Molecular Genetic Approaches in Conservation

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New York Oxford
OXFORD UNIVERSITY PRESS
1996

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Polymorphism of Genes in the Major Histocompatibility Complex (MHC): Implications for Conservation Genetics of Vertebrates

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Genes of the major histocompatibility complex (MHC) are emerging as important paradigms in the study of natural selection at the molecular level (Hedrick, 1994). Several decades of immunological and molecular genetic research have revealed that MHC genes play a critical role in the mounting of an immune response by vertebrate hosts to foreign pathogens. Additionally, more recent research in mice suggests that MHC genes influence individual odors in an allele-specific fashion and that these odors are used in mate choice and kin recognition (reviewed in Potts and Wakeland, 1993; Brown and Ecklund, 1994). The extraordinary polymorphism of MHC genes at the molecular level provides the basis of their ability to perform both of these diverse functions. This extreme polymorphism, and the nature of the forces generating and maintaining it, is the basis for recent claims that MHC genes should play a disproportionate role in the design of programs to conserve genetic diversity in captive populations and endangered species (Hughes, 1991a). Both the promise and the controversy surrounding the precise use of MHC genes in conservation genetics lie in our detailed, albeit incomplete, knowledge of proximate and ultimate mechanisms of selection on MHC genes in nature; we suspect that MHC genes have roles in disease resistance and reproductive success, yet there is considerable disagreement among biologists concerning basic mechanisms underlying these roles. In this regard, issues surrounding the use of MHC markers in conservation genetics are relevant to larger issues in conservation genetics concerning the importance of genetic diversity in general (Harcourt, 1992; Caro and Laurensen, 1994). In this chapter we review the data for MHC as a case example of these larger issues.

THE MHC FOR CONSERVATION BIOLOGISTS

Structure and Function

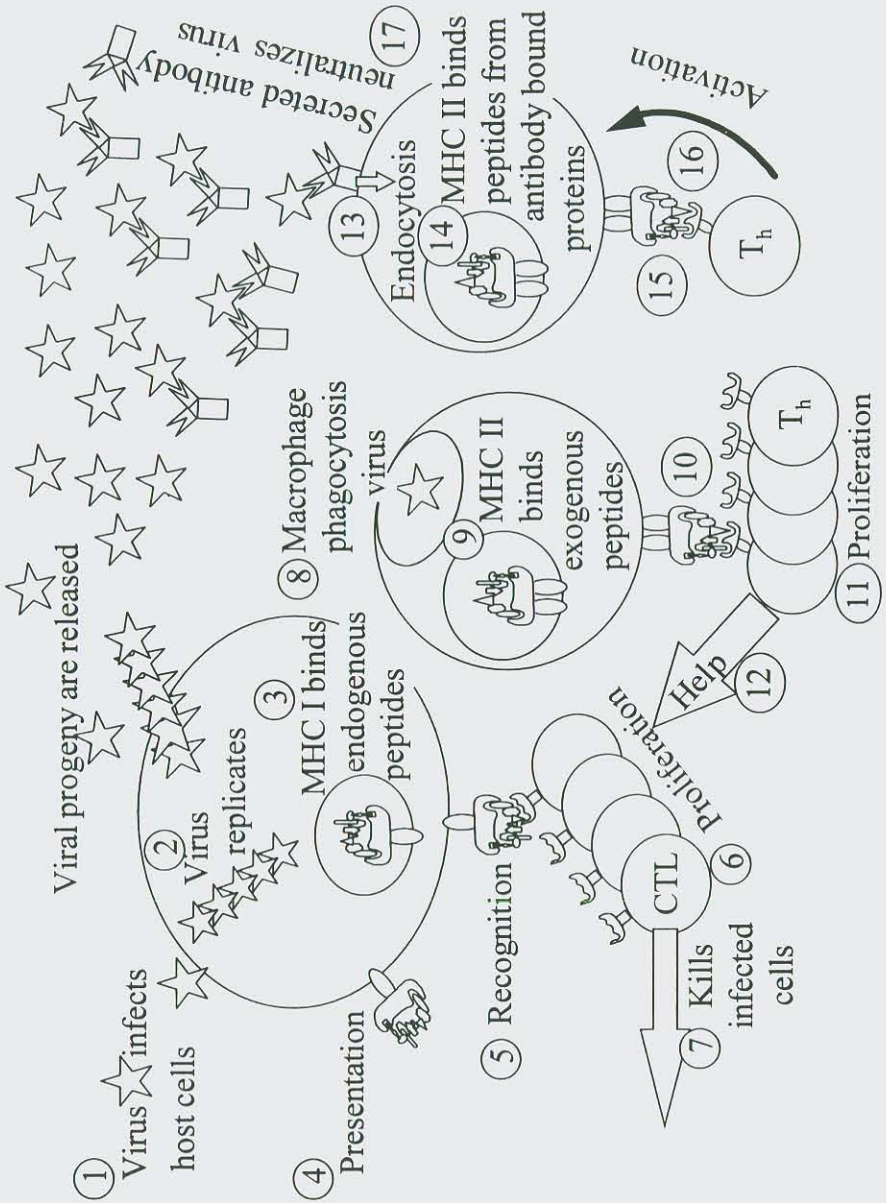
The MHC is one of three major multigene families contained within the immunoglobulin superfamily of metazoans (Klein, 1986; Hood and Hunkapiller,

Table 14-1 Characteristics of Mammalian MHC Class I and Class II genes

Character	Class I	Class II
Tissue distribtion	All nucleated cells	Immune system cells specialized for uptake and presentation of extracellular antigens (e.g., macrophages and B-cells)
Source site of antiegn	Intracellular	Extracellular
Number of loci (mouse/human)	3/3	2/5
Presents antigen to:	Cytotoxic T-lymphocyte (CTL or Tc)	Helper T-lymphocyte (T_h)
Function	Activates CTLs for killing of infected cells	Activates Th-cells which in turn activates appropriate B-cells for antibody production and appropriate CTLs for further proliferation beyond class I activation alone
Number of potentially polymorphic chains	1	2
Pattern of long-term evolution	Frequent gene deletion, duplication and death	More stable than class I

1991). In mammals, the MHC spans about 3500 kilobases of DNA and contains several hundred genes (Trowsdale et al., 1991; Trowsdale 1993). However, when one speaks of "MHC genes," one is usually referring to the few members of two classes in the MHC known as class I and II (Table 14-1). The class I and II molecules of mammals are the best-studied MHC genes and usually number about 2 to 3 in each class, although different species and haplotypes within species differ in these numbers. Class I and II MHC molecules are receptors that bind fragments of foreign proteins ("antigen" or "peptides") that have been truncated ("processed") into short amino acid chains (9–20 residues) by cellular machinery (Table 14-1; Figure 14-1). For any given MHC, a small subset of these peptides bind to a part of the MHC molecule called the antigen-binding site (ABS). These bound peptides are taken to the cell surface and "presented" to two important components of the vertebrate immune system, cytotoxic and helper T-cells; binding by T-cells to the peptide–MHC complex initiates the adaptive immune response (Figure 14-1). Recent studies of peptides purified from intact class I and II molecules from humans and mice reveal the importance of specific amino acid positions in the peptides which act as "anchor positions" for binding to particular MHC alleles, lending support to the idea that different MHC molecules bind different universes of peptides (reviewed in Germain and Margulies, 1993).

Although discovered prior to the human MHC (known as HLA), the MHC of chickens is much less thoroughly studied (Briles et al., 1948; Kaufman et al., 1990; Kaufman, 1995). As for other nonmammalian vertebrates, the overall function of the MHC in chickens (the "B" complex) is thought to be similar to that in mammals, but it is already clear that there are significant differences between avian and mammalian MHCs. For example, the B complex contains a whole family of receptor genes (B-G genes) with no known function or known homologues in



mammals (Kaufman and Salamonsen, 1992). Also, unlike any other vertebrate MHC, B-complex genes were recently shown to reside in at least two independent linkage groups (Briles, et al. 1993; Miller et al. 1994); thus it is almost certain that further comparative work within each vertebrate class will yield surprises, as has been the case with other compartments of the immune system (McCormack et al., 1991).

MHC molecules are currently known only from vertebrates, thus limiting use of MHC polymorphisms to the conservation genetics of this group. Nonetheless, a number of immunoglobulin superfamily molecules occur in invertebrates (Marchalonis and Schluter, 1990), and plants possess self–nonself recognition systems to which MHC molecules may in many respects be analogous (Dangle, 1992; Potts and Wakeland, 1993). Because of their importance in inbreeding avoidance and pathogen surveillance, such molecules may ultimately prove useful in the conservation genetics of these taxa as well.

POLYMORPHISM OF MHC GENES

MHC genes are the most polymorphic functional genes in vertebrates and possess a unique combination of features underlying this extraordinary diversity (reviewed in Hedrick, 1994). The most important conclusion that can be drawn from an analysis of each of these features is that some form of natural selection, specifically balancing selection, is required to explain each one. One of the first surprising features of MHC genes and molecules detected by immunologists was the extreme

Figure 14-1 (opposite) Outline of critical events and features of MHC-dependent immune recognition in response to a viral infection. Development of self/non-self discrmination by the adaptive immune system: Self/non-self discrimination is primarily achieved when T-cells that recognize MHC + self peptides are eliminated during T-cell maturation in the thynus. Consequently, activated T-cells (normally) only recognize MHC + non/-self peptides. Infection: Virus infects host cells (1) and replicates (2); viral progeny are released and new host cells are infected. Within infected host cells foreign and self proteins are being degraded by antigen processing pathways and MHC class I molecules are binding a subset of the resulting peptides (3). Activation of cytotoxic T-lymphocytes (CTL): MHC class I molecules bind a small subset of these peptide fragments from endogenously synthesized proteins (both viral and self) and present them on the surface of infected cells (4). Cytotoxic T-lymphocytes (CTL) that recognize (bind) MHC + viral peptides (5) proliferate (6) and begin killing infected cells (7). Activation of T-helper cells that enhance CTL activity: Macrophages at the site of infection phagocytose virus (8) and a subset of the resulting peptide fragments (derived from exogenous viral proteins) bind to MHC class II molecules (9) and are presented. T-helper (T_h) cells that recognize class II MHC + viral peptide fragments presented by these macrophages (10) respond by proliferating themselves (11) and also by enhancing the proloferation of nearby activated CTL via cytokine release (12). Activation of antibody producing B-cells: B-cells whose cell surface antibody binds to free virus. Endocytoses this virus (13), and a subset of the resulting peptide fragments (derived from these exogenous proteins) are bound (14) and presented by MHC class II molecules. T-helper cells that recognize class II MHC + viral peptide fragments presented by these B-cells (15) respond by proliferating themselves and also stimulating the presenting B-cells to proliferate (16) and release soluble antibodies that neutralize virus (17), CTL killing of infected cells and antibody neutralization of free virus continues until the infection is cleared.

number of alleles, which, for some MHC genes is known to exceed 100—a number too high to be explained by most neutral scenarios (Potts and Wakeland, 1990). This feature combined with suspiciously uniform allele frequencies and the excess of MHC heterozygotes in human populations, was among the first indications that some form of balancing selection was occurring in the MHC region (Hedrick and Thomson, 1983; Klitz et al., 1986; Potts and Wakeland, 1990; Hedrick et al., 1991; Hedrick, 1994). However, recent discoveries of MHC variation in isolated human populations suggest that the effects of balancing selection are not so strong as to override significant effects of genetic drift (Watkins et al., 1992; Belich et al., 1992; Titus-Trachtenberg et al., 1994).

Selection Favors Changes in the Antigen Binding Site

The diversity of MHC genes is largely, although not completely, confined to codons forming the ABS, that part of mature MHC molecules that binds foreign peptides. Hughes and Nei (1988, 1989) found that rates of nonsynonymous (amino acid-changing) substitutions were higher than rates of synonymous (non-amino acid-changing) substitutions in codons encoding the ABS, indicating that mutations that change the binding properties of MHC molecules often have a selective advantage when they arise. This pattern has been observed at all classical MHC genes for which one can infer by homology or otherwise the codons encoding the ABS (e.g., Sato et al., 1993). The extreme sequence divergence between MHC alleles is another hallmark of MHC polymorphism. For example, the mean divergence between most pairs of MHC loci can approach 10%, and some allelic pairs have diverged by as much as 30% (Gyllensten and Erlich, 1989; She et al., 1991). It is now known that both the extreme age of MHC allelic lineages maintained by balancing selection (Takahata, 1990, 1991; Takahata and Nei, 1990; Takahata et al., 1992; Klein et al., 1993) and the rapidity with which interallelic and interlocus gene conversion generates new variability (Melvold and Kohn, 1990; Kuhner et al., 1991; Gyllensten et al., 1991; She et al., 1991) cause this high sequence divergence. The age of MHC lineages within some species approaches 30 million years—a point illustrated dramatically by the pattern of relatedness of alleles in long-diverged species. In rodents and primates, pairs of MHC alleles found in distantly related species are often more related than all pairs within species (McConnell et al., 1988; Figueroa et al., 1988; Lawlor et al., 1988; Gyllensten and Erlich 1989; Gyllensten et al., 1990; She et al., 1990); phylogenetic trees of MHC alleles are in general much deeper than trees of the species from which the alleles are derived, or the trees of other nuclear genes for that matter (Klein, 1987; Golding and Felsenstein, 1990; Golding, 1993; Takahata, 1993). In addition, recent gene conversion events in and around the ABS codons have elevated the standing level of MHC diversity in several species, including humans (Erlich and Gyllensten, 1991; Belich et al., 1992; Watkins et al., 1992; Zoorob et al., 1993). Thus, both recombination and the long persistence times of MHC alleles make them unreliable indicators of the true ancestry of organismal lineages, suggesting that conservation plans focused on the geography of evolutionarily significant units (ESUs; Moritz, 1994) should rely on other, neutral markers for such purposes.

WHAT SELECTIVE MECHANISMS MAINTAIN MHC GENETIC DIVERSITY?

Models Based on Infectious Disease

Most models in which infectious disease plays a role in maintaining MHC genetic diversity, and in which there are immunological risks imposed by low MHC variability, are based on the idea that pathogens can evade those components of the immune response that are dependent on MHC molecules ("MHC-dependent" immune recognition or immunity), leading to a molecular evolutionary arms race between MHC molecules and pathogens (Doherty and Zinkenagel, 1975; O'Brien et al., 1985; O'Brien and Evermnann, 1988; Potts and Slev, 1995). In principle, pathogen variants that arise with mutations in peptides that are presented by host MHC molecules may escape MHC-dependent immunity because either MHC molecules no longer bind the variant peptide or T-cells no longer bind the peptide–MHC complex owing to "holes in the T-cell repertoire" (Figure 14-1). Frequency-dependent selection arises when most pathogens evade the most common host MHC genotypes, a situation in which common MHC alleles become disfavored and rare alleles become favored (Clarke and Kirby, 1966; Bodmer, 1972; Howard, 1991; Slade and McCallum, 1992). That pathogens do evolve in response to MHC alleles of the host is evident in experiments in which the mutations characterizing recent pathogen variants have arisen precisely in those portions of peptides that are presented by MHC molecules (Phillips et al., 1991; De Campos et al., 1993). Other types of MHC-pathogen interactions leading to MHC diversity have also been examined recently (Bertoletti et al., 1994; Klenerman et al., 1994).

Pathogen evasion can also give rise to heterozygote advantage for MHC genes because each MHC allele will be resistant to one set of pathogens and susceptible to another. In this model, resistances and susceptibilities are largely determined by past and ongoing pathogen evasion events. For example, specific MHC haplotypes proved resistant to pathogen infection in the case of Marek's disease, malaria, and other pathogens (Briles et al., 1987; Bacon, 1987; Lamont et al., 1987; Wakelin and Blackwell, 1988; Hill et al., 1991). MHC-linked *resistance* (via successful peptide presentation and T-cell recognition) is known to be dominant to susceptibility, so in theory individuals heterozygous for MHC alleles enjoy the combined set of resistances for both alleles and will in theory be more disease-resistant than either homozygote (Wakeland et al., 1989). One caveat to this hypothesis is that normally each allele expressed in an individual is responsible for deletion of a subset of T-cells during ontogeny of the immune system (see Figure 14-1), so it is possible that heterozygotes could suffer a net loss of overall immune recognition! This type of argument is usually used to explain why the duplication of MHC genes has not proceeded to the extent of other components of the immune system, such as the genes for T-cell receptors and antibodies (Figure 14-1), a situation that would make allelic diversity at individual MHC loci unnecessary (Hughes and Nei, 1988; Takahata, 1994).

Maintenance of MHC Diversity without Pathogen Evolution

Although theoretically attractive, pathogen evasion of MHC-dependent immunity has only indirect empirical support and may play only a minor role in MHC evolution (Tiwari and Terasaki, 1985). However, MHC diversity can in principle be maintained without evolution of invading pathogens: MHC heterozygotes will enjoy increased disease resistance simply because they can present a wider variety of antigens, completely independent of the occurrence of pathogen evasion events (Takahata and Nei, 1990; Hughes and Nei, 1992). Again there is no strong empirical support for this hypothesis and, as described above, it is possible that heterozygotes actually present fewer antigens than homozygotes. However, other models leading to frequency-dependent selection (Andersson et al., 1987b), in which the parasite can incorporate host MHC molecules and thereby immunize other hosts with any of the same MHC alleles, do have indirect support from immunization trials for SIV in macaques (Stott, 1991) and corroborating in vitro studies (Arthur et al., 1992).

Models Based on MHC-Based Mating Preferences

If MHC homozygosity is deleterious owing to increased susceptibility to infectious disease, then one would predict the evolution of reproductive mechanisms that allowed parents to preferentially produce MHC-heterozygous, disease-resistant offspring. Such mating preferences have in fact been experimentally demonstrated in house mice (*Mus musculus*), in which MHC-dissimilar mates are preferred both under laboratory conditions (Yamazaki et al., 1976, 1978, 1988, Egid and Brown, 1989) and in seminatural populations (Potts et al., 1991). These MHC-based disassortative mating preferences are diversity-maintaining and the strength of selection imposed by them was sufficient to account for the majority of MHC diversity observed in natural populations (Potts et al., 1991; Hedrick, 1992). Recent reports of MHC-based mating patterns in humans (Ober et al., 1993) suggest that this trait may have some generality in mammals and possibly other vertebrates. Olfactory mechanisms whereby individuals could evaluate the MHC genotypes of prospective mates have been convincingly demonstrated in both house mice (Yamaguchi et al., 1981) and rats (*Rattus norvegicus*, Singh et al., 1987). Although the precise molecular mechanisms are still unknown, MHC genes influence individual odors in an allele-specific fashion. Mutations in a single MHC gene alter the odor of those individuals (Yamazaki et al., 1983). Thus, the extreme genetic diversity of MHC antigen binding sites results not only in extensive variation in patterns of antigen presentation, but also in an extensive array of MHC-specific odor types.

The avoidance of inbreeding is an alternative, but not mutually exclusive, function for the evolution of MHC-based disassortative mating preferences (Brown, 1983; Partridge, 1988; Uyenoyama, 1988; Potts and Wakeland, 1990, 1993; Alberts and Ober, 1993; Brown and Ecklund, 1994). The extreme genetic diversity of MHC genes coupled with the olfactory ability to discriminate MHC-mediated odor types by rodents (Yamaguchi et al., 1981; Singh et al., 1987) and humans (Gilbert et al., 1986) makes it a potentially powerful system for recognizing

and avoiding mating with kin (Getz, 1981; Alberts and Ober, 1993; Brown, 1983; Potts and Wakeland, 1993; Partridge, 1988; Brown and Echlund 1994; Potts et al., 1994). For example, by avoiding mating with prospective mates who carry one or more alleles identical to those found in ones' own parents, all full- and half-sib matings and half of all cousin matings will be avoided (Potts and Wakeland, 1993). Furthermore, there is data indicating that MHC genes are being used as a kin recognition marker in contexts other than inbreeding avoidance (Manning et al., 1992). Finally, the only other genetic system exhibiting all the extreme genetic features of MHC genes are plant self-incompatibility genes (Potts and Wakeland, 1993), whose role in inbreeding avoidance is uncontested. This suggests that disassortative mating patterns could in principle contribute to, if not account for, the patterns of diversity observed at MHC genes.

MHC-Based Selective Fertilization or Abortion

One would expect selective fertilization or abortion mechanisms favoring zygotes that were heterozygous at MHC genes to evolve for the same reasons as discussed above for mating preferences. The data supporting the existence of such mechanisms in mammals is equivocal and has been recently reviewed (Alberts and Ober, 1993).

MHC VARIATION AND CONSERVATION GENETICS

Hypotheses explaining MHC diversity focus on either disease or reproduction (see above); both have fundamental implications for the welfare and longevity of small populations and hence are of fundamental importance to conservation biologists. The dramatic evidence for selection on MHC genes makes them of interest far beyond their use strictly as unusually polymorphic markers to keep track of lineages or measure overall heterozygosity. But does this constellation of unusual features of MHC genes warrant elevating their status in genetic studies of endangered species and captive breeding programs as suggested by Hughes (1991a) in a provocative article in *Conservation Biology*? In principle, the answer is irrefutable: *yes*, special attention should be given to genes affecting viability, fecundity, and mating. In practice, however, the situation is much less straightforward. The problem is twofold: First, *there is no empirical data indicating an unambiguous link between either MHC heterozygosity or MHC genotype of mates and the probability of successful reproduction* (Hill et al., 1991; Potts and Wakeland, 1993). Second, *any captive breeding scheme designed around a single linkage group will inevitably lose variation at other potentially important loci* (Vrijenhoek and Leberg, 1991; Gilpin and Wills, 1991; Miller and Hedrick, 1991). Hughes' (1991a) plea for a "radical reorientation" of captive breeding programs was based on a decade of population genetic research indicating selection for diversity at MHC loci and the known ability of MHC molecules to bind immunogenic peptides. However, whereas some workers insist that the balancing selection driving MHC polymorphisms must be overdominant selection (heterozygote advantage), many

others argue that other mechanisms are involved (e.g., Wills, 1991; Slade and McCallum, 1992). The intricacy and redundancy of the vertebrate immune system alone should discourage simplistic translations of MHC heterozygosity or monomorphism into a detailed immunological phenotype of an endangered species (e.g., Parham, 1991). Furthermore, the number of associations of HLA haplotypes and *autoimmune* diseases (at least in humans) far exceeds the number involving *infectious* disease; thus MHC variability is not by any means a panacea, and some haplotypes are overtly detrimental (Tiwari and Teraski, 1985; Potts and Wakeland, 1993). Understanding the precise mechanisms of molecular diversification is a prerequisite for constructing detailed conservation schemes around MHC loci. Such understanding is, unfortunately, very difficult to acquire and is likely to come about only after experimentation with natural and seminatural populations and rigorous controls.

Conserve Heterozygotes or Haplotypes?

In an ideal world in which our knowledge of MHC diversity was perfect, differences in hypothesized forces governing MHC diversity could translate into differences in practices for conservation biologists: the simplest models for the maintenance of MHC diversity suggest different schemes for short-term captive breeding or selection of individuals for reintroduced populations. If it were known that heterozygote advantage was the primary form of selection (a simple symmetrical overdominance model in which fitness is not conditioned on particular alleles; Hedrick, 1994), then a captive breeding scheme in which heterozygosity and allelic diversity is maximized would be favored (Hughes, 1991a). When choosing individuals to found new populations for reintroduction, heterozygous individuals could be chosen over homozygotes. By contrast, if it were known that particular rare alleles or haplotypes, and not heterozygosity per se, conferred greater fitness to individuals in a given place or time, as predicted by models of frequency-dependent selection, then preservation or reintroduction of these alleles might be a priority—at least for the short term. This latter scheme would result in substantially lowered allelic diversity at both MHC loci and at other loci in the genome in the short term in the same way that selection for particular haplotypes in nature would lower this observed level of polymorphism and heterozygosity (Hill et al., 1994). Indeed, Hedrick (1994) and Hedrick and Miller (1994) have shown that this type of management protocol would erode genome-wide diversity drastically compared to one based on preservation of overall levels of genetic diversity (see also Vrijenhock and Leberg, 1991; Gilpin and Wills, 1991; Miller, 1995). Another problem with the latter scheme is that under frequency-dependent selection, a "fit" allele today must have a lower fitness tomorrow; the same logic applies to fitness of alleles in particular localities. The unpredictability inherent in pathogen–MHC interactions necessitates a constantly evolving conservation scheme, one that would change on time-scales coincident with those driving the coevolution of fitness and particular alleles. In our ideal world both overdominant and frequency-dependent scenarios suggest conservation protocols minimizing loss of MHC diversity in the long term. Unfortunately, current knowledge of the forces

maintaining MHC diversity is not half so detailed for any vertebrate as to allow prescription of equally detailed conservation plans based solely on MHC.

Role of Mating Preferences in Conservation Genetics

Most of the authors participating in the MHC debate in *Conservation Biology* sparked by Hughes' (1991a) article (Vrijenhoek and Leberg, 1991; Gilpin and Wills, 1991), or subsequent discussions of the role of MHC in conservation genetics (Caro and Laurensen, 1994), ignored recent findings indicating the existence of MHC-based mating preferences and the use of MHC alleles as possible kin recognition markers in semi-natural populations (Potts et al., 1991; Manning et al., 1992). In principle, however, these two findings have dramatic implications for captive breeding programs. Say it was known that an endangered mammal used MHC-mediated olfactory cues to discriminate between possible mates and avoid inbreeding. Then by allowing MHC genetic diversity to become low we would be robbing a threatened species of an evolved mechanism to avoid inbreeding. If a species evolved an MHC-based inbreeding avoidance system in its evolutionary history, then it would be unwise to allow essential components of such a system, in this case MHC diversity, to be lost. The functional diversity of MHC genes for mating preferences could be lost in a few generations of inattention to this problem by managers of threatened species. It would be illogical to ignore the self-incompatibility system in the management of a threatened plant species; in so far as MHC genes function in ways similar to plant histocompatibility genes, the same logic could apply. In this case, appropriate management or breeding techniques would ensure MHC-dissimilarity among captive individuals or prospective founders of new populations and that sufficient variability was available for effective operation of an MHC-based inbreeding avoidance system (Manning et al., 1992; Brown and Ecklund, 1994).

Extrapolating from seminatural populations of mice to a typical endangered vertebrate may well be risky, and the rules and mechanisms by which mice process the information from MHC genes are still in question and may be different or nonexistent in other vertebrates. Contrary to textbook wisdom, many birds possess and use well-developed olfactory senses (Waldvogel, 1989), but we know nothing about their potential interactions with the avian MHC. It is our obligation at least to ask what the consequences would be were such genetic discrimination occurring in endangered mammals, and whether such consequences have fitness costs large enough to warrant attention. Could the inappropriate "social conditions" underlying low reproductive rates in captive cheetah populations have a component in MHC variation (Caro, 1993)? Could the success of programs designed to maximize hybridity (Templeton and Read, 1983; Templeton, 1987) be due in part to MHC genes? As our knowledge of important genetic systems becomes more precise, managers will be better able to assess the impacts of genes like those in the MHC on conservation goals; "conserving genetic diversity" in species already endangered may come to possess a logic ultimately more gratifying than the logic of preserving diversity at neutral

markers throughout the genome that are only surrogates of functional, fitness-related diversity.

CORRELATES AND TAXONOMIC DISTRIBUTION OF LOW MHC VARIABILITY

Case Studies of MHC Monomorphism

The most frequently mentioned cause for concern for species exhibiting low MHC variability is their possible increased susceptibility to epizootics (e.g., Hughes, 1991a). Some of the most thorough case studies in conservation genetics have voiced such a concern (O'Brien et al., 1985; O'Brien and Everman, 1988). Despite the usually abundant polymorphism at MHC genes, there are a number of species in which MHC genes exhibit low or no detectable polymorphism. Table 14-2 summarizes the known cases falling into this category, the causes of the low polymorphism hypothesized by the authors of those studies, and whether 14 significant health problems are known in each species. *None* of the studies referenced in Table 14-2 identified causes of low variability experimentally (see below), and none has established causal relationships between low MHC variability and disease susceptibility. Unfortunately, there is no unambiguous link between low intraspecific MHC variation and disease susceptibility (Caro and Laurenson, 1994), just as there is no link between typical levels of MHC polymorphism and "population health"; the recent viral-induced decline of a Serengeti population of lions (Morell, 1994)—population with average levels of MHC variability (Yuhki and O'Brien, 1990)—is a case in point. Perhaps the most celebrated case for a link between MHC variability and disease susceptibility is in cheetahs (O'Brien et al., 1985); nonetheless, cheetahs are low in variability throughout the genome (Yuhki and O'Brien, 1990), and other loci could be responsible for the numerous susceptibilities to disease recorded in cheetahs (Munson, 1993; Everman et al., 1993).

Two further points emerge from Table 14-2. First, even if the hypothesized cause of low MHC variation were correct in each case, no single cause could explain low MHC variation across taxa. Hypothesized causes range from the loss of polymorphism due to low pathogen loads in particular environments (e.g., southern elephant seals, naked mole-rats) or reproductive biologies (tamarins) to nonadaptive loss due to population bottlenecks (cheetahs, Asiatic lions). Second, low variability at MHC loci does not necessarily entail low variability at non-MHC loci (e.g., some marine mammals, beavers). Importantly, no study conducted at the level of nucleotide sequences has reported complete lack of variability, whereas several studies utilizing RFLP approaches have failed to detect any variability (see below). Most of the instances of low variability involve highly expressed class I loci, which are known sometimes to originate from ancestral loci exhibiting low polymorphism (Watkins et al., 1990b) or to undergo gene duplication much more frequently than class II loci (Rada et al., 1990; Hughes, 1991b; Nei and Hughes, 1991); both of these phenomena could influence the level of polymorphism detected in contemporary populations and could cloud understanding of the significance of MHC diversity.

Table 14-2 Low MHC Polymorphism in Mammalian Species and Its Hypothesized Correlates

Species (MHC Class I and/or II)	Method of Assay[a]	Low Variation at Other Loci?	Documented Health Problems?	Hypothesized Cause	Low Polymorphism Maladaptive?	Referen
Large-bodied terrestrial mammals						
Cheetah (I) (*Aconyx jubatus*)	RFLP	Yes	Yes	Bottleneck	Yes	1–3, 1
Asiatic lions (I) (*Panthera leo persica*)	RFLP	Yes		Bottleneck	Yes	4, 5
Small-bodied terrestrial mammals						
Naked mole-rat (I) (*Heterocephalus glaber*)	RFLP	Yes	No	Stable niche	No	6–8
Syrian hamster (I) (*Mesocricetus auratus*)[b]	Ser	?	No	Solitary, fewer parasites	Np	9
Helgoland Island mice (I) (*Mus musculus helgolandicus*)	Ser	?	No	Bottleneck	No	10
Balkan mole rats (I, II) (4 *Spalax* spp.)	RFLP	?	No	Bottleneck	No	11
Beaver (I, II) (*Castor fiber*)	RFLP	Yes/No	No	Reduced selection	No	12
Marine mammals						
Southern elephant seal (I, II) (*Mirounga leonina*)	RFLP	No	Yes	Fewer parasites	No	13
Sei whale (II) (*Balaenoptera borealis*	RFLP	No	No	Fewer parasites	No	14
Fin whale (II) (*Balaenoptera physalus*)	RFLP	No	No	Fewer parasites	No	14
Beluga whale (II) (*Delphinaptersus leucas*)	Seq	Yes	Yes	Reduced selection	No	19
Primates						
Cotton-top tamarin (I) (*Saguinis oedipus*)	Seq	?	Yes	Bone marrow chimeras, bottleneck, recent origin of gene	Yes/No[c]	15–1

[a] Method of assay: RFLP, restriction fragment length polymorphisms; Ser, serology (antibodies); Seq, nucleotide sequencing.

[b] Class I alleles of the Syrian hamster have been shown to be polymorphic by sequence analysis (Watkins 1990c) and is included here to augment the variety of causes of low polymorph hypothesized by researchers.

[c] Low polymorphism was hypothesized to facilitate rejection of fetuses sharing bone marrow, but is also thought to be associated with susceptibility to viruses.

References: 1. O'Brien et al. (1985); 2. Yuhki and O'Brien (1990); 3. Everman et al. (1993); 4. Wildt et al. (1987); 5. O'Brien et al. (1987); 6. Faulkes et al. (1990); Reeve et al. (1990); 8. Honeycu et al. (1991); 9. Streilein and Duncan (1983); 10. Figueroa et al. (1986); 11. Nizetic et al. (1988); 12. Ellegren et al. (1993); 13. Slade (1992); 14. Trowsdale et al. (1989); 15. Watkins et al. (1990a); Watkins et al. (1990b); 17. Hughes (1991); 18. Munson (1993); 19. Murray et al. (1995).

MINING THE VARIABILITY OF MHC GENES

Immunological Techniques

The techniques for isolating and characterizing MHC genes have evolved over the past three decades just as those for studying other genetic markers (reviewed in Parham, 1992). MHC molecules and allotypes were originally isolated with antibodies directed toward various conserved and variable regions of molecules. It was immediately clear from such methods that MHC molecules were polymorphic, and immunobiological methods are still of great value in isolating MHC molecules from novel species (Kaufman et al., 1990).

Restriction Fragment Analyses

RFLP techniques have been used extensively to characterize MHC diversity. A number of studies have successfully used cDNA probes from humans (Andersson et al., 1987a; Slade, 1992) or mice (Faulkes et al., 1990; Yuki and O'Brien, 1990) to reveal genomic variation in natural populations of other species via Southern hybridizations. Since estimates of nucleotide diversity via DNA sequencing are usually higher than when assayed via RFLPs, it is almost certain that the Southern blot approach using heterologous probes will usually underestimate the actual nucleotide diversity and heterozygosity. (The extent of this underestimation is not known for MHC genes, and most workers utilizing RFLPs have argued that it is negligible.) In natural populations it is also rarely known whether the observed RFLPs occur in functionally important regions of the MHC genes (e.g., exons 2 and 3 for class I or exon 2 for class II). Detailed conclusions about pathogen resistance or susceptibility based on RFLP variation alone would be at best inaccurate and at worst misleading.

Occasionally the use of probes from distantly related organisms can give ambiguous results. To determine patterns of realized reproductive success in red-winged blackbirds (*Agelaius phoeniceus*) by means of highly polymorphic markers, Gibbs et al. (1990) compared banding patterns of putative parents and chicks generated by Southern analysis using a mouse MHC class II cDNA probe. However, the polymorphism exhibited by these blots was extremely high even for an MHC locus, and it was unclear whether the bands generated by this probe represented bona fide MHC variation rather than polymorphism at an anonymous locus (Gibbs et al., 1991). (Surprisingly, use of probes from the chicken B complex failed to give reliable hybridization signals.) Nonetheless, probes from the human MHC were used to detect polymorphisms in putative MHC genes in chickens (Anderson et al., 1987a). Thus, while the use of heterologous MHC probes in Southern hybridizations has been extremely useful, caution should be exercised.

Hybridization probes from the focal species or a congener have frequently been used to detect MHC variation (e.g., Hala et al., 1988; Miller et al., 1988). We have developed a simple method utilizing PCR for rapidly generating probes

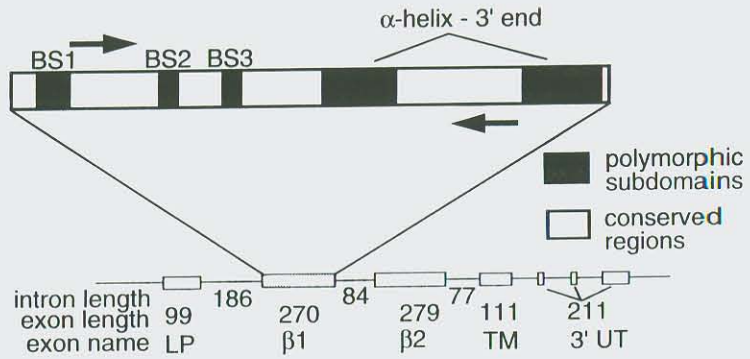


Figure 14-2 Position of degenerate PCR primers between polymorphic subdomains of the second exon of a chicken class II β-chain gene. Primers are indicated by bold arrows above the second exon (Edwards et al., 1995a). Approximate location of polymorphic subdomains in mammals appear in black in the enlarged second exon (above). The structure of a typical chicken class II β gene with lengths (in base pairs) of introns and exons are according to Xu et al. (1988). LP = leader peptide, TM = transmembrane, 3' UT = 3' untranslatedsequences.

specific for class II β-chain MHC genes for use in natural populations (Figure 14-2). By using degenerate primers we have amplified MHC class II β-chain genes from birds and crocodiles (Edwards et al., 1995a,b). These primers amplify approximately 210 bp of exon 2 and are targeted toward some of the same conserved regions of exon 2 that primer pairs in other studies have utilized (Gyllensten et al., 1990; Slade et al., 1994). Probes made from such PCR products cloned in TA-cloning vectors (Invitrogen) reveal abundant and specific MHC polymorphisms when used in Southern hybridizations (Edwards et al., 1995a), and are likely to prove useful in population surveys of natural populations.

PCR and Nucleotide Sequencing

Human HLA genes were one of the first to be amplified by the polymerase chain reaction (PCR; Horn et al., 1988), and all of the methods currently used to conduct population surveys of HLA genes are based on PCR. Such methods include sequencing of cloned PCR products amplified from cDNA (Ennis et al., 1990) and nonradioactive typing via hybridization with allele-specific oligonucleotides (ASOs) fixed to a nylon membrane (Saiki et al., 1989; Erlich and Bugawan, 1993). All of these methods rely on databases not only of primary structures of MHC molecules, but (for oligonucleotide typing) allelic variability in representative samples. Population surveys utilizing PCR sequencing or ASOs have shown that, while most of the major lineages of HLA molecules delineated by serology have survived scrutiny at the level of nucleotide sequences, some serological designations are inadequate reflections of differences at the molecular level (Parham, 1991). In a few cases, MHC molecules that were deemed monomorphic using serological or RFLP techniques have been found to be polymorphic, albeit at a low level, upon DNA sequencing (e.g., Syrian hamster, Watkins et al., 1990c; cheetah, Yuhki and O'Brien, 1994).

Determining variability at the nucleotide level for all relevant regions from specific MHC genes of most vertebrates can be difficult, and is usually attacked by constructing cDNA or genomic libraries (e.g., Ono et al., 1993). A drawback of sequencing studies using only primers directed toward conserved regions in exon 2 (Figure 14-2) is that at least one functionally important portion of exon 2 (β-strand 1 or BS1) will not be contained within the amplified product. Thus, what might appear to be similar alleles for the 210 bp amplified region might in fact be different alleles had exon 2 been sequenced in its entirety. To overcome this, we recommend utilizing the sequence data from the amplified portion of exon 2 to design further experiments to clone flanking exons. We have successfully used a recently described version of ligation-assisted primer amplification, termed ligation-anchored PCR (LA-PCR; Troutt et al., 1992), to obtain sequences, and hence primers, 5′ to exon 2. Sequences downstream of exon 2 can be obtained via conventional reverse-transcription PCR (RT-PCR) using primers targeted to conserved blocks in exon 4 (Kawasaki, 1990; Edwards et al., 1995b). A disadvantage of using either of the above techniques on endangered species is the need to isolate mRNA from tissue, usually from spleen in the case of class II genes (Table 14-1). However, methods are available for isolating sufficient mRNA from leukocytes to permit amplification of class I and II and MHC genes from approximately 0.5 ml of blood (J. Kaufman, personal communication).

It is already possible to amplify the entire exon 2 of class II genes of certain taxonomic groups using primers placed in flanking introns (e.g., Zoorob et al., 1993), and we suspect that PCR primers of wider taxonomic utility will be designed within the next few years that will amplify relevant exons of MHC genes in their entirety. Although some class II genes are so closely related that primers often amplify one or a few loci, it is often easy to distinguish cloned PCR products from different loci based on sequence motifs. Nonetheless, for studies employing DNA sequencing, is particularly important to have some criterion for stating that the sequences analyzed come from orthologous (identical by descent) loci, rather than from multiple loci. Such criteria, although well established in mammals, will be improved for other vertebrates through comparative studies of MHC gene organization.

MHC POLYMORPHISMS IN ENDANGERED SPECIES: POPULATION BIOLOGY FOR IMMUNOLOGISTS

A consensus is emerging that many important aspects of the dynamics of MHC evolution and of MHC-linked disease susceptibilities will almost certainly depend on analysis of relatively undisturbed human populations or natural populations of nonhuman vertebrates (e.g., Hill et al., 1991; Klein et al., 1993). Focus on natural populations removes many recent influences perturbing the action of natural selection, such as recent worldwide admixture of human populations and access to medical facilities in developed countries. Although the experimental data are lacking, it is plausible that MHC variation could have been intimately involved with some of the most dramatic examples of disease- or viral-induced selection in natural populations of vertebrates (e.g., Hawaiian birds, *Myxomatosis* in Australian

rabbits, etc.; O'Brien and Evermann, 1988). That the diseases mediating such selection in some cases are known to interact with MHC genes in humans makes such inferences even more compelling (Hill et al., 1991). We expect that many of the technical and analytical methods for identifying MHC-linked susceptibilities in model organisms will be useful in identifying such susceptibilities in natural and captive populations of vertebrates.

Our own studies are directed toward determining whether basic life history attributes are associated with MHC heterozygosity or particular alleles in both natural and seminatural populations. One such study is focused on the cooperatively breeding Florida scrub jay (*Aphelocoma caerulescens caerulescens*), a federally threatened subspecies of the scrub jay, which is widespread in western North America and Mexico. The total number of Florida scrub jays was recently estimated at 7000–10 000, and the ancient xeric scrub communities with which they are tightly associated are disappearing at an alarming rate owing to development and cessation of the frequent natural fires (Fitzpatrick et al., 1991). Several aspects of this subspecies are well suited to analysis of MHC correlates of life history variation and reproductive success: (1) The population at Archbold Biological Station has been the subject of one of the most detailed long-term studies of any songbird in North America; (2) this population is known to have undergone an episodic decline due to an epidemic in the late, 1970s; and (3) there are differences in reproductive success between groups that cannot be explained by differences in habitat quality or demographic variables (Woolfenden and Fitzpatrick, 1984). Unravelling the intricate relationship between MHC polymorphisms and reproductive success—a relationship which all the models for MHC polymorphism assume—will require large sample sizes (Hill et al., 1991) and, in some cases, long-term genetic and demographic data. The generation of knowledge pertaining to the function of MHC molecules in natural populations—including humans—will undoubtedly require a two-way communication between evolutionary biologists and immunologists (Hedrick, 1994). Monitoring of variability at MHC genes and other loci of immunological importance in captive and reintroduced populations could provide fertile ground for such communication.

CONCLUSION

There are abundant hints that the variation harbored in the vertebrate MHC could be of exceptional importance to conservation geneticists. Given the role of MHC genes in immunity and reproduction, it is likely that conserving MHC diversity will contribute to the vigor and longevity of populations in the long term. However, for most species, the immediate consequences of loss of MHC diversity are uncertain, and we lack the detailed knowledge required to predict these consequences in any one species. Considerable basic research should be devoted to evaluating the importance of MHC diversity in enough experimental and non-endangered natural populations that the importance of preserving MHC variability in endangered species can be ranked alongside other conservation genetic goals. As long as the roles of the MHC in disease resistance, reproductive efficiency,

and inbreeding avoidance remain viable possibilities, conserving MHC diversity should be an important consideration for conservation biologists.

ACKNOWLEDGMENTS

We thank E. Titus-Trachtenberg, P. Hedrick, M. Miller, and C. Moritz for helpful discussion and/or sharing of unpublished manuscripts. R. Ruff and P. Hedrick provided helpful comments on the manuscript. This work was supported by an Alfred P. Sloan Fellowship in Molecular Evolution to S.V.E. and support from the National Science Foundation to W.K.P. and from the National Institutes of Health to W.K.P. and E.K. Wakeland.

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