Latitudinal MHC variation and haplotype associated differential survival in response to experimental infection of two strains of *Batrachochytrium dendrobatitis* (Bd-GPL) in common toads

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Abstract

While both innate and adaptive immune system mechanisms have been implicated in resistance against the chytrid fungus *Batrachochytrium dendrobatitis*, studies on the role of specific MHC haplotypes on *Bd* infection are rare. Here, we studied latitudinal variation in MHC Class IIB loci along a latitudinal gradient from southern to northern Sweden in common toads, *Bufo bufo*. Swedish toad populations had fewer MHC Class IIB haplotypes compared to a previous study of populations in Britain. Furthermore, we found MHC diversity to decline from south to the north within Sweden. The low diversity may compromise the ability of northern populations to fight emerging disease, such as the chytrid fungus *Bd*. In a laboratory experiment, we infected newly metamorphosed toads with two strains of the Global Pandemic Lineage of the fungus (*Bd*-GPL) and compared survival with sham controls. We found *Bd*-infected toads had lower survival compared to controls. Survival was dependent on *Bd*-strain and whether experimental toads were collected in the south or the north of Sweden with lower survival in northern individuals. MHC diversity was lower in toads of northern origin, all northern animals being monomorphic for a single MHC haplotype, whereas we found seven different haplotypes in southern animals. Survival of infected animals was dependent on both *Bd*-strain and MHC haplotype suggesting differential infection dynamics depending on both *Bd*-strain and host MHC characteristics.

Introduction

Emerging infectious diseases are a severe threat to global biodiversity, causing population declines, and species extinctions, across a range of taxa [1]. Amphibian populations world-wide are faced with emerging diseases among them chytrid fungus *Batrachochytrium dendrobatidis* (Bd) which has been shown to infect more than 700 amphibian species and is implicated in the extinction of more than 100 species [2,3]. *Bd* consists of numerous strains and lineages, some of which are highly pathogenic while others are more benign [4, 5]. The infectious lineage *Bd*-GPL has evolved within the last century in south-east Asia and has since spread over the world infecting naïve amphibian species and populations [6]. *Bd*-GPL infections may cause mass mortality, while in other cases populations and species appear resistant to the disease [7]. Studies on the variation in the pathogenicity of the fungus and whether host populations vary in resistance are therefore vital, as is pin-pointing the immunological and genetic basis for such differences. Any amphibian species ability to resist the fungus will vary depending on a range of factors ultimately linked to immunogenetic variation and differences in pathogenicity among different lineages and strains of *Bd* [8].

In general, historical events such as postglacial colonization processes have had a profound impact on the geographical distribution of genetic diversity. During the Last Glacial Maximum (LGM), species inhabiting northern Europe were restricted to refugia in southern Europe ([9], [10]. After the LGM, species expanded northwards, a process which involved repeated founder events [10]. Population size bottle-necks caused by such founder events lead to loss of genetic variation [11]. Therefore populations residing at northern latitudes generally display less genetic diversity as compared to populations residing in former refugia [12,13, 14]. This is due to smaller effective population sizes and more fragmented populations at the edge of the expansion [15, 16].

MHC is a multigene family consisting of various number of genes coding for peptides involved in the adaptive immune defense of vertebrates [17]. It codes for proteins that are part of the adaptive immune system and is associated with disease and parasite resistance [18]. Previous studies of amphibians have shown a direct association between specific MHC alleles and *Bd*-resistance [19, 20].

Understanding geographic variation concerning immuno-genetic diversity is complicated by which mechanisms shape such diversity [21, 22]. For example, the exceptionally high of diversity at the Major Histocompatibility Complex (MHC) is believed to be maintained by various forms of balancing selection [23]. Because of the immune response enhancing properties of MHC, it is suggested that heterozygous individuals are more resistant to pathogens as they have a larger available repertoire to recognize pathogens [24]. However, immunogenetic variation may also be shaped by drift which vary in force in and among species [25, 26, 27].

In this study we focused on common toad (*Bufo bufo*) populations in Sweden, an area which was completely glaciated during the LGM. The aims of the study were to quantify MHC Class II variation along a latitudinal gradient, and to study whether *Bd*-mediated mortality depends on the *Bd*-strain, the presence of MHC Class IIB haplotypes or the geographic origin of the experimental animals. We experimentally infected newly metamorphosed common toads from the north and the south of Sweden with two strains of *Bd*-GPL, originating from Sweden and the UK, respectively, or treated toads with a sham control and recorded their survival over 30 days

Materials and Methods

MHC variation along the latitudinal gradient

For the analyses of geographical MHC-variation we collected tissue samples from 20 adult toads at 12 locations spread over four different regions along a latitudinal gradient across Sweden (Table 1) in April-May 2015. The toads were caught in breeding aggregations and a small piece of rear leg webbing was removed and immediately stored in 96 % ethanol. DNA was extracted from the tissue by using a DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. We amplified MHC Class II exon 2 loci (279 bp) by using the primers for Zeisset [28]. Both forward (2F347F: GTGACCCTCTGCTCTCCATT) and reverse (2R307R: ATAATTCAGTATATACAGGGTCTCACC) primers were modified for Illumina MiSeq sequencing with an individual 8 bp barcode and a sequence of three N to facilitate cluster identification [27], for details. The libraries were constructed by combining 13 different specific barcode tags, four forward primers and nine reverse primers [27].

PCR was done in a 2720 thermal cycler with the protocol: 97°C for 30 sec, followed by 35 cycles of 98°C for 10sec, 57°C for 20 seconds and 72°C for 15sec, and a final round of 72°C for 8 minutes until the samples are cooled down and stored at 4°C. PCR was conducted in a total volume of 30 µL: 0.5µL of DNA and 22.1 µL ddH₂O, 6 µL Phuside HF buffer, 0.6 µL dNTP, 0.75 µL of both forward and reverse primer and 0.3 µL Phusion Taq. After amplification samples were pre-pooled in five to nine pools in equimolar amounts and purified following the MiniElute Gel Extraction kit (Qiagen). For geographical analyses and for experimental animals eight libraries were prepared with a ThruPlex DNA-Seq Kit (Rubicon genomics) and sequencing was done with Illumina Miseq at NGI Uppsala (Uppsala Genome Center). In total 240 individuals for geography and 160 experimental animals were amplified. Replicates were selected randomly across populations. The Replication rate for the geographic analyses was ~40% (72 samples out of 160). Replication rate for the experimental individuals was 100%.

Miseq daata analysis

The sequence data was merged using FLASH [29]. The NextGen workbench (www.DnaBaser.com) was used to transform the files to '.fasta' format for further analysis. We demultiplexed the sample sequences with jMHC [30]. An Excel macro [31] was used to estimate allele variants per individual based on Degree of Change (DoC) calculations. The DoC criterion calculates cumulative amplicon sequencing depth among the variants in the amplicon. Plotting the sequencing depth per variant shows an inflection point where the true alleles contribute to a large proportion of the cumulative depth and all the artificial variants only contribute a minor proportion. We assigned the most frequent variants as valid MHC alleles that occurred in at least 3% of the reads [32, 33]. Amplicons with < 300 reads was discarded from analyses for quality reasons. We thoroughly checked for chimeras by eye and any of the valid sequences presented insertions, deletions or stop codons. Verified MHC class IIB exon 2 haplotypes were named in accordance with Klein [34], a species abbreviation followed by gene and a following number e.g. Bubu_DAB*01.

Experimental infections

Common taod eggs were collected in 2016 at two locations in Skåne (PM and PH, Table 1) and two locations in Luleå (LU1 and LU2). We carefully collected ~ 100 eggs from ~ 10 clutches per pond in order to reduce the risk of sampling related individuals. Eggs were brought to the laboratory in Uppsala and reared in 20 l containers in reconstituted soft water (RSW; NaHCO₃, CaSO₄, MgSO₄ and KCl added to deionized water; APHA 1985). RSW was then used throughout the experiment. Each clutch was kept in a separate container. At metamorphosis (stage 46; Gosner [35]1960), the tadpoles were removed from the containers and raised until completion of metamorphosis (stage 46; Gosner 1960) in terraria with access to water and shelter. Once the

experiments finished, metamorphosed toadlets were euthenized with an overdose of MS222, preserved in 96% ethanol and stored at 4 °C until DNA extraction.

The UK isolate of *Bd* was obtained from the Institute of Zoology, London and isolated from an Alpine newt (*Ichthyosaura alpestris*) from the UK in 2008. The Swedish isolate originated from a Green toad (*Bufo variabilis*) caught in Norra hamnen, Malmö municipality, Sweden in 2015.

The infection experiment was conducted and animals were housed in the sealed facilities at the Swedish Institute for Veterinary Science, Uppsala. We infected 50 (5 SK1, 14 SK2, 15 LU1, 16 LU2) post metamorphosed toadlets with a *Bd*-GPL strain from the United Kingdom (UK Mal 01, GPL-UK), 52 (4, 17, 16, 15) with an isolate from Sweden (SWED-40-5, GPL-SWED) and 52 (7, 17, 15, 13) with a sham control. Experimental animals were exposed to $12 \cdot 10^6$ zoospores of *Bd* in 30 ml of reconstituted soft water (RSW) (60000 zoospores/ml) for 5 h). Metamorphs in the control group were exposed to an equivalent volume of sterile media and RSW for the same time period, at the same stage of development.

All animals used in the experiment were typed for MHC Class IIB sequence variation according to the same protocol as described above. After experimental infection, the toadlets were kept individually in containers provided with water, shelter and food ad libitum. Survival was recorded daily and dead animals were preserved in 96% ethanol and stored at 4 °C. Thirty days after infection, the experiment was ended and surviving toadlets were euthanized with an overdose of MS222, preserved in 96% ethanol and stored at 4 °C until DNA extraction.

We used survival until the end of the experiment as a binomial response variable with *Bd*-strain treatment (GPL-UK, GPL-SWED and Control), geographic region / population (depending on the model), presence of various MHC haplotypes as independent variables as well as their interactions

in Generalised Linear Models. Due to underdispersion we used a quasibinomial error distribution with the logit link function where the dispersion parameter was estimated to 0.615. Geographic location was tested either as the actual population of origin or classified as region (north or south). Models followed the general structure:

Survival (Yes/No) $\sim$ *Bd*-strain treatment + Region/Population + MHC haplotype + Interactions

Model selection was based on Akaike's Information Criterion (AIC) and all analyses were run in the R packages "car" [36] and "nlme" [37].

Results

Geographic MHC variation

At least one MHC Class IIB sequence was successfully amplified in all but one individual. A total of 8 million reads were picked up by the two Miseq runs with on average 2 million reads per library. Thirteen unique sequences (hereafter haplotypes) were identified (Bubu_DAB*1 – Bubu_DAB*13). A maximum of four haplotypes per individual was found in 11 cases, in all other individuals we found one to three haplotypes.

Haplotype number varied between regions and populations (Fig.1) and decreased towards the north. We found significant differences in number of alleles among populations along the latitudinal gradient ($F_{3,8,010}=10.77$, $p=0.003$; Fig. S1). The observed number of different haplotypes in each region was (from south to north): Skåne 9, Uppland 5, Västerbotten 5 and Norrbotten 4. We found one haplotype unique to Västerbotten (Bubu_DAB*10 found in one individual). In Skåne we found 4 haplotypes unique to this region: Bubu_DAB*9, Bubu_DAB*8, Bubu_DAB*7 and Bubu_DAB*6. Bubu_DAB*01 was found in all individuals in all populations.

Experimental infections

Infection was confirmed by qPCR (for details see Meurling et al. in prep). Because of failed reactions infection could not be confirmed or denied in 14.9% of the individuals. One of the exposed individuals (with strain UK) proved to be negative to infection. Low levels of infection could be found in 19.2 % of control animals (Table. S1). All animals in the control treatment survived the 30-day experimental period. Of the 102 experimentally infected animals 54 died, 45 from the northern region and nine from the south. Of the six models tested, model 3 including “region” as a main effect and the interactions among “*Bd*-strain” and “MHC haplotype” and “*Bd* strain” and “region” had the highest AIC followed by a similar model excluding the “*Bd* strain/region” interaction (Table. 2).

We discovered a total of seven unique haplotypes: (Bubu_DAB*1, Bubu_DAB*2, Bubu_DAB*4, Bubu_DAB*6, Bubu_DAB*7, Bubu_DAB*9 and Bubu_DAB*11) in the experimental animals. All seven were found in the southern region, whereas in the northern region all individuals were monomorphic for Bubu_DAB*1.

Of the six models tested, model 2 was chosen having the lowest AIC (Table. 2). The model includes “Region”, “*Bd*-strain”, and “MHC-haplotype” as a main effects and the interactions among “*Bd*-strain” and “MHC haplotype” as well as “*Bd* strain” and “Region” (Table. 2). The result from the model are presented in table 3.

Of the interactions in the best model, a significant interaction among “*Bd*-strain” and “region” indicate that increased survival in the south depends on which *Bd*-strain the toads were infected with ($p=0.020$; $F=4.02$; Fig. 2). Furthermore, the haplotype Bubu_DAB*2 appears to provide protection against the Swedish *Bd* strain but this haplotype was detrimental when toads were infected with the UK-strain ($p=0.005$; $F=8.27$; Fig. 3a). The haplotype Bubu_DAB*9 had a detrimental effect on survival with a possible interaction with the *Bd*-strain treatment being

marginally less detrimental for individuals infected by the Swedish strain ($p=0.096$; $F=2.39$; Fig. 3b).

Discussion

Previous studies of latitudinal MHC variation in amphibians suggest neutral processes have had a strong influence on the geographic distribution of MHC diversity over large biogeographical areas. Thus MHC diversity tends to decline at northern latitudes when moving away from putative post glacial refugia in a number of species [38, 32, 39, 40, 27, 41]. A study of *B. bufo* MHC Class IIB variation in the UK found 17 unique sequences in 149 animals [28], and we found a total of 13 in 239 animals further to the north in Sweden, three sequences of which were also found in the UK. We also found lower MHC diversity at higher latitudes in Sweden.

Regional variation in MHC diversity may be shaped by both genetic drift and selection, and populations at different latitudes may face varying strengths and forms of selection [27, 42]. Varying selection may depend on differences in parasites and pathogen pressure [43], but also on historical demographic process such as population size fluctuations mediated through bottlenecks and founder events [11]. Generally, amphibian populations tend to be smaller and more fragmented at northern latitudes [44, 15], and since the force of genetic drift is inversely related to population size [45], northern populations are generally predicted to be more influenced by drift. Conversely in southern populations, selection is expected to be relatively more influential as populations tend to be larger and more connected. This has consequences for population's ability to combat disease and pathogens since immunogenetic diversity is predicted to be relatively less shaped by selection at northern latitudes [42].

In the experimental animals, we observed higher mortality in northern individuals. Whether this result depends on the observed lack of MHC variation or some other factor which differs among the northern and southern regions is impossible to discern with this study. Interestingly, we found a difference between the regions in response to the two different *Bd*-strains. *Bd* had a negative impact on survival regardless of strain but when infected with the UK strain, the southern individuals had almost complete survival whereas the northern ones suffered high mortality. When infected with the Swedish strain, both southern and northern individuals had high mortality albeit the rate was higher in the north. Unfortunately, due to lack of MHC variation in the northern animals it is impossible to discern whether this was due to differences in MHC variation or some other factor.

However, we were able to pick up some MHC-related differences in survival as presence of the haplotype Bubu_DAB*9 seemed to have a negative impact on survival when infected with the UK-strain, but the effect was limited when infected with the Swedish strain. Similarly, the haplotype Bubu_DAB*2 had a negative impact on survival when infected with the UK-strain but was marginally beneficial when infected by the Swedish. This difference among strains is somewhat surprising as both strains used in the experiment belong to *Bd*-GPL lineage which has emerged within the last century and is the cause of the extinction of more than 100 amphibian species [6]. Non GPL *Bd*-strains are rarely associated with chytridiomycosis, population declines and species extinction [6].

To our knowledge, this is the first report of differences in host mortality among *Bd*-GPL strains. Previous studies have shown MHC Class IIB-haplotype related differences in survival in *Lithobates yavapaiensis* [19, 20]. In *Litoria verreauxii*, experimental studies showed that certain MHC Class IIB amino acid sequences conferred a survival advantage and the differences were

ascribed to variation at the peptide-binding coding residues of the amino acid sequences [46]. The haplotypes which conferred a survival advantage in *L. verreauxii* were then compared to the most common haplotypes observed in species classified as either susceptible or resistant to *Bd* and *B. bufo* was grouped among the resistant species [46]. However, *B. bufo* have been shown to suffer high mortality in experimental infections with *Bd* [47]. Also, *Bd* has been shown to infect the sister species *B. spinosus* in a Spanish population (classified as *B. bufo* at the time of the study). While *Bd* was associated with mortality, no population declines were reported [48]. Yet, the species has declined in the study area since the time of the study [49].

The available evidence thus strongly suggests that susceptibility to *Bd* depends on a range of factors which may explain both intra and interspecific differences. Intraspecific differences may be ascribed to immuno-genetic competence and diversity. Populations which have a long history of exposure to *Bd* may have undergone selection favouring resistance alleles. Populations naïve to the disease may by chance have resistance alleles but populations further away from refugia and core areas of the distribution are often depauperate in genetic variation and less likely to harbour adaptive variation. Similar processes may act at the interspecific level as different species are shaped by different demographic events. For example, the moor frog in northern Europe harbours quite extensive MHC Class IIB variation, while the natterjack toad, *Epidalea calamita*, have lost most of its MHC-variation during the postglacial expansion to northern Europe [40, 50]. North American ranid frogs have been shown to show low hazard ratios while bufonids showed high when exposed to *Bd* [7]. The different strains of the fungus may also vary in susceptibility within and among populations. *B. dendrobatidis* and *B. salamandrivorans* have been shown to display quite different infection strategies, not surprisingly considering the deep divergence among these two chytrids [51]. As indicated by this study, also recently diverged *Bd*-strains may vary in

256 susceptibility although this is unlikely to be dependent on vastly different infection strategies.
 257 Nevertheless, the constant coevolutionary arms-race among hosts and pathogens is likely to lead
 258 to a range of susceptibilities and pathogenicity within and among species [52,53].

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Tables

Table 1. Spatial coordinates of the sampling sites. Tissue from adults from all populations were used in the latitudinal study. For the experiment we used toadlets collected as eggs from LU1 and LU2 (Norrbotten) and PM1 and PH (Skåne). For a map, see Fig. 1.

Region	Population and Coordinates (N, E)
<i>MHC genotyping sampling</i> <i>Latitudinal gradient</i>	
Norrbotten	LU1:65.583139,22.31945 LU2:65.56554,22.37404
Västerbotten	LU3: 65.582783,22.319320 UM1: 63.891378, 20.155378 UM2: 63.787126, 20.122895 UM3: 63.710052, 20.396955
Uppland	UP1: 59.827595, 17.668712 UP2: 59.845286, 17.598166 UP3: 60.178358, 17.854461
Skåne	SK1: 55.690073, 13.484828 SK2: 55.556388, 13.958642 SK3: 55.745295, 14.154159
<i>MHC genotyping sampling</i> <i>Experimental infections</i>	
Skåne	PM: 56.217897,13.731548 PH:55.539031,14.009702
Norrbotten	LU1:65.583139,22.31945 LU2:65.56554,22.37404

Table 2. Overview of the different models with main effects and the various interactions modelled. Akaike's Information Criterion (AIC) was used to select among models.

	Main effect	Interactions	Model df	AIC
Model1	Region + <i>Bd</i> -strain + MHC	<i>Bd</i> -strain:MHC	17	128.6
Model2	Region + <i>Bd</i>-strain + MHC	<i>Bd</i>-strain:MHC + <i>Bd</i>-strain:Region	19	127.7
Model3	Region + <i>Bd</i> -strain + MHC	<i>Bd</i> -strain:MHC + <i>Bd</i> -strain:Population	25	138.1
Model4	Population + <i>Bd</i> -strain + MHC	<i>Bd</i> -strain:MHC	19	131.4
Model5	Population + <i>Bd</i> -strain + MHC	<i>Bd</i> -strain:MHC + <i>Bd</i> -strain:Region	21	130.1
Model6	Population + <i>Bd</i> -strain + MHC	<i>Bd</i> -strain:MHC + <i>Bd</i> -strain:Population	25	138.1

Table 3. Results of GLM in the best model (model 2).

	SS	Df	F	Pr(>F)	
<i>Bd</i> -strain	62.19	2	50.59	0.000	***
Region	14.40	1	23.43	0.000	***
Bubu02	0.67	1	1.10	0.297	
Bubu04	2.33	1	3.79	0.054	.
Bubu06	0.54	1	0.89	0.348	
Bubu07	0.00	1	0.00	0.964	
Bubu09	2.84	1	4.62	0.033	*
<i>Bd</i> -strain:Region	4.94	2	4.02	0.020	*
<i>Bd</i> -strain:Bubu02	5.09	1	8.27	0.005	**
<i>Bd</i> -strain:Bubu04	0.00	1	0.00	1.000	
<i>Bd</i> -strain:Bubu06	0.00	2	0.00	1.000	
<i>Bd</i> -strain:Bubu07	3.14	2	2.56	0.081	.
<i>Bd</i> -strain:Bubu09	2.94	2	2.39	0.096	.
Residuals	81.75	133			

Figures:

Figure 1. Haplotype frequency distribution of MHC class II sequences in *Bufo bufo* 12 sites in 4 regions in Sweden.

436 **Figure 2.** Toads only carrying Bubu_DAB*1 showed an increased survival in the South compared to the
 437 North, but the increase depends on *Bd*-strain. Hence, the survival probability assumes that none of the
 438 MHC-alleles considered in the model are carried.

Figure 3. The predicted effect of the three alleles with potential *Bd*-strain interactions. a) The Bubu_DAB*2 haplotype provided protection against the Swedish *Bd*-strain, but was detrimental for individuals infected with the UK *Bd*-strain. The predicted values assume using a background of carrying the Bubu_DAB*6 and Bubu_DAB*7 haplotype as this was the case for all three individuals carrying the Bubu_DAB*2 haplotype. None of the three individuals with Bubu_DAB*2 were present in the control treatment, which is thereby excluded. b) Bubu_DAB*9 haplotype has a detrimental effect on survival for *Bd*-infected individuals, especially for the UK-strain. All predicted values assume the region south as this was the case for all individuals carrying these MHC-alleles.

a)

b)