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MHC variation compared to neutral variation in Swedish west coast populations of Natterjack toad (*Bufo calamita*) in the light of approaching chytridiomycosis

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Summary

Amphibians have been declining world-wide for the last three decades and despite a lot of effort the downtrend continues. A third of the 6000 known amphibian species are threatened or extinct; for another quarter the situation is unknown. In the light of these great population declines in amphibians the causes for this decline are very important to understand.

The fungal disease chytridiomycosis is one among many proposed causes to this decline. Originating from Africa, this disease has spread over the world since the early '90s, and infected populations on all continents where amphibians are present. The effect of the disease differs significantly among species and populations, ranging from mass die-offs in some populations to no observable effects in others. One of the causes for this is genetic differences in the major histocompatibility complex (MHC) – a gene complex involved in the vertebrate's immune defence.

In the light of the recent arrival of this disease to Sweden, I evaluated the MHC class II variation of the *Bufo calamita* (natterjack toad) populations on the west coast. These isolated populations are known to have low neutral genetic variation but there have been no studies on the adaptive genetic variation. In the seven investigated populations I found only two alleles, indicating that the adaptive genetic variation is also low. These two alleles were found in all populations, although in one population there was only one heterozygous individual, indicating that this population might be on its way to fixation for one allele. Of the two alleles, one was previously not described and thus contributed to the known diversity of MHC class II in the natterjack toad. The Swedish toads had even lower MHC variation compared to isolated British populations – two alleles compared to five in a lower number of individuals from two regions in the UK. No correlation of neutral genetic variation and MHC variation among the populations was found.

It became clear in this study that the reduced genetic variation in the Swedish natterjack toads is not restricted to the neutral markers. Variation at the MHC marker was likewise very low, but not correlated with neutral genetic variation. However, I found evidence for positive selection acting on the MHC gene and thus maintaining the little diversity there is. Moreover, one of the identified Swedish MHC alleles might be advantageous in terms of the chytridiomycosis disease, as that allele was strikingly frequent in individuals from infected British populations.

With amphibian populations declining all around the world, there is a great need for more knowledge. This is my contribution.

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Introduction

The amphibian decline

It is well known that we humans are affecting the Earth we live on, and that many of the species we share the planet with are declining is a sad truth. One of the most affected taxa is the amphibians which have suffered serious declines during the recent decades (Stuart *et al.*, 2004). The decline was first revealed in the '70s with mass mortalities reported in USA (e.g. Bradford, 1991) and rapid population declines recorded in, for example Australia (Laurance *et al*., 1996), USA (Sherman and Morton, 1993), and the UK (Beebee, 1977), but is now seen in every continent where amphibians are present (Stuart *et al.*, 2004). Today 32 % of the over 6 000 known amphibian species are globally threatened or extinct, and for a quarter of the total number the situation is unknown (Beja *et al*., 2011).

However, while the exact causes and extent of the decline in every specific region has been disputed (e.g. Pimenta *et al.*, 2005, Pounds *et al*., 2006a, Mendelson *et al*., 2006) the major conclusion is accepted: Amphibian populations are declining rapidly.

Among the suggested causes for this decline we find habitat loss, environmental change, UV-radiation, and pathogens (Beebee and Griffiths, 2005) – many caused or aggravated by human activity. Since the decline is global, scientists have searched for a world-wide explanation (Barinaga, 1990), but so far none has been found. It has been argued that it is unlikely that a single factor exists (Blaustein and Kiesecker, 2002). However, one identified factor linked to the decline of a large number of amphibian species is the rapidly spreading disease chytridiomycosis (Daszak *et al.*, 2003), caused by the fungus *Batrachochytrium dendrobatidis* (Bd, described by Longcore *et al.*, 1999).

Chytridiomycosis

The fungal disease chytridiomycosis was first found in the rain forests of Australia (1993-1994) and Central America (1996-1997) and described by Berger *et al*. (1998). It is an epidermal disease causing the outermost layer of the skin in the infected area to be altered and this may be the direct cause for the individual's death as it severely affects the skin breathing in the amphibian (Voyles *et al*., 2009).

The disease has been proposed to originate in Africa (Weldon *et al*., 2004), spreading over longer distances by collectors moving infected specimens and equipment, including, for example, boots. In addition to the native Africa and early studies in Central America and Australia, the fungus is also present in North America (e.g. Muths *et al*., 2003), South America (Hanselmann *et al.*, 2004), and Europe (e.g. Bosch *et al*., 2001). During the last years it has been reported in Asia (Savage *et al*., 2012), and is thus present on all continents with amphibians. In the spring of 2010, it reached Sweden (Lundquist and Rudbeck, 2010).

Even though chytridiomycosis has been proved to be closely linked to amphibian decline (Daszak *et al*., 2003) and proposed to be one of the major causes for amphibian population decline, the fungus causing the disease is found in both healthy and declining populations (e.g. May *et al*., 2011). Outbreaks of the disease seem to be dependent on environmental factors (James *et al*., 2009), such as temperature, as investigated by Pounds *et al*. (2006b) in South and Central America, where population disappearances were closely linked to years with increased temperatures.

Apart from the environmental factors influencing the disease progression, the individual's immune system plays a vital part in coping with pathogens. How the genetically dependent part of this complex system is acting in the case of chytridiomycosis is not known, but it is proposed that the second class of the major histocompatibility complex is involved (Richmond *et al*., 2009) and this is thought to be an important research area in the light of the approaching disease and declining amphibian populations.

Major Histocompatibility Complex

The major histocompatibility complex (MHC) is involved in an organism's immune defence and contains primarily two classes; the class I that binds and presents antigens derived from intracellular pathogens like viruses or cancer-infected cells, and the class II that corresponds to extracellular pathogens like bacteria or fungi (Piertney and Oliver, 2006). When coming across a pathogen that fits into the molecule it binds, it is transported to the surface and thus the immune defence is activated. While some parts of the MHC molecule are much conserved and are therefore under strong selection, (because these parts are responsible for the form and structure of the molecule), other parts show high levels of variation (Klein, 1986).

Due to its function in the immune system, the more variation found at the part of the molecule where the antigen is displayed (the peptide binding region, PBR), the more pathogens can be recognized and fought by the individual's immune system. Thus the parts of the molecule coding for these PBRs are proposed to be under strong diversifying selection, and the MHC has the most variable functional genes that have been described in vertebrates (Piertney and Oliver, 2006).

Exactly how the selective pressure is acting on the alleles with different PBRs is disputed (De Boer *et al*., 2004¸ van Oosterhout, 2009): heterozygote advantage (Doherty and Zinknagel 1975) rare-allele-advantage (Takahata & Nei, 1990), and host-pathogen coevolution (Borghans *et al*., 2004) have all been proposed. Another explanation could be disassortative mating preferences (Penn and Potts, 1999), as the famous t-shirt experiment showed (Wedekind *et al*., 1995) where male human students slept in t-shirts overnight and female students scored the t-shirts depending on how attractive they found the smell. It showed that the females were more likely to approve of the smell of men whose MHC was dissimilar to their own.

At the population level, it has been shown that a higher number of MHC alleles significantly correlates with, for example, lower parasite load (Meyer-Lucht and Sommer, 2009). It has also been shown that MHC heterozygosity or certain alleles can be correlated to survival from certain disease, in our case most interestingly from chytridiomycosis (Savage and Zamudio, 2011). With regard to the chytridiomycosis disease, MHC class II loci are the most likely candidate immune genes for two reasons: 1) they are the primary presenters of extracellular fungal pathogens and 2) class II-expressing dendritic and Langerhans lymphocytes are present in amphibian skin (Richmond *et al*., 2009), the primary location of BD infections (Savage and Zamudio, 2011).

In population genetic terms, MHC variation reflects adaptive genetic variation, i.e. genetic variation that confers an advantage in fitness. Their key function in the immune response and their characteristic extraordinary polymorphism place the genes of the MHC among the best markers to study molecular adaptation (Sommer 2005) and how genetic diversity at the MHC is directly related to a species' survival.

Routinely in population genetic studies, neutral genetic variation is measured to assess genetic diversity. However, genetic variation at coding genes can reflect evolutionary relevant adaptive processes. MHC variation reflects adaptive genetic variation because it directly affects an individual's fitness. The genes of the MHC are therefore among the best markers to study molecular adaptation processes (Sommer 2005).

Hauswaldt's *et al*. (2007) study of MHC in *Bombina bombina* was the first study of MHC in amphibians (except of the model species genus *Xenopus*). Apart from the *B. bombina* study and two studies of *B. calamita* (May and Beebee, 2009; May *et al*., 2011), there is in my knowledge no other MHC characterization in toads. As the number of MHC loci varies between taxa and even between individuals (e.g. 1-4 loci in humans, Bontrop *et al*. 1999) it is not known how many MHC class II loci the *Bufo calamita* has, but May and Beebee (2009) found at least two. It is not proven that both loci are functional.

Natterjack toad

B. calamita is one of three species of toads present in Sweden. Reproducing adults are around 5 cm and their characteristically short back legs give them a typical walking movement, although they climb and dig well (Andrén and Nilson, 1979).

The natterjack toad is common across Europe and in Sweden it reaches its northernmost boundary (see figure 1). Here the species is considered threatened (Tjernberg *et al*., 2010), found only on ~ 20 small islands in the west coast archipelago and on a few locations in Skåne (Andrén and Nilson, 1985a). Around Europe it is found in sandy ground, and as it is in Skåne. However, the west coast populations inhabit a rather unusual habitat on barren islands. It is unknown if these populations are relics from a previously larger distribution, or if they are the result of more recent dispersal events. Andrén and Nilson (1979) argues that even though they are not likely swimmers due to their short back legs, strings of eggs could be attached to the feet of seagulls and thus moved between islands. Another theory is that the toads came with sand from Denmark

when fishermen returning from fish market in Skagen brought sand for construction work (lighthouses etc.) to these islands (J. Höglund, personal communication). This theory has not been confirmed, but Andrén and Nilson (1985b) notes that most of the natterjack populated islands has old blocks of foundations from human settlements. The breeding ponds are often flooded by sea water and therefore the tadpole environment is sometimes saline (Andrén and Nilson,

1979). Even though the toads can breed in saline ponds, there is no evidence for adults swimming between islands and genetic studies indicates that the sea is a barrier between populations (Rogell *et al*., 2010a). The West Coast populations are thus very isolated and the neutral genetic diversity is low. But even though they are believed to be inbred, an experiment conducted by Rogell *et al*. (2010b) showed that even if the most inbred populations indeed had lower survival rate of larvae than populations with more genetic variation under favourable conditions, they performed equally or even better under more natural (stressful) conditions.

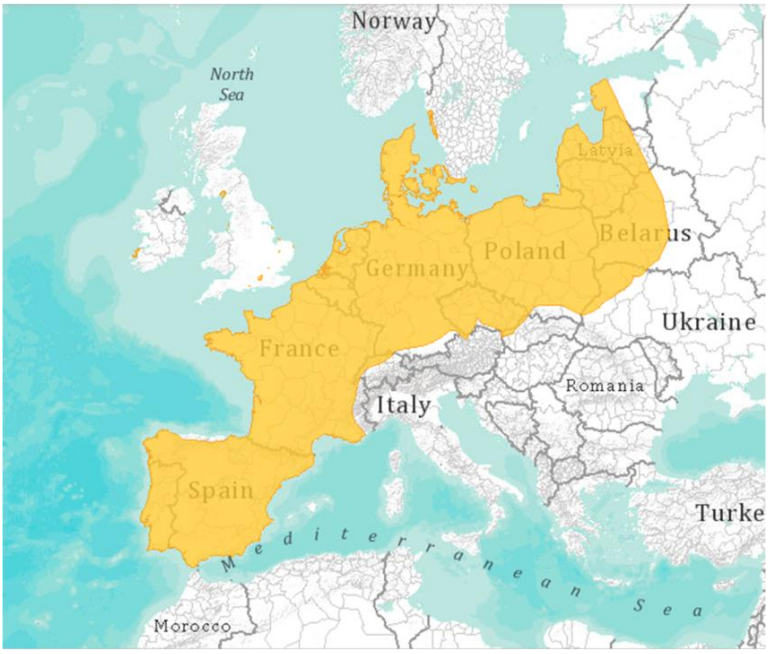

Figure 1. Distributation map of *Bufo calamita* (IUCN Redlist, Beja *et al*., 2009). Note that the distribution of continental populations might be exaggerated due to limited research.

Generally, low genetic diversity may cause an extinction threat especially for isolated populations and especially under changing environmental conditions. The ability of a population to adapt to new challenges is restricted and even small environmental changes could be devastating. However, even though the neutral genetic diversity is low, adaptive genetic variation, i.e. genetic variation that confers an advantage in fitness and therefore is under selection (Hedrick, 2001), maybe higher and the crucial factor for adaptation.

The most well studied populations of natterjack toad MHC are found in the UK. A recent study by May *et al*. (2011) showed a difference in allele frequencies of MHC Class II exon 2 in Bd-infected and uninfected populations in Britain. Of five alleles present in the uninfected populations, two were not found in the infected area and the frequency of one of the alleles was 93.6 % (compared to 32.5 % in the uninfected). Although there could be many reasons for this, this allele could play a part in coping with the disease (May *et al*., 2011).

Aims

Recently, the West Coast populations have been screened for neutral genetic variation using AFLP markers (Rogell *et al*. 2010a). The main aim of this study was to complement this knowledge with the variation in MHC class II, which is suggested to be associated with resistance to chytridiomycosis, using markers developed by May *et al*. (2011).

The specific objectives of my study were:

1. Assess MHC class II variation and compare to previously known (British) results.
2. On population level: Do the Swedish populations differ in MHC diversity?
3. Is there a correlation between neutral genetic diversity and MHC diversity?
4. If previously unknown alleles are found, how are they placed in a phylogeny with previously known alleles?
5. Speculate on the conclusions from these results with the regard to resistance to chytridiomycosis.

Materials and methods

Sampling and sampling locations

The DNA used in this study was previously extracted by Rogell *et al*. (2010a), including toads from seven populations. The biopsy samples (or in the case of one population; embryos) were collected in May 2006 on the West Coast islands of Altarholmen (58°1'N, 11°26'E), Buskär (58°21'N, 11°11' E), Fågelskär (58°6'N, 11°20'E), Hyppeln (57°45'N, 11°36'E), Måseskär (58°5'N, 11°19'E), Oxskär (58°7'N, 11°22'E) and Pater Noster (57°53'N, 11°27'E) (see figure 2). Of the extracted samples (protocol in Rogell *et al*. 2010a) DNA from 17-20 individuals per population were included in this study – a total of 132 individuals.

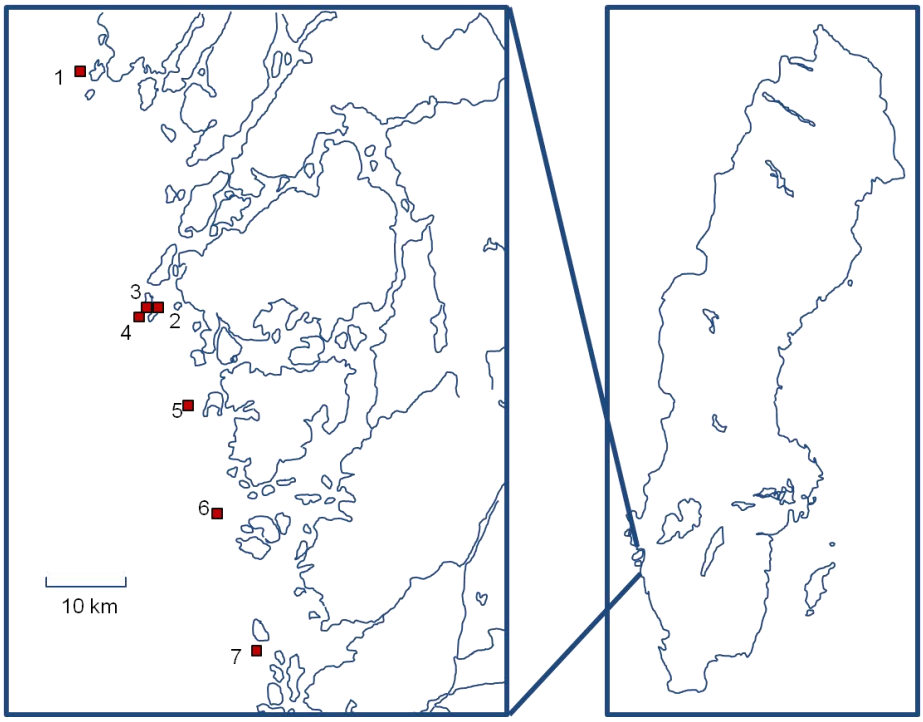

Figure 2. Map with the studied populations. From north to south: Buskär Oxskär, Fågelskär, Måseskär, Altarholmen, Pater Noster, and Hyppeln. Redrawn after Rogell *et al*., 2010a.

PCR

The PCR protocol was developed by May *et al*. (2011) for amplifying 285 bp of MHC class II exon 2 and the flanking introns. The protocol was slightly modified. The 30 µl-reaction contained 1.6 µl DNA, 21.2 µl water, 3 µl NH_4SO PCR buffer from Fermentas (St. Leon-Rot, Germany), 0.83 µl $MgCl_2$, 0.75 µl dNTP Mix, 0.15 µl of Taq-polymerase (Fermentas, St. Leon-Rot, Germany), 1.2 µl each of 2F347 forward primer and 2R307b reverse primer. This was run in a program with 94°C for 4 minutes for initial denaturation, followed by 35 cycles of denaturation at 94°C for 40 seconds, an annealing stage of 54°C for 40 seconds, and a primer extension stage of 72°C for 30 seconds. The last step was a final extension stage of 72°C for 5 minutes. The PCR products were controlled on a 1.5% agarose gel in 40 minutes at 80 Volts electrophoresis.

Direct sequencing

Before being sent for sequencing, the products were cleaned using a solution of 1.0 µl Exonuclease I and 2.0 µl FastAP Thermosensitive Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany) per 25 µl sample, incubated in 37°C for 30 minutes and 80°C for 30 minutes. The products were sequenced at Macrogen Inc. (Amsterdam, the Netherlands), initially in both forward and reverse direction. As all heterozygotes were unreadable in the reverse direction even when resequenced (see below), reruns were done only in the forward direction.

Cloning

To establish that the heterozygote genotypes contained the exact combination of known alleles, two individuals were cloned by using the TOPO TA kit (Invitrogen, Carlsbad, United States). I did a chemical transformation from fresh PCR-products. 1-4 µl of PCR-product were gently mixed with 1 µl TOPO vector and 1 µl salt solution, filling up with water to a total volume of 6 µl and incubating in room temperature for 5 minutes. Then 1-5 µl of DNA was mixed with 50 µl of DH5α component cells and incubated on ice for 30 minutes. The cells were heat shocked in a 42° C water bath and incubated on ice for an additional 2 minutes. To each sample, 950 µl of SOC medium was added and incubated at 37 ° C for 1 hour, rotating at 225 rpm. The solution was spread in two different amounts (80 and 160 µl) on LB plates containing 100 µg/ml ampicillin, incubated at 37 ° C for 16 hours and stored in the cold room for 1-2 days.

10 positive clones were picked and the bacteria were killed by heating to 99 ° C for 5 minutes, and the solution was used in PCR with the universal M13 primers by the following protocol: The 30 µl-reaction contained 3.75 µl DNA from colonies, 71.9 µl water, 3 µl NH_4SO PCR buffer from Fermentas (St. Leon-Rot, Germany), 2.3 µl $MgCl_2$, 1.5 µl dNTP Mix, 0.08 µl of Taq (Fermentas, St. Leon-Rot, Germany), 0.8 µl each of M13 forward primer and M13 reverse primer. This was run in a program with 95°C for 2 minutes for initial denaturation, followed by 30 cycles of denaturation at 95°C for 60 seconds, an annealing stage of 52°C for 60 seconds, and a primer extension stage of 72°C for 60 seconds. The last step was a final extension stage of 72°C for 10 minutes.

The PCR products were controlled for size on a 1.5% agarose gel in 40 minutes-80 Volts electrophoresis, checked for the correct size of the insert and cleaned using the protocol described above. 16 of the PCR-products were sent to Macrogen Inc. for sequencing.

Sequence analysis

The sequences were examined in Codon Code Aligner (v. 3.7.1, Codon Code Corporation); heterozygote sequences were marked in ambiguity codes. The exact sequences of the found alleles were transferred to MEGA (version 5, Tamura *et al*., 2011). All individuals were scored for which alleles they presented; alleles named starting with A. A summary for each population with the number of heterozygotes (and containing alleles) as well as homozygotes for each found allele were the basis for the population genetic analysis.

Neutral genetic variation

Data for comparison of neutral variation was taken from Rogell *et al*. (2010a). The authors had screened 30 individuals from each population for AFLP markers using 11 primer combinations, and found a total of 105 polymorphic loci. Of the seven populations, the authors found that Altarholmen had the highest proportion of polymorphic loci (91.4 %) and Måseskär the lowest (42.9 %) (see table 1). The values for pair wise Φ_{PT} are displayed in table 2. They also calculated a global F_{ST} of 0.157 (upper and lower 95% bootstrap confidence intervals 0.139 and 0.176).

Data analysis

Population genetic measures were done in Arlequin 3.5 (Excoffier and Lischer, 2010). Overall F_{ST} was calculated by computing a distance matrix with 100 permutations. The pair wise F_{ST} values model used 110 permutations. Comparisons with the neutral genetic markers by Rogell *et al*. (2010a) were performed in R (R Development Core Team, 2009); correlating pair wise Φ_{PT} from AFLP and pairwise F_{ST} from MHC using Mantel test (based on the Pearson correlation coefficient) and correlating MHC heterozygosity with the proportion of polymorphic AFLP locus for each populations using a Pearson correlation.

Phylogenetic trees were constructed using the neighbour joining method in mega, using the new alleles together with previously known *Bufo calamita* alleles. The proposed peptide binding regions were marked as described in Tong *et al*. (2006) and Brown *et al*. (1993) and marked and unmarked sites were tested respectively for synonymous and non-synonymous selection, and thus calculating the dN/dS ratio. Codon-based-Z-test of selection was used to determine the signification levels. The known alleles were transferred into a FASTA-file and recombination events were looked for in DNAsp (Librado and Rozas, 2009) with a minimum of recombination events calculated by the Hudson and Kaplan 4 gamete test (Hudson and Kaplan, 1985). Selection signal tests REL and SLAC were calculated using Datamonkey (www.datamonkey.org, Delport *et al*.

2010; Kosakovsky Pond and Frost, 2005a; Kosakovsky Pond and Frost, 2005b) with the HyPhy package (Kosakovsky Pond *et a.,* 2005) and model F81. Firstly, a GARD recombination test was done and this result was used for the further analyses of positive and negative selection at codon sites.

Results

Characterization of MHC class II variation

132 individuals from seven *Bufo calamita* populations were successfully sequenced and two alleles were found (A and B). The two alleles were of different length (282 respective 279 bp, see figure 3) due to an insertion of 3 bp towards the 3'-end of the allele. Therefore most of the end of the forward sequences for heterozygotes were polymorphic and the reverse sequences impossible to read. When marked with ambiguity codes all the heterozygous sequences were identical, and seemed to be contain of the exact alleles as the two homozygotes. This was confirmed with the result of the cloning as the cloned heterozygote, as the 16 sequences were either allele A or allele B. Interestingly, Allele B was earlier described by May *et al*. (2011).

Population differentiation

The two alleles were present in all populations (see table 1); although in Pater Noster allele B was represented by a single heterozygous individual. As with the neutral markers, a population structure was found with an overall F_{ST} of 0.174 ($P<0.001$), indicating that there was strong population differentiation. The pair-wise F_{ST} values were not always significant (see table 2); significant values ranged between 0.087 and 0.590. The greatest differences were found between populations Buskär and Hyppeln compared to Pater Noster.

Table 1. Allele frequencies and heterozygosity of MHC class II in seven Swedish West Coast populations of *Bufo calamita*. Neutral variation is per cent of polymorphic AFLP loci from Rogell *et al*., 2010a.

Population	N	Present alleles	Allele A Freq.	Allele B Freq.	Observed Heterozygosity	Expected Heterozygosity	Neutral variation
Altarholmen	19	A & B	0.68	0.32	0.42	0.43	91.4 %
Oxskär	20	A & B	0.83	0.18	0.35	0.29	77.1 %
Buskär	20	A & B	0.35	0.65	0.40	0.46	71.4 %
Fågelskär	17	A & B	0.74	0.26	0.41	0.39	57.1 %
Pater Noster	19	A & B	0.97	0.03	0.05	0.05	53.3 %
Hyppeln	18	A & B	0.47	0.53	0.39	0.50	49.5 %
Måseskär	19	A & B	0.71	0.29	0.58	0.41	42.9 %
Total	132	A & B	0.68	0.32	0.37	0.44	

Correlating neutral and MHC diversity

No significant correlation was found between neutral and MHC variation. For the mantel test of pair wise Φ_{PT}/F_{ST}, the confidence intervals ranged between -2.25 and 0.66 (n.s.). Neither was the comparison of AFLP polymorphism and MHC heterozygosity per

population (Pearson's product-moment correlation, r = -0.029, t=-0.29 df = 5, n.s.). When looking at the results, this is not surprising as the highest heterozygosity was found in the population with the lowest neutral variation (table 1).

Table 2: Pairwise F_{ST} for MHC in the lower left, pairwise Φ_{PT} from AFLP in upper right (from Rogell *et al*. 2010a); significant values (P<0.05) in bold.

MHC vs neutral	AL	BU	FA	HY	MA	OX	PA
AL		**0.065**	**0.153**	**0.063**	**0.170**	**0.056**	**0.096**
BU	**0.180**		**0.224**	**0.094**	**0.297**	**0.088**	**0.180**
FA	-0.022	**0.238**		**0.165**	**0.122**	**0.150**	**0.267**
HY	0.062	0.004	0.109		**0.160**	**0.071**	**0.106**
MA	-0.025	**0.210**	-0.027	**0.087**		**0.193**	**0.206**
OX	0.027	**0.362**	-0.004	**0.222**	0.011		**0.108**
PA	**0.237**	**0.590**	**0.190**	**0.469**	**0.210**	0.090	

Tests for positive selection

Analyses of the previously known alleles (May *et al*., 2011) and the Swedish alleles found here together show that the allele A differentiated from the other five alleles in sites where there has previously been no differentiation. To test for positive selection within the known MHC class II alleles of *B. calamita* I calculated the relative rates of synonymous (dS) and non-synonymous (dN) substitution (see table 3). Weak evidence for positive selection was found as the dN/dS ratio was not significant when combining proposed peptide binding regions from Tong *et al*. (2006) and Brown *et al*. (1993). When analysing only sites described in Tong *et al*. the dN/dS ratio was 1.99 (P=0.024) in the proposed PBRs, the non PBR sites did not give a significant result.

Table 3. Testing for selection in MHC class II alleles using relative rates of synonymous (dS) and non-synomous (dN). Table a) contains the result of peptid binding regions (PBR) as marked by Brown *et al*. (1993) and Tong *et al*. (2006); Table b) for PBR marked only according to Tong *et al*.

a)	dN	dS	dN/dS	Z-value	P-value
PBR sites	0.32	0.26	1.26	0.696	n.s.
Non PBR sites	0.07	0.10	0.79	-0.517	n.s.
All	0.13	0.13	0.96	-0.135	n.s.

b)	dN	dS	dN/dS	Z-value	P-value
PBR sites	0.42	0.09	1.99	1.997	0.024
Non PBR sites	0.09	0.02	0.73	-0.850	n.s.
All	0.13	0.13	0.96	-0.135	n.s.

I also identified a number of specific codon positions under positive or negative selection using the two different methods SLAC and REL (results are displayed in figure 3). Two negatively selected sites (SLAC) and 20 positively selected sites (REL) were found. The minimum number of recombination event was estimated at 10.

SLAC showed that two codons (42 and 73) were negatively selected (dN/dS ratio of -2.4 (P=0.09) and. -5.3, (P=0.09)), whereas REL found twenty positively selected sites (dN/dS values between 3.22-3.28, posterior probability 0.987-0.999, see figure 3).

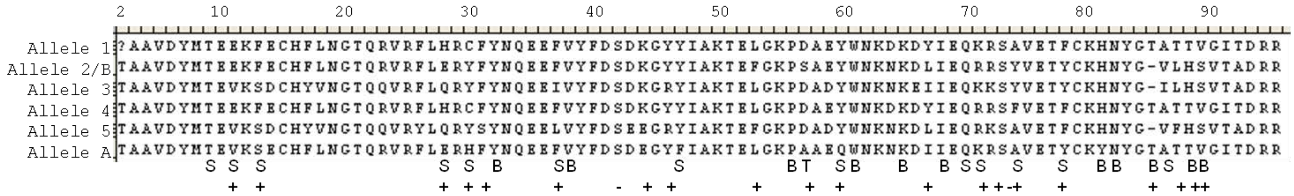

Figure 3. Alignment of amino acid sequences of MHC class II alleles. Proposed peptide binding regions are marked with B (sites from Brown *et al*., 1993), T (from Tong *et al*., 2006) and S (PBR sites according by both). Positive selection from REL is marked with +, negatively selected sites from SLAC analysis with -.

Comparison to the previously known MHC class II alleles of B. calamita

The two trees show the phylogeny for amino acid PBRs and nucleotide diversity respectively. In both phylogenies (figure 4), allele A clusters with allele 1 and 4, whereas B (2 in the British study) clustered with 3 and 5. The phylogeny of amino acid diversity in the PBR (figure 4a) had lower bootstrap values, as the few sequences make it hard to get high statistical power. The total number of segregating nucleotide sites was 63, and when comparing the amino acid sequences (figure 3), allele A added variation in three sites where the previously known alleles had the same amino acid.

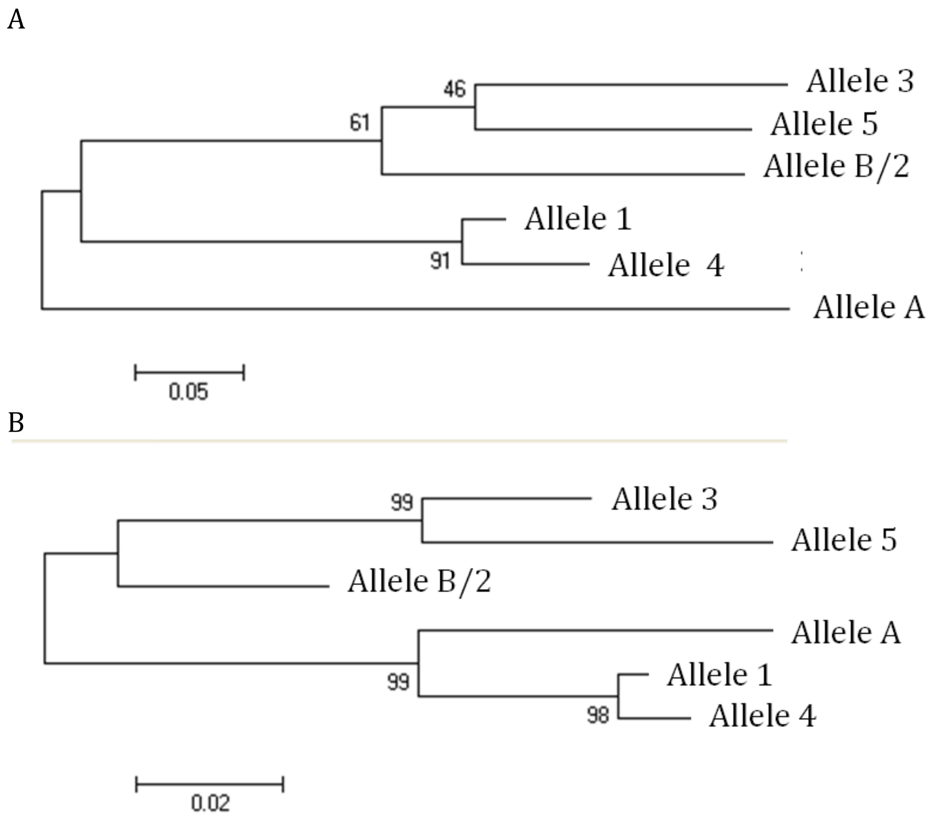

Figure 4. Phylogenies of MHC class II alleles, neighbor joining trees.
A: Tree based on the PBR sites in amino acid sequences.
B: Tree based on all nucleotide sequences.

Discussion

The seven populations showed a low level of variation at the MHC class II, as a total number of two alleles were found. These two alleles were found in all populations, with previously undescribed allele A being the most numerous overall and allele B corresponding to allele 2 in the study by May *et al.* (2011). In two populations, allele B was the most common, but in Pater Noster this allele was only present in one heterozygous individual, indicating that this population might be on its way to fixation for allele A. Compared to British natterjack toads, the Swedish toads have a low variation – in 132 individuals only two alleles were found, compared to five in 140 individuals in the two regions in the UK (May *et al*., 2011). However, in the Bd-infected areas in the UK study the allele corresponding to allele B in this study had a very high frequency and only two other alleles were present, compared to five in the uninfected area.

The overall F_{ST} for MHC corresponds well (0.174 to 0.157) to the global value from neutral variation (from Rogell *et al*., 2010a), confirming that there is population structure also considering adaptive genetic variation. Though pair-wise F_{ST} values were not always significant for the MHC alleles, the corresponding Φ_{PT} from the neutral markers have shown clear population structure even between geographically close populations. As it seems unlikely that only the MHC gene should have migrated, it is more probable that the selection pressure on the studied MHC gene in different locations is similar and thus selecting for certain alleles or heterozygosity. Evidence for this is demonstrated in, for example, the Måseskär population, where 11 of 19 individuals were heterozygotes but none were homozygotes for allele B.

It is clear that the reduced genetic variation in these toads is not restricted to the neutral markers, but even though the MHC variation was low, it was not correlated with low neutral genetic diversity in the studied populations. This is similar to the results from Rogell *et al*. (2010b) who did not find a correlation between high neutral genetic diversity and survival in their experiment of natterjack toad larvae survival under conditions simulating natural environment. The cause for the lack of correlation of neutral and MHC variation is not clear, but it is likely that the MHC might be under positive selection which acts to maintain what diversity there is present.

Combined with the results from studies of British natterjack toads (May *et al.*, 2011), typical characterizations of the MHC in the detected alleles was found, such as positive selection and recombination. Together the six alleles displayed 63 segregating sites. The proposed peptide binding regions of Tong *et al.* (2006) show a significantly higher rate of positive selection, and fourteen of twenty sites that showed positive selection were found in the proposed peptide binding regions.

May *et al.* states that the allele 2 (corresponding to allele A in this study) is present in many European populations (May *et al*., 2011). I can conclude that it is present also in the Swedish west coast population, but as it is not known how the toads established

populations in this area, the presence of these alleles might tell us the phylogeographic history of the toads. Due to their function in the immune system, MHC alleles can be retained over long evolutionary periods as well as lost rapidly due to selection. As the Swedish west coast populations are not believed to be very old, there is proposed (J. Höglund, personal communication) that they have been spreading by human hands during the last few centuries. It would be interesting to know what alleles are present in the Swedish Skåne population in the south (the only other location the natterjack toad is found in Sweden) and in northern Denmark. If the natterjack toads were more widespread in Sweden before being outcompeted by other toad species or natural causes, the Skåne and west coast populations should both carry a subset of the previously larger population's allelic richness. It would also have been interesting to involve Danish material to test the proposed theory that the toads came from Denmark might be more likely. The toads, it is proposed, came with the sand the West Coast fishing boats were filled when going home from the fish market in Skagen (North Denmark). The sand was used for construction work (lighthouses etc) on the small islands. It would be interesting to look for similarities between the west coast populations and other populations, but these descriptive MHC studies should also include neutral markers, as the MHC variation in the end always shows what alleles are selected for in the particular environment. This environment might differ between the present day West Coast populations and a suggested founding population.

In the light of the amphibian decline, estimating the pre-infection genetic variation might be the way of understanding how chytridiomycosis affects populations. The indications in Britain of low genetic diversity might be explained as the mortality of the disease might not be high, but the fitness impact great, which has lowered the reproductive success in the affected animals so genetic variation is lost. There have been no reports of mass mortality in natterjack toad due to chytridiomycosis, but some twenty years ago the natterjack toads, in the presence of Bd in a natural park in central Spain, declined from hatching in 19 ponds during the '80s to only 9 ponds in '99 (Martinez-Solano *et al*., 2003). Nor has May *et al*. (2011) found any evidence for reduced population size in the infected regions, but the MHC genetic variation is very low and might indicate that even though the population is not declining in number, the overall genetic variation has thus reduced the survival ability of the population in changing environment.

It is known, even in amphibians (Savage and Zamudio, 2011), that certain alleles correlate with increased survival rate in the presence of a pathogen. This correlation could be the reason that allele 2 in Britain has the allele frequency of 93.6 % in a region where chytridiomycosis is present, because it might be advantageous against the disease and therefore selected for. In a region without the disease, the allele frequency of that allele is 32.5 %, and out of the five alleles present in the uninfected region, only three occur in the infected region (May *et al.* 2011). There might of course be stochastic explanations for this, but this is an indication that would be interesting to further investigate. This allele 2 is identical to the allele B in this study, so one can speculate that

there might be a connection between the allele in an individual and the chances of it surviving in the presence of chytridiomycosis. There is hope for survival in all the West Coast populations, providing that the other circumstances do not interfere with the effect of the allele.

As the fungus causing chytridiomycosis now is found in Sweden, there is an upcoming natural experiment of how the populations will survive the infection. High mortality rates would of course have a devastating impact on the populations, but also the absence of mass mortalities might be crucial. Savage and Zamundio (2011) showed in their study of MHC class II correlation to chytridiomycosis survival that a certain allele and/or heterozygosity were strongly correlated to survival. If this is the explanation for the low MHC variation in the UK (May *et al*., 2011), not only the MHC variation but the overall genetic variation will be reduced. In the West Coast populations, where only two or three MHC class II genotypes are present, the affect that only individuals with a certain allele survive and reproduce would be reproducing would have a devastating effect on the overall genetic variation.

However, as the isolation cause by the sea barrier may have caused problems for the toads before, the same thing might be what could save them from the disease. However, many of these islands are frequently visited by humans (Andrén and Nilson, 1979); they might not be so safe anyway.

Conclusion

It became clear in this study that the reduced genetic variation in these toads is not restricted to the neutral markers. Variation at the MHC marker was likewise very low, but not correlated with neutral genetic variation. However, I found evidence for positive selection acting on the MHC gene and thus maintaining the little diversity there is. In the time of huge population declines, populations have to be studied today as they might be gone tomorrow.

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