Characterising MHC diversity in the platypus (*Ornithorhynchus anatinus*): An approach to species conservation

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The Major Histocompatibility Complex is a multigene family that plays a vital role in the innate and adaptive immune response of jawed vertebrates, as an interface between the immune system and infectious agents. Investigation of population diversity at the MHC genes is gaining attention to infer immunological fitness and adaptability of vulnerable populations to a changing environment. Microsatellite markers located in close proximity to MHC loci presents a simple, inexpensive and high throughput method of inferring genetic diversity at the MHC due to tight linkage with specific MHC alleles.

The platypus (*Ornithorhynchus anatinus*) faces an uncertain future from the impact of a variety of potential threats, including climate change, human encroachment causing habitat degradation and the infectious disease, mucormycosis. With platypus populations spread across the eastern and south-eastern Australian mainland and the islands of Tasmania, King Island and Kangaroo Island, the selective maintenance of MHC alleles is thought to vary between populations and characterisation of MHC diversity will prove valuable to current conservation efforts to infer the inherent evolutionary potential and immunological fitness of each surveyed population.

Keywords: *Ornithorhynchus anatinus,* Major Histocompatibility Complex, conservation genetics

Introduction

The platypus (*Ornithorhynchus anatinus*) is the sole survivor of a previously diverse and wide spread lineage of platypus-like, ornithorhynchid, monotremes. Their dependence on freshwater aquatic environments is a probable cause of their prehistoric range contraction as the climate changed (Miska et al. 2002; Grant 2007). With the current cycle of climate change and global warming, the modern platypus is once again threatened by environment modification and loss of suitable habitat (Grant and Temple-smith 2003; Warren et al. 2008). This has spurred on conservation efforts to investigate current distribution and genetic diversity between and within populations, so to provide instructive basis for concerted management. Environmental modification can isolate and fragment platypus populations, increasing the susceptibility of their populations to small population genetic effects, such as increased inbreeding and genetic drift (Frankham 1998; 1999). These genetic effects will reduce genetic variation, depress fitness related traits and severely decrease the species' ability to adapt in altered environments.

Genetic diversity can be assessed through several methods, but an approach gaining attention is using genes located in the Major Histocompatibility Complex (MHC), due to its close association with immunological fitness and therefore the population's survival potential (Bernatchez and Landry 2003; Aguilar et al. 2004; Santucci et al. 2007; Cheng et al. 2009a). Low MHC diversity may confer greater susceptibility to infectious disease and reduced adaptability to a changing environment, resulting in an increased risk of extinction (Boyce et al 1997; Piertney and Oliver 2006; Siddle et al. 2007). Utilising MHC diversity, studies in the platypus will allow researchers to assess the long-term stability of local populations in the face of altered environments and global climate change.

This review will firstly discuss the genetic diversity within and between isolated populations, such as island populations or populations affected by habitat fragmentation or isolation. Themes including neutral and positive genetic selection will be covered in this section. Subsequently, the MHC will be discussed with respect to mechanisms and drivers of positive selection, and as implemented as a molecular marker for genetic diversity and immunological fitness studies. Finally the current state of

knowledge about the platypus will be summarised in the context of conservation and MHC characterisation.

Genetic Diversity between and within Isolated Populations

Fifty percent of all the mammal species to have become extinct worldwide in the past 200 years have been lost from the Australian fauna, giving Australia the worst record for mammal conservation (Short and Smith 1994). These extinctions have mainly been attributed to changes to habitat as a result of introduced herbivores, increased agricultural practices and altered fire regimes, as well as the introduction and proliferation of exotic predators after European colonisation (Maxwell et al. 1996). While these human impacts mainly occurred on the mainland of Australia, offshore islands have often served to protect their isolated populations from the threatening European processes, in a form of passive conservation of mammal species (Maxwell et al. 1996; Frankham et al. 1997). Indeed, the conservation of many threatened species in Australia and around the world relies on populations persisting on islands, providing a source of founders for captive breeding and translocation (Mills et al. 2004).

Although this seems a good approach to species conservation, in small insular populations genetic drift tends to predominate over mutation and selection, making them prone to loss of genetic diversity, higher levels of inbreeding, lower reproductive fitness and compromised ability to evolve (Frankham et al. 2002). Genetic diversity is essential for populations to adapt, evolve and persevere through environmental challenges such as introduction of disease, pests, parasites, competitors, predators, pollution or climatic changes (Frankel and Soule 1981). As the preservation of genetic diversity is fundamental to conservation and evolutionary biology, many endangered species distributed in small isolated populations are susceptible to extinction due to the deleterious associations between genetic, demographic and environmental factors (Frankham et al. 2002).

Island populations have been shown to be at a much higher risk of extinction compared to mainland populations (Frankham, 1997; Frankham, 1998), a statement that could be extended to include fragmented, or isolated mainland populations. Frankham (1997; 1998) argues the critical predictor of extinction susceptibility is related to genetic factors including inbreeding depression, loss of genetic variation, accumulation of mildly deleterious mutations, and genetic adaptation to island environments, all of which diminish evolutionary potential. As a consequence, island populations under these effects are likely to have low genetic diversity and thus a limited genetic capacity to adapt to environmental change.

Evidence from studies encompassing mammals, birds, reptiles, insects and plants show that a significant majority of island populations have lower levels of genetic variation than mainland populations, as characterised by measures of heterozygosity, allelic diversity and percentage polymorphism comparisons (Frankham 1997). However, there are notable studies where island populations possessed greater genetic variation than mainland populations, such as the house mouse (*Mus musculus*) (Berry et al. 1981). These cases have been predominately attributed to characteristics of the species, such as effective dispersal ability or attributed to differences in climatic influences between mainland and island populations, impacting genetic diversity discretely (Frankham 1997). Furthermore, small insular populations have elevated inbreeding coefficients, within the known range where domestic and laboratory animals suffer elevated extinction rates (Frankham 1998). The resultant inbreeding depression, in combination with lower genetic diversity and genetic adaptation to island environments will interact with demographic and environmental stochasticity and catastrophes to increase the risk of extinction of island populations (Frankham 1998). Essentially, the genetic diversity of endangered island species may be more difficult to conserve than endangered mainland species due to their reduction of fitness components, such as survival, reproductive output, growth rates, and also an impaired ability to adapt to long-term changes in the environment (Sommer 2003). However, due to the smaller land area of islands, the elimination or reduction of threatening processes may be easier than in contiguous mainland areas, which confers the importance of islands as wildlife preserves.

Neutral Selection

Determining the inherent diversity within and across populations is one of the first steps in evaluating the genetic conservation value of a species. While there are many applicable methods, there are generally two predominant approaches. The first involves measuring genetic diversity at neutral loci, implementing methods such as microsatellite or mitochondrial DNA analyses. Generally, it is assumed that the amount of differential selection operating on these molecular markers is small so that variation is primarily determined by non-selective evolutionary factors such as genetic drift, gene flow and mutation (Boyce et al. 1997).

Microsatellites have frequently been used as a nuclear marker of choice for determining within population variation and relationships between closely related taxa (Boyce et al. 1997; Eldridge et al. 1999; Taylor et al. 1999; Aguilar et al. 2003; Eldridge et al. 2004; Mills et al. 2004; Santucci et al. 2007).

In such an application, mainland populations of three species of macropods were shown to have each retained substantial levels of genetic diversity, in stark contrast to the low genetic diversity identified from four respective island populations (Eldridge et al. 2004). This illustrates that while island populations have been insulated from the threatening processes impacting mainland populations, alternative forces specific to island populations have resulted in decreased genetic diversity.
This conclusion has been supported by a similar case study in dibblers (*Parantechinus apicalis*), a small dasyurid of Western Australian mainland and two small offshore islands. The mainland population had experienced range contraction over the last 200 years, however there was significant reduction of genetic variation within island populations when compared to the mainland (Mills et al. 2004). This study demonstrates that genetic uniformity in island populations would limit their use as founders for captive breeding and mainland reintroduction, as they would have limited ability to adapt to the new environment and diminished evolutionary potential as a new population. Therefore analyses to determine the remnant genetic diversity in island populations are vital when developing such programs.

Balancing Selection

Neutral loci cannot provide direct information on the fitness of individuals and the evolutionary potential of populations, which depend upon the levels and distributions of adaptive genetic variation (Miller et al. 2001). Therefore, the second approach to molecularly evaluate genetic diversity considers loci under selection, which may have patterns of variation that primarily reflect past selective events and are not necessarily consistent with population history or structure of the taxa (Boyce et al. 1997). This balancing selection is thought to counteract the effects of genetic drift and retard the rate of fixation of alleles within populations, but the effect on maintaining polymorphism depends on the product of selection intensity and effective population size (Sommer 2003).
Some of the best-characterised adaptive loci in vertebrate organisms are found in the Major Histocompatibility Complex (MHC) (Miller et al. 2001). Emerging evidence suggests that MHC studies currently represent the best approach available in vertebrates to investigate how natural selection promotes local adaptation at the gene level despite the actions of gene flow and genetic drift (Bernatchez and Landry 2003). Consequently, investigations into the variation at MHC genes are gaining attention to infer immunological fitness and adaptability of a population in the changing environment. Additionally, by contrasting variation at MHC with neutral markers, experimental accuracy is increased by the separation of the effects of balancing selection from neutral microevolutionary forces (Boyce et al. 1996; Aguilar et al, 2004; Santucci et al. 2007; Oliver et al. 2009). As Aguilar et al. (2004) demonstrated, positive selection can maintain diversity at the functional genes of the MHC despite strong neutral genetic forces generating monomorphism at neutral microsatellite markers.

Major Histocompatibility Complex

Structure and Function

The MHC is a multigene family encoding cell surface glycoproteins, which present the products of pathogen peptide (antigen) degradation to T cells, activating the adaptive immune response. The aim is to eliminate or neutralise both intracellular and extracellular pathogens (Janeway et al. 2001). MHC molecules therefore play a vital role in the adaptive immune response of all jawed vertebrates as the interface between the immune system and infectious disease (Bernatchez and Landry 2003), and are associated with autoimmune conditions and resistance to infection. The antigen-presenting molecules are divided into two major subfamilies, called Class I and Class II (Hughes and Yeager 1998; Kumanovics et al. 2003). The MHC is both polygenic and highly polymorphic, containing several different MHC Class I and Class II genes with multiple variants of each gene (Janeway et al. 2001). This provides a repertoire of MHC molecules with different ranges of peptide-binding specificity to effectively protect the host (Janeway et al. 2001). Not surprisingly, the MHC genes are the most polymorphic genes known in the genome (Piertney and Oliver 2006).
MHC Class I gene products are expressed on the surface of all nucleated cells, and act in the immune defence against intracellular pathogens by binding endogenously derived peptides from proteins in the cytoplasm and presenting the bound peptide to cytotoxic T cells (Jeffery and Bangham 2000). The Class I molecule is a heterodimer containing the class I heavy chain consisting of three extracellular domains (α1, α2, and α3) and a β2 microglobulin (Hughes and Yeager 1998). The peptide binding region (PBR) is formed by the α1 and α2 domains of the class I heavy chain and is able to bind peptides of 8-11 amino acids. MHC Class II genes are primarily expressed on antigen-presenting cells of the immune system, including B cells and macrophages, and are involved in surveillance of the extracellular environment by presenting exogenously produced peptide antigens to helper T cells (Jeffery and Bangham 2000). The Class II molecule is a heterodimer of an α chain and a β chain, with the PBR formed between both $\alpha$and

βchains (Hughes and Yeager 1998). MHC molecules are most polymorphic in the amino acids of the PBR, which in turn affects their peptide binding specificity, T cell response, and thus the host's immune response (Jeffery and Bangham 2000).
The Class I and II genes in mammals conform to the "birth-and-death" model, in which some duplication genes are retained in descendent genomes for long periods, whereas others are deleted or become pseudogenes (Edwards and Hedrick 1998; Nei and Rooney 2005). This generates genetic variability at MHC loci to defend the host from many new types of parasites. Class I loci undergo a faster rate of birth-and-death evolution than Class II loci, making it difficult to establish the orthologous relationships of different Class I genes among the different orders of mammals. In contrast, the longevity of Class II genes results in the retention of many orthologous loci, shared by different orders of mammals (Takahashi et al. 2000).
Much polymorphism seen at the MHC predates speciation, such that some MHC alleles arose and were subsequently retained during the evolutionary histories of many species (Figueroa et al. 1988). Similar MHC alleles are not only retained by different species of the same genus (*Mus*) but also by different genera of the same order (*Mus* and *Rattus*) (Figueroa et al. 1988). This retention of MHC alleles is characteristic of positive selection acting on these functional genes resulting in the long persistence time of MHC alleles (Hughes and Nei 1989). Appropriately, this persistence over long timespans provides a unique opportunity to probe the selective histories of divergent species (Edwards and Hedrick 1998). Furthermore, MHC variation is viewed as a valuable marker to conservation efforts due to its intimate association with factors that impinge on individual fitness, population viability and evolutionary potential in a changing environment (Piertney and Oliver 2006).

Balancing Selection at MHC Loci

Hughes and Nei (1988; 1989) provided early evidence for selective maintenance of MHC diversity to explain the usually high diversity of MHC loci. Specifically there is a greater rate of non-synonymous (amino acid altering; d_N) substitutions than that of synonymous (d_S) substitutions in the codons encoding the PBR, such that substitutions in this region are selected for in descendents. In addition, there is a high degree of heterozygosity at MHC alleles and long persistence of polymorphic alleles compared to neutral alleles. These features of the PBR increase its ability to bind a diverse array of antigens, maintaining a wide immunological surveillance (Sommer 2005b). In the remainder of the gene, outside the PBR, $d_S > d_N$, implying that the action of neutral selection is maintaining the structural composition of the MHC molecule. The observations of similar MHC alleles in different species and genera provided further evidence for strong selection of advantageous alleles at the MHC through speciation events (Figueroa et al. 1988; Edwards and Hedrick 1998). Accordingly, high polymorphism at MHC genes appears to be primarily generated by nucleotide substitution and balancing selection at the PBR (Nei and Rooney 2005) and many studies support the general hypothesis that allelic diversity at the MHC genes is maintained by parasite-mediated balancing selection (see sections 3.2.3 and 3.3.1).
Genetic variation in populations driven by natural selection often occurs in an episodic manner, making selection a potentially strong but often transient force (Elena et al. 1996). Indeed, it has been suggested that MHC loci may experience long periods of near-neutral evolution dominated by genetic drift, but also experience recurring episodes of evolution driven by natural selection (Bos et al. 2008). These periodic selective pressures serve to explain observations of low MHC diversity in several studies, where genetic drift prevails over low selection pressures to diminish allelic diversity at the MHC (Sommer 2003).
Furthermore, evidence has accumulated to suggest that MHC variation is affected by balancing selection, direction selection and drift, but the mode and relative strength of selection acting on MHC diversity varies spatially, temporally and across species, as it is dependent on selective intensity and effective population size (Sommer 2003; Oliver et al. 2009). In smaller populations, balancing selection may be so weak that neutral genetic forces such as gene flow, genetic drift and inbreeding have eroded its effect, constituting the predominant evolutionary forces shaping genetic variation (Boyce et al. 1997; Seddon and Baverstock 1999; Sommer 2003; Campos et al. 2006; Meyer-Lucht et al. 2008).
The specific behaviour of a species also impacts the nature of selective forces acting on MHC genes, such as population structure or demographic factors. Demographic factors influence sensitivity to fragmentation, and fragmentation-sensitive species experience higher population isolation compared to a fragmentation-tolerant species (Meyer-Lucht et al. 2008). These inherent demographic factors will lead to species-specific responses to environmental or climatic change, which will impact population structure and size, and thus effectiveness of balancing selection at the MHC genes. Conclusively, the importance of diversity at MHC for population survival in the wild will depend on several factors, including local conditions and the particular characteristics of the species in question (Campos et al. 2006).
The types of selection envisioned for MHC genes have traditionally included rare-allele advantage or heterozygote advantage. Both of these mechanisms are compatible with patterns of MHC polymorphism

and various selective scenarios involving disease resistance, mate choice and/ or reproductive incompatibilities between individuals (Edwards and Hedrick 1998). But with the progressive increase in MHC studies particularly in non-model species, these two hypotheses have given way to a new way of thinking.

Rare Allele Advantage (Frequency Dependent Selection)

The principle of rare allele advantage proposes that a pathogen will evolve to counteract the host immune system. Specifically, the pathogen evolves to evade the MHC-based immune response of the most common MHC genotype, which is producing the greatest selective pressure on the pathogen (Jeffery and Bangham 2000; Bernatchez and Landry 2003). This new pathogen variant will decrease the common host genotype and confer a selective advantage to individuals with new, rare MHC alleles (Jeffery and Bangham 2000; Bernatchez and Landry 2003). A virulent pathogen may exert strong selection on the host populations, whereby an allele that, by chance, provides better immunity against that pathogen will increase in frequency, and thus the pathogen can drive MHC diversity by fluctuations in the intensity of selection exerted (Jeffery and Bangham 2000). The time-lag nature of this antagonistic co-evolutionary relationship will lead to cycling of fitness values of different genotypes in both the pathogen and host, resulting in maintenance of high genetic diversity (Bernatchez and Landry 2003).

Overdominance/ Heterozygote Advantage Hypothesis

The hypothesis of heterozygote advantage (overdominant selection) proposes that individuals heterozygous at MHC loci are able to present a wider range of antigenic peptides from a pathogen to the immune system than homozygous individuals, thus enhancing immune surveillance (Jeffery and Bangham 2000). Hughes and Nei (1988; 1989) were the main proponents of the overdominance hypothesis as the simplest explanation of observed high degree of polymorphism, long persistence of polymorphic alleles in populations and the greater rate of non-synonymous substitutions at MHC loci. They argued that other hypotheses, such as selection for advantageous alleles and frequency-dependent selection, which were able to generate $d_N > d_S$, were not able to explain the high polymorphism and long persistence of alleles.

New Directions of Thought

From continued studies of MHC, it is evident that the previously mentioned traditional models cannot fully explain all observations (Piertney and Oliver 2006). Consistent associations between particular alleles and resistance to parasites, and discordance between measures from MHC and neutral markers, would indicate that fitness is based on dominant and not overdominant associations (Piertney and Oliver 2006, Tollenare et al. 2008; Blanchet et al. 2009). Indeed, the physiological mechanisms involved in the MHC-T-cell pathway may prevent an efficient adaptive immune response if an individual has too many different MHC alleles (Hughes and Nei 1989; Wegner et al. 2004; Kalbe et al. 2009). Even those studies that have inferred overdominance may in fact have detected heterozygote advantage through the effect of dominant resistant alleles rather than heterozygote superiority caused by true overdominance (Piertney and Oliver 2006). As even Hughes and Nei (1989) admitted, in practice the number of polymorphic loci is relatively small, below that expected for selection for overdominance.

Thus, current theoretical models predict that maximal pathogen resistance is achieved by an intermediate (optimal) rather than a maximal number of different MHC variants (Piertney and Oliver 2006). This has been supported by studies in teleosts (Landry et al. 2001; Kalbe et al. 2009). MHC genes of teleosts appear to have evolved and therefore act similar to those of their mammalian counterparts, thus inferences in teleosts can be used in mammalian MHC studies (Landry et al. 2001). Kalbe et al. (2009) found that intermediate MHC diversity maximises lifetime reproductive success, such that individuals with an optimal MHC basis are able to perform a shift from costly and self-damaging innate immune function towards a probably less costly and efficient adaptive immune strategy, and use the immunological mechanism more concertedly and economically. This also allowed optimal-MHC individuals to invest more into their reproductive period and thus their offspring. Conversely, fish with less optimal MHC diversity required a higher proportion of their resources allocated to immune defence mechanisms to maintain a tolerable parasite load.

In a complementary study in a mammal species (Tollenare et al. 2008), an association of two alleles with parasite load was found in the fossorial water vole (*Anvicola scherman*). Furthermore, the direction of parasite-meditated selection differed for the two MHC genes, challenging the common idea that the pattern observed at one locus can be used as an indicator of all MHC patterns. These results support the developing argument that particular alleles may be more important for disease resistance than heterozygosity.

Mechanisms of Balancing Selection

Critically, the processes that underpin selection at the MHC genes is yet to be concluded, although substantial evidence is building to confirm that a beneficial degree of individual MHC diversity is

maintained by parasite-mediated selection, and also suggest that this is amplified by MHC-based mate choice (Piertney and Oliver 2006; Kalbe et al. 2009).

Parasite Mediated Selection

With the general consensus accepting that MHC allelic diversity is driven by pathogen pressure, it can be assumed that as pathogen load in the environment changes across the landscape, so too will the selective pressures influencing local populations. Generally, high diversity of pathogens results in relatively high selection pressures for maintaining polymorphism at the MHC compared with environments with low rates of infectious disease (Bernatchez and Landry 2003). Low prevalence and transmission of pathogens in some environments, such as in domestic species, will result in low selective pressures for parasite-mediated polymorphisms at the MHC, which may generate insufficient strength to maintain allelic variation (Campos et al. 2006). As a consequence, the effect of variable parasite-mediated selection will result in local adaptation that promotes the maintenance of different subsets of MHC alleles in separate populations (Landry and Bernatchez 2001).

Determining the contribution of individual alleles against different haplotype backgrounds to a specific pathogen challenge is reportedly difficult, because all MHC haplotypes are likely to mount at least a minimal immune response to pathogen infection (Jeffery and Bangham 2000). However, studies to associate patterns of genetic differentiation with parasite prevalence across environments will nonetheless provide invaluable understanding of how local adaptation may be responsible for generating and maintaining MHC diversity (Bernatchez and Landry 2003).

In a study of MHC diversity in marine mammals, Slade (1992) reported low MHC polymorphism in the southern elephant seal (*Mirounga leonina*) and other marine mammals compared to outbred terrestrial mammals. As the distinctive difference between marine and terrestrial mammals is their external environment, it was proposed that the low MHC diversity found was the result of decreased exposure to microparasitic diversity. The relatively low pathogen exposure diminishes the selective pressure for diversity at immune response loci and as a consequence populations are at risk of high mortalities on exposure to pathogens with sufficient transmission potential to invade and persist.

A pronounced discrepancy between MHC and neutral microsatellite loci was identified by Landry and Bernatchez (2001) in comparisons involving Atlantic salmon (*Salmo salar*) from different spawning areas within one river system. Due to the distinct homing behaviour of salmon, this species forms genetically differentiated populations that may be locally adapted to their environment. The observed selective factors were suggested to predominate over drift and migration in shaping the population structure at this small geographical scale. Additionally, as parasites and pathogens can have a very local distribution and slow dispersal, there can be considerable difference in the pathogen prevalence in spatially isolated subpopulations and consequently difference at MHC loci.

MHC-Disassortative Mating

Mate choice has been suggested to interact with pathogen-mediated selection to shape MHC diversity. Studies examining MHC-related mate choice have emphasised that choice can be based on different criteria, those receiving the most attention include mate-choice for maximal MHC heterozygosity, minimal inbreeding or optimal alleles across several loci (Piertney and Oliver 2006). From studies in laboratory animals, Penn (2002) suggested that the main benefit of MHC-disassortative mating preference is to avoid inbreeding thereby minimising inbreeding depression. Studies into non-model organisms contradict this conclusion and instead have shown that mating preference may act to increase immunological fitness of offspring (Landry et al. 2001; Neff et al. 2008; Kalbe et al. 2009).

Several studies of MHC-associated mate choice in teleosts found no evidence for inbreeding avoidance but rather statistically significant support of non-random mating for amino-acid divergence at the PBR, suggesting this will increase the genotypic complexity of their offspring and thus generate optimal immune defence (Landry et al. 2001; Neff et al. 2008). This sexual selection was predominately observed in female teleosts, where female mate choice included two criteria to ensure optimal immunological fitness in offspring, including olfactory cues reveal a male's MHC variant diversity and colour to indicate the possession of currently protective alleles (Kalbe et al. 2009). Appropriately, in combination with balancing selection, disassortative mating may contribute to the maintenance of high polymorphism at PBR loci within a population. However, this is not reflected in all MHC-containing species, as evidence for lack of MHC-associated mating and even mate-choice for MHC-similar individuals have been observed (Sommer 2005a).

Molecular Methods for Inferring MHC Diversity

MHC diversity in populations is increasingly being used in a conservation context to identify populations that lack diversity (Piertney and Oliver 2006), attention focused on developing new, simplified molecular methods is accelerating.

Cloning and Sequencing

Cloning followed by sequencing of the MHC Class I and Class II PBR, was until recently the only means of accurately typing studied individuals, which is a particularly laborious and expensive exercise (de Groot et al. 2008). Sequencing the loci allows the precise polymorphisms to be located and compared within and across populations and species.

Single Strand Conformation Polymorphism (SSCP)

SSCP is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair), which results in a different secondary structure and a measurable difference in mobility through a gel. SSCP analysis therefore offers a simple, inexpensive and rapid method of screening population samples for sequence polymorphism at specified regions to greatly reduce the amount of sequencing necessary to determine the alleles present in a population (Woodward 2003). As the accuracy for SSCP analysis is typically within the range of 65% to 70%, which is highly dependent on physical factors, SSCP is best used as an indicative technique to direct further comprehensive sequencing (Weber et al. 2005).

MHC-linked Microsatellites

Due to the birth-and-death evolution of MHC genes, it is incredibly difficult to design locus-specific primers to assess MHC diversity (Cheng et al. 2009a, b) and thus molecular typing of class I and II genes is complicated and time-consuming (Penedo et al. 2005). The presence of hypervariable microsatellite loci densely distributed throughout all the vertebrate chromosomes, including the MHC region, presents an opportunity to determine MHC haplotypes indirectly by genotyping a series of these loci spread across the MHC (Meagher and Potts 1997). These loci display increased diversity owing to hitchhiking due to tight linkage with specific MHC alleles (Meagher and Potts 1997). For this reason, microsatellite markers within the MHC region are increasingly being used as simple and inexpensive proxies for reliably inferring levels of genetic diversity at the MHC (Aguilar et al. 2004; Penedo et al. 2005; Doxiadis et al. 2007; Santucci et al. 2007; de Groot et al. 2008; Cheng et al. 2009a, b).

A series of microsatellite markers within or closely flanking the MHC are powerful at discriminating haplotypes and when used in combination, provides a practical method for population comparison studies. Microsatellite allele numbers will be higher for loci near the PBR loci, due to the hitchhiking of neutral microsatellite alleles on selectively maintained MHC alleles (Meagher and Potts 1997). Such a set of MHC-linked microsatellite markers was employed by Cheng et al. (2009a), to determine the level of DBB diversity in populations of the Tammar wallaby (*Macropus eugenii*). This method revealed the highest level of β1 domain diversity seen in any marsupial species present in the tammar wallaby population in Kangaroo Island. Although this population has been isolated for an estimated 10,000 years, this proved that this island population was maintaining high genetic diversity at the MHC and had not undergone recent bottlenecks or significant genetic drift in the gene region.

Aguilar et al. (2004) also used MHC-linked microsatellite markers to effectively demonstrate the potential of balancing selection to maintain high heterozygosity in MHC loci through the severe population bottlenecks experienced by the San Nicolas Island fox (*Urocyon littoralis littoralis*). While this population was previously found to be monomorphic at neutral markers, the authors suggest that intense periodic balancing selection at the MHC may have allowed the persistence of variation in the foxes despite strong genetic drift evident at neutral markers. Both studies demonstrate the usefulness of using MHC-linked microsatellites as reliable and inexpensive proxies for MHC diversity studies in determining conservation value of endangered or vulnerable species.

MHC Diversity and Conservation Management

Although the implementation of MHC diversity studies has increased in modern times, the usefulness to a conservation management strategy remains in dispute. Hughes (1991) suggested a drastic reorientation in the management of endangered species was required, where efforts should concentrate on conserving allelic diversity at the small number of loci on which balancing selection operates in nature. As MHC represents the primary example in vertebrates, the preservation of maximal MHC diversity should constitute the main goal of conservation programs, as the loss of even a single allele at an MHC locus may seriously diminish a species' chances of survival (Hughes 1991).

A contrasting perspective advocates that the best general approach for maintaining genetic variation is a goal to minimise inbreeding over retaining MHC diversity, especially when the two aims are in conflict (Hedrick 2001). This approach is supported by the persistence of populations with significantly depauperate MHC diversity, indicating that lack of variation may not constitute a threat to population survival even in a relatively long time perspective (Babik et al. 2009). Inbreeding avoidance indeed has an important place in conservation management, but the imminent extinction threat of Devil Facial Tumour Disease faced by the Tasmanian devil (*Sarcophilus harrisii*) should serve as an example of the consequences of low MHC diversity for population survival (Siddle et al. 2007). Therefore, it is of key importance that MHC diversity be included in conservation management programs, such that a principle

of precaution can be employed to address this extinction risk to vulnerable populations. Comprehensive population studies of MHC diversity will enable monitoring of the spatial and temporal immunological fitness of populations and provide an informed basis for genetic rescue of low MHC diversity populations.
Genetic rescue has been defined as an increase in population fitness owing to immigration of new alleles, by more than can be attributed to the demographic contribution of immigrants (Tallmon et al. 2004). Genetic rescue can thus be used as a conservation tool to effect alleviation of inbreeding depression and increasing fitness in insular, inbred populations. Successful genetic rescue through controlled translocations has been documented as effective management strategies in endangered populations, by the removal of detrimental inbreeding-associated variation and restoring neutral variation at rapid rates (Hogg et al. 2006; Bouzat et al. 2009). However, corrective genetic measures through translocations can be costly, and minimising disease spread is often of greater concern than genetic viability over the longer term (Hogg et al. 2006). This is particularly important for the potential application of genetic rescue in populations with low MHC diversity. The reduction in allelic diversity at MHC loci exposes inbred populations to increased susceptibility to introduced disease through translocation. Therefore, while genetic rescue represented a potential conservation tool to counteract reduced MHC diversity and the associated reduction in immunological fitness, it should be approached with care.

Platypus

Phylogeny and Genome Features

The late Australian monotreme expert, Dr Mervyn Griffiths, once described the platypus as "the animal of all time". This small aquatic animal is one of the two genera that constitute the order Monotremata. Monotremata diverged from Theria about 166 million years ago (MYa) and are therefore the oldest branch on the mammalian tree (Bininda-Emonds et al. 2007). Theria subsequently diverged into marsupial and eutherian mammals (also known as placentals) about 148 MYa (Bininda-Emonds et al. 2007; Warren et al. 2008). The order constitutes the two extant lineages: Tachyglossidae, containing four echidna species, and Ornithorhynchidae, containing only the platypus.
Characteristic of Monotremata, platypuses possess distinct mammalian qualities, such as milk production, fur and thermoregulation, but also reptilian features in their skeletal structure and oviparity. Specifically, the platypus lays eggs but the young hatch at an early developmental stage, after which the platypus mothers suckle their young (Grant 2007). The platypus represents the last survivor of a highly diverse and widely distributed lineage of ornithorhynchid monotremes that previously occupied aquatic habitats in Australia, South America and Antarctica as long as 110 million years ago (Miska et al. 2002; Grant 2007; McMillan et al. 2007), emphasising the importance of their continued survival. Furthermore, studies into this model monotreme can assist in conservation efforts in the long-beaked echidna (*Zaglossus* spp), which only occurs in New Guinea and is considered endangered by the International Union for the Conservation of Nature and Natural Resources (IUCN).
The platypus genome also has characteristic mammalian and reptilian hallmarks, making genome studies of this model species particularly valuable (Warren et al. 2008). The platypus has a diploid genome with 52 chromosomes: 21 pairs of autosomes (6 large and 15 small) and 10 sex chromosomes. In females, there are 5 different pairs of X chromosomes, and in the male there are 10 unpaired elements: 5 X chromosomes and 5 Y chromosomes. These pair at the first meiotic division and form a sex chromosome chain during prophase I, which has an alternating order of X and Y chromosomes in the male platypus (McMillan et al. 2007). Pseudoautosomal regions within adjacent X and Y chromosomes are necessary to ensure pairing and segregation of the sex chromosomes into male-determining sperm with five Y chromosomes, and female-determining sperm with five X chromosomes (Dohm et al. 2007; Tsend-Ayush et al. 2009). The precise mechanism of sex determination is yet to be established, however studies have suggested the involvement of the DMRT1 gene and the GATA4 gene, which have roles in sexual differentiation in other vertebrates and are located on the sex chromosomes in the platypus (El-Mogharbel et al. 2007; Wallis et al. 2008; Grafodatskaya et al. 2007).

Habitat and Distribution

The platypus is endemic to eastern Australia, restricted to areas where permanent streams occur, and inhabiting these bodies of water and their riparian margins. It can be found along the eastern coast of Australia, as far north as Cooktown in Queensland, to southern and western Victoria, and infrequently in South Australian rivers (Grant 2007). Additional populations exist in isolation on Tasmania, King Island to the north of Tasmania, and Kangaroo Island off the coast of South Australia (Grant 2007). Tasmania, Australia's largest island covering 68401 km2, became cut off from the mainland by rising sea levels approximately 13,000 years ago, and the 1260 km2 King Island subsequently separated from the north of Tasmania about 3,000 years later (Hope 1973). The breeding population of Kangaroo Island, which has

an area of 4500 km2, was established in the island's western waterways from introduced Victorian platypuses during to the early 1940s (Grant 2007).

Due to the resulting isolation from the mainland, island populations may have experienced reduced genetic diversity, increased levels of inbreeding and numerous adaptations to island life with the cumulative effect of increasing vulnerability to extinction (Frankham 1997). But through isolation, these island populations have not experienced some of the threatening processes of human colonisation that have occurred on the Australian mainland, which may have resulted in the retention or emergence of unique island genotypes. Due to the environmental and geographical differences in these island environments, the interaction between neutral and balancing selection is expected to vary between the islands and vary from the mainland, potentially resulting in distinct allele frequencies and variable genetic diversity in each population. The platypuses on Kangaroo Island are seen as a relatively young island population, and may not have undergone the same extent or severity of island-associated genetic effects when compared to the long-separated King Island and Tasmania. Additionally, different genetic-demographic interactions are likely to be observed due to the varying land areas of each island (Frankham 1998). MHC diversity analysis across these island populations may therefore infer the short-term and long-term effects of balancing selection in populations on various island sizes.

Conservation

Platypuses are adapted to a semi-aquatic lifestyle, with webbed feet, a horizontally flattened tail and streamlined body. The platypus feeds predominately on benthic invertebrates, appearing to be non-selective in its prey choices, but nevertheless this makes the species heavily reliant on healthy freshwater ecosystems (Grant and Temple-Smith 2003; Serena et al. 2001). Platypuses are classified as "least concern" by the IUCN and are considered common in the wild, if rather reclusive. It appears that the overall distribution of the platypus has remained similar to pre-European times, but due to a lack of reliable quantitative data this remains unconfirmed (Grant and Temple-Smith 2003). However, they are considered vulnerable based on their dependence on both the riparian and aquatic environments of freshwater systems that are under stress from climate change and degradation resulting from human activities (Grant and Temple-Smith 2003; Warren et al. 2008).

European Colonisation

Although platypuses are adapted to the aquatic environments they inhabit, they are known to prefer terrestrial short cuts rather than meandering water canals when moving between water bodies during foraging activity. As their movement is not particularly efficient over land, many platypuses die as a result of being run over by motor vehicles or from predation by dogs and foxes (Munday et al. 1998). Modification of the riparian and aquatic environment through forestry, agricultural practices and dam constructions represent potentially significant threats to the survival of resident platypuses. The downstream effects of river regulation include temperature, flow and sediment changes, which impact on the abundance and diversity of benthic invertebrate species in streams (Grant 2007). Forestry and agricultural land use directly damages the riparian environment, which may reduce the availability of suitable burrow sites, and also impact the benthic prey of platypuses (Grant and Temple-Smith 2003).

Disease

Mucormycosis infections caused by the fungi *Mucor amphibiorum* represent the most significant disease in the platypus, causing severe ulcerative dermatitis and pneumonitis of platypuses in northern Tasmania (Munday et al. 1998). Severely ulcerated animals become debilitated and often flyblown, and mortalities have been recorded as a direct result of this disease. *M. amphibiorum* has been found on the mainland in the soil from Queensland and has also been reported from captive frogs held in Queensland, New South Wales, Victoria and Western Australia (Gust and Griffiths 2009). However the disease occurs only in Tasmanian platypuses and not Queensland platypuses. Genotyping at six neutral microsatellite loci has shown that Tasmanian populations form a distinct genetic subpopulation that is easily distinguishable from mainland populations (Akiyama 1998). Susceptibility to mucormycosis is probably due to the isolation of Tasmanian platypuses from the organism for more than 13,000 years, whereas the Queensland platypuses have likely co-evolved with it. The theory is that M. amphibiorum was introduced into Tasmania from Queensland with green tree frogs, co-transported with banana consignments and then with the frogs acting as vectors, the disease was able to spread along the river systems and infect the susceptible resident platypuses (Munday et al. 1998; Grant 2007; Gust and Griffiths 2009). Since the initial discovery of ulcerated platypuses in 1982, disease prevalence peaked in the mid 1990s with approximately 35% of captured platypuses were ulcerated and infected with mucormycosis in affected areas of Northern Tasmania (Gust and Griffiths 2009). This figure appears to have dropped to around 3% in recently sampled animals (Gust personal communication).

Neutral Genetic Diversity in Platypus Populations

A previous population survey documented that the King Island population was monomorphic for all six neutral microsatellite loci examined (Akiyama 1998). This may be an indication that founder effects and prolonged isolation have resulted in loss of genetic variability due to genetic drift and inbreeding. The microsatellite study suggested that the King Island platypuses are of mainland descent, as opposed to Tasmanian origins, and the Kangaroo Island population is of Victorian descent (Akiyama 1998). The use of neutral markers is effective for determining within population variation and relationships between the isolated platypus populations (Boyce et al. 1997). Further investigation of the MHC diversity within and between platypus populations will build upon this established information, inferring the immunological fitness and evolutionary potential of each population (Bernatchez and Landry 2003; Oliver et al. 2009). Results can direct conservation efforts to identify populations at risk from extinction due to low MHC diversity and construct management plans to ensure the continued survival of this iconic species.

Platypus MHC

Some class I (Miska et al. 2002) and class II (Belov et al. 2003) loci were recently characterised, and utilised in the construction of several MHC-containing Bacterial Artificial Chromosome (BAC) clones from the platypus (Dohm et al. 2007). These BACs allowed characterisation of two Class II genes, two Class I genes and three framework genes in the core MHC. The Class II genes were named DZA and DZB, and the Class I genes were named Class I-1 and Class I-2 genes. An additional MHC gene was found in an MHC block outside the core MHC, although this was an inactivated pseudogene (Dohm et al. 2007). Phylogenetic analyses with the characterised class I and class II loci in the platypus did not resolve orthologous relationships with corresponding marsupial or placental mammal MHC sequences (Miska et al. 2002; Belov et al. 2003). Instead, both classes are considered to be sister groups derived from gene duplication events prior to monotreme and therian divergence (Miska et al. 2002; Belov et al. 2003).

The platypus MHC genes are not contiguous and have been localised to different pseudoautosomal regions of platypus sex chromosomes, with the core MHC region, mapped to X3/Y3 and MHC block mapped to chromosome Y4/X5 (McMillan et al. 2007; Dohm et al. 2007). This is the first time that MHC has been localised onto the sex chromosomes in any mammal or vertebrate, as the MHC region is predominately located on a single autosome in other species (Dohm et al. 2007). These are among the smallest PARs in the platypus karyotype, which may result in high recombination frequency aiding to diversify MHC in offspring (Dohm et al. 2007).

MHC Diversity Within and Between Platypus Populations

MHC Class II-DZB diversity has been investigated in platypus, within and across its mainland and island populations (Woodward 2003). This study revealed that while the mainland and the Kangaroo Island populations existed at Hardy Weinberg Equilibrium, the Tasmanian population displayed significant homozygous excess, and the King Island was entirely monomorphic for a single allele at the domain related to the PBR. These results imply that even with selective forces present in the Tasmanian population that may have slowed the loss of genetic diversity, this cannot completely counter the effects of genetic drift. This is shown in its most extreme state in the allele fixation due to prevailing neutral genetic forces in the small island populations on King Island. The fact that the King Island platypuses lack diversity at these key immune genes raises conservation concerns about the long-term survival of this population, in the face of potential disease emergence or introduction, as well as climate change (Sanderson, unpublished data).

Conclusion

With its reliance on freshwater aquatic environments, the platypus faces an uncertain future where human influences and climate change may degrade and reduce the availability of suitable habitat. This vulnerability is driving an increased conservation effort to evaluate the susceptibility of each platypus population to these threatening processes, which involves assessment of adaptability to change, immunological fitness and evolutionary potential. Initial studies have used SSCP, cloning and sequencing of the platypus Class II DZB alleles, which is an accurate method for determining MHC diversity (Woodward 2003). However for comprehensive conservation efforts, high throughput and simple molecular methods are necessary to assess MHC diversity within and between platypus populations. Microsatellites located within or flanking MHC genes can infer allelic identity due to tight linkage, and therefore represent molecular tools that can be simply and efficiently implemented on large scales across the platypus distribution (Meagher and Potts 1997). The development of MHC-linked microsatellites in the platypus has also been assisted by the characterisation and sequencing of several MHC genes. From this, a set of microsatellite markers encompassing several MHC loci can be designed

and implemented in combination for broad MHC diversity comparisons across populations (Meagher and Potts 1997; Boyce et al. 1997; Aguilar et al. 2004).

Further investigations into the MHC diversity will prove valuable to current conservation efforts. Primarily, a population study of MHC diversity within and between platypus populations will infer the evolutionary potential, and the distinct and variable selective histories of individual populations. Comparisons between island and mainland populations may increase the state of knowledge of MHC diversity maintenance by positive selection in isolated populations. In addition, comparison of MHC diversity between Queensland and Tasmanian populations may enable characterisation of mucormycosis resistance and susceptibility, respectively, which may assist in diagnostics and assessment of mucormycosis vulnerability in other populations. In the critically depauperate population of King Island, investigating the MHC diversity at several loci will present wider evidence and allow better assessment of future population survival. Low MHC diversity does not commit this population to early extinction, but it is significantly endangered from introduced pathogens that cause extinction and will necessitate conservation management intervention.

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