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Evolutionary biology

Major histocompatibility complex selection dynamics in pathogen-infected túngara frog (*Physalaemus pustulosus*) populations

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Pathogen-driven selection can favour major histocompatibility complex (MHC) alleles that confer immunological resistance to specific diseases. However, strong directional selection should deplete genetic variation necessary for robust immune function in the absence of balancing selection or challenges presented by other pathogens. We examined selection dynamics at one MHC class II (MHC-II) locus across Panamanian populations of the túngara frog, *Physalaemus pustulosus*, infected by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd). We compared MHC-II diversity in highland túngara frog populations, where amphibian communities have experienced declines owing to Bd, with those in the lowland region that have shown no evidence of decline. Highland region frogs had MHC variants that confer resistance to Bd. Variant fixation appeared to occur by directional selection rather than inbreeding, as overall genetic variation persisted in populations. In Bd-infected lowland sites, however, selective advantage may accrue to individuals with only one Bd-resistance allele, which were more frequent. Environmental conditions in lowlands should be less favourable for Bd infection, which may reduce selection for specific Bd resistance in hosts. Our results suggest that MHC selection dynamics fluctuate in túngara frog populations as a function of the favourability of habitat to pathogen spread and the vulnerability of hosts to infection.

1. Introduction

Coevolution between pathogens and their hosts generates variability in host genomes that is vital for maintaining population viability in changing environments [1,2], but how evolution drives immunogenetic variation in natural populations remains subject to debate [3]. In the short term, disease may select for major histocompatibility complex (MHC) alleles in vertebrate hosts that confer resistance to specific pathogens [4]. Strong directional selection in response to highly virulent pathogens should deplete genetic variation and lead to a loss of heterozygosity [5]. However, MHC polymorphisms are needed to generate effective immune responses to the wide range of pathogens that organisms are likely to encounter over their lifetimes [6]. We thus expect to find evidence of both directional and balancing selection acting on host immunogenetic variation, depending on pathogen infectivity, the efficacy of host immune responses and population infection history.

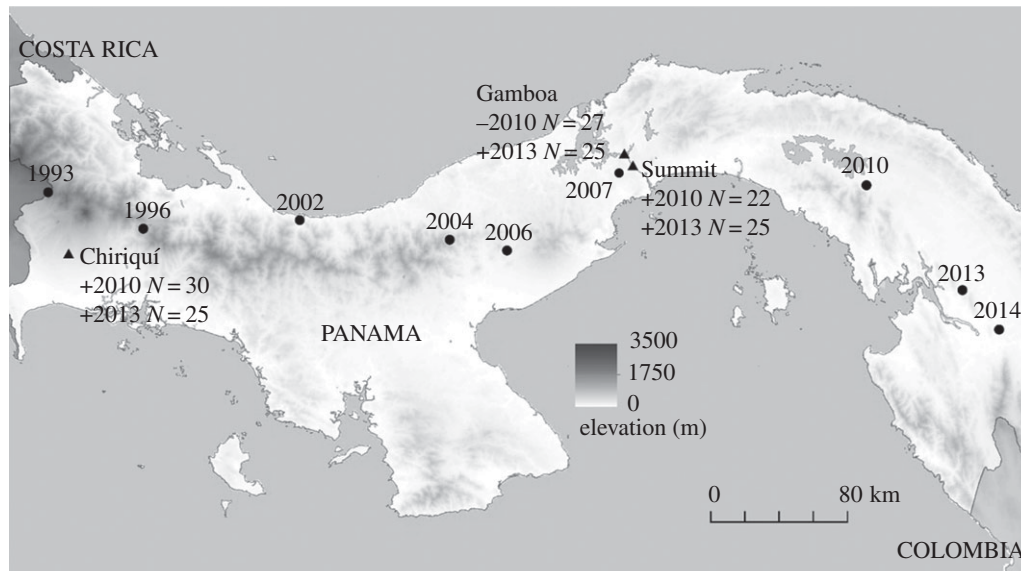


Figure 1. Túngara frog sampling localities. Sites sampled in 2010 and 2013 with sample sizes and infection status [13] (triangles). Other sites documented to have experienced Bd-related declines, following a wave from west to east (filled circles) [8–10].

Here, we examine how MHC selection dynamics in Panamanian túngara frog (*Physalaemus pustulosus*) populations may fluctuate with habitat suitability and the spread of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (denoted Bd) [7]. In western Panama, population extirpations followed Bd incursions during the last decade, especially at elevations above 200 m [8,9]. By contrast, no amphibian population declines attributable to Bd have been reported in lowland Panama [9,10]. We assessed how the Bd pathogen may affect natural selection on disease-resistance genes by comparing the MHC class II (MHC-II) $\beta 1$ domain of túngara frogs from western Panama with those from lowland central Panama, specifically examining fluctuating directional selection for MHC-II $\beta 1$ variants encoding conformations associated with Bd resistance [11,12].

2. Material and methods

(a) Fieldwork and disease status

We conducted fieldwork from June to August in 2010 and 2013 at sites in the western highlands (Chiriquí) and central lowlands of Panama (Gamboa and Summit, figure 1; electronic supplementary material, table S1). Chiriquí túngara frog populations were Bd-infected in both years. Gamboa populations were Bd-free in 2010 but, as the pathogen spread there, subsequently became infected [13]. Summit was infected by Bd as early as 2007 [9].

(b) Major histocompatibility complex-II $\beta 1$ and microsatellite genotyping

We extracted DNA from toe clips and amplified the $\beta 1$ domain of one MHC-II locus (see the electronic supplementary material, methods and table S2). We cloned PCR products and sequenced between 6 and 20 clones per transformation. After constructing an allelic library of 63 cloned individuals, we directly sequenced amplicons and determined MHC-II genotypes. We assessed neutral genetic differentiation within and among populations using a panel of five microsatellite markers [14] organized into two fluorescently labelled multiplex PCRs (protocols and primers in the electronic supplementary material, table S3).

(c) Analysis of population genetic differentiation

We checked the data for conformity to Hardy–Weinberg equilibrium. Then we analysed allelic richness, observed heterozygosity, expected heterozygosity, inbreeding index F_{IS} , relatedness and inbreeding-corrected relatedness among populations.

We quantified genetic differentiation between populations by calculating pairwise F_{ST} values and assessed the significance of these differences with permutation tests and a Bayesian individual clustering method.

(d) Detection of selection pressure

We compared F_{ST} values with those obtained under a simulated neutral distribution. Analyses were conducted for each region separately, combining data from 2010 and 2013, and for population pairs to identify region-specific differences in selection pressure.

We calculated the frequency of individuals with two, one or zero MHC-II $\beta 1$ alleles encoding potential Bd-resistant peptide-binding conformations of pockets 4, 6 and 9 [11]. We examined how frequencies of these Bd-resistance alleles changed as Bd spread.

3. Results

(a) Population genetic differentiation

(i) Microsatellites

We found between 13 and 32 alleles per locus, with heterozygosity between 0.19 and 0.96 (electronic supplementary material, table S3). Using Bayesian individual clustering, a clear separation emerged between the highland population of Chiriquí and the two lowland populations across sampling periods (electronic supplementary material, figure S1). F_{ST} values were significant for all pairwise comparisons involving Chiriquí populations (range: 0.059–0.092, $p < 0.01$; table 1; electronic supplementary material, table S4). F_{ST} values between Gamboa and Summit populations did not significantly differ from zero (range: 0.001–0.014, $p > 0.05$; table 1; electronic supplementary material, table S4). Chiriquí populations genetically differed between 2010 and 2013 ($F_{ST} = 0.027$, $p < 0.01$), but other populations did not genetically differ between years (range: 0.001–0.012, $p > 0.05$; table 1; electronic supplementary material, table S4).

Table 1. Genetic differentiation (F_{ST} values) at neutral markers (lower half) and at MHC-II exon 2 (upper half) among túngara frog populations. (Chi, Chiriquí; Gam, Gamboa; Sum, Summit; 10, 2010; 13, 2013; in *italics*, not infected by Bd. In **bold**, F_{ST} values significantly different from zero ($p < 0.05$, $**p < 0.01$). Based on permutation tests with 10 000 iterations. P -values were adjusted using a sequential Bonferroni correction.)

	Chi 10	Gam 10	Sum 10	Chi 13	Gam 13	Sum 13
Chi 10	—	0.105**	0.044**	0.049**	0.125**	0.070**
Gam 10	0.059**	—	0.047**	0.083**	0.032*	0.031*
Sum 10	0.071**	0.012	—	0.027**	0.057**	0.014
Chi 13	0.027**	0.073**	0.078**	—	0.095**	0.036*
Gam 13	0.079**	0.001	0.014	0.092**	—	0.022
Sum 13	0.074**	0.001	0.012	0.082**	0.002	—

Table 2. Detection of differential selection on the MHC among populations sampled in 2010 and 2013. Frequency of MHC alleles (MHC) and variants encoding the Bd-resistant P9 pocket conformation (P9). (Het, expected heterozygosity; Gam-Sum inf, includes only the populations of Gamboa (2013) and Summit (2010 and 2013), infected by Bd. In **bold**, values significantly higher than the simulated distribution (posterior probabilities, $*PP > 0.95$, $**PP > 0.99$) were considered to be under different selection regimens.)

locus	MHC		P9	
	Het	F_{ST}	Het	F_{ST}
Chiriquí	0.934	0.032	0.000	n.a.
Gamboa	0.828	0.032	0.321	0.194**
Summit	0.943	0.014	0.452	−0.010
Chi-Gam	0.934	0.093*	0.305	0.179**
Chi-Sum	0.956	0.047	0.310	0.219*
Gam-Sum	0.896	0.034*	0.393	0.090**
Gam-Sum inf	0.915	0.031	0.430	−0.022
all pop	0.930	0.053	0.305	0.179**

(ii) Major histocompatibility complex

We isolated 48 MHC-II $\beta 1$ alleles across populations (electronic supplementary material, table S1). Pairwise F_{ST} comparisons based on MHC genotypes significantly differed from zero (range: 0.027–0.125, $p < 0.01$) except among Bd-infected Gamboa and Summit populations (range: 0.014–0.022, $p > 0.05$; table 1; electronic supplementary material, table S4). Allelic diversity, heterozygosity, inbreeding coefficients and relatedness were similar among groups of populations tested (electronic supplementary material, table S5).

(b) Selection pressure on major histocompatibility complex-II $\beta 1$

Genetic variation between Chiriquí and Gamboa populations, and between Gamboa and Summit populations, was significantly higher at the MHC-II locus than expected under assumptions of neutrality (table 2). When analysing only Bd-infected populations (Gamboa in 2013 and Summit in both years), however, these effects no longer were apparent (table 2).

Most individuals in all the populations had at least one MHC-II $\beta 1$ allele encoding the P9 pocket conformation associated with Bd resistance in other amphibians [11] (figure 2;

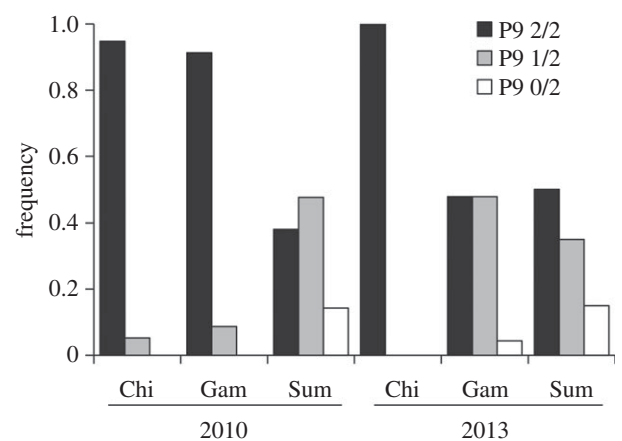


Figure 2. MHC-II $\beta 1$ genotypes with the P9 pocket conformation associated with Bd resistance in túngara frogs. The frequency of individuals with 2 (P9 2/2), 1 (P9 1/2) or no (P9 0/2) resistance alleles is shown for the Chiriquí (Chi), Gamboa (Gam) and Summit (Sum) populations in 2010 and 2013.

electronic supplementary material, table S1). All but one (46 out of 47) individual from the highland Chiriquí population possessed two alleles with this P9 conformation. We observed a significant decrease in the frequency of these alleles in Gamboa from 2010 (42/46, 0.91) to 2013 (32/46, 0.69, $z = 2.63$, $p = 0.008$), while frequencies did not vary in Summit (2010: 26/44, 0.59, 2013: 29/44, 0.66, $z = -0.66$, $p = 0.51$; figure 2). Between 85 and 100% of individuals in all populations had at least one MHC-II $\beta 1$ allele associated with Bd resistance. Alleles encoding P4 and P6 pocket conformations were either absent or present at low frequencies in Bd-infected populations (electronic supplementary material, figure S2).

Analyses of pocket 9 conformation frequencies revealed a signature of differential selection across populations, between Chiriquí and Summit, between Chiriquí and Gamboa, and between sampling years in Gamboa ($F_{ST} = 0.179$, 0.219, 0.179 and 0.194, respectively, posterior probability (PP) = 0.96–0.99, table 2). Differential selection on the P9 conformation between Gamboa and Summit also was apparent ($F_{ST} = 0.090$, PP = 0.96) but became non-significant when considering only Bd-infected populations ($F_{ST} = -0.015$, PP = 0.21; table 2; electronic supplementary material, table S6).

4. Discussion

Our results suggest that Bd-infected túngara frog populations experienced differential selection on their MHC-II alleles.

Bd-resistance alleles predominated in the Chiriquí population, in the western highland region of Panama where amphibian populations have been most severely affected by Bd. These MHC-II alleles encode peptide ligands with specific conformational properties that confer resistance to Bd [11]. MHC-II differentiation was higher than expected under assumptions of neutrality, consistent with directional selection for the advantageous variants rather than genetic drift.

In the lowland Gamboa and Summit sites, individuals presenting only one Bd-resistance allele appeared to benefit from a selective advantage. Indeed, after the 2013 incursion of Bd into Gamboa, individuals with one MHC-P9 allele increased in frequency, matching those found in Bd-infected Summit populations. However, species that are highly susceptible to Bd may need two doses of MHC molecules to offset the pathogen's mechanism of attack on the host [11]. Such appears to have been the case in túngara frog populations of Chiriquí where individuals with two MHC-P9 alleles predominate but not in lowland Panama, where those presenting one Bd-resistance allele are more abundant.

The differential selection on MHC-II alleles in Chiriquí compared to Gamboa and Summit may reflect environmental differences between the highland and lowland habitats. The warmer ambient temperature range in the lowlands is much less favourable to Bd growth [8] and should promote more efficient immune function [15]. Bd loads were 6–30 times higher in highland than lowland túngara frog populations [13], supporting this hypothesis.

As lowland frogs incur less risk of Bd infection, those populations may have been subject more to balancing selection for MHC heterozygosity and less to directional selection for adaptive immune responses to Bd. Evidence of directional or balancing pathogen-driven selection on amphibian MHC systems is not new [12,16,17]. However, our findings suggest that patterns of MHC diversity in túngara frogs reflect regimens of fluctuating selection that may be predicted by the Bd-exposure history of a population and environmental conditions that affect pathogen virulence. Given the variable effects that Bd imparts on amphibian populations around the world, further tests of this hypothesis are possible, which should clarify how immune responses to Bd have evolved.

Selection dynamics on the MHC are best interpreted in the context of the molecular interactions that occur between

MHC molecules and pathogenic antigens [18]. Our results add to the developing consensus that grouping MHC variants by their functional properties provides a more powerful approach to understanding selection dynamics than focusing on genotypes [11,18].

The strong selection for Bd-resistance alleles we observed, together with high levels of Bd infection in the Chiriquí population [13], suggests that túngara frogs infected by the pathogen in this region suffer a fitness cost. Most alleles present in the Chiriquí population encode one specific P9 pocket molecular conformation. This may decrease their capacity to bind antigens of other pathogens. Therefore, selection for Bd resistance may have made túngara frogs more susceptible to other diseases [6]. More generally, amphibians living in areas where Bd is enzootic may have evolved immunity to the pathogen but at the cost of reduced capacity to respond to other pathogens.

Chytrid fungus has had a global impact on amphibian diversity and the disease continues to spread unabated [7]. Our results raise the possibility that Bd differentially influences MHC selection dynamics within amphibian populations in response to environmental conditions that modulate pathogen virulence. Studies of MHC variation potentially can identify the cryptic impact of Bd on apparently healthy amphibian populations prior to the onset of precipitous population declines.

Ethics. Collection of samples was authorized by the Ministry of the Environment (ANAM), Panama, and experimental protocols were approved by the Smithsonian Tropical Research Institute (IACUC: 2011-0825-2014-02).

Data accessibility. MHC sequences: GenBank accession nos. KR083791–KR083833, and KT438737–KT438741. Alignment of MHC-II β 1 exonic and intronic sequences with primers used for their amplification (FASTA format), and genotyping data for microsatellite markers, have been deposited in the Dryad repository: <http://dx.doi.org/10.5061/dryad.cn90m> [19].

Authors' contributions. B.W., M.J.R. and A.B. designed the research; M.J.R. and S.R. collected the samples; T.A.K., A.B. and C.D. conducted laboratory benchmark; A.B., T.A.K. and J.A.E. analysed the data; B.W., A.B. and T.A.K. wrote the manuscript; B.W. and M.J.R. contributed reagents/materials; all authors revised and approved the manuscript, for which they agree to be held accountable.

Competing interests. The authors declare no competing interests.

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