, run temperature and current characteristics may affect the resolution of SSCP (Sunnucks *et al.* 2000; Kukita *et al.* 2002); usually some optimization is needed for establishing optimal SSCP conditions under which maximum resolution is achieved. Excellent results have been reported even with the gel-based SSCP, purported to be less sensitive (Garrigan & Hedrick 2001; Noakes *et al.* 2003). Moreover it should be noted that differences between alleles are commonly larger than 1 bp, and hence CE-SSCP is perfectly suitable for rapid, low-cost screening of a large number of samples with minimum optimization (e.g. Radwan *et al.* 2007).

The main problem with SSCP is common to all conformation-based techniques (see below), i.e. these methods become successively more problematic if alleles from multiple loci co-amplify in the sample. In such cases gels/chromatograms become difficult to interpret because bands representing various alleles may overlap due to similar mobility, and if amplification of some alleles is less efficient, then these alleles are very likely to be missed due to the confounding effect of an elevated background (Babik *et al.* 2009a; Lenz *et al.* 2009). Also, in cases when individual bands must be excised, most of the sequences resulting from amplification of bands may be mixed and thus not useable.

Denaturing Gradient Gel Electrophoresis & Temperature Gradient Gel Electrophoresis. Denaturing Gradient Gel Electrophoresis (DGGE) (Fisher & Lerman 1983; Myers *et al.* 1987; Knapp *et al.* 1997) is one of the most popular techniques in MHC genotyping. The rationale behind this method is that double stranded DNA molecules exhibit sequence-dependent denaturation characteristics. Double-stranded DNA fragments are electrophoresed in an acrylamide gel along a denaturing gradient formed of formamide and urea. When a DNA molecule begins to denature its mobility is altered and its migration rate decreases. Molecules differing in sequence will thus occupy various positions in a gel, forming discrete bands. A GC clamp at the 5′ end of one of the amplification primers is often used to avoid complete denaturation which would result in poor resolution because fully

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denatured DNA fragments of the same length will have equivalent mobility (Sheffield *et al.* 1989; Knapp 2005). DGGE has several advantages, including a broad range of fragment sizes over which mutations can be resolved (100–1000 but typically up to 500 bp, Knapp 2005) and the repeatability of banding patterns under identical conditions. For example, particular haplotypes can be distinguished by eye on the basis of DGGE patterns in macaques (Doxiadis *et al.* 2000). The greatest apparent advantage of this technique is that it typically produces a single band per allele, this band can be excised, reamplified and sequenced, thus revealing the allele sequence without cloning (e.g. Middleton *et al.* 2004). The method seems more advantageous in this respect than SSCP, especially for multilocus systems, because each allele produces two SSCP bands (plus possibly, some additional bands representing alternative conformations) resulting in more complex gel pictures. The combination of DGGE and direct sequencing of bands is in some situations claimed to be superior to cloning (Middleton *et al.* 2004). On the down side, the following issues should be mentioned: (i) individual banding patterns on DGGE can be extremely complex if multiple MHC loci are amplified in a single PCR reaction (Knapp 2005), although using DGGE has been used to to separate as many as seven MHCII-DRB (the most polymorphic β chain gene in mammals) alleles in a single rhesus macaque (Knapp *et al.* 1997); (ii) considerable initial optimization effort is usually required regarding gel concentration, denaturing gradient and electrophoresis time and voltage (Hayes *et al.* 1999; Miller *et al.* 1999; Knapp 2005); (iii) formation of heteroduplexes may be a problem because they show distinct denaturation profiles and may be erroneously scored as new alleles or misscored if their mobility is identical to that of some of the true alleles. Lastly, co-migration of bands representing different alleles would also compromise the resolution of DGEE (Knapp 2005).

Denaturing Gradient Gel Electrophoresis may be classified as a medium throughput method with typically tens to a few hundred individuals typed (Miller & Lambert 2004; Westerdahl *et al.* 2004; Bonneaud *et al.* 2006; Ekblom *et al.* 2007), although typing up to 20,000 samples has been reported (Miller *et al.* 1999) and limited multiplexing is possible (Miller *et al.* 2001). DGGE was found to be superior to RFLP in terms of the resolution in a study of Atlantic salmon (Langefors *et al.* 2000).

Temperature Gradient Gel Electrophoresis (TGGE) is a technique very similar to DGGE, but a temperature gradient replaces the denaturant gradient. This technique has been used in the Australian bush rat (Seddon & Baverstock 1999); a direct comparison in the study of MHC in salmons suggests its inferiority to DGGE (Miller *et al.* 1999), but there is not enough comparative data to fully evaluate the utility of this method for MHC genotyping.

Reference Strand Conformational Analysis. Reference Strand Conformational Analysis (RSCA) (Arguello *et al.* 1998) is based on the hybridization of sequences present in an amplicon to a fluorescently labelled reference (FLR) strand, and this method thus requires an automated DNA sequencer. The mobility of the resulting heteroduplex depends on the number and distribution of mismatches between the allele and the FLR and forms the basis for allele discrimination. FLRs are obtained by PCR amplification of a single allele (homozygote or cloned sequence) with a fluorescently labelled primer. The advantage of the method is the possibility of using more than one FLR, which increases the potential for distinguishing all or almost all alleles in a single set of experimental conditions. This sort of flexibility is not provided by SSCP. However, RSCA requires some initial knowledge about the study system, sufficient at least to produce several FLRs reasonably reflecting the variety of allelic lineages present in a given organism. Many divergent FLRs are usually tested before a few are finally chosen for large scale genotyping. Lenz *et al.* (2009) discuss factors which should be considered during the choice of FLRs. Their rigorous study on sticklebacks, a fish with complex MHC II consisting of several recently multiplicated loci which co-amplify and have to be genotyped together, demonstrated the utility of RSCA for typing of such complex MHC systems; RSCA results were confirmed by cloning, and only 0.2% of 3403 allele combinations were indistinguishable with three applied FLRs. However, gel-based SSCP outperformed RSCA for genotyping variation at a single MHC locus in a salmonid (Noakes *et al.* 2003). An important drawback of RSCA is that direct characterization of new alleles by excision of bands from the gel, reamplification and sequencing is not possible, and thus RSCA must be combined with a sequence-based technique.

Microsatellites for MHC genotyping

An interesting approach for MHC genotyping utilizes tightly linked polymorphic microsatellite loci located, for example, in the 3′ untranslated region or introns of MHC genes (e.g. Schwaiger *et al.* 1993; Meagher & Potts 1997; Hansen *et al.* 2007). The advantages of this approach include the availability of very simple and reliable methods for scoring differences in microsatellite allele length and the possibility of rapid processing of a large number of samples. The obvious disadvantage is the necessity of identifying the suitable microsatellite(s) and reliable data on the linkage of individual MHC and microsatellite alleles.

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Techniques involving DNA sequencing

Sequence-based typing. Sequence-based typing (SBT) is widely used for MHC genotyping in humans and domesticated animals (McGinnis *et al.* 1995; Kennedy *et al.* 2002; Miltiadou *et al.* 2003; Livant & Ewald 2005). Amplicons obtained with locus-specific or allele-group specific primers are directly sequenced. Heterozygous chromatograms are then interpreted in terms of diploid genotypes through a comparison with the database of all allelic combinations, for which special tools are available (Helmberg *et al.* 2004). Thus, the utility of SBT is again based on extensive knowledge of the polymorphism in humans and domesticated animals. A study by Bos *et al.* (2007) indicates that SBT may be applicable also to non-model organisms because it is often possible to correctly reconstruct haplotypes from diploid chromatograms containing heterozygous positions using the Bayesian inference method implemented in PHASE (Stephens *et al.* 2001; Stephens & Donnelly 2003). Although the non-neutrally evolving highly polymorphic MHC sequences clearly violate the assumptions of the coalescent model underlying PHASE, the method performed well in the analysis of real MHC data and was almost insensitive to the choice of priors. Not surprisingly, performance of the method was poorer for a dataset in which the number of heterozygous positions was high compared to sample size and virtually no homozygotes were observed. The results of the study by Bos *et al.* (2007) as well as other studies (Bos *et al.* 2008; Mona *et al.* 2008) are encouraging and indicate that Sanger sequencing together with computational inference of alleles may find widespread utility in non-model organisms in which the design of locus specific primers is possible. The clear advantage of this method is the single-base resolution and straightforward setup of the analyses which does not require extensive testing of experimental conditions. It should be particularly useful in situations in which variation is very high because it eliminates the time-consuming and costly validation of results of mutation detection methods. However, this approach is not applicable to multi-locus cases.

Cloning and sequencing of PCR products. Cloning of PCR products is an established procedure for the typing of multiallelic templates. An amplicon is ligated into a vector of choice, introduced into bacteria, and then inserts from individual colonies derived from a single DNA molecule are sequenced. Cloning and sequencing provide phase information and theoretically may be used for amplicons of any complexity. However there are two issues: (i) in addition to PCR artefacts, additional artefacts may be introduced by bacterial mismatch repair systems (see above), (ii) typically a large number of clones need to be sequenced for accurate genotyping and elimination of artefacts, consequently cloning is a labour intensive and costly procedure. This technique has been widely used in MHC studies and is likely to remain essential for initial assessment of variation and establishing allele sequences. The method is a crucial step in the development of some typing techniques such as RSCA in which cloned alleles usually serve as a template for the production of FLRs. Although sometimes used for medium-scale genotyping (Babik & Radwan 2007; Babik *et al.* 2009a), it is not well suited for large scale studies and finds use particularly at preliminary stages of research, usually in conjunction with other methods.

Next Generation Sequencing. The advent of ultra high throughput Next Generation Sequencing (NGS) technologies (reviewed in Shendure & Ji 2008) is likely to affect also the area of MHC genotyping. The most promising in this context is currently 454 technology (Margulies *et al.* 2005) because it provides a read length of 250–500 bp, sufficient to cover entire MHC exons. This technique is equivalent to the sequencing of clonally amplified products derived from a single DNA molecule in a cell-free system, thus avoiding formation of artefacts related to cloning in bacteria (see above). By using individually sequence-coded 'tagged' primers for polymerase chain reaction (PCR) amplification of the desired products, it is possible to sequence in a single 454 experiment PCR products derived from tens or hundreds of individuals (Binladen *et al.* 2007; Meyer *et al.* 2008; Babik *et al.* 2009b). Because sequencing is performed at a high coverage, the level of which can be adjusted as required, it is possible to obtain an accurate representation of all variants present in an amplicon, even those which are present in a low number of copies (Thomas *et al.* 2006). This technique was first applied for MHC genotyping in the bank vole (Babik *et al.* 2009b; Kloch *et al.* in press), a species with a complex multilocus MHC II DRB system. It has been shown that despite a high number of artefacts generated during 454 sequencing, reliable genotyping and discrimination of true alleles from artefacts is possible. The method appears particularly amenable for the genotyping of highly polymorphic, multilocus MHC systems, for which other, indirect genotyping methods fail or are not practical due to the high cost and amount of labour involved, such as cloning and Sanger sequencing. The advantages of NGS are: (i) relatively low cost, (ii) scalability – a single experiment may form a part of a sequencing run or multiple sequencing runs can be used in a large genotyping project, (iii) little initial optimization – once suitable primers are identified and tagged, genotyping can be performed quickly, (iv) coverage can be adjusted as required, enabling investigation of systems of various complexity, and (v) there is no two step procedure which would require further characterization of

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variants detected by mutation screening techniques. Disadvantages of the method include the cost of tagged primers (which however may be used multiple times and as such form a permanent resource), and the necessity of establishing efficient procedures for distinguishing true alleles from PCR and sequencing artefacts. If very high confidence is needed, all samples can be run in duplicates (Wegner 2009) and consensus genotypes may be obtained. Empirical validation of this genotyping method should always be performed by running a fraction of samples in duplicate (Babik *et al.* 2009b; Kloch *et al.* in press).

Comparison of methods and suggested strategies for MHC genotyping

It is true that 'No MHC typing technique is perfect' (Knapp 2005). It is however possible and useful to compare the strengths and weaknesses of available methods in situations frequently encountered in non-model vertebrates (Table 1).

If typing of individual Mendelian loci is possible, the researcher finds her/himself in a rather comfortable situation. Not only is the spectrum of available methods widest but also the detection of artefacts or other genotyping problems, e.g. the presence of null alleles, is usually easier. In many cases CE-SSCP coupled with direct PCR product sequencing followed by computational haplotype inference may be very fast and cost effective, particularly when SSCP is used for i) identification of homozygotes which are then directly sequenced (e.g. Babik *et al.* 2005) and ii) validation of the results of haplotype inference methods. Gel-based SSCP and DGGE followed by excision of bands corresponding to individual alleles may be useful when variation is high and poorly characterized. RSCA may be suitable for medium to high throughput screening. If the allelic variation is well understood, SSOP may be useful for large scale genotyping of known variation.

If multiple variable Mendelian loci are to be typed, next generation sequencing is worth considering as a rapid and medium to high throughput method, if either products from various loci are pooled or generic primers amplifying multiple loci are applied (the resulting sequences can then be assigned to individual loci on the basis of sequence similarity). Generic primers may reduce the number of manipulations and the cost of the batch of tagged primers. The use of generic primers might, however, potentially lead to less reliable genotyping results because of the increased frequency of artefacts in multitemplate PCR reactions and possible unequal amplification of various alleles.

Multilocus typing using SSCP or DGGE may still be reliable if the number of co-amplifying alleles is moderate (up to 4–6) (Knapp *et al.* 1997; Babik *et al.* 2008). However, multilocus SSCP and DGGE patterns may be very complex and genotyping becomes more problematic with an increase in the number of loci (Knapp 2005; Babik *et al.* 2009a). In such situations it may be beneficial to use motif specific primers for amplification of subsets of alleles (Westerdahl *et al.* 2004), and subsequently standard methods such as SSCP or DGGE can be used for genotyping. Multiple PCR reactions would be needed in order to genotype each individual, which increases cost, labour, and the number of manipulations. Traditionally, cloning has been used for resolving complex multilocus genotypes and is still considered as the gold standard. However, its use for routine high throughput genotyping would be costly and labour-intensive, as a high number of clones must be sequenced to obtain a good estimate of the allelic composition of the sample (Lenz & Becker 2008). A high frequency of cloning artefacts (see above) also needs to be taken into account and procedures reducing their frequency add to the complexity of the method.

Table 1 Major histocompatibility complex (MHC) genotyping methods commonly used in non-model vertebrates or likely to be popular in the near future and their properties that may influence the choice of a particular method

Method	Per sample cost	Resolution	Initial optimization effort	Suitability for multilocus typing	Suitability for single locus typing	Throughput	Straightforward characterization of detected variants
Gel SSCP	€	Medium to high	Medium	+	++	Low to medium	+
CE-SSCP	€	Medium to high	Low to medium	++	+++	Low to high	—
DGGE	€	Medium to high	High	++	+++	Low to medium	++
RSCA	€€	Medium to high	High	++	+++	Medium to high	—
SBT	€€	High	Low	—	+++	Low to medium	+++
Cloning	€€€	High	Low	++	+	Low	+++
NGS	€-€€€*	High	Low	+++	++†	Low to high	+++

*Dependent on the scale of the experiment and coverage required.
†The method becomes cost-effective when variation in single locus system is very high.

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Particularly difficult situations are expected to occur when a large number of similar alleles is expected, as in the cases when concerted evolution within species homogenizes the allelic composition of multiple MHC loci, as is the case in some birds (Hess & Edwards 2002; Westerdahl 2006) or amphibians (Sammut *et al.* 1999). A large number of similar alleles may make genotyping and even establishing the true number of alleles in an individual problematic. NGS is likely to be the method of choice here, although high coverage and the development of dedicated methods for filtering out artefacts are necessary (Babik *et al.* 2009b).

Another aspect which should be taken into consideration is the scale of the research on a given system. If the project involving MHC genotyping is likely to be relatively short-lived, e.g. designed to address a specific, well defined research question, then heavy investment in establishing high throughput, low cost genotyping method like SSOP or RSCA may not be the best option. In such cases NGS, SBT or even cloning seem viable alternatives. If, on the contrary, the system is likely to be used in long-term, large-scale studies then both speed and per-individual cost become essential and thus considerable initial effort into establishment of an optimal genotyping method is justified. CE-SSCP and RSCA well optimized for speed and sensitivity may be favoured in these cases. However, if the system is extremely complex in terms of the number of loci and/or allelic variation, large scale genotyping through NGS seems to be a viable option.

Future prospects

A number of current evolutionary and ecological studies require accurate MHC genotyping and this is almost certain to hold true in the future. A set of thoroughly tested and validated techniques widely applicable for non-model vertebrates exist, such as SSCP, DGGE, SBT, which are perfectly suitable and sufficient for accurate and efficient genotyping in many cases. However considerable challenges and pitfalls have been associated with genotyping of complex multilocus systems and much remains to be done to improve reliability of genotyping and eliminate typing artefacts in such cases. Additional genomic information is needed, enabling a more informed choice of primers. It would be extremely useful to obtain long-range haplotype information spanning multiple MHC genes, and there is a growing interest in the development of relevant methods (Guo *et al.* 2006; Dapprich *et al.* 2008). In the area of actual scoring of sequence variants attention should be given particularly to two areas – testing the utility of SBT involving amplicon sequencing and subsequent computational inference of alleles/gametic phase (Bos *et al.* 2007), and the broad applicability of next generation sequencing (Babik *et al.* 2009b). In the context of NGS studies, the assessment of the type and nature of potential errors and the establishment of procedures applicable for a wide spectrum of situations is needed, particularly if this approach is to achieve the identification of all true variants and the effective filtering of artefacts.

Acknowledgements

Jacek Radwan, Maciej Pabijan, Krystyna Nadachowska, Katarzyna Gac, Briana Gross, Christophe Eizaguirre and two anonymous reviewers provided valuable comments. The work was supported by the Polish Mininistry of Science and Higher Education grant PB 2893/P01/2007/33.

References

Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife immunogenetics? *Trends in Ecology & Evolution*, **21**, 433–438.

Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V, Polz MF (2005) PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Applied and Environmental Microbiology*, **71**, 8966–8969.

Aguilar A, Smith TB, Wayne RK (2005) A comparison of variation between a MHC pseudogene and microsatellite loci of the little greenbul (*Andropadus virens*). *Bmc Evolutionary Biology*, **5**, 47.

Antunes SG, de Groot NG, Brok H *et al.* (1998) The common marmoset: a new world primate species with limited MHC class II variability. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 11745–11750.

Apanius V, Penn D, Slev PR, Ruff LR, Potts WK (1997) The nature of selection on the major histocompatibility complex. *Critical Reviews in Immunology*, **17**, 179–224.

Arguello JR, Little AM, Pay AL *et al.* (1998) Mutation detection and typing of polymorphic loci through double-strand conformation analysis. *Nature Genetics*, **18**, 192–194.

Arnold C, Hodgson IJ (1991) Vectorette PCR: a novel approach to genomic walking. *Genome Research*, **1**, 39–42.

Babik W, Radwan J (2007) Sequence diversity of MHC class II DRB genes in the bank vole *Myodes glareolus*. *Acta Theriologica*, **52**, 227–235.

Babik W, Durka W, Radwan J (2005) Sequence diversity of the MHC DRB gene in the Eurasian beaver (*Castor fiber*). *Molecular Ecology*, **14**, 4249–4257.

Babik W, Pabijan M, Radwan J (2008) Contrasting patterns of variation in MHC loci in the Alpine newt. *Molecular Ecology*, **17**, 2339–2355.

Babik W, Pabijan M, Arntzen JW *et al.* (2009a) Long-term survival of a urodele amphibian despite depleted major histocompatibility complex variation. *Molecular Ecology*, **18**, 769–781.

Babik W, Taberlet P, Ejsmond MJ, Radwan J (2009b) New generation sequencers as a tool for genotyping of highly polymorphic multilocus MHC system. *Molecular Ecology Resources*, **9**, 713–719.

Bak EJ, Ishii Y, Omatsu T *et al.* (2006) Sequence analysis of major histocompatibility complex Class-II DQB 1 (Patr-DQB1) alleles

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in chimpanzees by polymerase chain reaction-based methods. *Human Immunology*, **67**, 655–663.

Ben-Shlomo R, Figueroa F, Klein J, Nevo E (1988) Mhc class II DNA polymorphisms within and between chromosomal species of the *Spalax ehrenbergi* superspecies in Israel. *Genetics*, **119**, 141–149.

Bernatchez L, Landry C (2003) MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? *Journal of Evolutionary Biology*, **16**, 363–377.

Biedrzycka A, Radwan J (2008) Population fragmentation and major histocompatibility complex variation in the spotted suslik, *Spermophilus suslicus*. *Molecular Ecology*, **17**, 4801–4811.

Binladen J, Gilbert MT, Bollback JP *et al.* (2007) The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. *PLoS ONE*, **2**, e197.

Binz T, Reusch TBH, Wedekind C, Milinski M (2001) SSCP analysis of Mhc class IIB genes in the threespine stickleback. *Journal of Fish Biology*, **58**, 887–890.

Bjorkman PJ, Saper MA, Samraoui B (1987) Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**, 506–512.

Bondinas GP, Moustakas AK, Papadopoulos GK (2007) The spectrum of HLA-DQ and HLA-DR alleles, 2006: a listing correlating sequence and structure with function. *Immunogenetics*, **59**, 539–553.

Bonneaud C, Sorci G, Morin V *et al.* (2004) Diversity of Mhc class I and IIB genes in house sparrows (*Passer domesticus*). *Immunogenetics*, **55**, 855–865.

Bonneaud C, Perez-Tris J, Federici P, Chastel O, Sorci G (2006) Major histocompatibility alleles associated with local resistance to malaria in a passerine. *Evolution*, **60**, 383–389.

Bontrop RE (2006) Comparative genetics of MHC polymorphisms in different primate species: duplications and deletions. *Human Immunology*, **67**, 388–397.

Borghans JAM, Beltman JB, De Boer RJ (2004) MHC polymorphism under host-pathogen coevolution. *Immunogenetics*, **55**, 732–739.

Bos DH, Turner SM, Dewoody JA (2007) Haplotype inference from diploid sequence data: evaluating performance using non-neutral MHC sequences. *Hereditas*, **144**, 228–234.

Bos DH, Gopurenko D, Williams RN, Dewoody JA (2008) Inferring population history and demography using microsatellites, mitochondrial DNA, and major histocompatibility complex (MHC) genes. *Evolution*, **62**, 1458–1468.

Bowen L, Aldridge BM, Gulland F *et al.* (2004) Class II multiformity generated by variable MHC-DRB region configurations in the California sea lion (*Zalophus californianus*). *Immunogenetics*, **56**, 12–27.

Bowen L, Aldridge BM, DeLong R *et al.* (2006) MHC gene configuration variation in geographically disparate populations of California sea lions (*Zalophus californianus*). *Molecular Ecology*, **15**, 529–533.

Briles WE, Briles RW, Taffs RE, Stone HA (1983) Resistance to a malignant lymphoma in chickens is mapped to subregion of major histocompatibility (B) complex. *Science*, **219**, 977–979.

Brown JH, Jardetzky TS, Gorga JC *et al.* (1993) 3-Dimensional structure of the human class-II histocompatibility antigen HLA-DR1. *Nature* **364**, 33–39.

Bryja J, Galan M, Charbonnel N, Cosson JF (2005) Analysis of major histocompatibility complex class II gene in water voles using capillary electrophoresis-single stranded conformation polymorphism. *Molecular Ecology Notes*, **5**, 173–176.

Bunce M, O'Neill CM, Barnardo MCNM *et al.* (1995) Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 and DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens*, **46**, 355–367.

Chelvanayagam G (1996) A roadmap for HLA-A, HLA-B, and HLA-C peptide binding specificities. *Immunogenetics*, **45**, 15–26.

Cline J, Braman JC, Hogrefe HH (1996) PCR fidelity of Pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Research*, **24**, 3546–3551.

Dapprich J, Ferriola D, Magira EE, Kunkel M, Monos D (2008) SNP-specific extraction of haplotype-resolved targeted genomic regions. *Nucleic Acids Research*, **36**, e94.

Delarbre C, Jaulin C, Kourilsky P, Gachelin G (1992) Evolution of the major histocompatibility complex: a hundred-fold amplification of MHC class I genes in the African pigmy mouse *Nannomys setulosus*. *Immunogenetics*, **37**, 29–38.

Doherty PC, Zinkernagel RM (1975) Enhanced immunological surveillance in mice heterozygous at H-2 gene complex. *Nature*, **256**, 50–52.

Doxiadis GGM, Otting N, de Groot NG, Noort R, Bontrop RE (2000) Unprecedented polymorphism of MHC-DRB region configurations in rhesus macaques. *Journal of Immunology*, **164**, 3193–3199.

Edwards SV, Potts WK (1996) Polymorphism of genes in the major histocompatibility complex: implications for conservation genetics of vertebrates. In: *Molecular Genetic Approaches in Conservation* (eds Smith TB & Wayne RK), pp. 214–237. Oxford University Press, New York-Oxford.

Edwards SV, Wakeland EK, Potts WK (1995) Contrasting histories of avian and mammalian MHC genes revealed by class II B sequences from songbirds. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 12200–12204.

Edwards SV, Hess CM, Gasper J, Garrigan D (1999) Toward an evolutionary genomics of the avian Mhc. *Immunological Reviews*, **167**, 119–132.

Eizaguirre C, Yeates SE, Lenz TL, Kalbe M, Milinski M (2009) MHC-based mate choice combines good genes and maintenance of MHC polymorphism. *Molecular Ecology*, **18**, 3316–3329.

Ekblom R, Saether SA, Jacobsson P *et al.* (2007) Spatial pattern of MHC class II variation in the great snipe (*Gallinago media*). *Molecular Ecology*, **16**, 1439–1451.

Ellegren H, Hartman G, Johansson M, Andersson L (1993) Major Histocompatibility Complex monomorphism and low levels of DNA fingerprinting variability in a reintroduced and rapidly expanding population of beavers. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 8150–8153.

Ellis SA, Morrison WI, MacHugh ND *et al.* (2005) Serological and molecular diversity in the cattle MHC class I region. *Immunogenetics*, **57**, 601–606.

Ennis PD, Zemmour J, Salter RD, Parham P (1990) Rapid cloning of HLA-A,B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 2833–2837.

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Feng C, Putonti C, Zhang MZ *et al.* (2009) Ultraspecific probes for high throughput HLA typing. *Bmc Genomics*, **10**, 85.

Fisher SG, Lerman LS (1983) DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 2092–2096.

Freeman-Gallant CR, Johnson EM, Saponara F, Stanger M (2002) Variation at the major histocompatibility complex in Savannah sparrows. *Molecular Ecology*, **11**, 1125–1130.

Froeschke G, Sommer S (2005) MHC class II DRB variability and parasite load in the striped mouse (*Rhabdomys pumilio*) in the southern Kalahari. *Molecular Biology and Evolution*, **22**, 1254–1259.

Furlong RF, Yang Z (2008) Diversifying and purifying selection in the peptide binding region of DRB in mammals. *Journal of Molecular Evolution*, **66**, 384–394.

Garrigan D, Hedrick PN (2001) Class I MHC polymorphism and evolution in endangered California Chinook and other Pacific salmon. *Immunogenetics*, **53**, 483–489.

Garrigan D, Hedrick PW (2003) Perspective: detecting adaptive molecular polymorphism: lessons from the MHC. *Evolution*, **57**, 1707–1722.

Grimholt U, Larsen S, Nordmo R *et al.* (2003) MHC polymorphism and disease resistance in Atlantic salmon (*Salmo salar*); facing pathogens with single expressed major histocompatibility class I and class II loci. *Immunogenetics*, **55**, 210–219.

Guo Z, Hood L, Malkkit M, Petersdorf EW (2006) Long-range multilocus haplotype phasing of the MHC. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 6964–6969.

Gyllensten UB, Lashkari D, Erlich HA (1990) Allelic diversification at the class II DQB locus of the mammalian major histocompatibility complex. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 1835–1839.

Hansen MM, Skaala O, Jensen LF, Bekkevold D, Mensberg KLD (2007) Gene flow, effective population size and selection at major histocompatibility complex genes: brown trout in the Hardanger Fjord, Norway. *Molecular Ecology*, **16**, 1413–1425.

Hashimoto K, Nakanishi T, Kurosawa Y (1990) Isolation of carp genes encoding major histocompatibility complex antigens. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 6863–6867.

Hashimoto K, Okamura K, Yamaguchi H *et al.* (1999) Conservation and diversification of MHC class I and its related molecules in vertebrates. *Immunological Reviews*, **167**, 81–100.

Hayes VM, Wu Y, Osinga J *et al.* (1999) Improvements in gel composition and electrophoretic conditions for broad-range mutation analysis by denaturing gradient gel electrophoresis. *Nucleic Acids Research* **27**, e29.

Hedrick PW (2002) Pathogen resistance and genetic variation at MHC loci. *Evolution*, **56**, 1902–1908.

Hedrick PW, Thomson G (1988) Maternal-fetal interactions and the maintenance of HLA polymorphism. *Genetics*, **119**, 205–212.

Hedrick PW, Parker KM, Miller EL, Miller PS (1999) Major histocompatibility complex variation in the endangered Przewalski's horse. *Genetics*, **152**, 1701–1710.

Helmberg W, Dunivin R, Feolo M (2004) The sequencing-based typing tool of dbMHC: typing highly polymorphic gene sequences. *Nucleic Acids Research*, **32**, W173–W175.

Hess CM, Edwards SV (2002) The evolution of the major histocompatibility complex in birds. *BioScience*, **52**, 423–431.

Hughes AL (1991) MHC polymorphism and the design of captive breeding programs. *Conservation Biology*, **5**, 249–251.

Hughes AL, Nei M (1988) Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. *Nature*, **335**, 167–170.

Hughes AL, Nei M (1989a) Evolution of the major histocompatibility complex: independent origin of nonclassical class I genes in different groups of mammals. *Molecular Biology and Evolution*, **6**, 559–579.

Hughes AL, Nei M (1989b) Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 958–962.

Janeway CA, Travers P, Walport D, Shlomchik MJ (2004) *Immunobiology: The Immune System in Health and Disease* Garland Publishing, New York.

Jarvi SI, Briles WE (1992) Identification of the major histocompatibility complex in the ring-necked pheasant, *Phasianus colchicus*. *Animal Genetics*, **23**, 211–220.

Jarvi SI, Tarr CL, McIntosh CE, Atkinson CT, Fleischer RC (2004) Natural selection of the major histocompatibility complex (MHC) in Hawaiian honeycreepers (Drepanidinae). *Molecular Ecology*, **13**, 2157–2168.

Judo MSB, Wedel AB, Wilson C (1998) Stimulation and suppression of PCR-mediated recombination. *Nucleic Acids Research*, **26**, 1819–1825.

Kanagawa T (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). *Journal of Bioscience and Bioengineering*, **96**, 317–323.

Kaufman J, Salomonsen J, Flajnik M (1994) Evolutionary conservation of MHC class I and class II molecules - different yet the same. *Seminars in Immunology*, **6**, 411–424.

Kelley J, Walter L, Trowsdale J (2005) Comparative genomics of major histocompatibility complexes. *Immunogenetics*, **56**, 683–695.

Kennedy LJ, Carter SD, Barnes A *et al.* (1999) DLA-DRB1 polymorphisms in dogs defined by sequence-specific oligonucleotide probes (SSOP). *Tissue Antigens*, **53**, 184–189.

Kennedy LJ, Carter SD, Barnes A *et al.* (2000) DLA-DQA1 polymorphisms in dogs defined by sequence-specific oligonucleotide probes (SSOP). *Tissue Antigens*, **55**, 257–261.

Kennedy LJ, Barnes A, Happ GM *et al.* (2002) Evidence for extensive DLA polymorphism in different dog populations. *Tissue Antigens*, **60**, 43–52.

Kikkawa EF, Tsuda TT, Sumiyama D *et al.* (2009) Trans-species polymorphism of the Mhc class II DRB-like gene in banded penguins (genus *Spheniscus*). *Immunogenetics*, **61**, 341–352.

Klein J (1986) *The Natural History of the Major Histocompatibility Complex*. Wiley & Sons, New York.

Klein J (1987) Origin of Major Histocompatibility Complex polymorphism - the transspecies hypothesis. *Human Immunology*, **19**, 155–162.

Klein J, Sato A, Nikolaidis N (2007) MHC, TSP, and the origin of species: from immunogenetics to evolutionary genetics. *Annual Review of Genetics*, **41**, 281–304.

Kloch A, Babik W, Bajer A, Siński E, Radwan J (in press) Effects of an MHC-DRB genotype and allele number on the load of gut parasites in the bank vole *Myodes glareolus*. *Molecular Ecology*, in press.

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Knapp LA (2005) Denaturing gradient gel electrophoresis and its use in the detection of major histocompatibility complex polymorphism. *Tissue Antigens*, **65**, 211–219.

Knapp LA, Cadavid LF, Eberle ME *et al.* (1997) Identification of new Mamu-DRB alleles using DGGE and direct sequencing. *Immunogenetics*, **45**, 171–179.

Ko WY, David RM, Akashi H (2003) Molecular phylogeny of the *Drosophila melanogaster* species subgroup. *Journal of Molecular Evolution*, **57**, 562–573.

Kukita Y, Higasa K, Baba S *et al.* (2002) A single-strand conformation polymorphism method for the large-scale analysis of mutations/polymorphisms using capillary array electrophoresis. *Electrophoresis*, **23**, 2259–2266.

Kulski JK, Shiina T, Anzai T, Kohara S, Inoko H (2002) Comparative genomic analysis of the MHC: the evolution of class I duplication blocks, diversity and complexity from shark to man. *Immunological Reviews*, **190**, 95–122.

Kumanovics A, Takada T, Lindahl KF (2003) Genomic organization of the mammalian Mhc. *Annual Review of Immunology*, **21**, 629–657.

L'Abbe D, Belmaaza A, Decary F, Chartrand P (1992) Elimination of Heteroduplex artifacts when sequencing HLA genes amplified by polymerase chain reaction (PCR). *Immunogenetics*, **35**, 395–397.

Langefors A, Lohm J, von Schantz T, Grahn M (2000) Screening of Mhc variation in Atlantic salmon (*Salmo salar*): a comparison of restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and sequencing. *Molecular Ecology*, **9**, 215–219.

Lee KR, Park E, Moon SH *et al.* (2008) Development and clinical evaluation of a microarray for HLA-A and-DRB1 genotyping. *Tissue Antigens*, **72**, 568–577.

Leffell MS (2002) MHC polymorphism: coping with the allele explosion. *Clinical and Applied Immunology Reviews*, **3**, 35–46.

Lento GM, Baker CS, David V *et al.* (2003) Automated single-strand conformation polymorphism reveals low diversity of a Major Histocompatibility Complex Class II gene in the threatened New Zealand sea lion. *Molecular Ecology Notes*, **3**, 346–349.

Lenz TL, Becker S (2008) Simple approach to reduce PCR artefact formation leads to reliable genotyping of MHC and other highly polymorphic loci - Implications for evolutionary analysis. *Gene*, **427**, 117–123.

Lenz TL, Eizaguirre C, Becker S, Reusch TBH (2009) RSCA genotyping of MHC for high-throughput evolutionary studies in the model organism three-spined stickleback *Gasterosteus aculeatus*. *Bmc Evolutionary Biology*, **9**, 57.

Livant EJ, Ewald SJ (2005) High-resolution typing for chicken BF2 (MHC class I) alleles by automated sequencing. *Animal Genetics*, **36**, 432–434.

Longeri M, Zanotti M, Damiani G (2002) Recombinant DRB sequences produced by mismatch repair of heteroduplexes during cloning in *Escherichia coli*. *European Journal of Immunogenetics*, **29**, 517–523.

Margulies M, Egholm M, Altman WE *et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature*, **437**, 376–380.

Marsh SGE, Albert ED, Bodmer WF *et al.* (2005) Nomenclature for factors of the HLA system, 2004. *Tissue Antigens*, **65**, 301–369.

McGinnis MD, Conrad MP, Bouwens AGM, Tilanus SGJ, Kronick MN (1995) Automated, solid-phase sequencing of DRB region genes using T7 sequencing chemistry and dye-labeled primers. *Tissue Antigens*, **46**, 173–179.

Meagher S, Potts WK (1997) A microsatellite-based MHC genotyping system for house mice (*Mus domesticus*). *Hereditas*, **127**, 75–82.

Meyer M, Stenzel U, Hofreiter M (2008) Parallel tagged sequencing on the 454 platform. *Nature Protocols*, **3**, 267–278.

Middleton SA, Anzenberger G, Knapp LA (2004) Identification of new world monkey MHC-DRB alleles using PCR, DGGE and direct sequencing. *Immunogenetics*, **55**, 785–790.

Mikko S, Roed K, Schmutz S, Andersson L (1999) Monomorphism and polymorphism at MHC DRB loci in domestic and wild ruminants. *Immunological Reviews*, **167**, 169–178.

Milinski M (2006) The major histocompatibility complex, sexual selection, and mate choice. *Annual Review of Ecology Evolution and Systematics*, **37**, 159–186.

Milinski M, Griffiths S, Wegner KM *et al.* (2005) Mate choice decisions of stickleback females predictably modified by MHC peptide ligands. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 4414–4418.

Miller HC, Lambert DM (2004) Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (Petroicidae). *Molecular Ecology*, **13**, 3709.

Miller KM, Ming TJ, Schulze AD, Withler RE (1999) Denaturing gradient gel electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. *BioTechniques*, **27**, 1016–1030.

Miller KM, Kaukinen KH, Beacham TD, Withler RE (2001) Geographic heterogeneity in natural selection on an MHC locus in sockeye salmon. *Genetica*, **111**, 237–257.

Miltiadou D, Law AS, Russell GC (2003) Establishment of a sequence-based typing system for BoLA-DRB3 exon 2. *Tissue Antigens*, **62**, 55–65.

Mona S, Crestanello B, Bankhead-Dronnet S *et al.* (2008) Disentangling the effects of recombination, selection, and demography on the genetic variation at a major histocompatibility complex class II gene in the alpine chamois. *Molecular Ecology*, **17**, 4053–4067.

Myers RM, Maniatis T, Lerman LS (1987) Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods in Enzymology*, **155**, 501–527.

Nei M, Rooney AP (2005) Concerted and birth-and-death evolution of multigene families. *Annual Review of Genetics*, **39**, 121–152.

Nei M, Gu X, Sitnikova T (1997) Evolution by the birth-and-death process in multigene families of the vertebrate immune system. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 7799–7806.

Noakes MA, Reimer T, Phillips RB (2003) Genotypic characterization of an MHC class II locus in lake trout (*Salvelinus namaycush*) from Lake Superior by single-stranded conformational polymorphism analysis and reference strand-mediated conformational analysis. *Marine Biotechnology*, **5**, 270–278.

O'Brien SJ, Evermann FF (1988) Interactive influence of infectious disease and genetic diversity in natural populations. *Trends in Ecology and Evolution*, **3**, 254–259.

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O'Brien SJ, Roelke ME, Marker L *et al.* (1985) Genetic basis for species vulnerability in the cheetah. *Science*, **227**, 1428–1434.

Olerup O, Zetterquist H (1992) HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens*, **39**, 225–235.

Oliver MK, Telfer S, Piertney SB (2009) Major histocompatibility complex (MHC) heterozygote superiority to natural multi-parasite infections in the water vole (*Arvicola terrestris*). *Proceedings of the Royal Society B-Biological Sciences*, **276**, 1119–1128.

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 2766–2770.

Oto M, Miyake S, Yuasa Y (1993) Optimization of nonradioisotopic single strand conformation polymorphism analysis with a conventional minislab gel electrophoresis apparatus. *Analytical Biochemistry*, **213**, 19–22.

Otting N, Heijmans CIC, Noort RC *et al.* (2005) Unparalleled complexity of the MHC class I region in rhesus macaques. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 1626–1631.

Penn DJ (2002) The scent of genetic compatibility: sexual selection and the major histocompatibility complex. *Ethology*, **108**, 1–21.

Penn DJ, Potts WK (1999) The evolution of mating preferences and major histocompatibility complex genes. *American Naturalist*, **153**, 145–164.

Penn DJ, Damjanovich K, Potts WK (2002) MHC heterozygosity confers a selective advantage against multiple-strain infections. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 11260–11264.

Piertney SB, Oliver MK (2006) The evolutionary ecology of the major histocompatibility complex. *Heredity*, **96**, 7–21.

Qiu XY, Wu LY, Huang HS *et al.* (2001) Evaluation of PCR-generated chimeras: mutations, and heteroduplexes with 16S rRNA gene-based cloning. *Applied and Environmental Microbiology*, **67**, 880–887.

Radwan J, Kawałko A, Wójcik JM, Babik W (2007) MHC-DRB3 variation in a free-living population of the European bison *Bison bonasus*. *Molecular Ecology*, **16**, 531–540.

Radwan J, Biedrzycka A, Babik W (2009) Does reduced MHC diversity decrease viability of vertebrate populations? *Biological Conservation*, doi: 10.1016/j.biocon.2009.07.026.

Sambrook JG, Figueroa F, Beck S (2005) A genome-wide survey of Major Histocompatibility Complex (MHC) genes and their paralogues in zebrafish. *Bmc Genomics* **6**, 152.

Sammut B, Du Pasquier L, Ducoroy P *et al.* (1999) Axolotl MHC architecture and polymorphism. *European Journal of Immunology*, **29**, 2897–2907.

Sato A, Figueroa F, Murray BW *et al.* (2000) Nonlinkage of major histocompatibility complex class I and class II loci in bony fishes. *Immunogenetics*, **51**, 108–116.

Schaschl H, Wegner KM (2007) Contrasting mode of evolution between the MHC class I genomic region and class II region in the three-spined stickleback (Gasterosteus aculeatus L.; Gasterosteidae : Teleostei). *Immunogenetics*, **59**, 295–304.

Schaschl H, Tobler M, Plath M, Penn DJ, Schlupp I (2008) Polymorphic MHC loci in an asexual fish, the amazon molly (*Poecilia formosa*; Poeciliidae). *Molecular Ecology*, **17**, 5220–5230.

Schwaiger FW, Buitkamp J, Weyers E, Epplen JT (1993) Typing of artiodactyl MHC-DRB genes with the help of intronic simple repeated DNA sequences. *Molecular Ecology*, **2**, 55–59.

Seddon JM, Baverstock PR (1999) Variation on islands: major histocompatibility complex (Mhc) polymorphism in populations of the Australian bush rat. *Molecular Ecology*, **8**, 2071–2079.

Sheffield VC, Cox DR, Lerman LS, Myers RM (1989) Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 232–236.

Shendure J, Ji HL (2008) Next-generation DNA sequencing. *Nature Biotechnology*, **26**, 1135–1145.

Snell GD (1968) The H-2 locus of the mouse: observations and speculations concerning its comparative genetics and its polymorphism. *Folia Biologica (Prague)*, **14**, 335–358.

Sommer S (2005) The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Frontiers in Zoology*, **2**, 16.

Sommer S, Schwab D, Ganzhorn JU (2002) MHC diversity of endemic Malagasy rodents in relation to geographic range and social system. *Behavioral Ecology and Sociobiology*, **51**, 214–221.

Stephens M, Donnelly P (2003) A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *American Journal of Human Genetics*, **73**, 1162–1169.

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics*, **68**, 978–989.

Stern LJ, Brown JH, Jardetzky TS *et al.* (1994) Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature*, **368**, 215–221.

Sunnucks P, Wilson ACC, Beheregaray LB *et al.* (2000) SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Molecular Ecology*, **9**, 1699–1710.

Susskind B (2007) Methods for histocompatibility testing in the early 21(st) century. *Current Opinion in Organ Transplantation*, **12**, 393–401.

Takahata N, Nei M (1990) Allelic genealogy under overdominant and frequency-dependent selection and polymorphism of Major Histocompatibility Complex loci. *Genetics*, **124**, 967–978.

Thomas ML, Harger JH, Wagener DK (1985) HLA sharing and spontaneous abortion in humans. *American Journal of Obstetrics and Gynecology*, **151**, 1053–1058.

Thomas RK, Nickerson E, Simons JF *et al.* (2006) Sensitive mutation detection in heterogeneous cancer specimens by massively parallel picoliter reactor sequencing. *Nature Medicine*, **12**, 852–855.

Thompson JR, Marcelino LA, Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Research*, **30**, 2083–2088.

Thursz MR, Thomas HC, Greenwood BM, Hill AVS (1997) Heterozygote advantage for HLA class-II type in hepatitis B virus infection. *Nature Genetics*, **17**, 11–12.

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Tong JC, Bramson J, Kanduc D *et al.* (2006) Modeling the bound conformation of Pemphigus Vulgaris-associated peptides to MHC cclass II DR and DQ alleles. *Immunome Research*, **2**, 1.

Trowsdale J (1995) 'Both man and bird and beast': comparative organization of MHC genes. *Immunogenetics*, **41**, 1–17.

Vincek V, Nizetic D, Golubic M *et al.* (1987) Evolutionary expansion of Mhc class I loci in the mole-rat, *Spalax ehrenbergi*. *Molecular Biology and Evolution*, **4**, 483–491.

Wedekind C, Walker M, Portmann J *et al.* (2004) MHC-linked susceptibility to a bacterial infection, but no MHC-linked cryptic female choice in whitefish. *Journal of Evolutionary Biology*, **17**, 11–18.

Wegner KM (2008) Historical and contemporary selection of teleost MHC genes: did we leave the past behind? *Journal of Fish Biology*, **73**, 2110–2132.

Wegner KM (2009) Massive parallel MHC genotyping: titanium that shines. *Molecular Ecology*, **18**, 1818–1820.

Wegner KM, Kalbe M, Kurtz J, Reusch TBH, Milinski M (2003) Parasite selection for immunogenetic optimality. *Science*, **301**, 1343.

Wenink PW, Groen AF, Roelke-Parker ME, Prins HHT (1998) African buffalo maintain high genetic diversity in the major histocompatibility complex in spite of historically known population bottlenecks. *Molecular Ecology*, **7**, 1315–1322.

Westerdahl H (2006) Passerine MHC; genetic variation and disease resistance in the wild. *Journal of Ornithology*, **147**, 48.

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.

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.

Wittzell H, Von Schantz T, Zoorob R, Auffray C (1994) Molecular characterization of three Mhc class II B haplotypes in the ring-necked pheasant. *Immunogenetics*, **39**, 395–403.

Wittzell H, Bernot A, Auffray C, Zoorob R (1999) Concerted evolution of two Mhc class II B loci in pheasants and domestic chickens. *Molecular Biology and Evolution*, **16**, 479–490.

Zhang F, Hu SW, Huang J *et al.* (2005) Oligonucleotide microarray for HLA-DRB1 genotyping: preparation and clinical evaluation. *Tissue Antigens*, **65**, 467–473.

Zhou JH, Ferencik S, Rebmann V *et al.* (2003) Molecular genetic and biochemical analysis of Woodchuck (*Marmota monax*) MHC class I polymorphism. *Tissue Antigens*, **61**, 240–248.

Zylstra P, Rothenfluh HS, Weiller GF, Blanden RV, Steele EJ (1998) PCR amplification of murine immunoglobulin germline V genes: strategies for minimization of recombination artefacts. *Immunology and Cell Biology*, **76**, 395–405.

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