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Characterization of MHC class IIB for four endangered Australian freshwater fishes obtained from ecologically divergent populations

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ABSTRACT

Genetic diversity is an essential aspect of species viability, and assessments of neutral genetic diversity are regularly implemented in captive breeding and conservation programs. Despite their importance, information from adaptive markers is rarely included in such programs. A promising marker of significance in fitness and adaptive potential is the major histocompatibility complex (MHC), a key component of the adaptive immune system. Populations of Australian freshwater fishes are generally declining in numbers due to human impacts and the introduction of exotic species, a scenario of particular concern for members of the family Percichthyidae, several of which are listed as nationally vulnerable or endangered, and hence subject to management plans, captive breeding, and restoration plans. We used a next-generation sequencing approach to characterize the MHC IIB locus and provide a conservative description of its levels of diversity in four endangered percichthyids: Gadopsis marmoratus, Macquaria australasica, Nannoperca australis, and Nannoperca obscura. Evidence is presented for a duplicated MHC IIB locus, positively selected sites and recombination of MHC alleles. Relatively moderate levels of diversity were detected in the four species, as well as in different ecotypes within each species. Phylogenetic analyses revealed genus specific clustering of alleles and no allele sharing among species. There were also no shared alleles observed between two ecotypes within G. marmoratus and within M. australis, which might be indicative of ecologically-driven divergence and/or long divergence times. This represents the first characterization and assessment of MHC diversity for Percichthyidae, and also for Australian freshwater fishes in general, providing key genetic resources for a vertebrate group of increasing conservation concern.

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1. Introduction

Genetic diversity is a key population requirement for adaptation to changing environments, and losing diversity due to restricted gene flow and genetic drift may have implications for population persistence and species viability [1,2]. Therefore, conservation programs typically strive to maintain genetic diversity by assessing variation at neutral DNA as a proxy for demographic processes affecting diversity. Adaptive genetic diversity is often neglected however, mostly due to the technical demands of appropriate genotyping [3–5]. One potentially adaptive marker that has proved important and tractable is the major histocompatibility complex, MHC [6,7]. However, information on MHC genes is lacking for many taxa of economic and conservation importance, such as the Percichthyidae, a family of Australian freshwater fishes targeted by recreational fisheries and conservation management plans.

The MHC is a hypervariable multigene family that encodes key proteins for the adaptive immune response in vertebrates primarily...
involved in the presentation of foreign antigens to T-cells [8,9]. The two classes of MHC proteins differ in function and expression pattern. MHC class I is expressed on all nucleated cells and presents antigens derived from intracellular pathogens, whereas MHC class II is expressed only on antigen-presenting cells, and displays antigens from extracellular and vesicular parasites. Unlike in other vertebrates, MHC class I and class II genes are not tightly linked in fish, because they reside on different chromosomes [10,11].

A major driver of the evolution of MHC genes is thought to be parasite-mediated balancing selection via rare allele advantage or heterozygote advantage maintaining high levels of polymorphism for these genes [12–16]. As a consequence, MHC lineages are typically maintained over long evolutionary periods, leading to the trans-species polymorphism commonly observed across all vertebrate taxa [17–20]. Another peculiarity of MHC is the substantial copy number variation of loci among and within species, ranging from no MHC II loci in Atlantic cod to greater than 10 loci in cichlids [21–25]. In the second exon of MHC II, diversifying selection is known to act on antigen-binding sites (ABS) which determine the specificity of the allele and are the source of the highest polymorphism [19,24,26]. Furthermore, intra- and interlocus recombination and gene conversion can shuffle standing genetic variation into new combinations and thereby into combinatorial diversity [27–29]. These diverse and surprising patterns found in teleost MHC II compared with other vertebrates (e.g. no physical linkage with MHC I, large variation in copy number) indicate that further study of this highly diverse group is essential to a general comprehensive understanding of the function and evolution of this gene.

The MHC is also implicated in mate choice and inbreeding avoidance. Mate choice for optimal diversity increases reproductive success and offspring fitness and maintains diversity at these genes [30–33]. Natural selection, enhanced by sexual selection, on MHC genes might lead to local adaptation and divergence of populations in response to prevalent parasite faunas [13,34], which will then have implications for translocation and captive breeding programs for reintroduction. A functional gene region such as the MHC may therefore act as a useful candidate for screening populations in conservation programs in addition to traditional neutral marker assays, such as those based on microsatellites.

The Percichthyidae, or temperate perches, is an ancient and diverse family [35] that dominates the temperate freshwater fish fauna of Australia and includes species of recreational and conservation concern (e.g. cods, pygmy perch). Nine of 20 described species of Australian Murray–Darling Basin Threatened Freshwater Fish Populations [51]. The individuals of the parental generation were collected from the lower Murray River (SA) and assigned into breeding groups based on microsatellite variation in order to maximize genetic diversity in the offspring and minimize inbreeding in the restoration program [53].

Starting material for DNA isolation from all specimens was a small portion of caudal fin. DNA was extracted using a modified salting out method [54]. In brief, tissue was digested with proteinase K (10 mg/ml) buffered in TNE solution (400 mM NaCl, 50 mM Tris pH 7.5, 0.5% SDS, 20 mM EDTA), centrifuged at 13,000 rpm. DNA was precipitated from the supernatant with 100% isopropanol before being pelleted and washed twice with 70% ethanol. The resulting pellet was resuspended in 30 μl of 0.1 × TE buffer. Quality and concentration of DNA extracts was estimated via spectrophotometry using a Nanodrop 1000 instrument (Sigma, Australia). Sample concentrations were then normalised to 15 ng/μl prior to amplification.
2.2. Amplification, cloning, and sequencing

As an initial exploratory step to assess the level of MHC class II diversity in this group of fishes, we genotyped a subset of samples of *N. australis* via a traditional cloning and sequencing approach. A 247 base pair (bp) fragment of exon 2 of the MHC IIB was amplified for four *N. australis* specimens using the primers GAIIE2F (GTCCTTTAATCCAGGACTCTGAG) and GAIIE2R (ACTCCCGGACTTATGACAG) [55]. Amplifications were performed in 10 μl volumes containing 1 × PCR buffer (New England Biolabs), 2 mM MgCl₂, 200 μM of each dNTP (Astral Scientific, Australia), 0.4 U Phusion DNA polymerase (New England Biolabs), 0.2 μM of each primer, 5% DMSO, and 15 ng of template DNA. Reaction conditions were 95 °C for 5 min followed by touchdown cycling of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 30 s for 10 cycles with the annealing temperature dropping to 52 °C for a further round of 25 cycles. The samples were transformed into TOP10 chemically competent cells using the TOPO® TA Cloning® Kit (Invitrogen) following the manufacturer’s instructions. After an overnight incubation on selective plates containing kanamycin, up to 12 clones per individual were picked into 25 μl deionized water and denatured at 99 °C for 10 min. The insertion size was checked by PCR amplification using the M13 forward and reverse primers provided by the distributor. Positive clones were sequenced on an ABI 3130 genetic analyzer (Applied Biosystems) at Flinders Medical School, Australia, using the gene-specific primer GAIIE2.

2.3. Next-generation sequencing

The results of the cloning work indicated that multiple alleles and perhaps duplicated loci existed for the MHC class II, at least for *N. australis*. Given the prevalence of similar findings for other fish species [22,25,56,57], we decided to continue the genotyping following a NGS approach to capture the full range of allelic diversity [58–60]. A 247-bp fragment of MHC IIB (203 bp without primers), encompassing 88% of the entire exon 2, was amplified using the primer pair GAIIE2F and GAIIE2R [55]. These primers were modified at the 5’ end with a series of unique 6 bp individual index tags (see Table S1). These index tags were used in specific forward and reverse combinations to allow for pooling of multiple individuals in a single sequencing run. PCR amplifications were carried out using the conditions described above but reducing the number of cycles to 10 and 20 for annealing temperatures of 55 °C and 52 °C, respectively.

PCR products were pooled in equimolar concentrations and forwarded to the Australian Genome Research Facility (AGRF) for sequencing. Sequencing adaptors were blunt-end ligated to the amplified fragments and unidirectional pyrosequencing was performed as two 1/8 plate runs of a Roche 454 GS FLX system. The samples were run twice to control for sequencing errors which can occur at relatively high frequencies [52,58]. The run included 129 samples from the four percichthyids (39 *G. marmoratus*, 44 *M. australasica*, 22 *N. australis*, and 24 *N. obscura*). The four individuals that had been previously typed via cloning and Sanger sequencing were included as control.

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2.4. Data analysis

2.4.1. Genotyping

There is a growing body of recommendations for analysing next-generation sequencing data [57–61], and the best analysis pipeline is very project-specific depending on, among other things, the initial question, the sampling design, and the sequencing platform. Therefore, we followed the genotyping suggestions by Sommer et al. [60] with some modifications (below). The raw data from each pyrosequencing run was filtered separately for high quality reads using the NGS QC Toolkit with default settings and a minimum read length of 200 bp [62]. Variants were then assigned to individuals according to their index tags in jMHC [63] and the tags and primers were trimmed. Then, variants occurring in only one amplicon of each individual were removed. Chimera detection was performed in UCHIME using the default settings and for the combined set of variants within a run as suggested by the authors [64]. Since the two pyrosequencing runs do not come from independent amplification products, we could not follow the recommendations by Sommer et al. [60]. Rather, we compared the variants found in parents and offspring of *N. australis* and *N. obscura*. Variants were considered putative alleles if they occurred in at least three different individuals, which is similar to the threshold used by Zagalka–Neubauer et al. [65]. The same criteria were used for discriminating between putative alleles and artefacts in G. marmoratus and *M. australisica*. We are aware that this approach is likely to underestimate the true allelic diversity due to the exclusion of rare alleles, however we did not aim for a full population genetic analysis but rather to give a first conservative description of MHC IIB diversity in this family.

2.4.2. Summary statistics

Sequences were aligned to MHC IIB alleles of other teleosts (*Dicentrarchus labrax* [Acc.no gi|111379942|], *Epinephelus coioides* [Acc.no gi|326632372|], *Gasterosteus aculeatus* [Acc.no gi|51449916|]) and the human DRB1*010101 as reference in BioEdit [66]. The best-fit nucleotide substitution models and estimates of genetic diversity were calculated in MEGA v6.06 [67] for each species or ecotype separately. A Z-test for positive selection over the entire exon 2 fragment was performed using the same program. Standard errors and significance were estimated by 10,000 bootstrap replications. Positively selected sites (PSS) were identified separately for each species or ecotype in OmegaMap v0.5 [68]. This method estimates positive selection in the presence of recombination using a population genetic approximation. The mutation rate (μ) and the transition/transversion ratio (κ) were chosen inefficiently prior distributions with starting values of 0.1 and 3.0, respectively. The selection parameter (ω), which was estimated independently for each codon, followed an inverse prior distribution between 0.01 and 20. The population recombination rate (ρ) also followed an inverse distribution with a range from 0.01 to 100; however, it was estimated for blocks of 10 codons. The minimum number of recombination was calculated using the four-gamete test implemented in DnaSP v5.10 [69,70].

2.4.3. Phylogenetic analyses

Phylogenetic relationships among MHC IIB alleles was inferred by Bayesian and maximum likelihood (ML) approaches. For the Bayesian analysis we used the program MrBayes [71] with settings adjusted to fit a Jukes–Cantor nucleotide substitution model with invariant sites and 5 discrete gamma categories. Two independent runs of four Markov chains Monte Carlo were run for 1 million generations and sampled at every 500th. A 25% fraction was discarded as burnin. The tree was visualized in MEGA v6.06 [67]. Additionally, an ML tree was constructed in MEGA v6.06 using the Jukes–Cantor nucleotide substitution model with a gamma distribution and invariant sites. Branch support was estimated by 500 bootstrap replications. MHC alleles often show mixed genealogies due to recombination and gene conversion, which can only be partly represented by phylogenetic trees. To circumvent this problem, a Neighbour-net network was constructed in SplitsTree v4.13 [72] using the Jukes–Cantor model with a gamma distribution and invariant sites.
3. Results

In total, 203 bp of exon 2 of the MHC IIB gene were sequenced for 122 individuals of four percichthyid species. One individual was excluded due to lacking overlap of the two sequencing runs. A further six individuals were excluded due to poor sequencing coverage of the variants for each fish and due to the requirement that variants occur in more than two fish. The coverage of both runs combined for each individual included in the analysis ranged from 41 to 6356 reads, with 115 individuals having a coverage of at least 100 reads. The 58,485 and 61,947 raw reads, from each run respectively, could be assigned to 81 different variants after using stringent filtering criteria. The 203 bp translate into 67 amino acids (aa) and none of the variants harboured premature stop codons (Fig. 1).

The sizes of the six population samples genotyped here range from 16 to 23 individuals, however, only 6 and 8 breeders were included for *N. australis* and *N. obscura*, respectively. A total of 6–21 and 3–16 unique nucleotide and amino acid sequences were detected per population, with *G. marmoratus* samples having very low numbers of variants (Table 1). The maximum number of alleles found per individual ranged from 4 to 14 across the four species, however, the median was 2–4 for all of them. Cloning of four *N. australis* individuals gave similar results to the NGS in terms of number of alleles per individual, however, the overlap of variants identified by both approaches was not complete. For the individuals SPBR2, SPBR23, and SPBR44, we identified 20 variants via cloning and 14 via NGS. The difference is probably due the lack of

![Amino acid alignment of MHC IIB alleles of the four percichthyid species to three additional teleost sequences (accession numbers: gi111379942, gi326632372, gi51449916) and the human DAB1. Positions marked with + and shaded in grey indicate to human ABS according to Brown et al.[73], PSS of the different species, populations, and ecotypes are shaded in different colours (*G. marmoratus*: light red — Shawns Creek Coonabarabran, dark red — Scrubby River, *M. australasica*: light green — Kowmung River, dark green — Yarra River, *N. australis* — dark blue, *N. obscura* — light blue. For the *Nannoperca* species only the breeders were included in the analysis, therefore, alleles that could not be recovered from the breeders are not shaded. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).]
quality filtering to detect PCR artefacts from the cloning approach combined with the conservative nature of our NGS filtering. More replication and higher sequence coverage may shed light on this discrepancy, however our results are in line with differences reported in other studies [60].

The nucleotide and amino acid diversities were between 0.119 – 0.155 and 0.200 – 0.268, respectively. The highest diversity was found in the introduced Yarra River population of M. australasica. There were no shared alleles among any of the species nor between ecotypes of G. marmoratus and M. australis. The nucleotide diversities between the ecotypes of G. marmoratus (π ± SE: 0.136 ± 0.015) and M. australis (π ± SE: 0.163 ± 0.016) are similar to the diversity recorded between the Nannoperca species (π ± SE: 0.142 ± 0.014).

A Z-test for positive selection over the exon 2 fragment was significant for the G. marmoratus ecotypes and the Nannoperca species. However, for both ecotypes of M. australis the signs of overall positive selection were very weak and non-significant (Table 1). A site-specific test of positive selection inferred positively selected sites (PSS) for all species and ecotypes with 8–13 sites showing signs of selection (Fig. 1, Table S2). For the Nannoperca species only the breeders were included for inference of PSS to avoid interdependencies due to parent-offspring relationships. Forty-four to 78% of the PSS correspond to human ABS de polymorphism that is a deletion compared with the present study [43,45,46](Cole et al. unpublished; Lean et al. unpublished). Also, for two breeding species, the nucleotide diversity (π ± SE), amino acid diversity (p-distance ± SE), ratio of non-synonymous to synonymous mutations (dS/dN), p-values are given for a Z-test of positive selection on exon 2, significant values are given in bold, minimum number of recombination events (Rm).

4. Discussion

We provide the first characterization of MHC genes in fishes of the family Percichthyidae, many of which show a decline in population size and are subjected to management and recovery actions [48,50,51].

The median number of alleles per individual in the four species, G. marmoratus, M. australis, N. australis, and N. obscura, indicates duplication of the MHC IIB locus in this family. This is a relatively common phenomenon of these genes, especially in fishes [22,25,56,57,74]. Furthermore, the differences in numbers of alleles among individuals indicate that there is copy-number variation for these species, which is also observed in other species [74,75]. However, our genotyping approach is likely to overestimate the extent of this variation, because rare or poorly amplified alleles may be excluded due to our strict filtering steps and low read depth for some individuals.

We did not detect any indel polymorphisms in the 81 identified alleles. This is in contrast to the Salmonidae, a phylogenetically distant family of teleosts in which MHC has been studied extensively. All species of this family studied to date exhibit a 3 bp indel polymorphism that is a deletion compared with the present study and human HLA [74,76–79]. This adds to the evidence that this functional polymorphism is specific to Salmonidae and not a common feature of teleost MHC II.

An interesting finding is the lack of shared alleles not only among species but also between the ecotypes of M. australis and G. marmoratus. This is in contrast to recently diverged ecotypes of a salmonid, lake whitefish (Coregonus clupeaformis), which share the same alleles [74]. The ecotypes of M. australis have been separated for several thousands of years [39] and might have adapted to their prevailing parasite fauna which could have led to the maintenance of diverged allele pools [80,81]. This could also be the case for the geographically isolated G. marmoratus populations. Further studies including assessments of parasite load would shed some light on the reasons for this divergence. However, a recent study on guppy ecotypes shows that divergent parasite communities might not lead to divergent MHC allele pools, but rather maintain a high and shared diversity among them due to stronger positive selection [82]. Our results suggest that adaptation of species-specific parasites on MHC has more influence on the alleles than the relatively weak positive selection. Whether our result is due to divergence of the ecotypes or the small number of populations included remains to be tested. It might, however, have implications for translocations and captive breeding programs.

All four percichthyids show reduced genetic diversity across their ranges as shown by population genetic surveys based on neutral markers [43,45,46](Cole et al. unpublished; Lean et al. unpublished). For instance, levels of microsatellite DNA variation in N. obscura are amongst the lowest reported for a fish species (e.g.
Fig. 2. Phylogenetic relationship of the MHC IIB alleles based on (a) Bayesian inference and (b) Neighbour-net network. Numbers on the branches are posterior probabilities. *G. marmoratus* alleles are labelled Gm01–Gm13. Light red alleles come from the Shaws Creek Coonabarabran population, dark red from Scrubby River. *M. australasica* alleles are labelled Ma01–Ma37. Light green and dark green are alleles present in the Kowmung River and the Yarra River, respectively. *N. australis* alleles are labelled Na01–Na14 and dark blue, *N. obscura* alleles are labelled No01–No17 and marked in light blue. Full names of the alleles are given in Table S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
mean heterozygosity of 0.318 and mean allelic richness of 1.92) [43]. Low levels of neutral genetic variation in Australian freshwater fishes is often explained by the continent’s naturally high hydrological instability that creates boom/bust cycles in population dynamics combined with impacts of recent anthropogenic activities that contribute to habitat fragmentation, population bottlenecks and disruption of metapopulation connectivity [46,49,83,84]. This overall pattern was not necessarily reflected in the diversity of MHC IIb genes reported in this study. Although we used very strict filtering conditions that likely removed true alleles (as shown in comparisons between parents and offspring of the two Nannoperca species), we still detected 81 unique alleles in a sample of 122 individuals from three different percichthyid genera. While we found fewer than 10 unique alleles for G. marmoratus, the M. australasica ecotypes and the Nannoperca species had 2–3 times as many alleles in a similar sample size. However, for all species and ecotypes, the total number of alleles and their diversity are lower than those reported for non-endangered fish species with more than one MHC IIb locus [20,27,74,85]. Among the four percichthyid species, G. marmoratus did not show especially low nucleotide or amino acid diversity despite strong bottlenecks in the source populations. Together with the fact that this species had the strongest signal of positive selection (Tables 1, 2), this indicates the report of recent genetic diversity at this functionally important gene region. Interestingly, for M. australasica genetic diversity was much higher in the Yarra River (an introduced population) than in the Kowmung River (native range) which might be related to the translocation activities (e.g., multiple sources) undertaken in the Yarra River [39].

Positive selection and recombination play an important role in the evolution of MHC genes [24,26–28]. Similarly, these processes seem to be involved in the evolution of MHC IIb genes in the four percichthyid species. Although overall positive selection was not significant in M. australasica populations, a site-specific test inferred positively selected sites (PSS) in all four species of which about 55–75% correspond to human antigen-binding sites (ABS). The crystallographic structure of teleost MHC molecules is unknown to date and several studies indicated that teleostean PSS might not necessarily be identical to human ABS [24,74,85–87]. In particular, the PSS residue 30 (which corresponds to 53 in HLA of Brown et al. [73]) was also found to be positively selected in salmonids [74,79,87]. The consistent pattern of positive selection in such distantly related teleost strongly suggests that this site is part of the teleost ABS. Interestingly, the positions of PSS in the two G. marmoratus samples were quite different from each other. However, in this taxon, our sample of six and seven alleles per population is rather small and this peculiarity might not hold true for larger samples.

The alleles cluster in a genus-specific manner with G. marmoratus, M. australasica, and Nannoperca species forming three distinct clusters. Within the genera, however, the alleles do not sort according to species and/or ecotypes despite the absence of allele sharing. If considering the ecotypes as potential species [39–41] then the three genera, Nannoperca, Gadopsis, and Macquaria, show trans-species, but not trans-generic polymorphism. For two of the Nannoperca breeding groups, there are alleles missing from the offspring genotypes when compared to their parents. Whether these alleles are missing due to disassortative mating cannot be disentangled from the possibility that alleles were missed due to strict filtering; this is further complicated by the common occurrence of copy number variation in fish MHC [25,57]. Also, two alleles of the offspring were not present in the parental genotypes, which is likely due to the filtering procedure used here. Since MHC is reported to be involved in mate choice and reproductive success [30,31,88], diversity at this locus might have implications for the success of captive breeding currently under way for the Nannoperca species [51,53]. Further experimental studies are needed to assess population-wide MHC diversity and inheritance in these species. The latter might enable planning breeding designs for optimal immunogenetic diversity, which is likely to increase offspring fitness and adaptation to local selection regimes.

In conclusion, we provide evidence that MHC might be a useful tool for studying adaptive diversity in these species and that it might have implications for management strategies such as translocations and captive breeding. The species, ecotypes, and populations are likely adapted to their distinct parasite fauna, a process that might have contributed to the divergence observed within percichthyid species. We hope that these resources will enable the assessment of population level MHC diversity in future studies of Percichthyidae and that such information be used in combination with other appropriate genetic data [e.g. 5] in policies and practices relevant to conservation management of Australian fishes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.06.009.

Data accessibility

GenBank accession numbers: KT163149–KT163229.

Author contributions

LBB conceived the study, SM, LBB and PS designed experiments. SEB and SM generated the data. MH collected samples. SEB, SM, and SP analysed the data. SEB and SM led the writing. SEB, SM, MH, SP, PS and LBB wrote and edited the final manuscript.

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