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ORIGINAL ARTICLE

Characterization of MHC class IA in the endangered southern corroboree frog

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Abstract Southern corroboree frogs (*Pseudophryne corroboree*) have declined to near extinction in the wild after the emergence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in southeastern Australia in the 1980s. A major captive breeding and reintroduction program is underway to preserve this iconic species, but improving resistance to *B. dendrobatidis* would help the wild population to be self-sustaining. Using 3′ and 5′ rapid amplification of complementary DNA ends (RACE), we characterized the major histocompatibility complex (MHC) class IA locus in this species. We then used sequences generated from RACE to design primers to amplify the peptide-binding region (PBR) of this functional genetic marker. Finally, we analysed the diversity, phylogeny, and selection patterns of PBR sequences from four *P. corroboree* populations and compared this with other amphibian species. We found moderately high MHC class IA genetic diversity in this species and evidence of strong positive and purifying selection at sites that are associated with putative PBR pockets in other species, indicating that this gene region may be under selection for resistance to *Bd*. Future studies should focus on identifying alleles associated with *Bd* resistance in *P. corroboree* by performing a *Bd* laboratory challenge study to confirm the functional importance of our genetic findings and explore their use in artificial selection or genetic engineering to increase resistance to chytridiomycosis.

Keywords Major histocompatibility complex · *Pseudophryne corroboree* · *Batrachochytrium dendrobatidis* · Genetic variation · Chytrid fungus · Amphibian declines

Introduction

The southern corroboree frog (*Pseudophryne corroboree*) is an iconic Australian amphibian endemic to the Snowy Mountains region of the state of New South Wales (Hunter et al. 2009). Since the emergence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in southeastern Australia in the early 1980s, this once abundant species has been in steady decline, with only a handful (<50) of individuals currently remaining in the wild (Hunter et al. 2010a; McFadden et al. 2013). An intensive captive breeding and reintroduction program for *P. corroboree* has been in place since 2010 (Hunter 2012; McFadden et al. 2013); however, this species may remain reliant on conservation management unless resistance to *Bd* increases.

Bd susceptibility is known to be influenced by multiple factors including climate (Raffel et al. 2012), host immunity (Richmond et al. 2009), and host behaviour (Richards-Zawacki 2010); yet it is difficult to determine which factors are most important in influencing population disease outcomes. Evidence of within-species variation of disease susceptibility suggests that immunogenetics is a major factor influencing host survival (e.g. Savage and Zamudio 2011). Among immune genes, the major histocompatibility complex

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(MHC) is likely to influence disease resistance as this highly polymorphic gene complex plays a critical role in several components of the adaptive immune system (Klein and Figueroa 1986). The two major classes of classical MHC molecules are class I and class II. MHC class I molecules are expressed on all nucleated somatic cells and mainly present antigens from intracellular pathogens to cytotoxic T cells (T_C), while MHC class II molecules are only present on antigen-presenting cells where they primarily present antigens from extracellular pathogens to helper T cells (T_H) (Bernatchez and Landry 2003).

In amphibians, MHC variation has been associated with resistance to *Aeromonas* bacteria (Barribeau et al. 2008), *Ranavirus* (Teacher et al. 2009), and *Bd* (Savage and Zamudio 2011; Bataille et al. 2015; Savage and Zamudio 2016). Because *Bd* is an intracellular pathogen that occurs within epidermal cells and is not known to be phagocytosed by immune cells, MHC class IA may play a key part of the immune response to *Bd*. Indeed, preliminary evidence indicates that *Bd*-infected tissue has increased apoptosis and cell death (Ellison et al. 2014; Brannelly 2016). MHC class IA molecules bound to pathogen-derived antigens activate T_C, thus inducing apoptosis by the Fas ligand pathway or by the release of cytotoxic granules (Kagi et al. 1994; Ashkenazi and Dixit 1998; Goldsby et al. 2002), and may therefore induce apoptosis in *Bd*-infected cells. Activated T_C also secrete a variety of cytokines with known antifungal capabilities such as IFN-γ and TNF-α (Roilides et al. 1998; Stevens et al. 2006); thus, T cell activation, mediated by MHC class IA, may contribute to *Bd* resistance.

The two major types of MHC class I genes are the highly polymorphic class IA (classical MHC) and monomorphic class IB (non-classical MHC) (Janeway et al. 2005). In this study, we investigated variation of classical MHC class IA because it has higher levels of genetic diversity and expression than that of class IB and is known to be associated with pathogen immunity in many species (e.g. Teacher et al. 2009; Wang et al. 2014; Aguilar et al. 2016). MHC class IA molecules are comprised of an α chain and a β2 microglobulin chain (Janeway et al. 2005). The α chain contains a cytoplasmic region, transmembrane region, and three extracellular domains (α1, α2, and α3), which are encoded by MHC class IA exons 2–4. The peptide-binding region (PBR), at which most MHC genetic diversity is found, is located in the α1and α2 domains, corresponding to exons 2 and 3. Within the PBR, the highest amino acid diversity is found in the antigen-binding pockets—due to their direct interactions with pathogen peptides (Matsumura et al. 1992). Changes in the amino acid sequence of these pockets result in structural modifications that can alter the pathogen binding affinity (Matsumura et al. 1992; Zhang et al. 1998). Locations of MHC class IA pockets in amphibians have not been confirmed with x-ray crystallography, but can be predicted by alignments of sequences from other species and by identification of codons under selection (Flajnik et al. 1999; Kiemnec-Tyburczy et al. 2012).

The goal of this study was to characterize the MHC class IA region of *P. corroboree* for future investigations of genetic associations with immunity to *Bd*. Our specific aims were to develop complementary DNA (cDNA) primers to characterize the hypervariable PBR and to investigate the genetic diversity of the MHC class IA region of this species.

Materials and methods

Samples

RNA for cDNA synthesis was extracted from liver tissue collected from 16 adult *P. corroboree* individuals (euthanized from a separate study). Tissue was preserved with RNAlater (Qiagen) until extraction. Frogs were excess to the captive breeding program and were delivered to James Cook University from the Amphibian Research Centre. They were collected from the wild as eggs from four different populations (Manjar, $N = 5$; Jagumba, $N = 2$; Snakey Plains, $N = 5$; Cool Plains, $N = 3$; and unknown population, $N = 1$; Fig. 1) and ranged in age from 5 to 8 years old. RNA was extracted using a RNeasy Kit (Qiagen) following the manufacturer's protocols for tissue extraction. After weighing, 20 to 30 mg of tissue was immediately placed in buffer RLT plus 2 M DTT, ground up using a micro pestle, and then homogenized with an 18-gauge needle and syringe. The final elution step was performed using 16 (Micro Kit) or 30 μl (Mini Kit) of RNase-free water. Concentration and quality of RNA were measured with a Nanodrop (ND-1000), and the final product was stored at −80 °C until use. Genomic DNA was extracted from the skin and muscle from 11 of the previously sequenced *P. corroboree* individuals, along with one additional individual from the Cool Plains population, with a Bioline Genomic DNA Extraction Kit and preserved at −20 °C until analysis.

cDNA synthesis and RACE

Because no sequence information was available for conserved regions of the MHC class IA for *P. corroboree*, we used 3′ and 5′ rapid amplification of cDNA ends (RACE) (Scotto-Lavino et al. 2006a, b) to characterize MHC class IA of this species. This method is useful when working with poorly characterized gene regions because it allows the generation of sequences when only a small part of the sequence is known (Scotto-Lavino et al. 2006b). After performing RACE, we developed primers that amplified the most variable regions of MHC IA, exons 2 and 3. All primers were designed using Primer 3 (v. 2.3.4) in Geneious (v. 7.1.5) and were manufactured by Macrogen. Primer sequences and

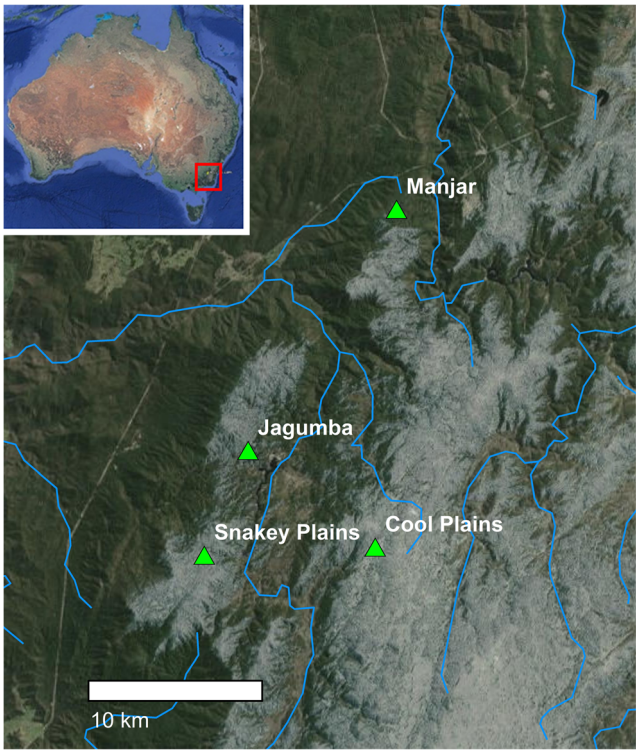

Fig. 1 Map of populations sampled for our study. The base map was prepared using ArcGIS (v 10.2.2); *inset* was taken from Google earth (v 7.1.5.1557; imagery date 12/14/15)

reaction information can be found in Table 1. Sequences were annotated using the chicken genome (*Gallus gallus*; AB426152). cDNA synthesis reactions and RACE PCR followed the protocol described in Scott-Lavino et al. (2006a, b). For minor modifications due to reagent differences, see Online Resource 4. Briefly, total RNA was used to synthesize cDNA using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara) and RACE primers (5′ RACE primers varied, 3′ RACE primer: Q_t alt). 5′ RACE had an additional step of appending a poly(A) tail to the cDNA. This was accomplished by first purifying cDNA with a gel/PCR Purification Mini Kit (Favorgen) to a final elution volume of 20 μl and then A-tailing purified cDNA with the following reaction: 20 μl cDNA, 3.5 μl 10× tailing buffer, 5.0 μl $CoCl_2$, 4.0 μl dATP, and 0.3 μl Tdt with the thermal cycler program: 10 min at 37 °C and then 5 min at 65 °C. All RACE and PCRs were performed with an ABI Veriti thermal cycler.

Polymerase chain reaction of MHC class IA

PCR primers designed from RACE sequences were used to amplify exons 1 to 4 of *P. corroboree* MHC class IA cDNA. PCR amplification was performed in 25 μl reactions containing 0.2 μl of Ex Taq (Takara), 2.5 μl of 10× buffer, 1.25 μl of each primer (10 μM), 2.0 μl of dNTP mix (2.5 mM), 16.8 μl of PCR grade water, and 1.0 μl of cDNA template. Thermal cycler conditions consisted of an initial activation step of 95 °C for 3 min; followed by 30 cycles of 95 °C for 3 min, 55 °C for 30 s, and 72 °C for 1 min; and a final elongation step of 72 °C for 10 min. The elongation step was increased to 1 min to minimize the formation of PCR artefacts. To confirm genotypes, independent PCRs were performed with the 11 cDNA-sequenced individuals using genomic DNA (gDNA) exon 2 primers (PcIAex2-2F1 and PcIAex2-2F1; Table 1).

Cloning and sequencing

PCR products resulting from RACE or PCR amplification, along with a 100-bp DNA ladder (Takara), were separated by gel electrophoresis on a 1% agarose gel at 110 V. Bands of the correct size were excised from gels and extracted with a FavorPrep Gel Purification Kit (Favorgen) following the manufacturer's protocol. PCR products were cloned with the RBC T&A cloning vector kit, and recombinant DNA was transformed using HIT-DH5α competent *Escherichia coli* cells (RBC Bioscience). Cells were grown on LB agar plates (with ampicillin, X-Gal, and IPTG) for 16 to 18 h at 37 °C. We used blue-white screening to select from 24 to 80 clones from each transformation and amplified them with M13 primers using standard reaction conditions. PCR products were purified for sequencing by a cleanup reaction of 10 μl of PCR product, 1 U of Antarctic phosphatase, 1 U of exonuclease, and 2.6 μl of RNase-free water and the thermal cycler program: 37 °C for 30 min, 80 °C for 20 min, and 4 °C for 5 min. Purified PCR products were Sanger-sequenced by Macrogen, and sequences were analysed with Geneious (v. 7.1.5). Sequence identity was confirmed by BLAST and alignment with human and amphibian MHC class IA sequences from GenBank. Alleles included in analyses were replicated across at least two independent PCRs. Genomic DNA from an individual from another study (11c, unpublished data) was genotyped to validate allele 21.

Analyses

Genetic divergence of nucleotide and amino acid sequences was analysed with MEGA 7 (Kumar et al. 2016). A Kimura 2-parameter gamma-distributed model (K2+G; Kimura 1980) was used to analyse nucleotide substitutions, and a Jones-Taylor-Thornton gamma-distributed model was used for amino acid sequences (JTT+G; Jones et al. 1992). Tests for recombination and selection were implemented with programs from the Datamonkey server (Delport et al. 2010). The genetic algorithm recombination detection (GARD) method was used to detect evidence of recombination in our dataset (Kosakovsky Pond et al. 2006).

We tested for evidence of positive selection in the entire alignment with the partitioning approach for robust inference

Table 1 RACE and PCR primers

Name	Sequence	Product size (bp)	Reaction
Qi	GAGGACTCGAGCTCAAGC	n/a	3′ RACE, R2; 5′ RACE, R2
Qo	CCAGTGAGCAGAGTGACG	n/a	3′ RACE, R1; 5′ RACE, R1
Qt(alt)	CCAGTGAGCAGAGTGACGAG GACTCGAGCTCAAGCTTTTTTTTTTT TTTTTT	n/a	3′ RACE, cDNA; 5′ RACE, R1
BgIAx4R2	AATCCGTACACCTGGCAGTG	n/a	5′ RACE R1
BgIAex3-4bridgeR	GGCYGAACTCTCCTCTCCAG	546	5′ RACE R2
PcIAx2R1	TCTCTCTCCCAGTACTCCGC	248	5′ RACE R2
PcIAx2R2	GCCGTACATCCACTGGTAGG	294–351	5′ RACE R2
PcIAx1F1.5	CACAGGAGGACGTCACCCYA	n/a	3'RACE R1
PcIAx1F3	TTATTCTGGGGGTGTCAGGC	824–948	3'RACE R2
PcIAex1F1	ACTGCTTATTCTGGGGGTGTC	588–698	cDNA PCR exons 2–3
PcIAex4R1	GTGAAGCTTTGTGACCTCGC	588	cDNA PCR exons 2–3
PcIAex4R2	GTCAGGATGGGGGAGGATCT	698	cDNA PCR exons 2–3
PcIAex2-2F1	TCTGGTTGAAGCGGCTCATC	213	gDNA PCR exon 2
PcIAex2-2R1	GCTGRGAGATGACGGCAGCA	213	gDNA PCR exon 2

of selection (PARRIS) method; this maximum likelihood method detects evidence of positive selection across an alignment and is robust to the presence of recombination (Scheffler et al. 2006). Next, we tested for evidence of selection at the codon level using four methods: SLAC, FEL, REL, and MEME. Single likelihood ancestral counting (SLAC) is the most conservative method, fixed-effects likelihood (FEL) is intermediate and considered the best method for overall performance, random-effects likelihood (REL) is the most powerful method but prone to false positives, and mixed-effects model of evolution (MEME) excels at detecting evidence of episodic diversifying selection (Kosakovsky Pond and Frost 2005; Murrell et al. 2012). Lastly, we compared the *P. corroboree* sites under selection with those from other published studies on MHC class IA (Flajnik et al. 1999; Kiemnec-Tyburczy et al. 2012; Lillie et al. 2014). By comparing our results with those of others, we were better able to determine sites under selection in *P. corroboree*.

Evolutionary relationships of *P. corroboree* nucleotide sequences and other vertebrate taxa were inferred by constructing maximum likelihood (ML) phylogenetic trees with the complete nucleotide dataset in MEGA 7. Evolutionary distances were computed using the K2+G method and tree node support was estimated via 500 bootstrap replicates (Felsenstein 1985).

Results

RACE

The first successful amplification of *P. corroboree* MHC class IA sequences was obtained using the 5′ RACE primer pairs, BgIAx4R2 and BgIAex3R12 and BgIAx4R2 and BgIAex3-4bridgeR (GenBank accession nos. KX372239 and KX372241; Table 1; Fig. 2; Online Resource 1), which amplified 357 to 546 bp long segments of exons 1 through 3 from two individuals. These primers were first developed for *Bufo gargarizans* (Didinger et al. 2017) from published MHC class IA sequences of *Espadarana prosoblepon* (accession numbers JQ679332 and JQ679341) and *Smilisca phaeota* (accession numbers JQ679380 and JQ679390). We aligned the three *P. corroboree* sequences and designed two new primers (PcIAx2R1, PcIAx2R2). Then, we used 5′ RACE to amplify a 248- to 351-bp region of exons 1 through 3 from three individuals (KX372233, KX372235, and KX372237). Next,

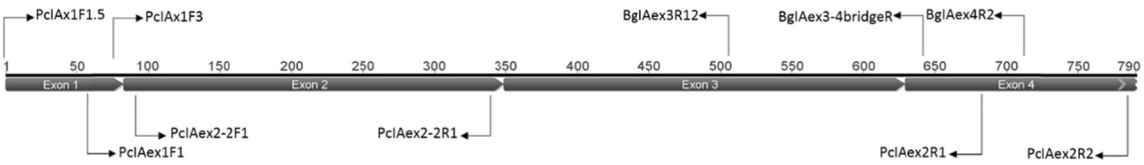

Fig. 2 RACE and PCR primer locations. Primers positioned *above* the gene fragment were RACE primers and those *below* were PCR primers

we aligned all of the previous *P. corroboree* sequences and designed 3′ RACE primers (PcIAx1F1.5, PcIAx1F3) that amplified the majority of class IA exons for three individuals (KX372234, KX372238, KX372242). This primer combination amplified exons 1 through 6 and produced the longest contiguous sequence (948 bp) that we obtained in our study.

cDNA sequencing

We aligned the RACE sequences and designed PCR primers (PcIAex1F1, PcIAex4R1, and PcIAex4R2) to amplify cDNA of the entire MHC class IA peptide-binding region. These primers successfully amplified cDNA from 11 of 16 *P. corroboree* individuals from four different populations. Initially, we used primer pair PcIAex1F1 and PcIAex4R2 for PCR amplification because this combination produced longer amplicons (698 vs. 588 bp). However, we found that primer PcIAex4R2 produced more non-specific amplification and worked in a lower percentage of individuals than PcIAex4R1, so we sequenced the remainder of individuals using reverse primer PcIAex4R1.

Number of expressed MHC class IA loci

After eliminating single-copy alleles, we recovered sequences from two to eight unique alleles per individual for a total of 15 unique alleles (GenBank accession nos. KX372221 and KX372232 and KYO72979 and KYO72985; Table 2; Online Resource 2). A nucleotide BLAST search indicated that these alleles were most similar to published anuran sequences for *Pelophylax nigromaculatus*, *Rana temporaria*, and *Rana yavapaiensis*. We could not confirm the total number of MHC class IA loci for this species without a reference genome, but we suggest a conservative estimate for the minimum number of loci in this species is four based on the maximum number of alleles observed in an individual divided by two. All alleles are likely functional because they were transcribed, they align with other MHC class IA sequences, and there was no evidence of deletions, insertions, or stop codons in any of the sequences.

Genetic variation and phylogeny of MHC class IA sequences

Genetic divergence in *P. corroboree* MHC class IA sequences was towards the higher end of that observed in other amphibian species (Table 3). Mean divergence of the full length 588 bp sequences in *P. corroboree* was 0.124 nucleotides and 0.271 amino acid residues. Sequence divergence was higher in exon 2 than exon 3 at both the nucleotide (0.162 vs. 0.146) and amino acid levels (0.299 vs. 0.289), corresponding to results in other amphibians (Kiemnec-Tyburczy et al. 2012). The ML tree of MHC class IA sequences was monophyletic with respect to *P. corroboree* (bootstrap value = 74%; Fig. 3). Alleles, 5, 7, 8, and 21 and 13, 15, and 23, clustered together with 92 and 100% bootstrap support respectively, indicating that these clusters may comprise two of the four putative MHC class IA loci.

Tests of selection

We found evidence of recombination at one site in our alignment (nucleotide site 194). Selection tests were performed using a GARD-inferred trees model to account for these breaks. The PARRIS method found strong evidence for positive selection in our alignment of 15 *P. corroboree* MHC class IA alleles ($P < 0.000001$). Four other methods (SLAC, FEL, REL, and MEME) found evidence of positive and purifying selection acting on different codons (Fig. 4; Online Resource 3). The most conservative method, SLAC, found evidence of positive selection acting on two codons ($P < 0.1$). In contrast, the least conservative method, REL, found evidence of positive selection acting on 37 codons (Bayes factor > 50). In total, eight codons were under positive selection in at least one test $P < 0.05$ (28, 47, 68, 69, 72, 98, 125, and 165) and four of these were significant $P < 0.05$ in more than one method (28, 47, 68, and 98). Six of these sites (28, 47, 68, 72, 98, and 165) corresponded to sites previously identified as putative peptide-binding sites in other anurans (Flajnik et al. 1999; Kiemnec-Tyburczy et al. 2012; Lillie et al. 2014).

The SLAC method found evidence of purifying selection acting on four sites ($P < 0.05$) and the FEL model on 14 sites ($P < 0.05$). There were a total of 14 sites under purifying selection in at least one test $P < 0.05$ (14, 18, 21, 42, 57, 73 78, 85, 107, 109, 119, 137, 139, 169, and 171) and were five sites (14, 18, 42, 78, and 169) with evidence of purifying selection $P < 0.05$ in more than one method (Fig. 3). Three of these sites (78, 109, and 119) correspond to sites under positive selection in other anurans (Flajnik et al. 1999; Kiemnec-Tyburczy et al. 2012; Lillie et al. 2014).

Discussion

We found moderately high genetic variation in the MHC class IA in southern corroboree frogs compared with other amphibian species. This contrasts with the results of population genetic studies in *P. corroboree* of polymorphic enzymes and microsatellite markers which found that genetic diversity was low in comparison to other amphibian species, including the closely related *Pseudophryne pengilleyi* (Osborne and Norman 1991; Morgan et al. 2008). This is consistent with observations that selection may maintain MHC variation in severely bottlenecked populations where variation at neutral loci has been depleted (Aguilar et al. 2004).

Table 2 Summary of *Pseudophryne corroboree* allele variants

ID	Population	Clutch	MHC IA alleles								N sequenced
			I	II	III	IV	V	VI	VII	VIII	
5c	Cool plain	6	4	5	7	8	9	15			34
7c	Cool plain	7	4	5	7	9	15				39
11c	Cool plain	3	4	8	9	15	21				30
12j	Jagumba	9	4	8	9	11	13				31
24j	Jagumba	5	1	4	5	7	9	15	16	17	40
13m	Manjar	2	1	2	5	7	9	16	17		80
17m	Manjar	1	1	5	16	17	23				24
6m	Manjar	14	2	4	8	9	13				37
17s	Snakey	21	1	5	14	16	17				36
23s	Snakey	18	1	4	8	9	17	21	23		51
6s	Snakey	22	1	5	7	8	14	16	17		40
9s	Snakey	20	11	13							24

We also found evidence of positive and purifying selection acting on regions corresponding to putative peptide-binding pockets in other anurans indicating that these codons may be under selection for resistance to *Bd*. Although the association of MHC class IA alleles with *Bd* resistance has not yet been tested, associations have been identified between specific MHC class IIB residues (e.g. proline at codon 46) and *Bd* resistance in multiple species such that residues may have better binding affinity for *Bd*-derived peptides (Savage and Zamudio 2011; Bataille et al. 2015). Experimental infection studies will be needed to investigate the influence of candidate MHC class IA residue identities on *Bd* susceptibility.

Despite their moderate *Bd* prevalence in the wild and susceptibility to *Bd* in the laboratory, populations of *P. corroboree* have steadily declined since *Bd* was introduced in the 1980s (Hunter et al. 2010b). This small species requires high exposure doses of *Bd* to become experimentally infected, and when exposed to 1×10^6 zoospores, they took an average of 7–8 weeks to die (Brannelly et al. 2015; S. Cashins unpub data). Population resilience to *Bd* is known to be influenced by multiple factors, including climate, host behaviour, reproductive effort, and immune genotype (Raffel et al. 2006; Richards-Zawacki 2010; Savage and Zamudio 2011; Bataille et al. 2015; Brannelly et al. 2016). Climate and behaviour are unlikely contributors to the prolonged persistence of *P. corroboree* because this alpine

Table 3 MHC class IA genetic divergence among anurans

Species	Nucleotide divergence			Amino acid divergence			*N*	N Alleles	Reference
	Exon 2	Exon 3	Full-length[b]	α1	α2	Full-length[b]			
Pseudophryne corroboree (cDNA)	0.162	0.146	0.124	0.299	0.289	0.271	11	9	This study
P. corroboree (cDNA, gDNA)	0.263	na	na	0.508	na	na	12	15	This study
Agalychnis callidryas	0.304	0.212	0.202	0.320	0.349	0.287	5	19	Kiemnec-Tyburczy et al. 2012
Espadarana prosoblepon	0.233	0.145	0.153	0.329	0.230	0.225	5	12	Kiemnec-Tyburczy et al. 2012
Lithobates catesbeianus	0.287	0.115	0.132	0.377	0.201	0.197	5	12	Kiemnec-Tyburczy et al. 2012
Lithobates clamitans	0.292	0.113	0.130	0.367	0.196	0.193	5	16	Kiemnec-Tyburczy et al. 2012
Lithobates yavapaiensis	0.115	0.080	0.076	0.219	0.129	0.134	5	9	Kiemnec-Tyburczy et al. 2012
Smilisca phaeota	0.141	0.122	0.102	0.218	0.182	0.160	5	11	Kiemnec-Tyburczy et al. 2012
Pelophylax nigromaculatus	0.201[a]		0.106	0.257[a]		0.183	25	40	Gong et al. 2013
Polypedates megacephalus	0.341	0.115	0.156	0.486	0.213	0.243	11	7	Zhao et al. 2013
Rhacophorus omeimontis	0.319	0.132	0.143	0.452	0.240	0.229	27	20	Zhao et al. 2013

[a] PBR sites analysed together

[b] Contains segments of PBR and other regions

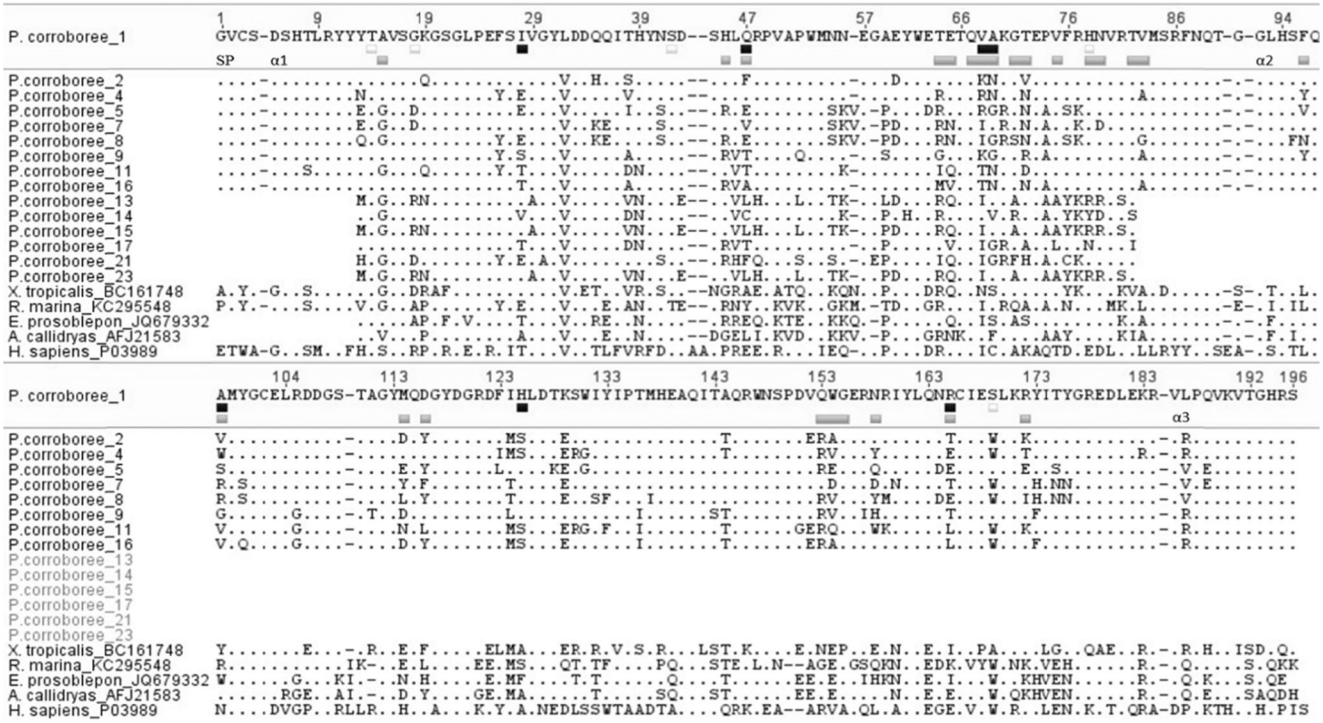

Fig. 3 Amino acid alignment of MHC class IA. Geneious alignment of MHC Class IA from *Pseudophryne corroboree*, *Xenopus tropicalis*, *Rhinella marina, Espadarana prosoblepon*, *Agalychnis callidryas*, and *Homo sapiens*. *Black boxes* indicate sites under positive selection in *P. corroboree*; *white boxes* indicate sites under purifying selection; *grey boxes* indicate putative peptide-binding residues from Flajnik et al. (1999) and Lillie et al. (2014)

species lives within the thermal growth range of *Bd* year-round (Hunter et al. 2009; Murray et al. 2011). However, it is possible that its terrestrial life history as an adult reduces exposure and transmission of *Bd*.

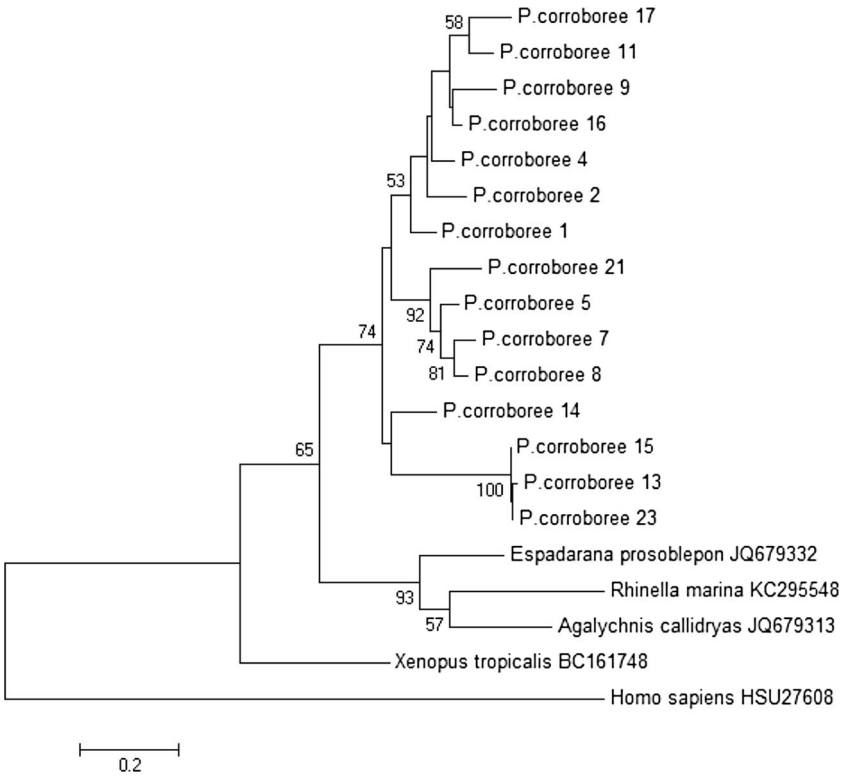

Fig. 4 A phylogenetic tree comparing MHC Class IA nucleotide evolution in *P. corroboree* and other vertebrates. The evolutionary history was inferred using the maximum likelihood method, and evolutionary distances were computed with the Kimura 2-parameter method. Gamma distribution was used to model rate variation among sites. Bootstrap percentage values above 50% are shown on *branches* (500 replicates). The *scale bar* represents the number of base substitutions per site. Sequence titles include GenBank accession numbers

If *Bd* resistance genes were present in the *P. corroboree* population before the introduction of *Bd*, declines such as those observed would be expected as the population contracted from susceptible individuals dying off while resistant individuals persisted. This may explain the prolonged persistence of this species in the wild in the presence of *Bd*. A recent study by Savage and Zamudio (2016) in a natural population of lowland leopard frogs found that alleles associated with *Bd* resistance were under positive selection while those not associated with resistance were not, indicating that this species may be evolving *Bd* resistance. Although we found evidence of selection on the MHC of *P. corroboree*, it is not possible to disentangle whether this is due to recent population declines or selection on the MHC for pathogen resistance. However, our evidence that MHC variation is relatively high in contrast with earlier findings that neutral variation is relatively low suggests that recent selection has occurred at the MHC.

In the phylogenetic analysis of MHC class IA of *P. corroboree* and other anurans, *P. corroboree* sequences clustered with strong bootstrap support. Furthermore, a subset of *P. corroboree* alleles formed two supported clusters, indicating that these sequences may represent distinct loci.

Even if they have evolved resistance to *Bd*, *P. corroboree* has continued to decline in the wild, likely due to various extrinsic and intrinsic factors that reduce population resilience such as sympatric reservoir hosts, drought, narrow distributional range, and low fecundity (Hunter et al. 2010b; Scheele et al. 2016). The species is functionally extinct in the wild, and how the captive breeding and reintroduction program is managed will have important implications on the long-term sustainability of the species. Currently, this species is being managed to maintain the genetic diversity of the founder population (Lees et al. 2013). Because *Bd* is unlikely to be eradicated from the region, *P. corroboree* should be managed in a manner that increases the likelihood of survival in the presence of *Bd*.

While maintaining genetic diversity may be important to ensure population persistence in the long term, if species are under severe threat from a single pathogen, gene variants that confer resistance to that pathogen should be targeted and promoted in the population (Scheele et al. 2014; Kosch et al. 2016). Whether MHC variation itself confers resistance to *Bd* or whether this variation has been retained due to selection from other pathogens is unclear. Ideally, specific MHC variants that confer resistance to *Bd* can be identified, and their frequencies increased via selective breeding or gene editing technology. Further challenge experiments are necessary to distinguish between the long-term benefits of specific variants versus increased MHC polymorphism. Caution is prudent when selecting for specific variants, as any reduction in overall MHC polymorphism may make the population vulnerable to pathogens other than *Bd* (Kosch et al. 2016).

Our preliminary investigation of *P. corroboree* MHC class IA indicated that this species has a minimum of four MHC IA loci. However, in the absence of a reference genome, it is not possible to be confident of individual genotype without more extensive sequencing. The criteria suggested by Galan et al. (2010) indicate that for a system with four loci, a minimum of 90 clones need to be sequenced in order to achieve 95% confidence that all variants have been sampled. Thus, it is likely that we are underestimating MHC class IA genetic variation in this species. Future work, ideally using high throughput sequencing (e.g. Illumina), can use the genetic information from this study as a baseline to investigate the influence of an MHC class IA genotype on *Bd* resistance through a *Bd* challenge experiment. This will allow for the identification of potential *Bd* resistance alleles, which can inform future management decisions for this species concerning selective breeding or genetic manipulation to increase *Bd* resistance and improve survival rates in the wild. Although natural selection for disease resistance in *P. corroboree* may have occurred, it has been insufficient to stabilize this population and continued intervention is required to prevent extinction.

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