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THE ROLE OF IMMUNOGENETICS IN AMPHIBIAN SUSCEPTIBILITY TO CHYTRIDIOMYCOSIS

INTRODUCTION:

The fungus *Batrachochytrium dendrobatidis* (Bd) has led to the global decline of amphibian populations by causing the epidemic respiratory disease chytridiomycosis.

Bd is the main driver of amphibian extinction, exceeding the extinction rate of all vertebrates (Fisher & Garner, 2020). This has led to a huge loss of amphibian biodiversity. Previous research in this area (Bataille et al., 2015) has discovered an allele of the major histocompatibility complex (MHC) class II gene in amphibians that confers resistance to chytridiomycosis.

Here, we examined the frequency of the Bd-resistant MHC class II allele in global amphibian species through phylogenetic and sequence alignment analyses. The allele is highly prevalent in a random sample of 32 amphibian species, consistent with a possible positive selection pressure driving the resistant allele to potential fixation in wild *Anura* populations in chytridiomycosis afflicted areas.

MAIN OBJECTIVES:

- To survey the genetic diversity of the MHC class II beta gene across various species of the amphibian order *Anura.*
- To conduct a multiple sequence alignment of the collected *Anura* MHC class II beta sequences to examine genetic diversity at this gene and to ascertain the presence/absence of the Bd-resistant allele across various *Anura* species.
- To review the phylogenetic relationships between the aligned sequences to study the consequences of evolutionary relationships on the presence of the resistant allele to Bd infection and to produce a frequency table for a specific amphibian species having the resistant genotype.

EXPERIMENTAL METHOD:

The MHC class II antigen beta chain partial protein sequence of the amphibian species *Rana yavapaiensis* (Genbank accession number: ANQ37108) was used in BLAST to locate the nucleotide sequence of the MHC class II gene in other members of the *Anura* order. tBLASTn was used whereby the *Rana yavapaeinsis* protein query was used to search translated nucleotide Genbank databases.

Sequences were aligned using the MACSE (Multiple Alignment of Coding Sequences Accounting for Frameshifts and Stop Codons) alignment tool and converted from nucleotide sequences into a translated protein sequence alignment. Previous research by (Bataille et al., 2015) identified specific amino acid substitutions at four sites, amino acid positions 37, 56, 57, and 60, as responsible for resistance. We specifically surveyed the presence of the resistant amino acids at these sites, tyrosine/phenylalanine, proline, aspartic acid/glutamic acid and tyrosine respectively, as a method of inferring Bd resistance.

AliView alignment viewer and editor was used to trim the sequences (524 total) to fit the parameters of the reference sequence of *Litoria verrauxii* (Accession number: KJ679288) used in the original study (Bataille et al., 2015). A table was produced of the frequency of the amino acids responsible for the resistant P9 antigen binding pocket of the MHC being at the known resistance-conferring positions for each amphibian species (32 total).

Evolutionary relationships between the species was estimated using PHYML to infer phylogeny based on maximum likelihood. A phylogenetic tree was produced using the application iTOL (Interactive Tree of Life).

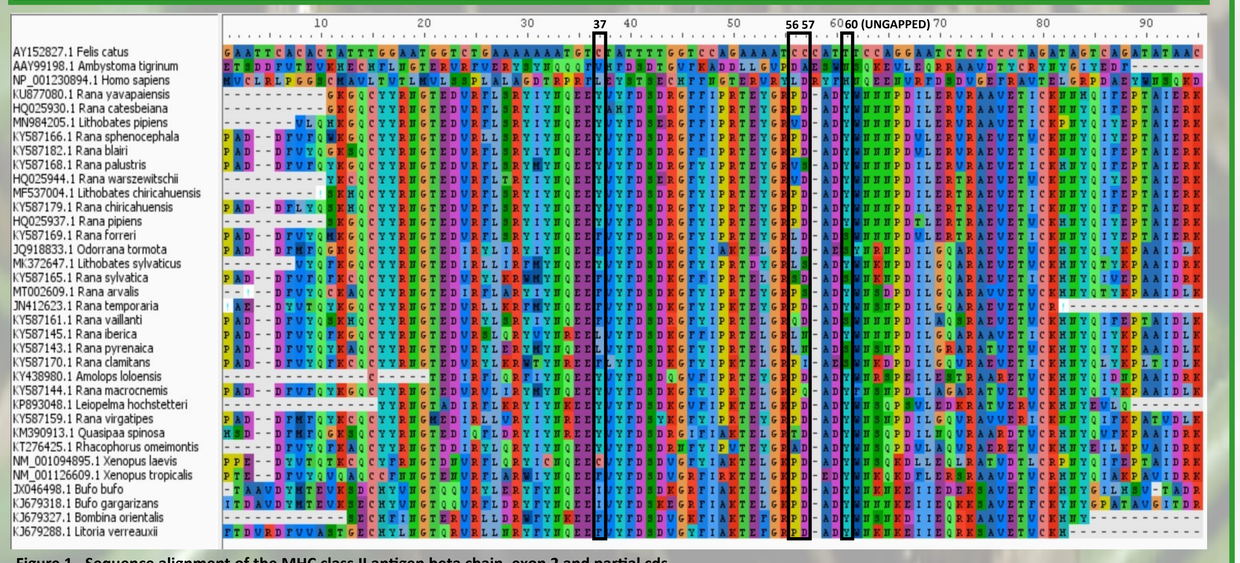

Figure 1. Sequence alignment of the MHC class II antigen beta chain, exon 2 and partial cds.

The alignment included 32 *Anura* species and 3 outgroups *Felis catus, Ambystoma tigrinum,* and *Homo sapiens (*Species name and Accession number left*).* Black box indicates known resistant amino acid positions in the sequence.

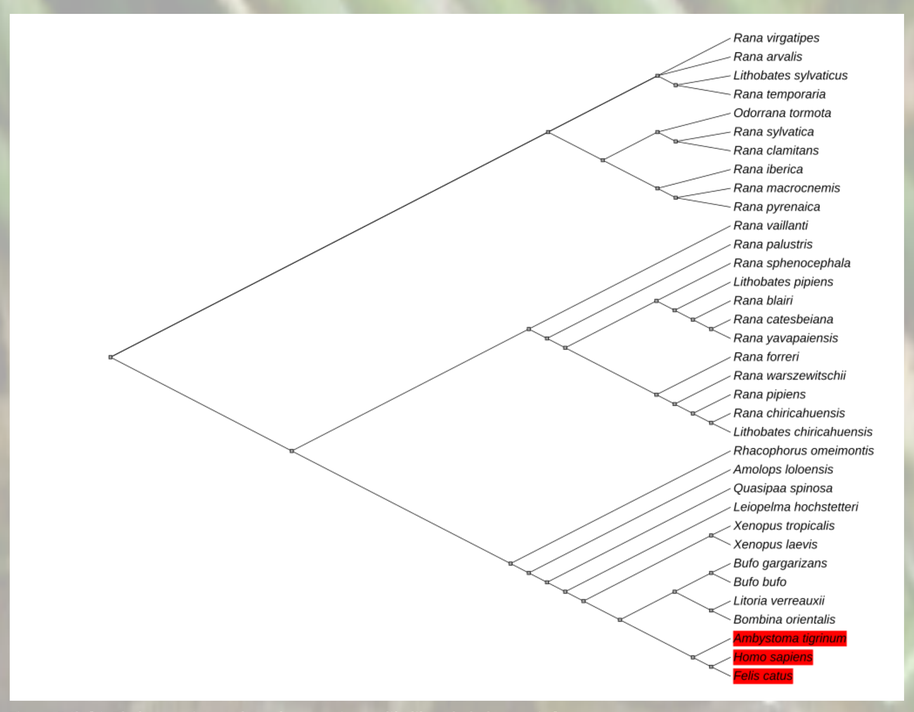

Figure 2 (left) Phylogenetic tree based on maximum likelihood phylogeny inference

Tree contains one sequence each from 32 *Anura* species including 3 outgroups *Felis catus*, *Ambystoma tigrinum,* and *Homo sapiens* (shaded in red).

Table 1 (right) Table of amino acid positions associated with resistant P9 pocket conformation.

The amino acids associated with Bd-resistance include Tyr/Phe37β, Pro56β, Asp/Glu 57β and Tyr60β. Contains species name with number of sequences available for analysis in brackets. 524 sequences were examined.

Shading represents strength of resistant amino acid frequency at positions 37, 56, 57, and 60 in the MHC Class II beta gene encoding for the MHC Class II beta 1 P9 binding pocket. Green shading represents observed frequency, where dark green is highest frequency. Grey shading represents species with a small number of samples available (1), thus accurate conclusions of amino acids present for these species could not be made. Table constructed using Microsoft Excel.

Amino Acid Positions Associated with Resistant P9 Pocket Conformation				
Species Name:	**pos37**	**pos56**	**pos57**	**pos60**
Rana virgatipes (1)	1	1	1	1
Rana arvalis (71)	0.887	1	0.887	0.437
Lithobates sylvaticus (17)	1	0	0.765	0.471
Rana temporaria (4)	0.25	0.5	0.5	0.75
Odorrana tormota (29)	1	0.241	0.759	0
Rana sylvatica (5)	1	0	0.6	0.4
Rana clamitans (2)	1	1	0.5	0
Rana iberica (1)	0	0	0	1
Rana macrocnemis (1)	1	1	0	1
Rana pyrenaica (1)	0	0	0	0
Rana vaillanti (1)	1	0	1	0
Rana palustris (4)	1	0.25	0.5	1
Rana sphenocephala (1)	1	1	1	1
Lithobates pipiens (37)	1	0.351	0.972	0.513
Rana blairi (4)	1	0.75	0.75	0.75
Rana catesbeiana (4)	1	0	1	0.5
Rana yavapaiensis (116)	0.991	0.431	0.991	0.905
Rana forreri (1)	1	0	1	0
Rana warszewitschii (2)	1	0	1	1
Rana pipiens (1)	1	1	1	1
Rana chiricahuensis (5)	1	0.2	1	0.4
Lithobates chiricahuensis (5)	1	0.6	1	0.4
Rhacophorus omeimontis (162)	0.994	0	0.944	0.981
Amolops loloensis (11)	0.722	1	0.909	0.909
Quasipaa spinosa (11)	1	0.45	1	0.9
Leiopelma hochstetteri (4)	1	1	0.75	1
Xenopus tropicalis (3)	0.3	1	1	1
Xenopus laevis (5)	0.6	0.6	0.4	0.8
Bufo gargarizans (1)	0	1	1	1
Bufo bufo (1)	0	1	1	1
Litoria verreauxii (11)	1	1	1	1
Bombina orientalis (1)	1	1	1	1
Total (524 seqs)	0.954	0.376	0.912	0.75

RESULTS:

332 of sequences (Total 524) from 32 *Anura* species had the amino acid phenylalanine at β37. 168 species had tyrosine at this position. 95.42% of total sequences surveyed contained these resistant amino acid variants. Two sequences (0.38%) had an aromatic tryptophan at β37 and the remaining 24 sequences consisted of leucine (2.67%), histidine (0.38%), isoleucine (0.38%) and cysteine (0.76%) at this position.

194 sequences had resistant proline at position β56 (37.6%) and 153 had alanine (29.2%). The remaining 174 sequences either had valine (8.97%), leucine (20.61%), serine (2.29%), glutamine (0.19%), or threonine (1.15%) at position β56.

478 sequences examined had aspartic acid associated with resistance at position β57, amounting to 91.22%. 36 sequences had serine at this position (6.87%). The remainder consisted of the amino acids glutamine (0.38%), asparagine (0.76%), resistant glutamic acid (0.19%), histidine (0.19%), isoleucine (0.19%) and glycine (0.19%).

At position β60, 393 sequences had the resistant amino acid tyrosine (75%). 116 sequences had serine at this position (22.14%). The remaining 15 sequences had either the amino acid asparagine (0.38%), histidine (0.38%), threonine (1.72%), phenylalanine (0.19%) or aspartic acid (0.19%) at position β60 in the sequence.

DISCUSSION:

Based on the findings (Table 1), it appears that for positions 37 and 57 in the MHC class II beta protein sequence the majority of the 524 sequences examined from 32 *Anura* species had the amino acids associated with Bd-resistance, phenylalanine/tyrosine (95.4%) and aspartic acid/glutamic acid (91.2%) respectively, at these sites. This suggests that the common ancestor of all *Anura* species may have possessed the known amino acids which confer resistance to Bd infection at these sites and negative selection, due to the threat of fatal chytridiomycosis infection, has acted to reduce the frequency of amino acids at positions β37 and β57 which lead to more susceptible conformations of the P9 binding pocket. During this research, the evolutionary relationships between species under examination was assessed. Further research is necessary to decipher the MHC class II beta genotype for the common ancestor of the order *Anura* and to assess its phylogeny in relation to the 32 species examined in this study.

At position β60, there appears to be a lower frequency of the known resistant amino acid tyrosine at 75%. Positive selection for the resistant MHC II allele may still be at play here, increasing the frequency of tyrosine in *Anura* populations as susceptible alleles are removed due to mortality from chytridiomycosis. Further research into the ancestral state is necessary to determine whether this frequency is due to this amino acid being present in the ancestral state or whether the selection pressure of Bd infection has led to the allele increasing in frequency in multiple *Anura* populations.

Position β56 had the highest variability of the four sites, with only 37.6% of sequences examined containing the resistant amino acid proline. According to previous studies, the protective MHC P9 binding pocket conformation relies on an aromatic β37 residue, proline at β56, aspartic acid/glutamic acid at β57 and a hydrophobic residue at β60, with all sites needed to confer Bd resistance. The low frequency of Proβ56 could suggest that this proline residue was not present in the ancestral state and may have increased in frequency in current *Anura* populations due to positive selection acting in individuals which displayed this proβ56 mutation in populations due to its role in conferring a resistant P9 binding pocket conformation.

CONCLUSION:

- In researching the presence of certain amino acids known to promote Bd-resistance in 32 *Anura* species, it has been found that there are varying levels of frequency across the four sites of interest, with positions β37, β57 and β60 showing high levels of resistant amino acid frequency and position β56 with a low level. Further research on a larger sample of each species and the common ancestor of the *Anura* order is needed to understand the evolutionary origins of this discovery and to further explore the relationships between the species examined and their status of resistance to chytridiomycosis, based on their P9 pocket conformation.

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