理學博士學位論文

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Biological Sciences

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Coevolution of Asian Amphibian and Chytrid Fungus, a Globally Emerging Pathogen

by

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Under the supervision of

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Abstract

Coevolution of Asian Amphibian and Chytrid Fungus, a Globally Emerging Pathogen

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Infectious diseases have been known as huge threats to biodiversity, global health and economy. About 60% of human emerging infectious diseases derived from zoonotic pathogens, and among which, about 70% had wild animal reservoirs. Therefore, studying wild life diseases dynamics is vital for understanding propagation patterns and interaction dynamics of pathogens and hosts in order to control them globally. Chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), has been implicated in population declines and species extinctions of many amphibians around the world, but not in Asia. In Asia, the Bd pathogen and its amphibian hosts have co-evolved over 100 years or more. Thus, resilience of Asian amphibian populations to infection might result from attenuated virulence of endemic Bd lineages, evolved immunity to the pathogen, or both. BdAsia-1, isolated from South Korea amphibians (*Bombina orientalis*), was suggested to be evolutionarily basal to recombinant global

pandemic lineages (BdGPL), which associated with worldwide amphibian population declines. However, the virulence of BdAsia-1 was still not clear. Although previous papers have suggested Asian amphibians were more resistant, there was no infection experiments performed to prove it. In this project, I firstly compared the virulence between BdAsia-1 and BdGPL using a known Australasian susceptible species, *Litoria caerulea*, then tested susceptibility of three Korean amphibians (*Bufo gargarizans, B. orientalis, and Hyla japonica*) using both lineages. Subjects became infected in all experimental treatments, but Korean species rapidly cleared themselves of infection, regardless of Bd lineage. Individuals of Asian species survived with no apparent secondary effects. By contrast, *L. caerulea,* after infection by either BdAsia-1 or BdGPL, suffered deteriorating body condition and carried progressively higher Bd loads over time. Subsequently, most subjects died. Comparing their effects on *L. caerulea*, BdAsia-1 induced more rapid disease progression on *L. caerulea* than BdGPL. My results clearly indicated that two Korean Bd isolates (KBO327 and KBO347) belonging to BdAsia-1 lineage were both hypervirulent to *L. caerulea*, with no sign of attenuation than BdGPL. All Korean amphibians I tested were resistant to or tolerant of both Bd lineages. The pathogen's virulence may have driven strong selection for adaptive immune responses in endemic Asian amphibian host species.

Susceptibility to the disease varies both within and among species, most likely attributable to inheritable immunogenetic variation. Major histocompatibility complex (MHC) is one of the priorities for investigating the mechanisms of disease

resistance selection. In previous research, MHC I and MHC II genes of some susceptible species were shown to be up-regulated following host infection by Bd, but resistant species exhibited no comparable changes in transcriptional expression. Bd-resistant species shared similar pocket conformations within the MHC-II antigen-binding groove. Among susceptible species, survivors of epizootics bear alleles encoding these conformations. Individuals with homozygous resistance alleles appear to benefit by enhanced resistance, especially in environmental conditions that promote pathogen virulence. Subjects that are repeatedly infected and subsequently clear Bd can develop an acquired immune response to the pathogen. Strong directional selection for MHC alleles that encode resistance to Bd may deplete genetic variation necessary to respond to other pathogens. Resistance to chytridiomycosis incurs life-history costs that require further study.

However, almost all MHC genotyping in previous amphibian research was based on single PCR derived traditional cloning based sequencing, which has limitations to identify all alleles efficiently and provide reliable results. This greatly reduced the reliability of the former findings- the association of MHC II with Bd resistance. Therefore, I amplified three amplicons independently for each sample, then applied next generation sequencing to genotype MHC II in *L. caerulea* and *B. gargarizans*. A number of MHC II alleles were identified in both species. Each allele had differential abundance and relative gene expression in both individual and organ level. MHC II was under balancing selection in both *L. caerulea* and *B. gargarizans*, but some codons in peptide binding region and WuKa sites of MHC II alleles were under strong positive selection in *L. caerulea*. Moreover, Wu-Kabat

variability was significantly higher in *L. caerulea* than that in *B. gargarizans,* indicating a higher protein variability in *L. caeurlea* as well. Moreover, most of the positive selected PBR and WuKa sites in *L. caerulea* were completely conserved in *B. gargarizans*, suggesting these sites might associate with Bd resistance. However, no clear gene expression pattern of different MHC II alleles was observed regarding to Bd treatment and susceptibilities. Although there were five unique MHC II alleles found in two resistant *L. caerulea* individuals, I did not observe clear patterns of these alleles relating to resistance owing to limited resistant sample size in *L. caerulea.* Further studies should be performed to demonstrate the roles of the positively selected sites in *L. caerulea*.

Keywords: *Batrachochytrium dendrobatidis*, Asian amphibians, pathogen virulence, coevolution, host resistance, MHC II, concerted evolution

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Chapter 1 . Introduction

1.1 Infectious Diseases

Infectious diseases negatively impact the global economy and cause serious public health problems in human populations. The emergence, or re-emergence of infectious disease often causes unexpected epidemics, or even pandemics (Altizer et al., 2003; Kate et al., 2008). Viruses are particularly dangerous because of the high mutation rates in viruses and subsequent rapid co-evolution. For instance, Avian influenza caused by influenza type A virus has caused over 750 million dollars in damage since 2003 in South Korea alone (Kim, 2017). An estimation of the economic damage to annual influenza in United States was approximately 2.5 million USD (Mao et al., 2012). A sudden emerge of Severe acute respiratory syndrome (SARS) due to the SARS coronavirus (SARS-CoV) in China has caused many people (about 10000) to suffer from disease and death (nearly 1000), and caused a country-wide and subsequent worldwide panic between 2012 and 2013 (Smith, 2006). The outbreak of Middle East respiratory syndrome (MERS) caused by ERS-coronavirus (MERS-CoV) in South Korea caused at least one death per day in June 2015 (Jung and Sung, 2017), and induced tourism depression with loss of around approximately $10 billion in revenue.

Infectious diseases threaten biodiversity, and induced huge population decline or even extinction as reviewed (Daszak et al., 2000). Canine distemper 3 caused by Canine distemper virus (CDV) (morbillivirus) caused the extinction of some populations of black-footed ferrets (*Mustela nigripes*) (Thorne and Williams, 1988) and African wild dogs (*Lycaon pictus*) (Ginsberg et al., 1995) and also threated

to Serengeti lions (*Panthera leo*) (Roelke-Parker et al., 1996) and wolves (*Canis simensis*) in Ethiopia (Laurenson et al., 1998). *Yersinia pestis* is lethal to Utah prairie dogs (*Cynomys parvidens*) in the United States (Hoogland et al., 2004). *Aphanomyces astaci* has caused mass mortality in crayfish population in Europe (Alderman, 1996).

Among human emerging infectious diseases, around 60% are derived from zoonotic pathogens, among which, about 70% have wild animal reservoirs (Karesh et al., 2012; Wolfe et al., 2007). All the diseases mentioned above, e.g. Influenza A, SARS, MERS, have non-human animal hosts. Infection of SARS-like coronavirus was identified in alive animals (such as, *Paguma larvata* and *Nyctereutes procyonoides*) from a live animal market of Guangdong, China (Guan et al., 2003). Whole genome sequencing revealed that the isolated SARS-like coronavirus shared 99.8% similarity with human SARS-CoV (Guan et al., 2003). An intermediate host of MERS-CoV was suggested to be camels since raw camel milk was consumed in the first reported case of MERS in Yemen (Sharif-Yakan and Kanj, 2014; WHO, 2014) and MERS-CoV was identified in raw camel milk (Reusken et al., 2014). Understanding the transmission models between animals and humans are critical for controlling disease. One of the most cost-effective way in controlling human disease could be researching on animal diseases. For instance, vaccination against rabies in domestic dogs is far preferable to post-exposure treatment in humans, in which the disease has nearly 100% mortality (Zinsstag et al., 2015).

In order to improve public health and strengthen the global economy, it is vital to improve our understanding of wild epidemiology, potential transmission

pathways to humans, and the evolutionary dynamics of pathogens and hosts (Fig .1).

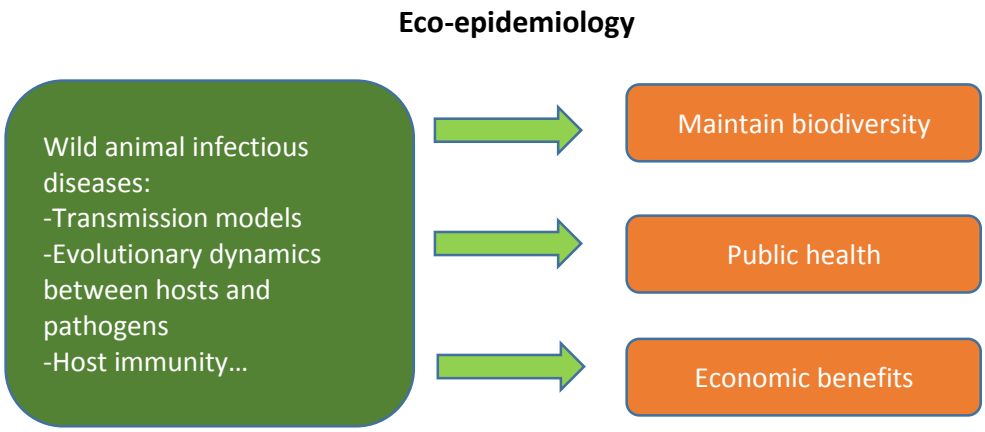

Figure 1.1 Importance of studying wild animal infectious diseases: Eco-epidemiology. Investigation of wild animal infectious diseases including transmission models, evolutionary dynamics between hosts and pathogens, host immunity will improve maintenance of biodiversity, public health and global economics.

1.2 Chytridiomycosis

Amphibians consume insects and are also prey for tertiary and quaternary consumers. Amphibians are particularly sensitive to environmental disturbances relative to other vertebrates, thus amphibian populations are a good indicator of ecosystem health (Hopkins, 2007). However, amphibian populations have recently been threatened with extinction not only because of habitat destruction, climate change and pollution, but also because of emerging infectious diseases (Berger et al., 2009; Kolby and Daszak, 2016; Laurance et al., 1996; Longcore et al., 1999; Waldman and Tocher, 1998).

Chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Bd), is a notorious amphibian fungal disease, and also is a good model for understanding wild animal infectious diseases (Kolby and Daszak, 2016; Matthew et al., 2012). In 1998 amphibian chytrid fungus was isolated from sick and dead frogs in Australia and Central America for the first time, providing the first evidence that *Bd* could lead to high morbidity and mortality of frogs (Berger et al., 1998; Longcore et al., 1999; Waldman and Tocher, 1998). When susceptible frogs were infected by *Bd*, they usually showed lethargy, inappetence, cutaneous erythema, irregular skin sloughing and failure of righting reflex before heart failure, possibly due to damaged osmoregulation ability and loss of electrolyte balance by Bd infection (Berger et al., 2005; Voyles et al., 2007). Bd zoospongia and zoospores can be examined via real time PCR and histology in infected skin (see Fig 2.5).

Subsequently, the presence of Bd was reported worldwide in 50 countries including South Korea (Bataille et al., 2013), Japan (Goka et al., 2009; Tamukai et al., 2014) and China (Bai et al., 2010; Bai et al., 2012; Zhu et al., 2016). In about 20 years since its initial identification, Bd has caused population declines or even extinction in Australia and Central America (Berger et al., 2016; Berger et al., 1998; Fu and Waldman, 2017; Kolby and Daszak, 2016; Longcore et al., 1999; Waldman and Tocher, 1998). However, no mortality or population decline due to Bd has been reported in Asia. As more Asian countries were surveyed, amphibian populations in Asia did not seem to be influenced by Bd (Swei et al., 2011). During our field trip survey, no Korean frogs exhibited clinical signs of chytridiomycosis when infected by Bd. Surprisingly, it is still unknown why this difference exists. Therefore, it is critical to find out why Bd causes population declines in other part of the world, but not in Asia, especially in Korea.

1.3 Bd Origin and Propagation

Bd was first proposed to originate from South Africa via international trade of *X. laevis* since the first and only case of chytridiomycosis (in 1938) until 2004 was from South Africa (Weldon et al., 2004). However, specimen from museums in several countries have since been sampled and tested and Bd was identified in an Illinois amphibian collected in 1888 (Talley et al., 2015), and in 1894 (Brazil) (Rodriguez et al., 2014), 1902 (Japan) (Goka et al., 2009), 1911 (North Korea) (Fong et al., 2015), 1933 (Cameroon) (Soto-Azat et al., 2010).

Indeed, as more Bd isolates became available, the age of Bd was estimated to be around 10,000 to 40,000 years-old via molecular clock analysis based on genomic sequencing (Rosenblum et al., 2013). A recent study using whole genome sequencing indicated that BdAsia-1 (from South Korea) was the basal Bd lineage compared with other lineages from African, Europe, and Brazil, South America, Australia, including global pandemic lineages (BdGPL) (O'Hanlon et al., 2018), consistent with a previous hypothesis (Bataille et al., 2013). The current Asian Bd lineages are limited to Korea, and has not been observed in China or Japan, although this may be due to the fact that it is difficult to identify Bd lineages in low Bd prevalence populations. The Asia-origin hypothesis has stimulated thought about how the basal lineage evolved within local amphibian populations in Korea and how this is possibly related to the fact that amphibian populations in Asia are, in general, not in decline due to Bd infection.

The pet trade was the most likely route for the spread of Bd in early 1900s (O'Hanlon et al., 2018; Weldon et al., 2004). In addition, bird migration may have played a role in the spread of Bd since Bd was also identified in the toes of aquatic birds, such as *Branta canadensis* and *Anser anser domesticus* (Garmyn et al., 2012). *Caenorhabditis elegans* might be additional hosts in the wild since Bd can cause high mortality to this species (Shapard et al., 2012).

1.4 Research Contents

1.4.1 Why was no amphibian population decline found in Asia with presence of ancestral Bd?

In South Korea, we have uncovered the highest diversity of endemic lineages of the pathogen found anywhere in the world (Bataille et al., 2013; O'Hanlon et al., 2018), which were the only Asian Bd linages isolated yet. Whole genome comparison of all fungal strains isolated from Africa, Brazil, Europe, as well as South Korea, showed that strains that were isolated from fire-bellied toads (*Bombina orientalis*) (local Korean frog) were the oldest identified thus far (O'Hanlon et al., 2018). Korean Bd lineage (BdAsia-1) seems to be more stable than other lineages, such as the global pandemic lineage (GPL) and hybridized lineages (O'Hanlon et al., 2018). It is possible that both BdAsia-1 evolved to be more virulent, and Asian amphibians more resistant, over time in a co-evolutionary arms race. It is also possible that BdAsia-1 evolved to be less virulent, at least partially explaining why Asian amphibian did not crash upon Bd infection.

Asian amphibians seem to be resistant to Bd, and studies regarding the mechanism of resistance to Bd have been published, such as genotype comparisons of the major histocompatibility complex (MHC) (Bataille et al., 2015; Lau et al., 2017), Toll Like receptor (TLR) (Lau et al., 2018) and transcriptomic comparisons (Lau et al., 2017). Nevertheless, no direct laboratory evidence, such as infection experiments, have been performed to prove that Asian amphibian are indeed resistant or tolerant to Bd. Therefore, the first part of my thesis (Chapter 2) addresses these problems. My research represents a large step forward in resolving the enigma of why Bd infection causes clinical signs of chytridiomycosis in some parts of the world but not others.

1.4.2 What is the mechanism of resistance to Bd infection?

If we know the mechanism of resistance to Bd infection, it is possible to protect susceptible species from the disease and also take measures to prevent it effectively.; Thus, it is possible for Bd to be controlled in the future and these initial studies can provide a good model for emerging infectious diseases, with an overall aim of protecting biodiversity. Therefore, it is critical to know how the resistant species tolerate Bd infection while susceptible species do not.

In amphibians, innate immunity has been studied more intensively than acquired immunity. Granular glands can secrete bioactive substances, such as peptides, into mucus as a first line of defense, and some of these enzymes have synergistic effects; different species seem to have different varieties of antimicrobial peptides (Rollins-Smith, 2009; Rollins-Smith and Conlon, 2005). AMPs could inhibit Bd growth in vitro, and serve as a key component of the innate immune response to Bd (Ramsey et al., 2010; Rollins-Smith et al., 2011). Different species could have different AMPs, which was suggested to be associated with differential susceptibility to Bd (Rollins-Smith, 2009; Rollins-Smith and Conlon, 2005; Woodhams et al., 2007). Various skin peptides have been identified in *B. orientalis* (Chen and Zhao, 1987; Gibson et al., 1991; Hou et al., 2015; Nagalla et al., 1996; Yasuhara et al., 1973; Zhou et al., 2018), *B. gargarizans* (Lee et al., 1998; Park et al., 1996), *H. japonica* (Kawasaki et al., 2008; Zhu et al., 2014), and *L. caerulea* (Stone et al., 1993).

An adaptive immune response, both humoral and cell-mediated, can be stimulated by various components of the innate immune system (Murphy and Weaver, 2016), and the relative functions of each aspect of the immune response

are integrated in comparative studies, thus both kinds of immune response should be examined when investigation mechanisms of disease defenses (Flajnik and Du Pasquier, 2004; Richmond et al., 2009). In a resistant species, *Xenopus laevis*, it was suggested that both innate and adaptive immunity were involved in the resistance to Bd infection because impairment of either innate or adaptive immunity can cause more susceptibility (Ramsey et al., 2010). However, there was no more robust immune response in resistant species (*Bufo marinu*s) as susceptible species (*Bufo boreas*) did on 18th day post Bd exposure in transcriptional level (Poorten and Rosenblum, 2016). In susceptible species, it seemed both innate and adaptive immunity was transcriptionally activated in the late infection stage, but somehow failed (Ellison et al., 2014; Grogan et al., 2018a). Interestingly, a relatively resistant population in susceptible species (*Litoria verreauxii alpine*) showed more robust immune response than other weaker populations in the early stage (Grogan et al., 2018a), which suggests a quicker and stronger immunity may be the key to be more resistant. However, there is still a lack of studies in the early response in both resistant species and susceptible species. Therefore, it is necessary to compare more hosts immune systems with different susceptibilities.

Major histocompatibility complex (MHC) is known to be critical involved in immunity, which can bind to self and pathogen-derived peptides, and present to T cells, thus activating T cell mediated adaptive immunity (Richmond et al., 2009). MHC was mainly divided into two classes, MHC I and MHC II primarily based on their protein structure and antigen recognition. In human, MHC I protein is a heterodimer of a large transmembrane subunit and a small non-transmembrane subunit,

recognized by $CD8^+$ T cells and expresses almost ubiquitously in nucleated cells; while MHC II is a heterodimer of two transmembrane proteins, recognized by $CD4^+$ T cells and expresses only in antigen-presenting cells (including dendritic cells, macrophages, B cells) (Mak et al., 2014). In frog model, *Xenopus*, MHC I and MHC II was found to express differentially from larval to adult (Du Pasquier and Flajnik, 1990; Salter-Cid et al., 1998). MHC Ia was detected in tadpole lung, gill, and intestine, skin, and then MHC Ia gene expressions increased dramatically after metamorphosis and were detected in most adult tissues, especially in intestine; while Class Ib gene expressions were only detected in adult and relatively lower and more restricted compared to class Ia. MHC II was detected only on B cells, macrophages, spleen reticulum, thymus epithelium, the pharyngobuccal cavity in tadpoles, while detected in all T cells in adult frogs.

Based on previous research (Bataille et al., 2015; Kosch et al., 2016; Lau et al., 2017; Savage et al., 2015; Savage and Zamudio, 2011), it was suggested that genetic variation in the MHC class II PBR associates with resistance to chytridiomycosis, reviewed last year (Chapter 3) (Fu and Waldman, 2017). However, since the transcriptional regulation mechanims of resistance (such as MHC genes) seemed to be quite complicated and inconsistant based on current studies, and also not full β chain of MHC II was characterized for most of the species, mostly importantly, the genotyping methods (traditional cloning bases sequencing) does yield efficient and reliable results as next generation sequencing does, it is still yet to decide how much percentage of the exact role of MHC II plays. Therefore, in Chapter 4, I applied next generation sequencing method to genotype MHC II in *L.*

caerulea, and *B. gargarizans* for further understanding the resistant mechanisms and finding out biomarkers for the disease.

1.5 Research Strategies

To test the three hypotheses for illustrating the reasons of 'resistant' Korean amphibians, 1) H1: Korean amphibian developed better immune system to both local and alien strain, 2) H2: Korean amphibian developed better immune system only to local Bd, 3) H3: Attenuated Bd lineage in Asia is the only reason (Fig 1.2), I infected disease-free subjects of a known susceptible Australasian species, *L. caerulea*, and three Korean native species, *B. gargarizans*, *B. orientalis*, and *H. japonica*, to BdAsia-1, BdGPL, or a sham inoculate. And then I compared the virulence between BdAsia-1 and BdGPL, and the resistance between different hosts.

If BdAsia-1 has similar or higher virulence compared to BdGPL while Korean frogs survived to both lineages, it refers to Korean frogs are more resistant or tolerant than imported species, such as *L. caerulea*. Next, I would identify the mechanisms behind Bd resistance by performing RNA-Seq and MHC II genotyping.

If BdAsia-1 is less virulent, and Korean frogs are susceptible to BdGPL, I would focus on virulence comparisons of the two Bd lineages at the genetic level.

In summary, the goals of this project are to determine (1) whether endemic Asian Bd strains are less virulent than GPL that is causing amphibian population declines elsewhere, (2) whether immune responses differ upon Bd infection in *L. caerulea* and Korean species, such as, *B. gargarizans*, *B. orientalis*, *H. japonica*, and, (3) the potential mechanisms for the resistance to Bd infection (Fig 1.2). This

research can help solve the issue of why the disease causes sickness in some parts of the world but not others. This, in turn, will assist in the development of management plans for the disease and preservation of wild frog populations.

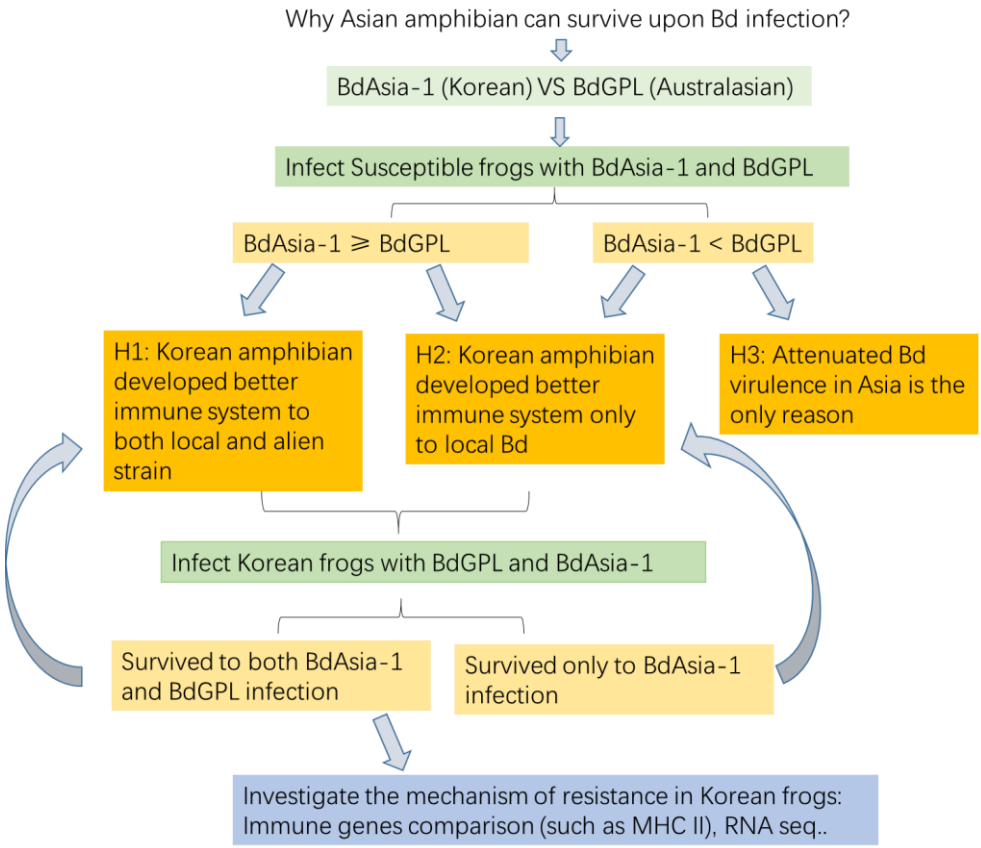

Figure 1.2 Research strategies in this study. To test why Korean amphibian can survive upon Bd infection, firstly, I infected disease-free subjects of a known susceptible Australasian species with BdAsia-1 and BdGPL to compare the virulence between BdAsia-1 and BdGPL. If BdAsia-1 has similar virulence compared to BdGPL, or even more virulent, while Korean frogs survived to both lineages, it should be hypothesis 1, that it is because of better immune system in Korean species so than they can survive upon Bd infection. Then I will try to figure out mechanisms behind this resistance by performing RNA-Seq, and MHC II genotyping. If BdAsia is less virulent, I will focus on virulence comparison of two Bd lineages in genetic level.

Chapter 2. Ancestral Chytrid Pathogen Remains Hypervirulent Following Its Long Co-evolution with Amphibian Hosts

Abstract

Many amphibian species around the world, except Asia, suffer morbidity and mortality when infected by the emerging infectious pathogen, *Batrachochytrium dendrobatidis* (Bd). A lineage of the amphibian chytrid fungus isolated from South Korea amphibians (BdAsia-1) is evolutionarily basal to recombinant global pandemic lineages (BdGPL) associated with worldwide amphibian population declines. In Asia, the Bd pathogen and its amphibian hosts have co-evolved over 100 years or more. Thus, resilience of Asian amphibian populations to infection might result from attenuated virulence of endemic Bd lineages, evolved immunity to the pathogen, or both. We compared responses of an Australasian amphibian, *Litoria caerulea,* known to be highly susceptible to BdGPL, with those of three Korean species, *Bufo gargarizans*, *Bombina orientalis*, and *Hyla japonica*, after inoculating subjects with BdAsia-1, BdGPL, or a blank solution. Subjects became infected in all experimental treatments, but Korean species rapidly cleared themselves of infection, regardless of Bd lineage. They survived with no apparent secondary effects. By contrast, *L. caerulea,* after infection by either BdAsia-1 or BdGPL, suffered deteriorating body condition and carried progressively higher Bd loads over time. Subsequently, most subjects died. Comparing their effects on *L. caerulea*, BdAsia-1 induced more rapid disease progression than BdGPL. The results suggest that the ancestral Bd lineage evolved to be hypervirulent in the absence of genomic recombination with other lineages. The pathogen's virulence may have driven strong selection for adaptive immune responses in endemic Asian amphibian

host species.

Keywords: amphibian population declines, chytridiomycosis, co-evolution, disease resistance, host-parasite dynamics, pathogen virulence

2.1 Introduction

The relentless struggle between pathogens and their hosts is a key factor that shapes global biodiversity. As pathogens evolve mechanisms to exploit their hosts, hosts evolve defenses to resist and tolerate infection, driving perpetual cycles of adaptation and counter-adaption (Schmid-Hempel, 2011). Steady-state periods of mutual adaptation and persistence can result from reciprocal selection on pathogens and their hosts (Anderson and May, 1979). Co-adaptation may be transient, however, as the evolution of host defenses typically lags innovations in pathogen's attack strategies (May and Anderson, 1983). Rather, dramatic declines and extirpations of host populations are expected to occur periodically as their defense strategies catch up. In certain circumstances, pathogens can drive hosts to extinction (Francisco and Benjamin, 2005; Wilber, et al., 2017). Rapidly changing environments that disrupt host homeostasis may accentuate these effects (Lively, 2006). Such appears to be the case with populations of amphibians that are declining in many parts of the world.

The emerging infectious disease chytridiomycosis, caused by pathogenic chytrid fungi, is ravaging amphibian populations in Europe, Australia, and the Americas, probably exacerbated by environmental degradation and climate change (Berger et al., 2016; Van Rooij et al., 2015). Asia accommodates a rich, diverse amphibian fauna, with new species being discovered regularly just as others disappear (Meegaskumbura et al., 2002). Amidst the rapid industrialization and spewing smokestacks of the continent, amphibians face enormous problems. Yet,

epizootics and population extirpations associated with the disease elsewhere in the world have not been witnessed on the continent nor have clinical signs of amphibian chytridiomycosis been reported. Many Asian amphibians harbour low infection loads of the amphibian chytrid pathogens, *Batrachochtyrium dendrobatidis* (hereafter denoted Bd) and *Batrachochytrium salamandrivorans* (Bataille et al., 2013b; Laking et al., 2017; Swei et al., 2011; Thorpe et al., 2018). Their effects on hosts, if any, are sublethal, may affect host life histories (An and Waldman, 2016) and, potentially, long-term population stability (Valenzuela-Sanchez et al., 2017).

Asian amphibian hosts have been co-evolving with chytrid pathogens over the past century if not longer. Chytrid infections have been identified in Korean frogs collected in 1911(Fong et al., 2015), from Chinese frogs collected in 1933(Zhu et al., 2014b), and possibly as early as 1902 in Japanese salamanders (Goka et al., 2009). Asian endemic Bd lineages are highly diversified as compared to those found on other continents (Bataille et al., 2013b; Goka et al., 2009). Deep sequence analyses point to four main lineages of the fungus, of which three are distributed globally. The fourth lineage is found only in Korea, on frogs native to the region, and most closely resembles the common ancestor from which global lineages were derived, probably between 50 and 150 years ago (O'Hanlon et al., 2018).

Korean amphibians are infected largely with endemic Bd lineages, and many populations show relatively high infection prevalence. Less frequently, native frogs and especially introduced American bullfrogs (*Rana catesbeiana*) bear infections of the hypervirulent global pandemic Bd lineage (Bd-GPL) (Bataille et al., 2013b).

Korean amphibian populations have persisted despite being infected, and sometimes co-infected, by a wide array of Bd lineages. Signatures of recombination between local and more recently derived international lineages are apparent in some Bd genomes, which potentially may make these lineages especially virulent to susceptible hosts (Farrer et al., 2011; Greenspan et al., 2018; Jenkinson et al., 2016; Phillips and Puschendorf, 2013; Schloegel et al., 2012).

As Bd has co-evolved with its amphibian hosts over many generations, possibly Korean Bd lineages have attenuated in virulence. The infectivity of the pathogen might be raised if, for example, infected hosts live longer and engage in more social interactions. Because Korean amphibians are infected largely by endemic lineages, the persistence of their populations might simply be a consequence of reduced pathogen virulence. Alternatively, in their long period of co-evolution, Korean amphibians may have evolved resistance to the pathogen. Previous research has demonstrated that both innate and adaptive immunity for resistance to Bd can rapidly evolve (Bataille et al., 2015; Savage and Zamudio, 2011; Voyles et al., 2018). In this scenario, recombination may not be a prerequisite for the evolution of hypervirulence. Indeed, endemic Bd lineages may have increased in virulence over time as their hosts evolved resistance (Alizon et al., 2009; Elsworth et al., 2014; Lively, 2006).

To discriminate between these possibilities, I infected disease-free subjects of a known Bd-susceptible Australian species, *Litoria caerulea*, and three Korean native species, *Bufo gargarizans*, *Bombina orientalis*, and *Hyla japonica*, with a Korean Bd lineage (BdAsia-1), a BdGPL lineage, or a sham inoculate. If Korean Bd

lineages have evolved reduced virulence, as commonly expected (Morgan and Koskella, 2011), species such as *L. caerulea* that are susceptible to BdGPL might survive infection by BdAsia-1. A finding that *L. caerulea* is vulnerable to infection by BdAsia-1 would support the alternative hypothesis that the resistance of Asian amphibians to chytridiomycosis is attributable to evolved immune defenses. This study also allows us to assess the specificity of evolved immune defenses of amphibians to particular Bd lineages (James et al., 2015), which may be important for planning efficacious disease mitigation strategies (Chalkowski et al., 2018).

2.2 Methods

2.2.1 Animal collection and husbandry

Asiatic toads (*Bufo gargarizans*) were collected in February 2017 from Geumsan and Jeonju, South Korea. Oriental fire-bellied toads (*Bombina orientalis*) were collected in Hwacheon, South Korea, during July and August 2017. Japanese tree frogs (*Hyla japonica*) were collected in Nakseongdae, Seoul and Goyang, South Korea during July and August 2017. White's tree frogs (*Litoria caerulea*) were caught from the wild in New Guinea in November and December 2016 and shipped to South Korea.

All frogs were to test for Bd infection immediately upon collection (Korean species) or on arrival in Korea (*L. caerulea*) (methods below). As expected, based on our lab's previous findings (Bataille et al., 2013), some subjects of the Korean species (< 20%) were infected by Bd so were excluded from further use. None of the *L. caerulea* imported from New Guinea tested positive for Bd.

All four species are largely terrestrial, so frogs were housed individually in closed plastic containers appropriate to their size (290×90×200 mm for *L. caerulea*, *B. gargarizans*, *B. orientalis*; 235×72×165 mm for *H, japonica*) with moistened paper towels and small open water reservoirs. Water was carbon-filtered and UV-treated to ensure it was pathogen-free. Containers were cleaned and water replaced three times weekly under sterile conditions before the inoculation and twice weekly thereafter. Subjects were fed with mealworms (*Tenebrio molitor* larvae) that had been dusted with amphibian nutrient powder (Superworm, Seoul, South Korea; 20%

calcium, 25% crude protein, Vitamin D3, and minerals) ad libitum. Subjects were held in a containment room at 21-22 °C on a 12 L:12 D photoperiod at 21-22 °C with a relative humidity of 40%.

Prior to beginning treatments, I conducted both nested PCR (Goka et al., 2009) and real time PCR (Boyle et al., 2004) on each subject to ensure that it was free of Bd infection (methods below). Any infected subjects were excluded from further experimental work. Sample sizes are given in Table 2.1. The mass and body length of each subject were measured (Table 2.2), and assigned subjects randomly to three treatments: BdAsia-1 (KBO347) infection, BdGPL infection, or control.

To verify the virulence of BdAsia-1, I used same amount of another Bd Asia-1 strain, KBO 327 (passage 9) for infection experiment in *L. caerulea*. KBO327 was also isolated from *B. orientalis* (Bataille et al., 2013a), belonging to BdAsia-1 lineage (O'Hanlon et al., 2018). The sample size is 15 (44.79g ± 15.97) and 10 frogs (49.02g±22.29) for Bd infection and control, respectively.

Study subjects were collected in the wild, under permission of local government authorities of Geumsan, Jeonju, and Hwacheon.

Table 2.1 Sample sizes and survivorship across treatments.

	Control	BdGPL	BdAsia-1
L. caerulea	12 (2)	15 (13)	15(15)
B. gargarizans	15 (0)	14 (0)	16 (0)
B. orientalis	15 (1)	13 (1)	15 (0)
H. japonica	9 (0)	13 (0)	12 (0)

Numbers of subjects that died during the experiment are indicated in brackets.

Table 2.2 Experimental infection protocols and animal sizes of the subjects.

		Litoria caerulea	*Bufo gargarizans*	*Bombina orientalis*	*Hyla japonica*
Snout-vent length (mm)	Mean (SD)	66.5 (7.0)	79.2 (10.8)	38.6 (2.8)	31.6 (2.3)
	Range	54.9 – 84.6	66.5 – 111.1	32.3 – 42.4	28.6 – 38.9
Body mass (g)	Mean (SD)	19.73 (5.73)	92.14 (33.76)	7.79 (1.29)	5.06 (1.05)
	Range	9.40 – 36.71	51.22 – 202.52	4.80 – 10.90	3.66 – 7.36
Number of zoospores + UV filtered water (ml)		$5*10^5 + 50$	$6\sim8*10^6 + 95$	$8*10^5 + 10$	$6*10^5 + 5$
Container size used for Bd infection (D, diameter; H, height; L, length; W, width; in mm)		120*80 (D*H)	120*80 (D*H)	70*25*70 (L*H*W)	60*30*40 (L*H*W)

2.2.2 Infection experiment

BdAsia-1, the basal Bd lineage that emerged to cause worldwide epizootics of chytridiomycosis (O'Hanlon et al., 2018), were previously isolated from *B. orientalis* in South Korea. This culture was cryopreserved upon isolation and revived prior to use with 5 passages. A second isolate, Abercrombie R - L. booroolongensis, was isolated from frogs in New South Wales, Australia by Lee Berger in 2009, cryopreserved, and underwent 7 passages. This isolate is one of the most virulent known to cause clinical signs of chytridiomycosis, and falls within the global pandemic lineage (Bd-GPL) associated with worldwide epizootics (O'Hanlon et al., 2018).

BdGPL and BdAsia-1 were cultured in TGhL broth (6.4 g tryptone, 1.6 g gelatin hydrolysate (Peptone-E), 3.2 g lactose monohydrate, 800 ml distilled water) for 4-5 days, and then transferred 1 ml of culture to each TGhL plate (10% agar) (Longcore et al., 1999). After 1 week, each plate with flooded 1-2 ml sterilized water and let the plate stand for 20 min before collecting zoospores from it. Then zoospore activity was quantified by counting the number of active zoospores under a microscope using a haemocytometer.

Subjects were inoculated with between 500,000 and 800,000 zoospores, increasing dose with average species mass (Table 2). Larger numbers of zoospores were used to inoculate the Korean species than *L. caerulea*, which is known to be highly susceptible to Bd. To infect the frogs, subjects were put into closed containers with prepared inoculum solutions of zoospores in sterilized water. Control groups

were treated in the same way but without Bd zoospores. In both experimental and control treatments, inoculum solutions were gently swirled around the subjects to ensure adequate exposure to potential infection. After 24 h, each individual was transferred back into its original container.

After Bd treatment, we monitored subjects for clinical signs of chytridiomycosis, such as lethargy, cutaneous erythema, inappetence, and skin sloughing (Berger et al., 2009), at least once daily. Subjects were considered dead when they failed to show a righting reflex and their heart stopped beating. The experiment was ended after three months, when all surviving subjects in infected groups tested negative for Bd infection. All survived individuals are being maintained in the lab and taken good care of regularly.

Experiments were performed in accordance with regulations of the Institute of Laboratory Animal Resources (permit ILAR-17-04-118) and the Institutional Biosafety Committee (permit SNUIBC-R170502-1) of Seoul National University.

2.2.3 Bd screening and pathogen loads

Prior to beginning the experiment, all subjects were observed for clinical signs of disease and screened at least twice for Bd infection by nested PCR, using protocols modified from Goka (2009) (Goka et al., 2009). To non-invasively detect infection, I swabbed frogs (MW-113 rayon swabs, Medical Wire and Equipment, Corsham, UK) along their ventral skin including legs and feet 10 times using a standardised protocol (Hyatt et al., 2007). We extracted DNA from each swab using 50 µl PrepMan Ultra (Applied Biosystems, Foster City, CA, USA) according to the

manufacturer's protocol, and stored it at -20 °C. Bd18SF1 (5′-TTTGTACACACCGCCCGTCGC-3′) and Bd28SR1 (5′-ATATGCTTAAGTTCAGCGGG-3′) as primers for the initial reaction, and Bd1a (5′-CAGTGTGCCATATGTCACG-3′) and Bd2a (5′-CATGGTTCATATCTGTCCAG-3′) were used for the second reaction. The PCR conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, and a final extension at 72 °C for 7 min. PCR was run in a Verity 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA).

To determine Bd loads carried by infected subjects, I analyzed the DNA that were extracted from swabs by real-time PCR with ITS1-3 Chytr (5′-CCTTGATATAATACAGTGTGCCATATGTC-3′) and 5.8S Chytr (5′-AGCCAAGAGATCCGTTGTCAA-3′). Thermocycler conditions consisted of an initial uracil-DNA glycosylase (UDG) incubation step at 50 °C for 2 min, followed by polymerase activation at 95 °C for 10 min, then by 50 cycles of 10 s at 95 °C and 1 min at 60 °C and lastly a melt curve of 95 °C for 15 s, 55 °C for 15 s, and 95 °C for 15 s (modified from Boyle et al. (Boyle et al., 2004)). Each sample was run in duplicate and infection intensity was estimated using a standard curve prepared by serial dilution of a Bd ITS standard of 3520, 352, 35.2, and 3.52 gene copies. Negative controls prepared by 1:5 dilution of PrepMan Ultra were included on every plate. Real-time PCR was run in an Illumina Eco Real-Time PCR system (Illumina, San Diego, CA, USA).

2.2.4 Body condition measurements

Changes in conditions of all subjects were tracked during the course of the experiment. For *L. caerulea*, I measured the mass of each individual immediately before infection and when each individual died or at the conclusion of the experiment. For *B. gargarizans*, *B. orientalis*, and *H. japonica*, I measured the body mass before infection, and then subsequently every week 2 days after feeding. Subjects were gently blotted with unbleached paper towels to remove excess moisture before taking mass measurements.

2.2.5 Histology

I dissected skin from both pelvic and ventral regions of all dead frogs (N = 28) that tested Bd-positive by PCR. Tissue was fixed in freshly made 10% neutral buffered formalin (formaldehyde 10 ml, distilled water 90 ml, sodium phosphate monobasic dehydrate 0.65 g, sodium phosphate dibasic anhydrous 0.4 g) for 24 h at 4 °C. After 2 to 6 h washing by running distilled water, the fixed tissue was processed in an automatic tissue processor for dehydration and then embedded in paraffin, sectioned at 5 μm, and placed onto silane-coated slides for hematoxylin and eosin staining. Stained samples were observed under fluorescence microscopy (Axio Observer Z1, Carl Zeiss, Göttingen, Germany).

2.2.6 Statistics

A Cox's proportional hazard model (Cox, 1972) were used to compare survival of experimental and control treatments, treating mass and snout-vent length (SVL) of subjects measured prior to inoculation as covariates. Differential survival among treatments were analyzed by the nonparametric Kaplan-Meier procedure. Analyses

were conducted with the coxph and survfit functions, respectively, in the R package survival.

Body mass change was compared in *L. caerulea* during the course of the experiment among treatments by one-way analyses of variance, after verifying that the data were normally distributed. Then I conducted post-hoc analyses of the differences by Tukey HSD multiple comparisons of means. R functions aov and TukeyHSD were used for these computations.

Semi-parametric regressions were performed using spline smoothing models (Brumback and Rice, 1998) to examine changes in mass of the three Korean species over the course of the experiment. Infection loads of *L. caerulea* and the Korean species after infection until death or the end of experiment were compared using the same approach. Infection loads were first transformed by adding one and computing base-10 logarithm scores. In each case, data was smoothed with cubic spline models and compared treatments with double hierarchical generalized linear mixed models based on a Gaussian distribution (Lee et al., 2006). Species and treatment time were treated as fixed effects and subject variation as a random effect. The control group was not included for the infection intensity analysis as all subjects tested negative for Bd infection. Wald tests were used to quantify the significance of terms included in the models. Analyses were conducted using the R function dhglmfit (Lee et al., 2017).

All computations were made with R ver. 3.3.1(R Foundation for Statistical Computing, Vienna, Austria).

2.3 Results

2.3.1 KBO347 strain from BdAsia-1 lineage is lethal to *L. caerulea*

After 31 days, frogs in the BdAsia-1 treatment group started to die, and BdGPL-treated frogs started to die thereafter (Fig 2.1). The Bd-inoculated subjects died in significantly larger proportions than the controls (Cox proportional hazards model, $z = 4.29$, $P < 0.00002$). This result was confirmed by Kaplan-Meier survival analysis (log rank test, $\chi^2 = 23.0$, 2 df, $P = 0.00001$). Initial size of subjects, measured either in length ($z = 1.30$, $p = 0.19$) or mass ($z = 0.54$, $p = 0.59$) did not affect the proportion of survivors.

BdAsia-1 caused mortality more rapidly than BdGPL (log-rank test, $\chi^2 = 4.3$, 1 df, $P = 0.04$). All BdAsia-1 infected frogs died, while two from the BdGPL infected group survived. Clear clinical signs of chytridiomycosis, including behavioral disorientation, skin sloughing and body wasting, were observed in morbid subjects (Fig 2.2).

Subjects in BdAsia-1 and BdGPL treatment groups lost mass while those in the control group gained mass during the course of the experiment ($F_{2, 28} = 29.79$, $P < 0.00001$) (Fig 2.3). Post-hoc analyses demonstrated significant differences in mass change between Bd-treated and control groups ($P < 0.001$), but not between BdGPL and BdAsia-1 treated group ($P = 0.89$).

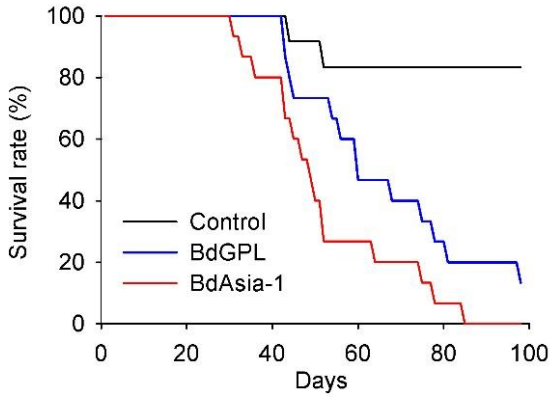

Figure 2.1 Comparison of survival rate among *L. caerulea* subjects exposed to BdGPL, BdAsia-1 (KBO347), and sham inoculates.

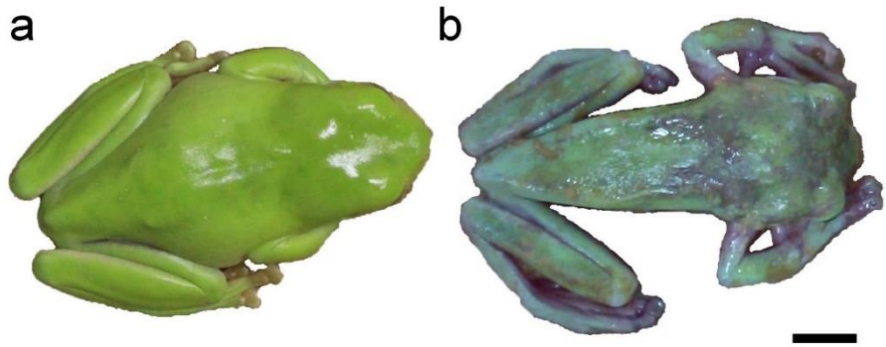

Figure 2.2 Phenotypic change in *L. caerulea* during course of infection. a, Subject prior to Bd inoculation. b, Subject 60 days after infection. Scale bar, 10 mm.

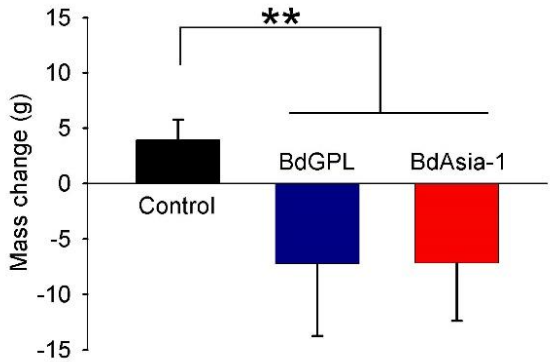

Figure 2.3 Change in mass of *L. caerulea* subjects exposed to BdGPL, BdAsia-1 (KBO347), and sham inoculates. Error bars denote standard errors of the mean. ** $P < 0.01$

2.3.2 Pathogen load and histological confirmation in *L. caerulea*

Infection intensities, measured as log-transformed zoospore genomic equivalents (log_{10}ZGE), differed between BdGPL and BdAsia-1 (KBO347) treatments when survivors are included ($z = 4.87$, $P < 0.01$) (Fig 2.4). Censuring the data to exclude survivors, infection loads still trended higher in BdAsia-1 treatment groups ($z = 1.94$, $P = 0.053$). We confirmed that Bd-inoculated subjects were infected by histology. In the stratum corneum of infected subjects, we found zoosporangia filled with zoospores as well as empty zoosporangia (Fig 2.5), indicating that the subjects were infectious.

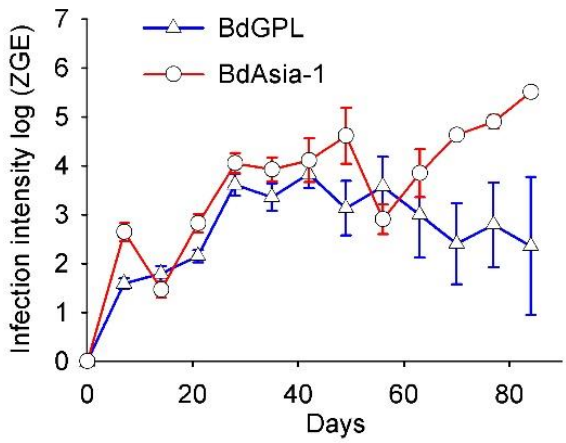

Figure 2.4 Bd infection intensity in *L. caerulea* during the course of the experiment. Infection intensity is given in base 10 log-transformed zoospore genomic equivalents (ZSE). Error bars denote standard errors of the mean. Here BdAsia-1 specified for KBO347. Semi-parametric regression, z = 1.94, P = 0.053.

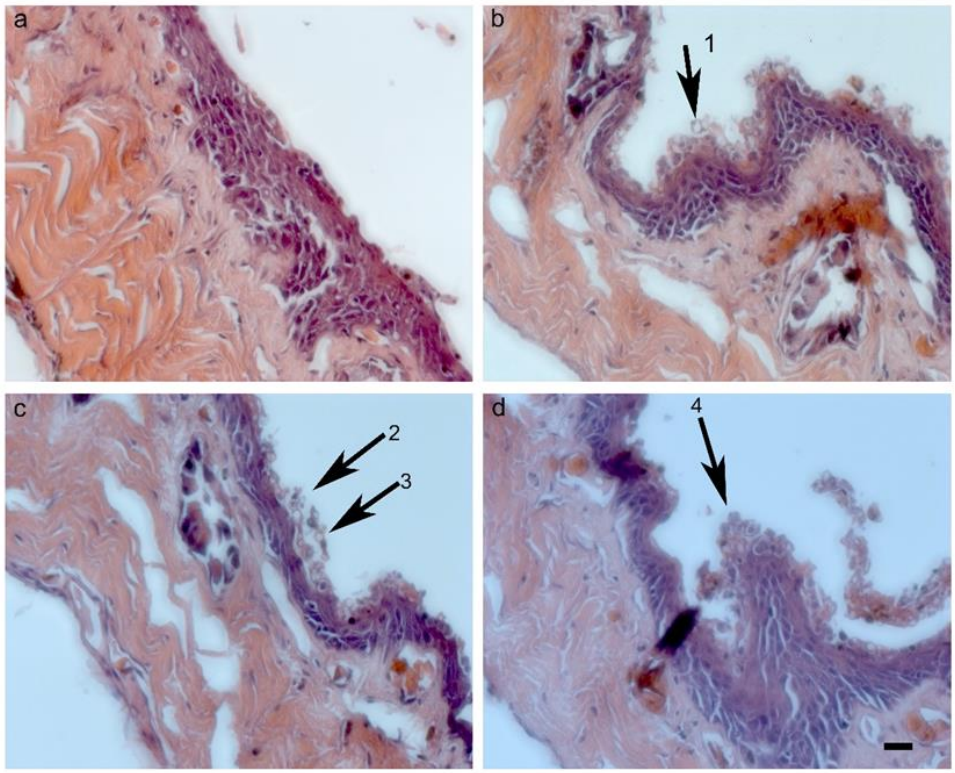

Figure 2.5 Skin histology of infected *L. caerulea*. Selected photos were from BdAsia-1 (KBO347) treated groups. a, Less infected skin region in which thickened epidermis can be mainly observed. b-d, Severe skin infection micrographs in individuals with high Bd infection loads. 1, A nearly empty zoosporangium. 2, Mature zoosporangium with zoospores in sloughing skin. 3, Empty zoosporangium that has already released zoospores in sloughed skin. 4, Mature zoosporangium with about eight zoospores, which were about to be released to external environment from epidermis. All four images are at the same magnification. Scale bar, 20 µm.

2.3.3 Virulence verification of BdAsia-1 using another BdAsia-1 strain (KBO327)

In the 34th days after KBO327 infection, *L. caerulea* started to die, and full mortality occurred within 78 days, but not one died in control group (Fig 2.6 a). The mortality was significantly induced by KBO327 treatment, and the initial mass variation did not affect this result (Cox proportional hazards model, z=-1.122, P=0.26). This result was further confirmed by Kaplan-Meier survival analysis (log rank test, χ^2 = 15.6, 1 df, P = 7.75 $\times$ 10^{-5}).

KBO327 infection also caused significant loss of body mass compared to Control group ($F_{1, 19}$ = 10.34, P = 0.00455) in the end of the experiment (Fig 2.6 b). Post-hoc analyses further demonstrated significant differences in mass change between Bd-treated and control groups (P = 0.0045513).

Comparing virulence between KBO327 and KBO347 by survival analysis, the results indicated there was no statistical difference between them (log rank test, χ^2 = 0, 1 df, P = 0.91), the initial body mass also had no significant difference between two groups (P=0.18). Bd infection was also monitored until the end of the experiment (11 weeks) exhibiting an increasing pattern (Fig 2.6 c). This experiment using another BdAsia-1 strain further confirmed that Korean Bd linage remains virulent to susceptible species, such as *L. caerulea.*

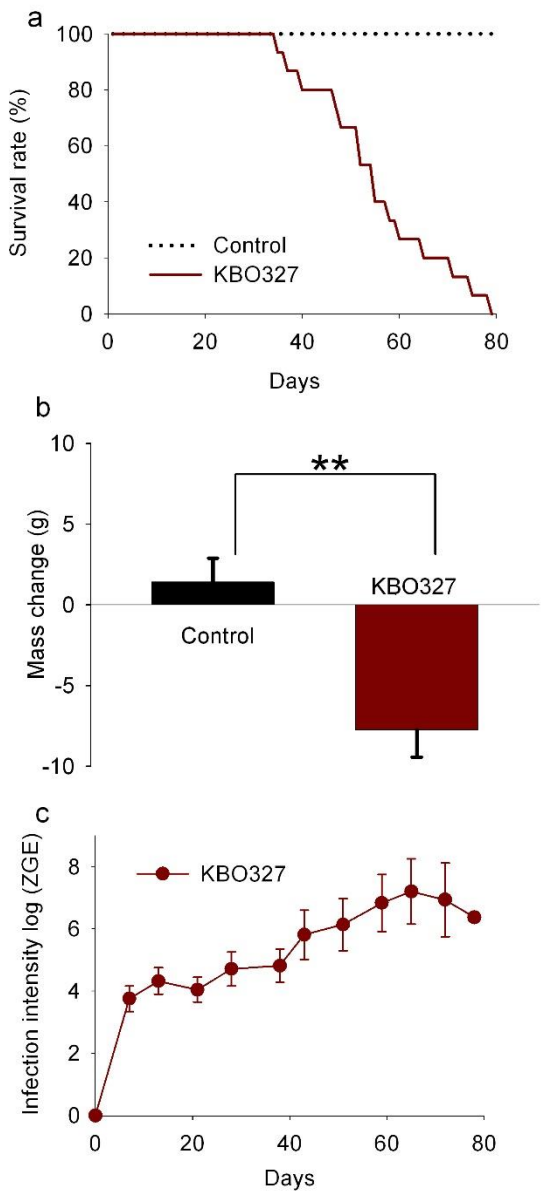

Figure 2.6 KBO327 also exhibited hypervirulence in *L. caerulea.* Survival rate (a), Mass changes (b), and infection intensity (c) of subjects exposed to KBO327 and sham inoculates. Infection intensity is given in base 10 log-transformed zoospore genomic equivalents (ZSE). Error bar denotes standard deviation of the mean. ** P < 0.01

2.3.4 Korean amphibians showed resistance both to BdAsia-1 and BdGPL

Unlike *L. caerulea*, the mass of Bd-inoculated subjects of *B. gargarizans* remained stable whereas *B. orientalis* and *H. japonica* significantly gained mass during the course of the experiment (Fig 2.7). For each Korean species, mass change did not significantly differ between experimental and control groups (*B. gargarizans*, z = 1.77, P = 0.08; *B. orientalis*, z = 0.76, P = 0.45; *H. japonica*, z = 1.37, P = 0.17).

In Bd-inoculated subjects of each species, Bd loads increased during the first week but then decreased each week subsequently. All *B. gargarizans* and *H. japonica* subjects fully cleared themselves of the pathogen but most *B. orientalis* retained very low loads at the conclusion of the study. For *B. gargarizans* and *B. orientalis,* infection intensity over time did not differ between subjects inoculated with BdGPL and BdAsia-1 (*B. gargarizans*, z = 1.01, P = 0.31; *B. orientalis*, z = 1.88, P = 0.06) but for *H. japonica* infection intensity varied slightly between the two Bd lineages (z = 2.11, P = 0.04).

Two *B. orientalis*, one control and one experimental subject inoculated with BdGPL, died during the course of the experiment. Neither subject presented clinical signs of chytridiomycosis and when tested by qPCR, we detected no evidence of Bd infection. For *L. caerulea*, *B. gargarizans*, and *H. japonica*, all subjects in experimental treatments became infected with Bd after inoculation. However, 8 % (1 / 13) of *B. orientalis* subjects inoculated with BdGPL and 27 % (4 / 15) of those inoculated with BdAsia-1 never became infected. Taken together, these results show that Korean frogs exhibited resistance to and tolerance of both BdAsia-1 and BdGPL. *Bombina orientalis* especially appears to demonstrate tolerance of the

pathogen.

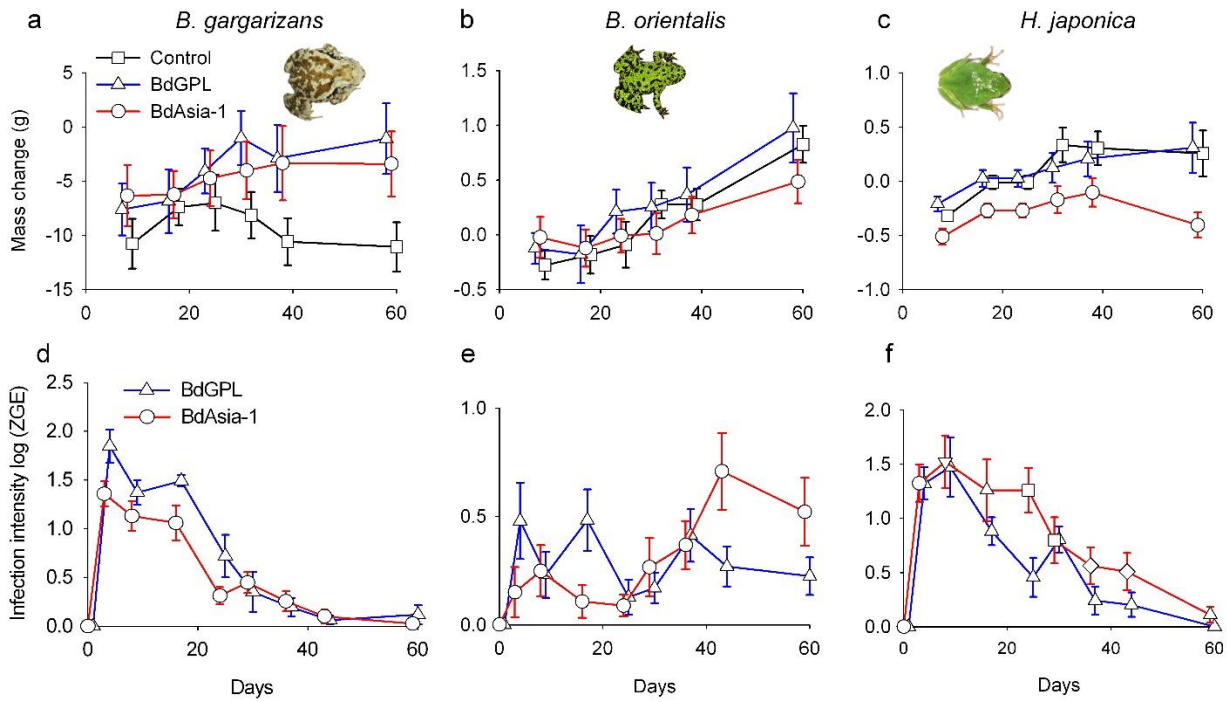

Figure 2.7 Korean toad and frogs are resistant both to BdAsia-1 and BdGPL. Mass changes during infection experiment (a, b, c). Bd intensity (d, e, f) in three species: *B. gargarizans* (a, d), *B. orientalis* (b, e), *H. japonica* (c, f). Error bars denote standard errors of the mean. Semi-parametric regression, a ($z = 1.77$, $P = 0.08$), b ($z = 0.76$, $P = 0.45$), c ($z = 1.37$, $P = 0.17$), d ($z = 1.01$, $P = 0.31$), e ($z = 1.88$, $P = 0.06$), f ($z = 2.11$, $P = 0.04$).

2.4 Discussion

This study reciprocally examines the resistance of Asian and Australasian amphibian host species and the virulence of endemic Bd lineages isolated from them. I found that *L. caerulea* is susceptible to an Australasian endemic BdGPL lineage and two BdAsia-1 strains with which it shares no evolutionary history. Yet, the three Korean host species that I tested are susceptible neither to Australian BdGPL nor BdAsia-1. By examining how responses to infection by the amphibian chytrid pathogen vary as a function both of Bd lineage and host species susceptibility, the question why Asian amphibian populations appear resilient to Bd infection even as Australasian species succumb to infection by the same pathogens can be addressed.

Contrary to the commonly held expectation that pathogens evolve toward benign co-existence with their hosts (Ewald, 1994; Morgan and Koskella, 2011), the results provide no evidence that endemic Asian Bd has attenuated in virulence over time. Indeed, the virulence of BdAsia may have dramatically risen over its long evolutionary history with Asian amphibian hosts. This results are consistent with recent findings that amphibians evolve effective physiological responses to tolerate or clear pathogen burdens, as discussed below. The evolution of immunity to chytridiomycosis may have driven selection for escalated pathogen virulence as hosts populations increasingly presented resistance to and tolerance of the disease (Fenton et al., 2006; Gandon and Michalakis, 2000; Miller et al., 2006).

2.4.1 Differences in susceptibility of Asian and Australasian host species to Bd

lineages

After inoculation with BdAsia-1, all *L. caerulea* suffered morbidity and mortality. However, two *L. caerulea* inoculated with BdGPL survived through to the end of the experiment. Disease progression was more rapid for subjects infected with BdAsia-1 than for those infected with BdGPL. As their clinical signs became more severe, subjects in both treatments lost mass at comparable rates. However, pathogen loads of subjects infected by BdAsia-1 continued to rise above 10,000 ZGE even as those infected by BdGPL levelled off substantially below this critical threshold level (Kinney et al., 2011). By all measures, BdAsia-1 caused more consistent pathogenesis in *L. caerulea*, even when compared to responses of Australian *L. caerulea* to BdGPL infection as previously described (Voyles et al., 2009).

By contrast, all three Korean host species that I tested showed no apparent effects after infection with either BdAsia-1 or BdGPL. Unlike *L. caerulea,* Korean host species gained mass after Bd exposure with the exception of *H. japonica* infected with BdAsia-1, which showed decreases in mass toward the end of the monitoring period. Although I confirmed all subjects inoculated with Bd indeed became infected, peak infection loads were very low (< 70 ZGE) as the experiment began. Then, all subjects rapidly cleared themselves of infection, except for *B. orientalis* which appeared to tolerate low pathogen burdens (< 3 ZGE). Although subjects infected by Bd-ASIA1 bore higher loads than those infected by Bd-GPL, these measurements were so low as to be barely detectable.

2.4.2 Virulence varies among Bd lineages

Virulence is best viewed in an ecological context. How pathogens affect hosts may be affected by environmental factors, population structure, and community dynamics (Savage, et al., 2015; Lively, 2006). Even in common garden experiments, where these conditions are held constant, effects of pathogens on hosts can vary dynamically, perhaps owing to differential expression of virulence genes by density-dependent mechanisms such as quorum sensing (Albuquerque and Casadevall, 2012). Additionally, genomic evolution of traits associated with virulence has been demonstrated in Bd lineages over extremely short timescales (Refsnider et al., 2015). Thus, comparisons of virulence among lineages with different isolation and passage histories are problematic. Certainly, my data are suggestive of BdAsia-1's higher virulence, but additional studies are needed to confirm my findings.

Recent attempts to assess the relative virulence of Bd lineages have generated conflicting results. At metamorphosis, larvae of North American wood frogs, *Rana sylvatica*, infected by a Brazilian Bd lineage (BdBrazil) suffered mortality comparable to those infected by BdGPL lineages endemic to their species range, but larvae appeared resistant to a Panamanian BdGPL lineage (Becker et al., 2017). Brazilian *Dendropsophus minutes*, some already Bd-infected when captured as adults, appeared largely Bd-tolerant, but subjects infected with BdGPL or hybrids between BdGPL and BdBrazil carried higher infection burdens than those infected with BdBrazil (Greenspan et al., 2018). One of these hybrid lineages proved most virulent infecting *Brachycephalus ephippium*, but the parental BdGPL lineage was most virulent when tested on *Ischnocnema parva*. With each host species, BdBrazil caused the least mortality and lowest infection burdens (Greenspan et al., 2018).

To compare the virulence of BdAsia-1 with Swiss (BdCH), South African (BdCape), and BdGPL lineages, O'Hanlon et al. (O'Hanlon et al., 2018) assessed life-history measures and survival to metamorphosis of British common toads (*Bufo bufo*) inoculated as larvae with each lineage. Subjects inoculated with BdGPL and BdAsia-1 were more likely to become infected and, together with those inoculated with BdCH, were less likely to survive and smaller at metamorphosis. However, less than 5 % of larvae inoculated with BdCH became infected, and even for the more virulent lineages, most subjects remained uninfected. Previously, a former lab member inoculated Korean *Bufo gargarizans* (= *Bufo bufo gargarizans*) larvae with BdGPL, using comparable procedures, but none of the subjects became infected (Chung, 2015). Inoculated after metamorphosis, BdGPL caused significantly more mortality in *B. gargarizans* than did BdAsia-1 or the other lineages. Taken together, these results suggest that BdAsia-1 is not especially virulent to larval and juvenile British toads but also that virulence may be modulated by ecological factors.

2.4.3 Co-evolution of Asian pathogen and its hosts

Rather than attempting to rank different lineages by their virulence, my purpose in conducting this study was to establish whether BdAsia-1 became less virulent through co-evolution with its hosts over many decades. By comparing responses of Korean and Australasian species to Bd lineages with which they evolved and those to which they are naïve, I can conclude that BdAsia-1 is at least as virulent as BdGPL to susceptible hosts. BdAsia-1 is the basal lineage from which hypervirulent BdGPL strains originated, possibly though hybridization and genetic

recombination (O'Hanlon et al., 2018). However, consistent with the substantial genomic variation evident among BdGPL lineages (Rosenblum et al., 2013), my results demonstrate that the evolution of hypervirulence in this chytrid pathogen is not dependent on recombination events. Rather, I would suggest that the emergence of hypervirulence drove the evolution of host resistance, which selected, in turn, for fungal strategies to subvert host immunity (Fites et al., 2013).

Extant Asian amphibians have overcome fungal counter-strategies so they are immune not only to the endemic strains with which they evolved but also to exotic recombinant strains that are decimating amphibian populations elsewhere in the world. By contrast, *L. caerulea* presented clinical signs of disease not only when infected with BdAsia-1 but also with a Bd lineage that infects hosts in its natural range. *L. caerulea* lives in lowland tropical forest in Australia and New Guinea where the climate is not especially favorable to Bd growth or transmission. In these conditions, frogs may not be affected by chytridiomycosis. In the absence of prior selection for resistance or tolerance, *L. caerulea* appears highly susceptible to the pathogen and quickly succumbs to infection by either lineage.

Mass die-offs of amphibians infected by Bd have been reported in Australia, New Zealand, the Americas, and parts of Europe. But even amidst the carnage caused by chytridiomycosis, some species seem to thrive even as others around them perish. Meanwhile, populations of some threatened species thought to have been extirpated now are slowly recovering (Kolby and Daszak, 2016; Scheele et al., 2017). And some species feared to be extinct in the wild now are beginning to reappear in their former ranges despite the continued presence of virulent Bd

lineages harbored by reservoir species (Voyles et al., 2018). As predicted by laboratory studies (Bataille et al., 2015; Savage and Zamudio, 2011), strong selection for physiological mechanisms that confer resistance to, and tolerance of, the pathogen appears to have rapidly occurred in the wild. As Bd transitions from an epizootic to an enzootic phase, it may pose less of a threat to species that evolve capabilities to cope with it.

2.4.4 Mechanisms of host defense against the pathogen

The first line of defense against chytridiomycosis lies in amphibian skin, which contains glands that secrete antimicrobial peptides (AMPs). Serving as a rapid but generalized innate immune response to infection, these AMPs inhibit Bd growth in vitro, and confer on some species resistance to chytridiomycosis. Variation in susceptibility to Bd among amphibian communities corresponds to interspecific differences in AMPs, as some lack effectiveness against Bd (Woodhams et al., 2007; Woodhams et al., 2006). Symbiotic bacteria also may play a role in innate immunity (Duffy et al., 2003), and their metabolites can exert a synergistic inhibition effect with AMPs to inhibit *Bd* growth in vitro (Myers et al., 2012). Possibly, peptides in the skin of *B. orientalis* and *B. gargarizans* have higher antimicrobial activity than those in *L. caerulea* (Apponyi et al., 2004; Park et al., 1996; Simmaco et al., 2009; Stone et al., 1993) but their efficacy against Bd is unknown except in *L. caerulea*.

Chytridiomycosis may select for stronger innate immune responses. In the early 2000s, several frog species disappeared from Panamanian rainforests. Long considered extinct, some species recently were rediscovered in their previous

natural ranges. As the disease initially emerged, Bd had been isolated and cryopreserved from infected frogs. Although epizootic survivors show reduced disease prevalence, the Bd pathogen appears just as virulent now as it had been prior to initial disease outbreaks (Voyles et al., 2018). However, the rebounding populations secrete AMPs with stronger anti-Bd properties than those collected, prior to the epizootic, from individuals naïve to the pathogen. Moreover, AMPs isolated from survivors in the wild more effectively inhibit Bd growth than those of frogs held in ex situ insurance colonies (Voyles et al., 2018). Although controls for possible degradation of AMPs over time are lacking, the results suggest that rapid natural selection has occurred for effective innate immune responses to Bd.

If infection persists, adaptive immune responses directed specifically toward Bd should follow. Amphibians, like most vertebrates, stochastically generate a wide repertoire of antigen receptors through somatic recombination, hypermutation and selection. Certain major histocompatibility complex (MHC) molecules bind to Bd epitopes, processing them for presentation by antigen-presenting cells to T lymphocytes. When T cells with receptors specific to the antigen-MHC complex are activated, adaptive immune responses are triggered that target the pathogen.

Comparative studies of Bd-susceptible and resistant amphibians worldwide reveal that resistant species, including *B. orientalis* and *B. gargarizans* that I used in this study, present similar conformations of the P9 pocket in the MHC class II β1 domain (Bataille et al., 2015). Within Australian *Litoria verreauxii* populations, only individuals with this MHC conformation survive chytridiomycosis (Bataille et al., 2015). North American *Rana yavapaiensis* show similar patterns of MHC-based

resistance (Savage and Zamudio, 2011, 2016). Panamanian *Engystomops pustulosus* are more likely to show such conformations in highland habitats favourable to Bd growth and transmission, as frogs there are most at risk of infection and disease (Kosch et al., 2016). Both field and laboratory studies thus suggest that adaptive immunity can evolve in amphibian populations following Bd incursions into their habitat. The resistance mounted by Korean species to Bd in my study thus might be attributable to their evolved innate immunity, adaptive immunity, or both.

Immunological mechanisms underlying Bd-resistance are complex, and research to date is beset with inconsistencies (Fu and Waldman, 2017). Transcriptome analyses reveal that genes encoding adaptive immune function generally are not up-regulated in Bd-resistant species (Rosenblum et al., 2012). Yet, in species susceptible to the disease, these genes often are up-regulated but to no effect as morbidity and mortality quickly follow (Ellison et al., 2014; Eskew et al., 2018; Grogan et al., 2018a). When a host is naïve to pathogens, the recruitment, activation, and proliferation of T and B cells proceeds slowly. On subsequent infection, the host's immune system is already primed to respond quickly to the pathogen. Yet, evidence of such immunological memory to Bd is mixed, with some studies reporting evidence of clearance or reduced infection load in repeated infections but others showing no such effects (Cashins et al., 2013; McMahon et al., 2014; Stice and Briggs, 2010).

As amphibians evolved adaptive immune responses against the pathogen, Bd apparently evolved counter-measures to evade host immune responses. Soluble

factors released by the pathogen inhibit lymphocyte proliferation and induce apoptosis although these effects may lessen with repeated infections (Fites et al., 2013; McMahon et al., 2014; Rollins-Smith et al., 2015). Innate immune responses to Bd appear to remain unaffected by these factors, as innate leukocyte proliferation remains intact (Fites et al., 2014; Young et al., 2014). Active suppression of immune function by Bd may make adaptive immune responses costly for susceptible species. Then, paradoxically, rather than conferring resistance, adaptive immunity acts to accelerate disease progression.

2.4.6 Conclusion

Pathogen virulence is not static. Infectivity and disease signs vary based on immune responses of hosts, which in turn are modulated by the organism's condition and the social and physical environment in which it lives. Moreover, whether infection culminates either in disease or subclinical effects may depend on interactions between host and pathogen genotypes. Thus, some inconsistencies among studies are inevitable. Yet, my study clearly demonstrates, for the first time, that Bd has not attenuated in virulence over a long period of co-evolution with its amphibian hosts. Indeed, BdAsia-1 appears more virulent than BdGPL when tested on hosts that are naïve to it.

Amphibian hosts that have co-evolved with endemic Bd express resistance generally to the pathogen in its many forms, including recombinant BdGPL lineages. This finding raises the possibility that effective immunization strategies might be developed, using Bd lineages with low virulence to induce adaptive immune

responses to more highly virulent lineages. Asian amphibians are traded internationally and carry endemic lineages, including BdAsia-1, that may threaten amphibian biodiversity. My research represents a large step forward in resolving the enigma of why Bd infection causes clinical signs of chytridiomycosis in some parts of the world but not others. This, in turn, should assist in the development of management plans for the disease.

Chapter 3. Major Histocompatibility Complex (MHC) Variation and the Evolution of Resistance to Amphibian Chytridiomycosis

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Abstract

Chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), has been implicated in population declines and species extinctions of amphibians around the world. Susceptibility to the disease varies both within and among species, most likely attributable to heritable immunogenetic variation. Analyses of transcriptional expression in hosts following their infection by Bd reveal complex responses. Species resistant to Bd generally show evidence of stronger innate and adaptive immune system responses. Major histocompatibility complex (MHC) class I and class II genes of some susceptible species are up-regulated following host infection by Bd, but resistant species show no comparable changes in transcriptional expression. Bd-resistant species share similar pocket conformations within the MHC-II antigen-binding groove. Among susceptible species, survivors of epizootics bear alleles encoding these conformations. Individuals with homozygous resistance alleles appear to benefit by enhanced resistance, especially in environmental conditions that promote pathogen virulence. Subjects that are repeatedly infected and subsequently cleared of Bd can develop an acquired immune response to the pathogen. Strong directional selection for MHC alleles that encode resistance to Bd may deplete genetic variation necessary to respond to other pathogens. Resistance to chytridiomycosis incurs life-history costs that require further study.

Keywords: Amphibian population declines; *Batrachochytrium dendrobatidis*; Chytridiomycosis; Heterozygosity; Life-history trade-offs; Major histocompatibility complex

3.1 Introduction

Worldwide declines of amphibian population declines first became apparent about 30 years ago, as researchers from around the world compared notes about the difficulty they were experiencing finding their study organisms (Wake, 1991). Fear that disappearing amphibians represented an early sign of an impending sixth mass extinction event (Wake and Vredenburg, 2008) gave rise to a flurry of research to ascertain the causes of amphibian population crashes. Amphibian population declines can result from numerous factors including habitat modification and fragmentation, exotic predators and competitors, over-exploitation, climate change, and emerging infectious diseases (Berger et al., 2016; Kolby and Daszak, 2016; Rollins-Smith, 2017).

In the 1990s, the most dramatic, precipitous declines were witnessed in pristine habitat in Central America and Australia, which could not easily be explained by anthropogenic factors. Although researchers had previously described the spread of amphibian population extinctions occurring in waves suggestive of pathogen spread (Laurance et al., 1996), the hypothesis was met with skepticism owing to the lack of knowledge of the causal agent. Subsequently, a new fungal pathogen, initially misidentified as a Perkinsus-like protist, and later recognized to be a chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter denoted *Bd*), was identified in victims of epizootics in Australia and Central America (Berger et al., 1998).

Initially, *Bd* was thought to be a recently emerged clone that spread globally

(Morehouse et al., 2003) to cause population declines. Later, endemic *Bd* strains were identified in many localities around the world (Kolby and Daszak, 2016; Olson et al., 2013). Although *Bd* was thought to be a novel pathogen of amphibians, the fungus has now been identified in amphibians collected over the past 100 years or more (Fong et al., 2015; Goka et al., 2009; Rodriguez et al., 2014; Talley et al., 2015). Recent population crashes in European salamanders have been linked with a second chytrid pathogen, *Batrachochytrium salamandrivorans* (denoted *Bsal*) (Cunningham et al., 2015; Martel et al., 2014; Spitzen et al., 2013).

3.2 How does *Bd* damage hosts?

Bd infects the keratinized mouthparts of larvae, which may interfere with their feeding behavior, as well as the keratinized skin of adults, which serves as an important respiratory organ for amphibians. Infection might cause hyperkeratosis, thereby impeding respiration or water balance, or hosts might be poisoned by a fungal toxin (Berger et al., 1998; Pessier et al., 1999). Adult amphibians showing clinical signs of chytridiomycosis may experience lower plasma concentrations of sodium and potassium electrolytes, which may induce asystolic cardiac arrest (Voyles et al., 2009). However, individuals bearing low infection loads, although potentially suffering some deficits from aclinical chytridiomycosis, show no significant impairment in metabolic measures such as rehydration rates (Carver et al., 2010). These results suggest that tolerance of, in addition to resistance to, the pathogen may be selected in amphibians living in habitats with enzootic *Bd* strains (Barribeau et al., 2008; Savage and Zamudio, 2016).

As *Bd* infects only superficial epidermal tissue (Rooij et al., 2012), infection might rapidly induce innate immune responses against it. Indeed, amphibian skin contains many mucous and granular glands, which secrete functional mucins and antimicrobial peptides (AMPs) (reviewed by (Rollins-Smith et al., 2011). These antimicrobial peptides (AMPs) inhibit *Bd* growth in vitro, and serve as a key component of the innate immune response to *Bd* (Ramsey et al., 2010; Rollins-Smith et al., 2011). AMP repertoires vary among species, perhaps correlated with differential susceptibility to *Bd* (Rollins-Smith, 2009; Rollins-Smith and Conlon, 2005; Woodhams et al., 2007). Symbiotic bacteria also may play a role in innate immunity (Duffy et al., 2003), and their metabolites can exert a synergistic inhibition effect with AMPs to inhibit *Bd* growth (Myers et al., 2012).

In susceptible species, *Rana muscosa*, *Rana sierra*, and *Atelopus zeteki, Bd*-infected individuals express reduced levels of keratin and collagen, key components of structural skin integrity (Ellison et al., 2014; Rosenblum et al., 2012). By contrast, in several resistant species, genes needed to maintain skin integrity, such as collagen, are up-regulated after subjects are infected with *Bd* (Ellison et al., 2014; Poorten and Rosenblum, 2016). Decreased expression of ion channel genes and increased expression of potassium/chloride genes in *Bd*-susceptible species are consistent with the observation of electrolyte imbalance directly preceding death (Voyles et al., 2009), attributable perhaps to physical disruption of the epidermis (Rosenblum et al., 2012).

Soluble factors released by *Bd*, possibly derived from the cell wall, can actively interfere with adaptive immunity and prevent clearance of the pathogen. In vitro,

Bd inhibits the proliferation of splenocytes, which leads to lymphocyte apoptosis in susceptible species (Fites et al., 2013). Immunodeficiency thus occurs as insufficient T-lymphocytes and B-lymphocytes are present to respond to and eliminate *Bd*. In vivo, lymphocyte genes are down-regulated in spleen of susceptible but not resistant or tolerant species (Ellison et al., 2014). Effectors associated with *Bd*, such as CRN13, can induce necrosis in infected tissues (Ramirez-Garces et al., 2016). Patterns of transcriptional changes upon *Bd* infection vary widely among anurans (Ellison et al., 2014; Ellison et al., 2014; Poorten and Rosenblum, 2016; Price et al., 2015; Rosenblum et al., 2012), but species resistant to *Bd* generally show evidence of stronger innate and adaptive immune system responses.

Adaptive immune responses appear to supplement innate immunity in conferring resistance to *Bd*. In African clawed frogs (*Xenopus laevis*), a species highly resistant to infection, AMPs of frogs which subject to X-irradiation remain stable, yet *Bd* infection loads rise as spleen leukocyte numbers decrease (Ramsey et al., 2010). IgM, IgY, and IgX antibodies bind to *Bd*, further suggesting a role for adaptive immune responses in conferring resistance (Ramsey et al., 2010). *Bd*-infected *Litoria caerulea*, a highly susceptible species, become immunocompromised as evidenced by lower total white blood cell and serum protein counts together with reduced splenic lymphocyte and immunoglobulin responses (Young et al., 2014).

Adult anurans are more susceptible to chytridiomycosis than tadpoles, perhaps because larvae lack keratinized tissues except in their mouthparts, which can be infected by *Bd*. Nonetheless, larvae of some species suffer reduced growth and even mortality when infected by or exposed to *Bd* (Blaustein et al., 2005;

Langhammer et al., 2014; Luquet et al., 2012). Ontogenetic changes in *Bd* resistance or tolerance correlate with aspects of immune system maturation, but the relationship, if any, remains to be studied. Metamorphosis entails radical reorganization of many tissue and organ systems including those involved in immune function, with a period of immunosuppression at metamorphic climax (Robert and Ohta, 2009; Rollins-Smith, 1998) during which infected individuals are most vulnerable to the disease.

3.3 How does *Bd* infection alter MHC expression?

If *Bd* triggers an adaptive immune response, we might expect to find changes in transcriptional expression of MHC genes following infection. In most susceptible species that have been studied, such as *Rana muscosa*, *Rana sierra*, and *Atelopus zeteki*, MHC genes appear to be up-regulated in the skin after infection (Ellison et al., 2014; Rosenblum et al., 2012). But in most resistant species being studies, such as *Xenopus (Silurana) tropicalis, Craugastor fitzingeri*, *Agalychnis callidryas*, and *Rana temporaria*, no changes in MHC transcriptional expression have been found (Ellison et al., 2014; Price et al., 2015; Rosenblum et al., 2009) (Table 3.1). However, down-regulation of MHC in spleen and liver in some susceptible species may be symptomatic of more general suppression of adaptive immune function as previously discussed.

In these studies, although up- and down-regulation of MHC transcriptional expression was measured, translational levels were not determined and might not coincide with transcriptional differences or remain unchanged. Moreover,

discordant results among studies may reflect other variables, including temperature effects on pathogen infectivity or host immune function, ages and background of hosts, harvest timing for RNA isolation, and even sex or individual differences which might be especially important given the small sample sizes (e.g. (Ramsey et al., 2010; Zhu et al., 2014a)), which was summarized in Table 3.2.

Table 3.1 MHC transcriptional regulation in susceptible and resistant amphibians after Bd infection.

Species	Susceptibility	Tissues examined	MHC expression (compared with control)		Reference
			MHC I	MHC II	
Xenopus tropicalis	Resistant	Liver, skin, spleen	–	–	Rosenblum et al. 2009
Rana muscosa *Rana sierrae*	Susceptible	Liver, skin, spleen	Up in skin (late), down in spleen (early)	Up in skin (late), down in spleen (late)	Rosenblum et al. 2012
Atelopus zeteki	Susceptible	Skin, spleen, small intestine	Up in skin	Up in skin	Ellison et al. 2014a
Atelopus zeteki	Susceptible	Skin, spleen	–	–	Ellison et al. 2014b
Atelopus glyphus			–	–	
Craugastor fitzingeri	Resistant		–	–	
Agalychnis callidryas			–	–	
Rana temporaria	Resistant	Liver	–	–	Price et al. 2015
Anaxyrus marinus *Anaxyrus boreas*	Resistant Susceptible	Liver, skin, spleen	– Down in liver	Down in liver Down in liver	Poorten and Rosenblum 2016

– no difference in expression

Table 3.2 Conditions from different researches for examining MHC transcriptional regulation by Bd.

Species	Age	Obtaining methods	Size	Bd strain	Bd amount used for treatment (zoospores)	Bd treatment	Infection temperature	Tissue harvest time after Bd treatment	Gender	Sample size	Method	Paper
Xenopus tropicalis	adult (age unknown)	unknown	unknown	unknown	unknown	unknown	18℃	3 days, show clinical signs	unknown	24	microarray	Rosenblum et al. 2009
Rana muscosa *Rana sierrae*	adult (age unknown)	wild eggs, bred in lab	unknown	Sierra-Nevada Bd	>1000000 in 100ml water	24hr; repeated 3 days	18℃	3 days,16 days	Mixed	6	pyrosequencing, microarray	Rosenblum, Poorten et al. 2012
Atelopus zeteki	18 months	captive-bred	unknown	JEL-423	300000 in 30ml Bd solution	10hr	21-22℃	33 days	female	9	RNA sequencing	Ellison, Savage et al. 2014
Atelopus zeteki	unknown	captive-bred	unknown	JEL-423	3000	10hr	18–19℃	33 days	unknown	11	RNA sequencing	Ellison, Tunstall et al. 2014
Atelopus glyphus		captive-bred			300000	10hr		62 days		12		
Craugastor fitzingeri		wild (Gamboa)			500000	24hr		41 days		9		
Agalychnis callidryas		wild (Ocelot Pond)			500000	24hr		41 days		10		
Rana temporaria	metamorphs	wild eggs, bred in lab	unknown	JEL-423	3000000 active zoospores in 30 ml	4hr	18-21ºC	4 days	unknown	30	RNA sequencing	Price, Garner et al. 2015
Bufo marinus	unknown	captive-bred	53.94 g to 122.20 g	JEL275	average: 2000000 in 10 mL of Holtfreter's solution	24hr	20℃	18 days	9male, 11female	20	Microarray	Poorten and Rosenblum 2016
Bufo boreas			35.47 g to 56.53 g		average: 1000000 in 10 mL of Holtfreter's solution			18 days	male	20		

3.4 MHC genetic diversity and susceptibility to *Bd* infection

Susceptibility to chytridiomycosis varies among regions of the world. In localities where *Bd* has a history of endemism (e.g., parts of Asia, South America, North America), many species appear resistant to, or tolerant of, *Bd*, showing no clinical signs of disease. Epizootics typically occur in areas soon after initial incursions of *Bd*, but among the subsequent carnage, some species may thrive even as others around them perish. Even in susceptible species, however, variation in resistance often is apparent. Natural selection presumably favors resistant variants, and populations of some species, thought to be extinct, now appear to be slowly recovering (Knapp et al., 2016).

Previous studies showed that MHC variants confer resistance on amphibians to virulent pathogens that cause epizootics and mass mortality events (Barribeau et al., 2008; Teacher et al., 2009). Recent studies have found that differential survival of amphibians infected by *Bd* maps to genetic variation in the MHC class II PBR (Bataille et al., 2015; Kosch et al., 2016; Savage and Zamudio, 2011, 2016; Tracy et al., 2015). The demonstration of adaptive immune responses to *Bd* was somewhat surprising given that infections often are limited to superficial epidermal cells (Richmond et al., 2009).

Acquired resistance to *Bd* is associated with particular amino acid residue positions situated in exon 2 of the MHC class II B gene, which encodes the β1 segment of the antigen-binding groove that presents epitopes to T cells. The stability of the antigen-MHC complex depends mainly on deep pockets within the

binding groove that interact directly with antigen residues. Nine pockets (P1-P9) are revealed by crystallography, of which four (P1, P4, P6 and P9) are polymorphic in the PBR and differ in how they bind to aliphatic, hydrophobic and hydrophilic amino acids (Brown et al., 1993; Dai et al., 2008; Stern et al., 1994). Alleles that encode particular configurations of the P9 pocket appear key to *Bd* resistance among amphibians (Fig 3.1) (Bataille et al., 2015). Specific properties of the P4 and P6 pockets also correlate with *Bd* resistance widely, but not universally, perhaps because of immune system differences among species, variation among *Bd* strains, or differences in environmental factors (Bataille et al., 2015).

The MHC of lowland leopard frogs (*Rana yavapaiensis*) is highly polymorphic, with 84 unique MHC class II PBR alleles identified (Savage and Zamudio, 2016). In laboratory infection studies, individuals bearing “allele Q” were resistant to *Bd*. Population genetic analyses detected a significant signal of positive selection acting on codon 46 in pocket 9 (Savage and Zamudio, 2011). Sampling in the field across several localities similarly found that this allele was positively associated with survival rates, but unlike in the laboratory experiments, heterozygosity conferred no advantage in natural conditions (Savage and Zamudio, 2016).

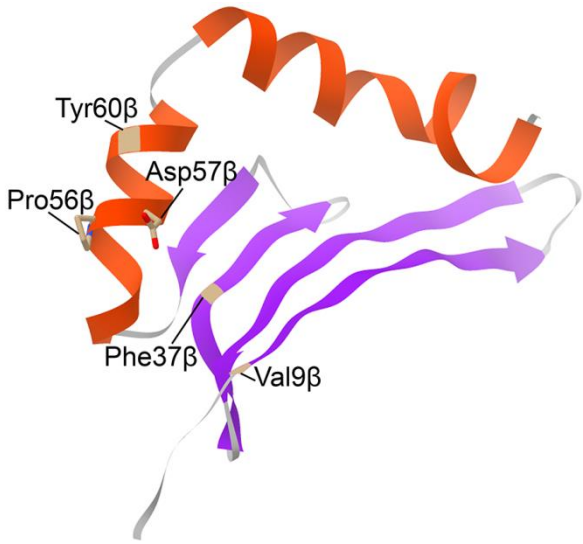

Figure 3.1 Homology modelling of MHC class II β1 domain allele Buga-1.

Display of the typical three-dimensional structure of the vertebrate MHC class II β1 domain, with α-helices represented as red helical ribbons and β-sheets as purple thick arrows. The peptide binding residues of the P9 pocket are indicated, with atomic structure for residues 56β and 57β.

Alpine tree frogs (*Litoria verreauxii alpine*) from populations with a history of *Bd* endemism appear more resistant to experimental infection with *Bd* than those from populations that are naïve to *Bd*. Comparing disease progression among three populations, infection loads were lower and survivorship higher in subjects from one locality that had been infected *Bd* for many decades. Subjects from a site in which *Bd* has been historically absent had higher infection loads and succumbed more quickly to infection, but so too did subjects from another *Bd*-infected locality. Twenty-two MHC IIβ alleles were recovered among the populations. Of 84 infected frogs, 6 survivors bore 8 alleles containing identical residues at 5 codon positions, all present within pocket 9. Only subjects homozygous for alleles encoding this pocket conformation benefited from this resistance (Bataille et al., 2015). Similar pocket conformations of the P9 pocket in the MHC II β1 domain appear to be fixed in *Bd*-resistant *Bufo* species and *Bombina orientalis* in Korea (Bataille et al., 2015).

In Panama, highland and lowland frog populations appear to have evolved different immunogenetic responses to the pathogen. *Bd* has been enzootic in highlands since the late 1990s but has only recently reached lowland populations. Highland populations of the túngara frog (*Physalaemus pustulosus*) live in amphibian communities that suffered declines when *Bd* initially spread to them. Surviving individuals tend to have MHC class II alleles with the resistant P9 configuration, and most individuals are homozygous for these alleles. By contrast, temperature regimens in lowlands are not favorable for the transmission of *Bd*, population declines have not been observed, and individuals tend to bear these same alleles but in the heterozygous state. Selection for resistance alleles thus

appears to fluctuate based on environmental conditions that affect pathogen spread.

Class II molecules present antigens to helper T cells from extracellular pathogens such as *Bd*, so most amphibian disease studies have focused on class II. MHC class I molecules are expressed on all nucleated somatic cells and mainly present antigens from intracellular pathogens to cytotoxic T cells. Thus, class I would seem unlikely to be involved in responses to extracellular *Bd*, but recent studies suggest that *Bd* sometimes penetrates into cells (Rooij et al., 2012). Endangered Corroboree frogs, *Pseudophryne corroboree*, which are facing steep population declines in the wild attributable to *Bd*, show high MHC class IA diversity. Genetic evidence points to strong positive and purifying selection at class IA sites that are associated with PBR pockets in resistant species (Kosch et al., 2017). Comparably high levels of genetic diversity in MHC class I in Japanese *Rana* species similarly raise the possibility of selection for *Bd* resistance in those species as well (Lau et al., 2016).

3.5 *Bd*-induced immunological memory

If amphibians demonstrate an adaptive immune response to *Bd*, immunisation protocols might be developed to enhance their resistance to the pathogen. Results to date on the inducement of robust acquired immunity have been mixed. Exposure to formalin-kllled *Bd*, by injection, did not reduce susceptibility of juvenile yellow-legged frogs (*Rana muscosa)* to subsequent infection (Stice and Briggs, 2010). Boreal toads (*Anaxyrus boreas*) injected with

heat-killed *Bd* also survived no better than control subjects when infected with *Bd* (Rollins-Smith et al., 2009). Nonetheless, inoculation with *Bd* induced a systemic response evident in circulating antibodies specific to *Bd* in *X. laevis* (Ramsey et al., 2010).

As *Bd* infects the skin, inoculation by injection might not be appropriate for gauging the development of an adaptive immune response. Partial immersion of boorolong frogs (*Litoria booroolongensis*) in solution containing *Bd* zoospores readily caused subjects to become infected (Cashins et al., 2013). Frogs then were cleared of infection by treatment with the fungicide itracanazole and subsequently reinfected using the same procedure, but these individuals survived in no higher numbers than control group that were first infected (Cashins et al., 2013). Control for immunosuppressive properties of itracanazole were lacking, however, and a reanalysis of the same data revealed that previously inoculated frogs demonstrated increased resistance upon reinfection (McMahon et al., 2014).

As *Bd* is viable only within a limited temperature range, hosts can be cleared of the disease by potentially less invasive heat treatments. Using such a procedure, Cuban treefrogs (*Osteopilus septentrionalis*) inoculated with *Bd* showed decreased infection loads in each successive cycle of infection and clearance by heat treatment. This acquired immunity was attributable to increased abundance and proliferation of splenic lymphocytes rather than changes in skin peptide abundance (McMahon et al., 2014)). Furthermore, prior exposure to *Bd* not only activated a strong immune system response but also induced a behavioral response, as *Bd*-exposed oak toads (*Bufo quercicus*) showed an aversion to substrate contaminated by *Bd* zoospores

that was lacking in *Bd*-naïve subjects (McMahon et al., 2014).

3.6 MHC resistance alleles incur tradeoffs

MHC loci in natural populations often are highly polymorphic, presumably because multiple alleles encode many PBR conformations that bind to a wide array of epitopes. This variation can be favored by heterozygote advantage or negative fluctuating selection. As the MHC is co-dominantly expressed, in the former case, heterozygotes potentially can present twice as many PBR conformations as homozygotes. In the latter case, hosts can present PBR conformations to which pathogens have had no opportunity to adapt. Adaptive immune responses are thought to be enhanced with increased levels of immunogenetic variation, although exceptions exist (Slade, 1992). In some systems maximum resistance is achieved by having an optimal number of MHC class IIb alleles, less than the maximum possible, when hosts are infected by multiple parasites (Wegner et al., 2003). This might result from deletion of self-reactive T cells, which restricts the pool of available T cells (Nowak et al., 1992).

Studies on the dynamics of immunogenetic responses to *Bd* are consistent with the hypothesis that that when infected by a new, virulent pathogen, strong directional selection over the short term may favor particular alleles that confer resistance to it. However, the advantages of effective immune response to particular pathogens such as *Bd* may be offset by reduced immune capacity to respond to other pathogens. Selection for particular resistance alleles thus is expected to vary as a function of environmental conditions that promote *Bd*

virulence and the efficacy of immune responses to the pathogen. In boreal toads (*A. boreas*), a species highly susceptible to chytridiomycosis (Carey et al., 2006), more heterozygous individuals were found to be infected by *Bd* in surveys of natural populations (Addis et al., 2015). Although the MHC was not characterized, this result is consistent with the expectation that *Bd* exerts strong selection for homozygosity of resistance alleles. This selection may vary among populations living in different habitat properties that affect pathogen virulence and host resistance. Shifts in selection regimens may explain the variable results found in field and laboratory studies, and between populations living in differing habitats, even within species.

In evolutionary terms, immune responses can incur fitness costs. Alleles conferring resistance not only to *Bd* but also other pathogens, including ranavirus (Teacher et al., 2009) and the bacterial pathogen *Aeromonas hydrophila* (Barribeau et al., 2008) have been identified in amphibians. Bacterial pathogens, especially *A. hydrophila*, frequently have been linked to epizootics of amphibian populations but now are thought to represent secondary infections of individuals infected by *Bd* or other pathogens. In experimental studies, *X. laevis* tadpoles bearing MHC alleles that conferred resistance to *A. hydrophila* grew slower than those with susceptible MHC alleles (Barribeau et al., 2008). These results point to a possible tradeoff between growth and immune function. Rapid growth rates of tadpoles and larger size at metamorphosis typically accrue strong fitness advantages in amphibians. Thus, while certain MHC alleles confer disease resistance, individuals that bear them may have lower reproductive success. In environments in which a pathogen

like *Bd* does not represent a severe threat to amphibians, balancing selection rather than strong directional selection is expected.

3.7 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) can recognize pathogen-associated molecular patterns (PAMPs), structures shared by many pathogens (Hayashi et al., 2001; Rollins-Smith et al., 2009). TLRs play a critical role in early pathogen recognition and defense pathways (Beutler and Rietschel, 2003). Activation of TLR-mediated cell signaling pathways can induce the up-regulation of genes that orchestrate immune responses, leading to cytokine production, proliferation and survival (Misch and Hawn, 2008; Rollins-Smith et al., 2009). In turn, this can activate cellular pathways culminating in the production of transcription factors that target MHC promoters, regulating gene expression (Mak et al., 2014).

In *Bd*-infected *A. zeteki*, several TLR genes, such as TLR2 type-1-like and TLR5, were up-regulated in the skin, spleen and intestine (Ellison et al., 2014). TLR2 recognizes fungal derived ligands, while TLR5 is known to recognize bacterial flagella (Akira and Takeda, 2004). Among the up-regulated TLRs, TLR5 was most up-regulated upon *Bd* infection, suggesting that naïve-infected animals were possibly responding to increased secondary bacterial infections during the course of *Bd* infection. Secondary bacterial infections, such as *A. hydrophila,* have been linked to mortality during fungal infections in amphibians (Taylor et al., 1999). However, the possibility that this contributes to mortality in *Bd*-infected amphibians remains untested. Studies on other susceptible amphibians found no evidence for

TLR activation during infection (Rosenblum et al., 2012; Rosenblum et al., 2009).

3.8 Concluding Remarks

Chytridiomycosis caused by *Batrachochytrium dendrobatidis*, has been threatening amphibian populations. There were species which showed severe susceptibility with high mortality after *Bd* infection, but also species which showed relatively resistant or tolerant after *Bd* infection. Previous researches have been dedicating to characterize the defending mechanisms of *Bd* infection in both susceptible species and resistant or tolerant species.

Transcriptome comparison between before and after *Bd* infection in different species revealed complicated transcriptional responses. MHC transcriptional levels were also differently regulated in different studies and different species upon *Bd* treatment, which might due to intrinsic reasons of hosts or developmental stages, sequencing techniques, or even tissue harvesting time points. However, it is more likely that MHC class I and MHC class II are up-regulated upon *Bd* infection.

By analyzing genotypes and structure of MHC class II peptide binding regions in *Bd* infected frogs, allele Q and pocket 9 were identified to be positively associate with resistance from laboratory experiments. Researches in the wild were also performed indicating allele Q was under directional selection driven by *Bd* infection. Not only MHC class II might be associated with Bd resistance, but also MHC class I, which also exhibited high or moderately high in some anuran species. Moreover, combination of certain MHC class I and MHC class II might be also important for further investigation, instead of solely MHC class II or class I in one population.

Besides, in each research, researchers had better provide how MHC gene and protein level were regulated in response to *Bd* in that species prior to MHC genotyping. If even gene expression level was not up-regulated by *Bd*, then it is difficult to trust all the predictions only based on only on MHC genotypes.

Heterozygosity is not always an advantage, which is supported in *Bd* infection both in wild. Although certain MHC alleles were suggested to be beneficial to pathogens, it is not the more alleles the better. Costs, such as growth inhibition could also occur. However, costs of possessing resistance alleles resisting to *Bd* have yet characterized. Thus, more researches, especially comparative researches of other wild species with different disease tolerance should be further investigated. Moreover, it is very important to look deeply in skin under microscopy to search how *Bd* infects cells in skin and make a new model of activation of acquired immunity.

Chapter 4 Characterization of MHC IIß1 in Bd Susceptible and Resistant Amphibian

Abstract

Population of many amphibians in the world was threatened by *Batrachochytrium dendrobatidi*s (Bd), except in Asia. Differential susceptibilities of host might due to differential genetic variations. Major histocompatibility complex (MHC) genes exhibits the most polymorphism among all the genes identified yet and MHC II was suggested to be associated with Bd resistance previously. However, previous amphibian MHC genotyping was based on mostly a single PCR derived traditional cloning based sequencing, which largely reduced the reliability of the concluded role of MHC II in Bd resistance. In this study, I applied a more reliable genotyping method based on next generation sequencing derived from three amplicons independently for each sample to compare MHC IIß1 genetic variation in Bd – susceptible (*Litoria caerulea*) and – resistant (*Bufo gargarizans*) species. First, next generation sequencing was confirmed to be more reliable than traditional cloning based sequencing in amphibian MHC genotyping. Second, I found high copy number of variation of MHC II in both species with similar average amount of alleles for each individual. Third, each allele differs in frequency among individuals, and differential MHC II allele gene expression was observed in both species, but no clear expression pattern was found regarding to Bd treatment and different susceptibilities. Different organs in same individual has MHC II allele variation and spleen tends to possess more MHC II alleles than liver. Besides, MHC II in both species were under balancing selection and seemed following concerted evolution pattern. Nevertheless, Wu-Kabat variation was significantly higher in *L. caerulea*

than that in *B. gargarizans*, and seven PBR and WuKa sites of MHC II in *L. caerulea* were under strong positive selection. More interestingly, four among the seven positive selected site in *L. caerulea* were completely conserved in *B. gargarizans*, which might associate with Bd resistance, but could also be other diseases. Although there were unique alleles in two Bd – resistant *L. caerulea*, no clear pattern was observed due to small sample size. This study provided a much more reliable MHC genotyping result in regard to Bd resistance, but further studies should be performed to confirm the role of MHC II in Bd resistance.

Keywords: chytridiomycosis susceptibilities, MHC II genetic variants comparison, next generation sequencing, differential gene expression, copy number of variation, allele frequency, positive selection, concerted evolution

4.1 Introduction

Amphibians are vital components of food chains in many ecosystems and are key indicators of biodiversity and ecosystem health (Myers et al., 2000; Vitt et al., 1990). Biochemicals from amphibians are also used in medicine (Clarke, 1997) and cosmetics (He et al., 2018), and amphibians are often important cultural symbols due to their charismatic nature. Chytridiomycosis, caused by the chytrid fungus, *Batrachochytrium dendrobatidi*s (Bd), has triggered population declines or even extinction of many amphibian populations in the past few decades, possibly even earlier (Kolby and Daszak, 2016), however, Asian amphibian populations have remained largely unaffected.

Encoding the most polymorphic genes identified yet, major histocompatibility complex (MHC) is known as a generalist involved in numerous essential immunological activities (Rock et al., 2016) and behavior including mate choice (Havlicek and Roberts, 2009; Milinski, 2006) and kin recognition (Manning et al., 1992; Zelano and Edwards, 2002). The unusually high level of polymorphism found in MHC genes is thought to be generated and maintained by pathogen-driven selection, although mate choice has also been implicated (Havlicek and Roberts, 2009; Milinski, 2006). Because the MHC is responsible for binding and presenting peptides to T-cells, it is generally thought that increased MHC variation is associated with increased potential to recognize potential pathogens and thus increased immunocompetence (Milinski, 2006; Sommer, 2005). This unusually high genetic

variation also makes the MHC one of the most frequently used genetic markers in the study of evolutionary dynamics between host and pathogen.

Susceptibility to chytridiomycosis in amphibians has been suggested to be associated with MHC II genotype and subsequent protein conformation (Bataille et al., 2015b; Fu and Waldman, 2017; Savage and Zamudio, 2011). However, MHC genotyping approaches in amphibians are still far behind those of other vertebrates. Until recently, most MHC studies in amphibians relied on dubious genotyping methods based on a single PCR amplification and traditional cloning based sequencing (TCBS). Given the MHC's unusually high genetic variation combined with frequently observed gene copy number variation in individuals, replicate PCRs would seem to be required for accurate genotyping, yet are rarely done in published studies. Confounding this weakness, most studies use only a fragment of a single exon, often less than 150 bp, which makes variant verification and subsequent genotyping and estimates of true heterozygosity difficult. Even among the few studies that used next generation sequencing with two independent PCRs (Savage and Zamudio, 2016), PCR chimeras were not assessed. These weaknesses in MHC genotyping have greatly reduced the ability to test MHC II's potential role in resistance to Bd infection, and relegated most behavior MHC studies of frogs' suspect. Although next generation sequencing (NGS) has been widely applied to MHC genotyping in other vertebrates with a developed and reliable methodology (Ekblom and Galindo, 2011; Hosomichi et al., 2015; Sommer et al., 2013), these newer, more reliable methods have yet to be applied to frog MHC studies (Grogan et al., 2018b). Although *Litoria caerulea* is known to be susceptible to Bd infection,

and several studies have investigated various aspects of innate immunity (Berger et al., 2005; Voyles et al., 2009; Young et al., 2014), the role of MHC II was not known yet. *Bufo gargarizans* was suggested to be resistance species before (Bataille et al., 2015; Bataille et al., 2013) proved to be a resistant species in my previous study (Chapter 2), MHC II genotyping was performed based on TCBS (Bataille et al., 2015), thus, it is necessary genotype *B. gargarizans* (resistant) again, along with *L. caerulea* (susceptible), using NGS and more sophisticated genotyping methods (e.g. Sommer et al. 2013) in order to accurately assess whether MHC II is associated with Bd resistance.

In the study, I used Illumina next generation sequencing to genotype the MHC II ß1 chain in *L. caerulea* from New Guinea and *B. gargarizans* from South Korea. Next, I analyzed overall genetic diversity of MHC II ß1, including peptide binding sites (PBR), in both species in order to understand the association of MHC II to host susceptibilities *de-novo*. Tests for selection were conducted at the MHC II in both species to verify whether purifying or balancing selection is acting on *B. gargarizans*, and whether positive selection is acting on *L. caerulea.* Genetic variants were also compared among 38 *L. caerulea* (only one was resistant to Bd) as well as with 35 *B. gargarizans* in order to identify potential MHC II genotypes associated with Bd resistance. Moreover, phylogenetic trees were constructed to assess MHC II variants lineages and any possible variant clusters that include potential ancestral conservation or possible convergent evolution at the MHC II region.

4.2 Methods

4.2.1 Samples collection

L. caerulea were obtained from a commercial wild animal distributor in South Korea. These animals were documented as wild-caught in New Guinea in November and December 2016. *B. gargarizans* were collected in February 2018 from Jeonju, South Korea, with permission from the necessary government agencies of South Korea. All animals used for this study were also for other studies, no animals we sacrificed specifically for this part of the project. All the liver or spleen samples from *L. caerulea* (N = 37) were collected from animals that died during the previous infection experiment (Chapter 2) with permission from the Institute of Laboratory Animal Resources (permit ILAR-17-04-118) and the Institutional Biosafety Committee (permit SNUIBC-R170502-1) of Seoul National University. All the liver samples from *B. gargarizans* (N = 35) were harvested from animals that are presumed to have died naturally. A total of two *L. caerulea* survived in the previous BdGPL infection experiment (Chapter 2), one individual, Lit24, was sacrificed for separate experiment unrelated to this project. The other surviving frog, Lit40, was still alive as of 5 December, 2018, therefore a buccal swab was collected.

4.2.2 RNA isolation

All organs or buccal swab were freshly harvested from each dead individual and stored in RNA*later*™ Stabilization Reagent (Qiagen) before isolating RNA. Total RNA from each sample was isolated using either TRIzol reagent (Favorgen Biotech Corp) or Qiagen RNeasy mini kits according to manufacturer's instructions.

4.2.3 MHC II genotyping by cloning based sequencing

RACE (rapid amplification of cDNA ends) PCR was used for amplification of the full length of cDNA from mRNA based on a short known sequence (Scotto-Lavino et al., 2006). This method is remarkably useful for genotyping from non-model organisms. I used four RNA samples from four individuals to generate primers from exon 1 (49F) and exon 3 (KJR3) of MHC II in *L. caerulea* by RACE. And then I used primers 49F and KJR3 for amplification of DNA encoding MHC II ß1 (part of exon 1, full exon 2 and part of exon3, 392bp) for all individuals. KJR3 was designed based on 3' RACE, and 49F was designed based on 5' RACE. The primers used in this study are listed in Table 4.1. The PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 15 s at 58 °C, 50s at 72 °C, and a final extension at 72 °C for 5 min. PCR was run in a Verity 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The detailed methodology are as followings.

First, MHC II exon 2 sequences of *Litoria verreauxii alpina* were aligned in Geneious V7.1.4 used for designing primer, KJF1, KJF2 (Kearse et al., 2012). The 1st strand cDNA was synthesized with Qt using TAKARA PrimeScript™ 1st strand cDNA Synthesis Kit. Partial exon 2 and entire exon 3, exon 4 was successfully amplified with 3' RACE using KJF1 and Qo. Then a second PCR was performed using KJF2 and Qi, using TAKARA ex taq polymerase. The acquired MHC II fragments were purified, ligated to TA vector followed the instructions of RBC T&A cloning vector kit (RBC Bioscience, Taipai, Taiwan) and transformed to DH5a competent Escherichia coli cells (RH617, RBC Bioscience, Taipei, Taiwan). Cells were grown on LB agar plates for

16-18 h at 37 ℃, and blue-white screening was used to select white colonies which were supposed to be positive transformants. About 16-20 clones per individual were picked with correct sizes for sequencing using Applied Biosystems 3730xl DNA Analyzer (Macrogen, South Korea) with M13F and M13R primers. The sequences were confirmed as MHC II by NCBI BLAST.

Based on the sequences obtained from 3'RACE, the reverse primers KJR1, KJR2, KJR3 from exon 3 were designed for 5' RACE. After first strand cDNA was synthesized with KJR1, a poly (A) was added to the of 5' end of the new first-strand cDNA. All of exon 1 and about half of exon 2 were amplified by another round of nested PCRs. With first PCR using KJR2 and Qt, second PCR using KJR3 and Qi, followed by the same cloning procedure, the exon1 region was sequenced and a forward primer was designed from exon 1:49F. The PCR conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 15 s at 58 °C, 2min at 72 °C, and a final extension at 72 °C for 5 min.

For *B. gargarizans*, the same procedure was followed as above, and BugaF and BugaR were generated to amplify the entire exon 2 and part of exon 3 of MHC II (a total of 340bp).

4.2.4 MHC II genotyping by next generation sequencing

Although I sequenced about 20 clones for each individual, variant validation was still difficult as both species appear to exhibit copy number variation in the MHC II. Thus, accounting for PCR, cloning and sequencing error, 20 clones was not sufficient to attain the required statistical power (> 0.95) needed to validate alleles.

False positives can occur due to mutation during cloning or during PCR, including the common generation of chimeric sequences (Kanagawa, 2003; Lenz and Becker, 2008). Therefore, in order to obtain a more reliable result with higher statistical confidence, I completed three independent PCRs for each sample from both *L. caerulea* (49F and KJR3) and *B. gargarizans* (BugaF and BugaR). These amplicons were then sequenced using Illumina chemistry at GENEWIZ (Suzhou, China). The PCR conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 15 s at 58 °C, 50s at 72 °C, and a final extension at 72 °C for 5 min. PCR was run in a Verity 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). Bar codes were added to both forward and reverse primers. Amplicons were then run on a gel for concentration estimates and then pooled to roughly equal molar amounts for later purification and Illumina library construction at Genewiz.

The purified PCR products first passed quality control test using Qubit® dsDNA HS, then after constructing library, QC was performed again. The amplicons were sequenced in both forward and reverse direction. Each step was under strict monitoring and QC to guarantee the NGS results. A total of 2,439,408 reads were generated, and each amplicon had an average of 12000 reads, nearly 600 times more reads than sequencing 20 clones in traditional genotyping.

The allele verification method followed 1) de-multiplex the raw data from company using jMHC to extract only the matched sequence between forward and reverse primers for each read (Stuglik et al., 2011), 2) extract sequences only with precise read of both barcodes, 3) remove singletons, 4) remove sequences that

differed by only one or two bases from putative alleles, 5) remove PCR chimeras, as described by Sommer et al. (2013). The final allele validation for each individual was to compared with one or two replicates (from independent PCR products) for each individual. Only alleles that appeared repeatedly in two or three replicates were regarded as true alleles (i.e. validated variants).

Table 4.1 Primers used for MHC II genotyping in *L. caerulea* and *B. gargarizans.*

Primer		Sequences (from 5' to 3')	Reference
Qt		CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TTT TGC	Scotto-Lavino et al., 2006
Qo		CCA GTG AGC AGA GTG ACG	
Qi		GAG GAC TCG AGC TCA AGC	
KJF1	Forward primer	TCA CAG ATG TCA GAG ACT TCG T	This study
KJF2	Forward primer	GTC AGA GAC TTC GTA GWA GC	
KJR1	Reverse primer	CTG CCC ATT CCG GTA CCA TT	
KJR2	Reverse primer	TCC GGT ACC ATT TCA CCT CG	
KJR3	Reverse primer	AGG AGG ATG TGC TGC TTT GT	
49F	Forward primer	GGC TCG CTT CTT GCA TCT TAC	
M13F	Forward primer	GGT TTT CCC AGT CAC GAC G	
M13R	Reverse primer	TCA CAC AGG AAA CAG CTA TGA C	
BugaF	Forward primer	TGC AGA TTG TCA GAT GCC GT	
BugaR	Reverse primer	TGC TTG GCT CTT CAT GTC GA	

4.2.5 Amino acid structure comparison and PBR annotation

Validated MHC II variants from both species were aligned using MUSCLE (Geneious V. 11.0), translated and BLASTed in NCBI for confirmation as MHC II sequences. The PBR was annotated by comparing the PBR of HLA DR1 in *Homo sapiens* (accession number: D50889.1) and MHC II in *Falco peregrinus* (accession number: EF370947.1) (Alcaide et al., 2007; Brown et al., 1993a). However, the PBR in amphibians might be different than that of human (Eimes et al., 2015). Therefore, a Wu-Kabat amino acid variability analysis (Wu and Kabat, 1970) was performed to further investigate codons that may also be under selection. If the Wu-Kabat variability value of amino acids was twice or more than the average for that site within species, then that codon was regarded as a potential site (denoted as WuKa site) under selection (Bos and Waldman, 2005; Wu and Kabat, 1970).

4.2.6 Selection tests

PBR or WuKa sites in MHC II in species (*B. gargarizans*) resistant to Bd were assumed to be positive selection, likely some form of balancing selection. *De novo* PBR sites that were not variable in *B. gargarizans* are likely subject to purifying selection, as these sites are usually highly polymorphic in other vertebrate taxa (Sommer, 2005). In the susceptible species (*L. caerulea*), I expected to find more polymorphism at *de novo* PBR sites as well as more WuKa sites, as this species is likely under more intense selective pressure from Bd than the resistant Asian species. To test this hypothesis, four tests were carried out on: complete DNA sequences, PBR sites only, non-PBR sites only, WuKa sites, and non-WuKa sites for both species using Mega v. X (Kumar et al., 2018).

First, Tajima's neutrality test was performed to test nucleotide diversity and mutation rate (Tajima, 1989). Second, a codon-based Z test of neutrality (HA1: dN=dS) was performed to compare the numbers of synonymous (dS) and nonsynonymous substitutions (dN) using the Nei-Gojobori method (Nei and Gojobori, 1986). The variance of the difference was computed using the bootstrap method (500 replicates). Third, single likelihood ancestor counting (SLAC) was performed using a maximum likelihood ancestral state reconstruction and minimum path substitution counting to estimate site - level dS and dN, and applies a simple binomial-based test to test if dS differs from dN in HyPhy (Hypothesis Testing using Phylogenies) package (Kosakovsky Pond and Frost, 2005). The value of dN-dS generated by this test can used for detecting codons under selection and P value for neural evolution null hypothesis is calculated (if P is less than 0.1, the site was regarded as significant positive selected sites) (Kosakovsky Pond and Frost, 2005; Suzuki and Gojobori, 1999). This method is especially applicable for large samples compared to empirical or hierarchal Bayesian approaches (Yang et al., 2000), although it has risks to underestimate the substitution rate (Kosakovsky Pond and Frost, 2005). Finally, a second Z-test was carried out to test for positive selection (HA = dN > dS). This method provides a P value for positive selection and is thus a common method to test for selection.

4.2.7 Phylogenetic trees construction

Phylogenetic trees derived from the entire MHC II ß1 fragments, the concatenated PBR and WuKa sites from both *L. caerulea* and *B. gargarizans* were constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The

bootstrap consensus tree inferred from 500 replicates reflects the evolutionary history of the MHC II in the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (i.e. bootstrap values) (Felsenstein, 1985). The evolutionary distances were computed using the P value method (Nei and Kumar, 2000) and are in the units of the number of base substitutions per site. This analysis involved 102 nucleotide sequences. All positions with less than 100% site coverage were eliminated, i.e., fewer than 0% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 267 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018) and FigTree v 1.4.4 (Rambaut, 2012) was used to construct radial trees. The same methods were used for constructing amphibian MHC II ß1 tree after downloading all available MHC II DNA sequences (relating to this study) from NCBI.

4.2.8 Statistics

To compare the difference of allele number and Wu-Kabat amino acid variability value in *L. caerulea* and *B. gargarizans*, I used one-way analyses of variance (‘aov’ function). All computations were made with R ver. 3.3.1 v (R Foundation for Statistical Computing, Vienna, Austria).

4.3 Results

4.3.1 NGS has distinct advantages in amphibian MHC genotyping

An average of 3 -12 MHC II ß1 alleles were identified in both species using NGS with at least two PCR replicates (Table 4.2). This is in striking contrast to the 1-2 alleles previously identified using single PCR-cloning methodology; statistical significance increased fivefold when applying NGS over cloning (Table 4.3) ($F_{1,26}$ = 32.12, P < 0.0001). NGS identified many more variants that traditional cloning, thus improving the entire analysis of this study (Table 4.2).

4.3.2 *L. caerulea* smd *B. gargarizans* have a similar number of alleles per individual as well as copy number variation (CNV) at MHC II ß1

In total, 57 alleles were identified from 39 samples (37 frogs) in *L. caerulea* (297-300bp) and 39 alleles (270-273bp) identified from 35 samples in *B. gargarizans* using NGS sequencing and improved bioinformatics. Although the average number of alleles that each frog possessed (Number of total alleles/Number of frogs = 1.46) in *L. caerulea* was slightly higher than that of *B. gargarizans* (1.11), each individual in the two populations possessed a similar number of alleles (Table 4.4), as the difference was not statistically significant ($F_{1, 72}$ = 1.109, P = 0.296).

Detailed information of verified alleles in each sample are shown in Table 4.5 for *L. caerulea*, and Table 4.6 for *B. gargarizans*. Both species exhibited CNV ranging from 3 - 12 alleles (Table 4.5, Table 4.6). Alleles were not distributed equally in either population. In *L. caerulea*, the LicaeII7 allele (300 bp) had highest abundance (N = 36) and LicaeII8 (N = 26) (297 bp) ranked second, and 75.4% alleles (N = 43) were

unique to different individuals (Table 4.5, Fig 4.1a). In *B. gargarizans*, BugaIIB3 (N = 34) ranked highest, and BugaIIB1 (N = 33) ranked second, and 82.05% alleles (N=28) were unique to individuals (Table 4.6, Fig 4.1b).

Table 4.2 MHC II genotyping comparison in *L. caerulea* between TCBS and next NGS.

	Cloning based sequencing			Next generation sequencing					
				Repeat 1		Repeat 2			
ID	Total read	Uniques	Repeated sequences	Total uniques	After deleting singlets	Total uniques	After deleting singlets	Alleles	Identical alleles
Lit19	16	11(1pd)	2(3,3)*	1037	80	1985	170	5	0
Lit20	20	11	1(10)	1922	271	2770	336	6	1
Lit21	16	8	2(7,3)	1034	38	1283	75	9	0
Lit22	20	6	1(14)	1855	297	3170	545	12	1
Lit23	23	11	2(8,2)	2879	449	3386	554	10	1
Lit24	21	13	2(6,4)	2017	374	2917	459	9	1
Lit25	20	15(1 pd)	1(5)	1462	334	1668	374	3	0
Lit26	19	7	1(11)	1577	341	2886	561	3	1
Lit27	20	11	1(8)	3095	566	3806	682	10	1
Lit28	23	12	1(12)	1419	223	3337	730	5	1
Lit29	17	5	2(9,3)	3564	576	4110	641	4	1
Lit30	20	10	1(10)	1167	250	1492	309	4	0
Lit31	21	13	2(6,4)	1718	370	1786	357	4	2
Lit32	20	11	1(10)	653	126	1468	344	3	1

* stands for the number for each repeat.
pd, pseudogene.

Table 4.3 MHC II genotyping results comparison between TCBS and NGS in *L. caerulea*.

	Min	1st Qu.	Median	Mean±SD	3rd Qu.	Max
TCBS	1	1	1	1.429 ± 0.513	2	2
NGS	3	4	5	6.214 ± 3.118	9	12

TCBS: traditional cloning based sequencing
NGS: next generation sequencing

Table 4.4 Summary of alleles in *L. caerulea* and *B. gargarizans* via NGS.

	Sample sizes	Total alleles	Min	1st Qu.	Median	Mean ± SD	3rd Qu.	Max
L. caerulea	*39*	57	3	4	5	5.615 ± 2.402	6	12
B. gargarizans	*35*	39	3	4	5	5.397 ± 2.159	6	12

SD, standard deviation. Qu, quartile. 1st Qu refers to 25% quantity. 3rd Qu refers to 75% observations below this quantity. Mean, alleles per individual.

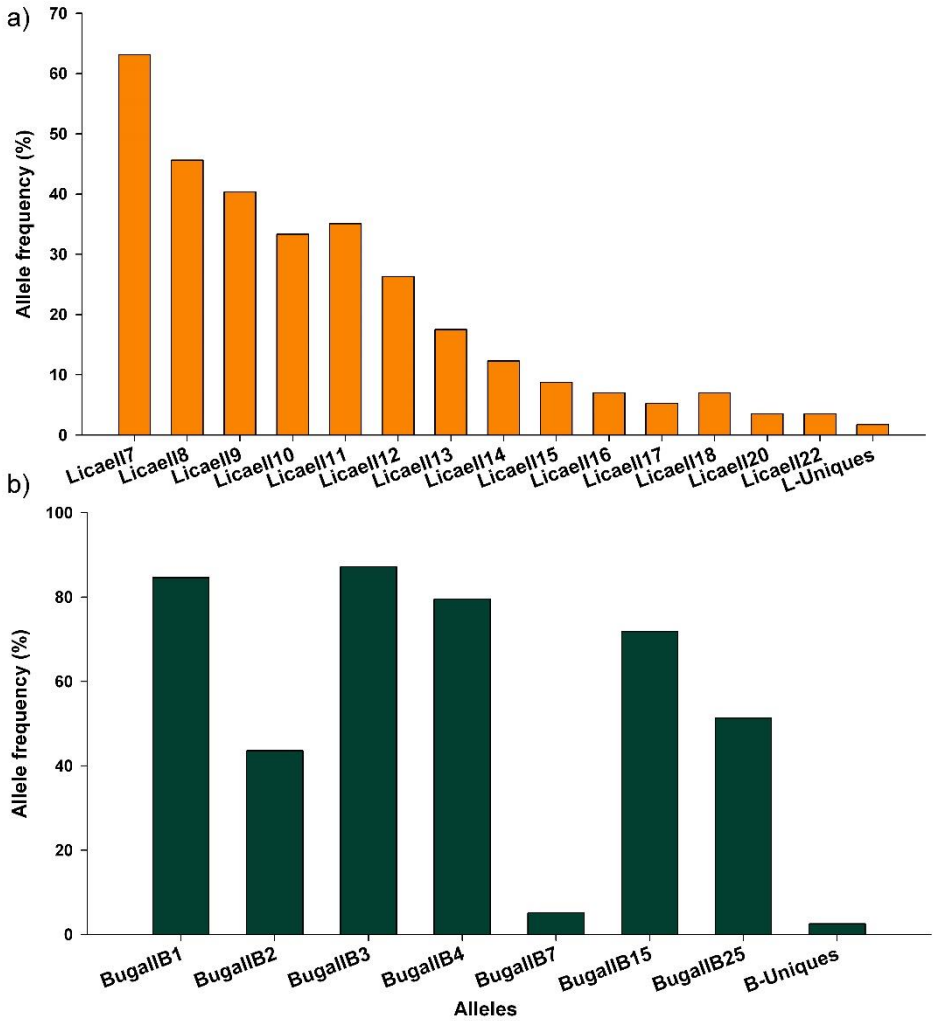

Figure 4.1 Allele frequency in *L. caerulea* and *B. gargarizans*. a) Allele frequency for *L. caerulea*. b) Allele frequency for *B. gargarizans*. L-Uniques represents only once appearance *L. caerulea*. B-Uniques represents once appearance in *B. gargarizans*.

Table 4.5 Genotypes of 39 samples for *L. caerulea*.

ID	NO of alleles	Licae II1	Licae II2	Licae II3	Licae II4	Licae II5	Licae II6	Licae II7	Licae II8	Licae II9	Licae II10	Licae II11	Licae II12	Licae II13	Licae II14	Licae II15	Licae II16	Licae II17
Lit1	4							1	1			1				1		
Lit2	8							1	1	1	1	1	1	1			1	
Lit3	6							1	1		1	1	1				1	
Lit4	6							1	1	1		1		1	1			
Lit5	3							1	1								1	
Lit6	6							1	1	1		1	1					
Lit7	5							1			1	1	1		1			
Lit8	4							1	1	1					1			
Lit9	3								1			1						
Lit10	4							1		1				1	1			
Lit11	5							1	1		1	1		1				
Lit12	3							1	1					1				
Lit13	4							1		1				1				
Lit14	5							1	1	1	1							
Lit15	6							1	1		1	1	1		1			
Lit16	6							1			1		1					1
Lit17	4							1	1	1						1		
Lit18	10							1	1	1	1	1		1				1
Lit19	5							1	1	1	1							
Lit20	6							1	1	1		1						
Lit21	9							1	1	1	1						1	
Lit22	12	1	1	1	1	1	1	1	1	1		1		1				
Lit23	10							1		1	1	1	1					1
Lit24	9							1	1	1					1	1		
Lit25	3								1									
Lit26	3							1		1					1			
Lit27	10							1	1	1	1	1						
Lit28	5							1	1	1		1		1				
Lit29	4							1		1								
Lit30	4								1	1	1							
Lit31	4							1			1	1	1					
Lit32	3							1			1		1					
Lit33	3							1	1							1		
Lit34	6							1			1		1	1				
Lit35	5							1			1	1	1					
Lit36	6							1		1	1	1	1			1		
Lit37	5							1	1	1		1	1					
Lit38	5							1	1	1		1	1					
Lit39	10							1			1		1					
	Total	1	1	1	1	1	1	36	26	23	19	20	15	10	7	5	4	3

Table 4.5 Genotypes of 39 samples for *L. caerulea*.

ID	LicaeII18	Licae II19	Licae II20	Licae II21	Licae II22	Licae II23	Licae II24	Licae II25	Licae II26	Licae II27	Licae II28	Licae II29	Licae II30	Licae II31	Licae II32	Licae II33	Licae II34	Licae II35
Lit1																		
Lit2																		
Lit3																		
Lit4																		
Lit5																		
Lit6			1															
Lit7																		
Lit8																		
Lit9																		
Lit10																		
Lit11																		
Lit12																		
Lit13																		
Lit14			1															
Lit15																		
Lit16	1																	
Lit17																		
Lit18					1													
Lit19															1			
Lit20													1					
Lit21	1															1		
Lit22	1																	
Lit23	1	1			1													
Lit24				1		1	1	1										
Lit25																		
Lit26																		
Lit27																		
Lit28																		
Lit29																		1
Lit30																		
Lit31														1				
Lit32																		
Lit33																		
Lit34											1							
Lit35																	1	
Lit36																		
Lit37																		
Lit38																		
Lit39									1	1		1						
Total	4	1	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 4.5 Genotypes of 39 samples for *L. caerulea*.

ID	Licae II36	Licae II37	Licae II38	Licae II39	Licae II40	Licae II41	Licae II42	Licae II43	Licae II44	Licae II45	Licae II46	Licae II47	Licae II48	Licae II49	Licae II50	Licae II51	Licae II52	Licae II53	Licae II54	Licae II55	Licae II56	Licae II57
Lit1																						
Lit2																						
Lit3																						
Lit4																						
Lit5																						
Lit6																						
Lit7																						
Lit8																						
Lit9										1												
Lit10																						
Lit11																						
Lit12																						
Lit13								1														
Lit14																						
Lit15																						
Lit16													1									
Lit17																						
Lit18																				1	1	
Lit19																						
Lit20					1																	
Lit21											1					1						
Lit22																						
Lit23																		1				
Lit24																						
Lit25			1												1							
Lit26																						
Lit27		1				1			1					1			1					
Lit28																						
Lit29	1																					
Lit30																						
Lit31																						
Lit32																						
Lit33																						
Lit34							1															
Lit35																						
Lit36																						
Lit37																						
Lit38																						
Lit39				1								1							1			1
Total	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 4.6 Genotypes of 35 samples for *B. gargarizans.*

ID	NO of alleles	BugaII B1	BugaII B2	BugaII B3	BugaII B4	BugaII B5	BugaII B6	BugaII B7	BugaIIB 8	BugaII B9	BugaII B10	BugaII B11	BugaII B12
Bg1	3	1		1									
Bg2	5	1	1	1									
Bg3	4	1		1	1								
Bg4	4	1		1	1								
Bg5	4	1		1	1								
Bg6	4	1	1	1	1								
Bg7	5	1		1	1								
Bg8	5	1	1	1	1								
Bg9	4	1		1	1								
Bg10	5	1		1	1								
Bg11	3			1	1								
Bg12	6	1		1	1								
Bg13	5	1		1	1			1					
Bg14	6	1		1	1								
Bg15	5	1		1	1								
Bg16	8	1	1	1	1								
Bg17	5	1	1	1	1								
Bg18	4	1		1									
Bg19	8	1	1	1	1			1					
Bg20	6				1		1						1
Bg21	8	1	1	1	1					1			
Bg22	5	1	1	1	1								
Bg23	4	1		1									
Bg24	5	1	1	1	1								
Bg25	5	1		1	1								
Bg26	5	1	1	1	1								
Bg27	6	1	1	1	1								
Bg28	6	1	1	1	1								
Bg29	6	1	1	1	1								
Bg30	6	1	1	1	1								
Bg31	6	1	1	1	1								
Bg32	7	1	1	1	1								
Bg33	11	1		1	1				1				
Bg34	11	1	1	1	1						1		
Bg35	9	1		1	1	1						1	
	Total	33	17	34	31	1	1	2	1	1	1	1	1

Table 4.6 Genotypes of 35 samples for *B. gargarizans*.

ID	BugalIB 13	BugalI B14	BugalI B15	BugalI B16	BugalI B17	BugalI B18	BugalI B19	BugalI B20	BugalIB 21	BugalI B22	BugalI B23	BugalI B24	BugalI B25
Bg1			1										
Bg2											1		
Bg3			1										
Bg4			1										
Bg5			1										
Bg6													
Bg7			1										
Bg8			1										
Bg9			1										
Bg10	1		1										
Bg11													1
Bg12			1			1							1
Bg13													1
Bg14			1						1				1
Bg15			1										1
Bg16			1										1
Bg17													
Bg18			1										1
Bg19			1					1					1
Bg20			1										1
Bg21			1									1	1
Bg22			1										
Bg23			1										1
Bg24			1										
Bg25			1										1
Bg26			1										
Bg27			1										1
Bg28			1										1
Bg29			1										1
Bg30			1										1
Bg31			1										1
Bg32			1										
Bg33		1		1	1								1
Bg34							1			1			1
Bg35			1										1
total	1	1	28	1	1	1	1	1	1	1	1	1	20

Table 4.6 Genotypes of 35 samples for *B. gargarizans.*

ID	BugalI B26	BugalI B27	BugalIB 28	BugalI B29	BugalIB 30	BugalIB 31	BugalI B32	BugalI B33	BugalIB 34	BugalIB 35	BugalI B36	BugalI B37	BugalI B38	BugalIB 39
Bg1														
Bg2													1	
Bg3														
Bg4														
Bg5														
Bg6														
Bg7			1											
Bg8														
Bg9														
Bg10														
Bg11														
Bg12														
Bg13														
Bg14														
Bg15														
Bg16					1			1						
Bg17														
Bg18														
Bg19														
Bg20							1							
Bg21														
Bg22														
Bg23														
Bg24														
Bg25														
Bg26														
Bg27														
Bg28														
Bg29														
Bg30														
Bg31														
Bg32		1							1					
Bg33				1						1				
Bg34	1										1	1		
Bg35						1								1
total	1	1	1	1	1	1	1	1	1	1	1	1	1	1

4.3.3 Differential gene expression patterns of MHC IIß1 between *L. caeruela* and *B. gargarizans*

Since the sequencing results were generated from mRNA, the reads should reflect relative gene expression levels. Genotyping revealed several interesting patterns. First, in general, one or two alleles were usually dominant in an individual (reads > 1000) while two or more validated alleles were expressed at a much lower level. For example, Lit8 had two dominant alleles, LicaeII7 (3086 reads) and LicaeII14 (2597 reads), yet also expressed other alleles (LicaeII8 and LicaeII9) to a much lower degree (reads = 2) (Table 4.7, Fig 4.2).

Second, the same allele of MHC II was differentially expressed between different individuals within species. For example, the most abundant allele, LicaeII7 dominated in most of the samples as shown in Fig 4.2, but its expression was largely inhibited in Lit14 (reads = 2). This observation further supports the idea that indicated low read count (e.g. read = 2), is common with "true" alleles. This observation would have been missed using traditional cloning methods. In addition, some alleles, such as LicaeII20 were dominantly expressed in Lit14, but were only found in two individuals.

There were six samples (Lit8, Lit9, Lit10, Lit11, Lit16, Lit18) from three individuals (frog37, frog45, frog40). Lit8 and Lit10 were from the same individual (frog37) but different organs (liver and spleen, respectively), and they exhibited different genotypes (Table 4.8). Lit9 and Lit11 were also from different organs but same individual, frog45. Spleen tended to express more alleles than liver for the same individuals in *L. caerulea*, but mostly dominant alleles were shared in liver and

spleen for same individual except allele LicaeII8 in Lit9 (2903 reads) and Lit11 (4 reads) (Table 4.7).

Frog37 and frog45 were in the control group of my previous infection experiment for transcriptome analysis, and lit14 to lit18 were in Bd infected groups with different durations of infection (Table 4.7). There were also samples from the previous infection experiment for comparing virulence of different Bd lineages (Chapter 2). Among these, 19 individuals (Lit19, Lit20, Lit21, Lit22, lit25, Lit26, Lit27, Lit28, Lit 29, Lit30, Lit31, Lit32, Lit33, Lit34, Lit 35, Lit36, Lit37, Lit38, Lit39) died of Bd infection, while one survived from Bd infection (Lit24) which was considered as resistant individual. I compared relative gene expression corresponding to Bd treatment, however, there was no obvious trend observed in control samples and Bd treated samples (Table 4.7, Fig 4.1), nor in the resistant individual (Lit24) or among the five confirmed susceptible individuals (Fig 4.3). Lit24 has four unique alleles (LicaeII21, LicaeII23, Licae24, Licea25) (Table 4.5, Fig 4.3), which might be related to Bd resistance. However, only one resistant individual was genotyped via NGS. The other resistance survivor (Lit40) was genotyped through TCBS. LitcaeII58 to LitcaeII63 alleles were also unique compared to all other alleles (Fig 4.4).

In *B. gargarizans*, three pairs of mating *B. gargarizans* were collected from the wild in Jan. 2018. Interestingly, each pair (all RNA from liver) had different MHC II genotypes indicating that they may disassortatively mate with regard to MHC II genotype or prefer specific alleles that are more highly expressed in the mate versus themselves (Table 4.9).

Table 4.7 Relative gene expression of different alleles in different organs with or without Bd treatment in *L. caerulea*.

Treatment	Original ID - source	Current ID	LicaeII 7	LicaeII 8	LicaeII 9	LicaeII 10	LicaeII 11	LicaeII 12	LicaeII 13	LicaeII 14	LicaeII 15	LicaeII 17	LicaeII 19	LicaeII 20	LicaeII 22	LicaeII 48	LicaeII 55	LicaeII 56
5D - Con	37 - liver	Lit8	3086	2	2					2597								
	37 - spleen	Lit10	1706		13				3	1206								
	45 - liver	Lit9	2903	3			2											
	45 - spleen	Lit11	4	5461		9	17		7									
5D - Bd	4 - liver	Lit14	2	19	4	1042								1406				
10D - Bd	25 - liver	Lit15	24	3		9	2650	2028		2								
	36 - spleen	Lit17	8	23	2						636							
	40 - liver	Lit16	1863			1604		9				2	2			3		
	40 - spleen	Lit18	2234	2	4	2435	5		7			4			3		2	4

I applied the value of reads as the relative gene expression for each allele in different samples. Con, control. D, day.

Table 4.8 MHC genotypes in different organs in three *L. caerulea*.

Original ID	Sample source	Current ID	No of alleles	Expressed alleles
37	Liver	Lit8	4	LicaeII7, LicaeII8,LicaeII9, LicaeII14
	Spleen	Lit10	4	LicaeII7, LicaeII9,LicaeII13, LicaeII14
45	Liver	Lit9	3	LicaeII8, LicaeII11,LicaeII45
	Spleen	Lit11	5	LicaeII7,LicaeII8,LicaeII10, LicaeII11, LicaeII13
40	Liver	Lit16	6	LicaeII7, LicaeII10, LicaeII12, LicaeII17, LicaeII18, LicaeII48
	Spleen	Lit18	10	LicaeII7, LicaeII8,LicaeII9, LicaeII10, LicaeII11, LicaeII13, LicaeII17, LicaeII22, Licae56, Licae57

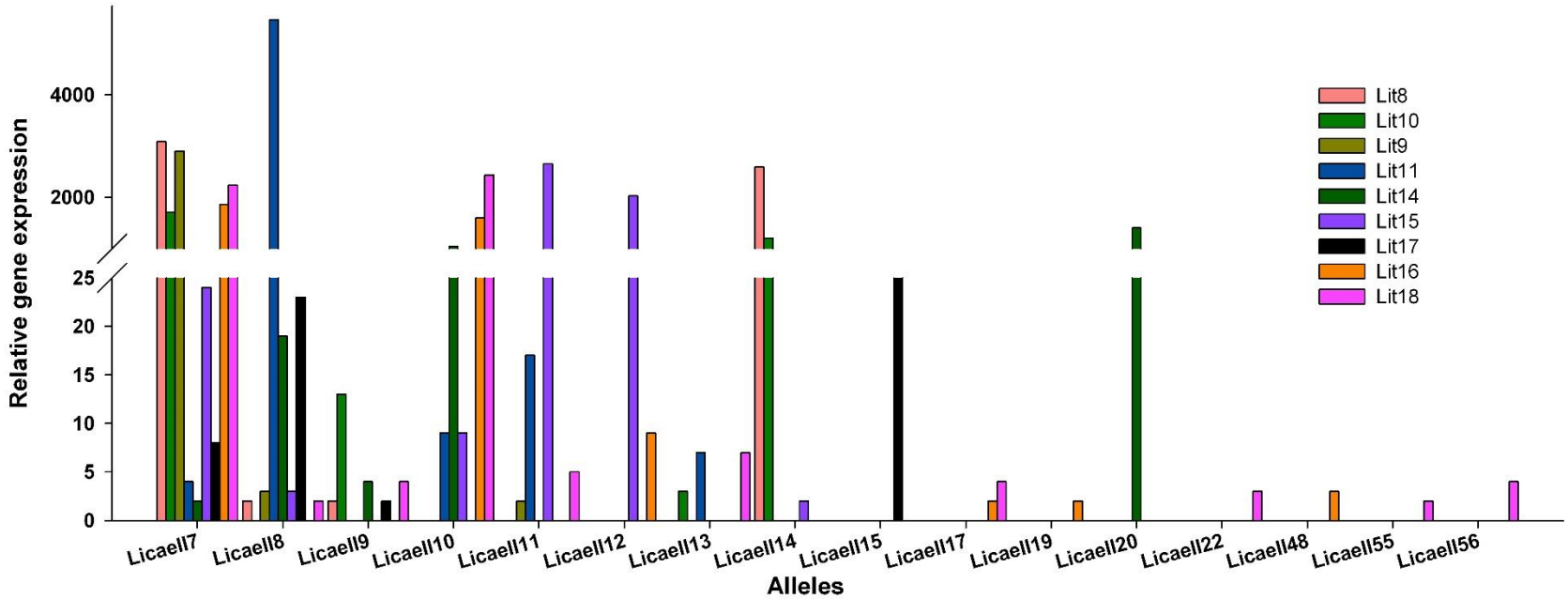

Figure 4.2 Comparison of relative gene expression of different alleles with or without Bd treatment in *L. caerulea*. The relative gene expression, I applied the value of reads for each allele in different individuals. Li8, Lit9, Lit10, Lit11, were in control group while the others were in Bd treated group.

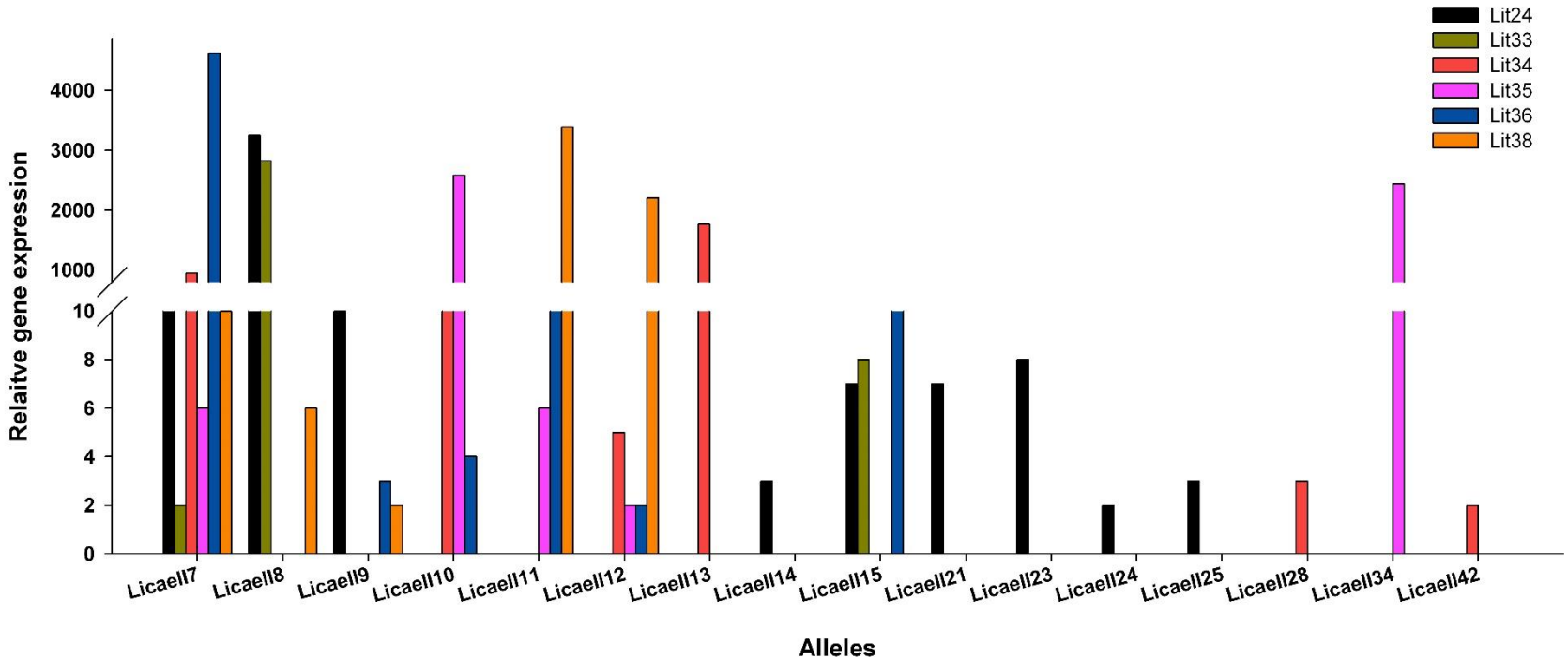

Figure 4.3 Comparison of relative gene expression of different alleles in survived and dead individual from Bd infection in *L. caerulea*. The relative gene expression, I applied the value of reads for each allele in different individuals. Lit24 survived from Bd infection, while the other five individuals died from Bd infection.

Table 4.9 MHC II genotypes and relative gene expression in mating *B. gargarizans*.

Original ID	Current ID	No of alleles	Expressed alleles							
			BugaII B1	BugaII B2	BugaII B3	BugaII B4	BugaII B7	BugaII B15	BugaII B20	BugaII B25
Pair 1	Bg9	4	23		3955	6		51		
	Bg24	5	37	1190	4570	73		54		
Pair 2	Bg15	5	2021		1694	2		96		5
	Bg19	8	14	13	964	41	63	2218	16	2
Pair 3	Bg25	5	1168		33	7		1478		8
	Bg26	5	17	10	4	24		1990		

4.3.4 MHC IIB is under strong balancing selection in both species, but some sites were under strong positive selection in *L. caerulea*

Although the MHC II ß1 chain of the other resistant *L. caerulea* (Lit40) that survived infection was genotyped via TCBS, two batches of independent PCR were performed and there was one consistent allele, LicaeII58. LicaeII59 and LicaeII60 had two reads independent single PCR. Although LicaeII61, LicaeII62, LicaeII63 had single reads from limited sample sizes, the variation observed could encode unique amino acids, and therefore might be maintained by balancing selection. A total of 63 alleles were found in *L. caerulea* from 38 individuals and were used for further selection tests.

The 291bp (N = 14) and 294 bp (N = 49) allele nucleotide sequences of *L. caerulea* and 270 (N=34) and 273 bp (N = 5) alleles of *B. gargarizans* were used for selection tests. While amino acid sequences of *L. caerulea* were trimmed to the same or nearly same length as that in *B. gargarizans*, as human and bird MHC II were used as references (Fig 4.4). there was a near perfect alignment without gaps between frog, bird, human until the last PBR site (site 84), and all of these disparate taxa shared 18 conserved sites (19.8%) (Table 4.10). As expected, identical sites were much higher within species, and *B. gargarizans* (60.9%) had more than that of *L. caerulea* (42.9%).

In total, 23 PBR sites (sites: 4, 6, 8, 23, 27, 33, 32, 42, 51, 55, 56, 60, 63, 65, 69, 73, 76, 77, 80, 81, 83, 84) were denoted for *L. caerulea* and *B. gargarizans* based on HLA PBR (Table 4.11).

There were 18 WuKa sites (Wu-Kabat variability > 2.86; sites: 6, 10, 11, 12, 13, 21, 23, 32, 42, 62, 65, 66, 69, 72, 80, 81, 82, 83) found in *B. gargarizans* while 16 WuKa sites (Wu-Kabat variability > 5.16; sites: 7, 8, 23, 42, 43, 44, 46, 52, 66, 68, 69, 73, 81, 83, 84, 86) found in *L. caerulea* (Table 4.11, Fig 4.5). *L. caerulea* had a higher Wu-Kabat variability than *B. gargarizans* significantly ($F_{1,180}$ = 8.367, P = 0.00429). It had six WuKa sites overlapping with those of *B. gargarizans* (sites: 23, 52, 66, 69, 81, 83) and eight sites with PBR sites (8, 23, 42, 69, 73, 81, 83, 84). Site 23, 69 and 81 were overlapping in both species as PBR and WuKa sites.

Tajima's neutrality test suggested that the MHC II in both *L. caerulea* and *B. gargarizans* were under balancing selection (D > 0) (Table 4.12). The PBR and WuKa sites were under positive selection in the codon-based Z test in *L. caerulea.* Moreover, MHC II exhibited strong positive selection at specific codons (eight codons were detected under positive selection) in *L. caerulea* while no positively selected sites were found in *B. gargarizans* when analyzed by a HyPhy test which tests selection using phylogenies (Table 4.12). This suggests that there is strong positive selection at specific codons in *L. caerulea* compared to *B. gargarizan*s. Among the positively selected sites in *L. caerulea*, four sites were denoted before as PBR sites (4, 23, 33, 69) and five sites were denoted as WuKa sites (7, 23, 44, 52, 69) (Table 4.11, Table 4.13). Two sites (23, 69) among these were overlapping in both methods. More interestingly, site 4, 7, 33, and 44 were completely conserved in *B. gargarizans*, which suggest that these sites might be important for pathogens that have a long history with *B. gargarizans*.

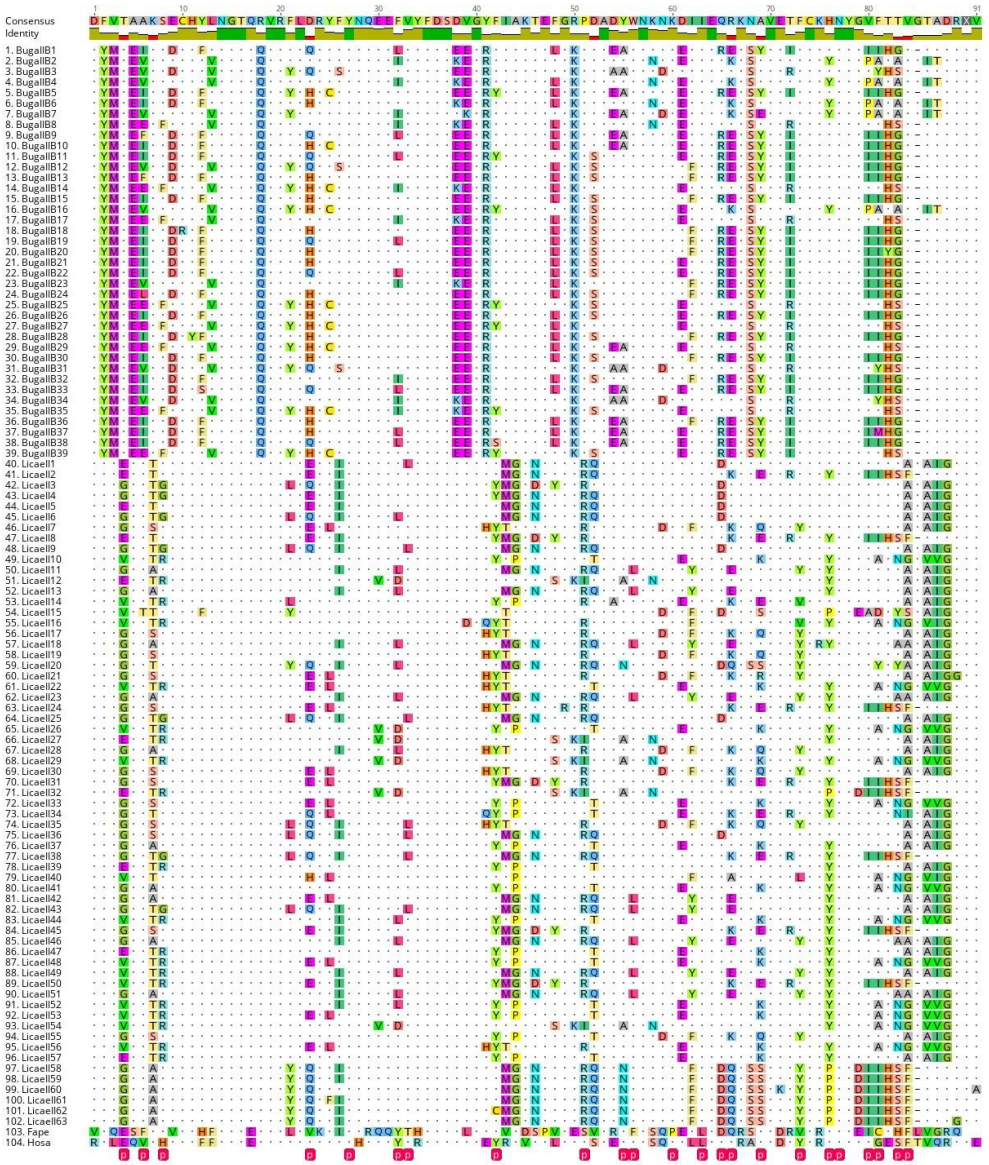

Figure 4.4 Amino acid alignment of MHC II ß1 chain. 1-39 are corresponding translations of each allele for *B. gargarizans*. 40-102 are corresponding trastlations of each allele for *L. caerulea*. Fape, *Falco peregrinus* (accession number: EF370947.1). Hosa, *Homo sapiens* (accession number: D50889.1). Red boxes in the bottom with a “P” inside refer to PBR identifed in HLA.

Table 4.10 Summary of protein statistics.

	Length	Sequences	Identical sites	Pairwise identity	Pairwise positive (BLSM62)	Ungapped lengths		
						Mean ± SD	Min	Max
B. gargarizans	91	39	55 (60.4%)	84.60%	90.80%	90.1 ± 0.3	90	91
L. caerulea	91	63	39 (42.9%)	78.50%	85.50%	90.8 ± 0.4	90	91
All four species	91	104	18 (19.8%)	69.40%	80.50%	90.5 ± 0.5	89	91

Table 4.11 PBR sites and WuKa sites in *L. caerulea* and *B. gargarizans*.

	No of sites	Sites
PBR	23	4, 6, 8, 23, 27, 33, 32, 42, 51, 55, 56, 60, 63, 65, 69, 73, 76, 77, 80, 81, 83, 84
WuKa-L	16	7, 8, 23, 42, 43, 44, 46, 52, 66, 68, 69, 73, 81, 83, 84, 86
WuKa-B	18	6, 10, 11, 12, 13, 21, 23, 32, 42, 62, 65, 66, 69, 72, 80, 81, 82, 83

WuKa-L refers to WuKa variability in *L. caerulea*, and WuKa-B refers to WuKa variability in *B. gargarizans*.

.

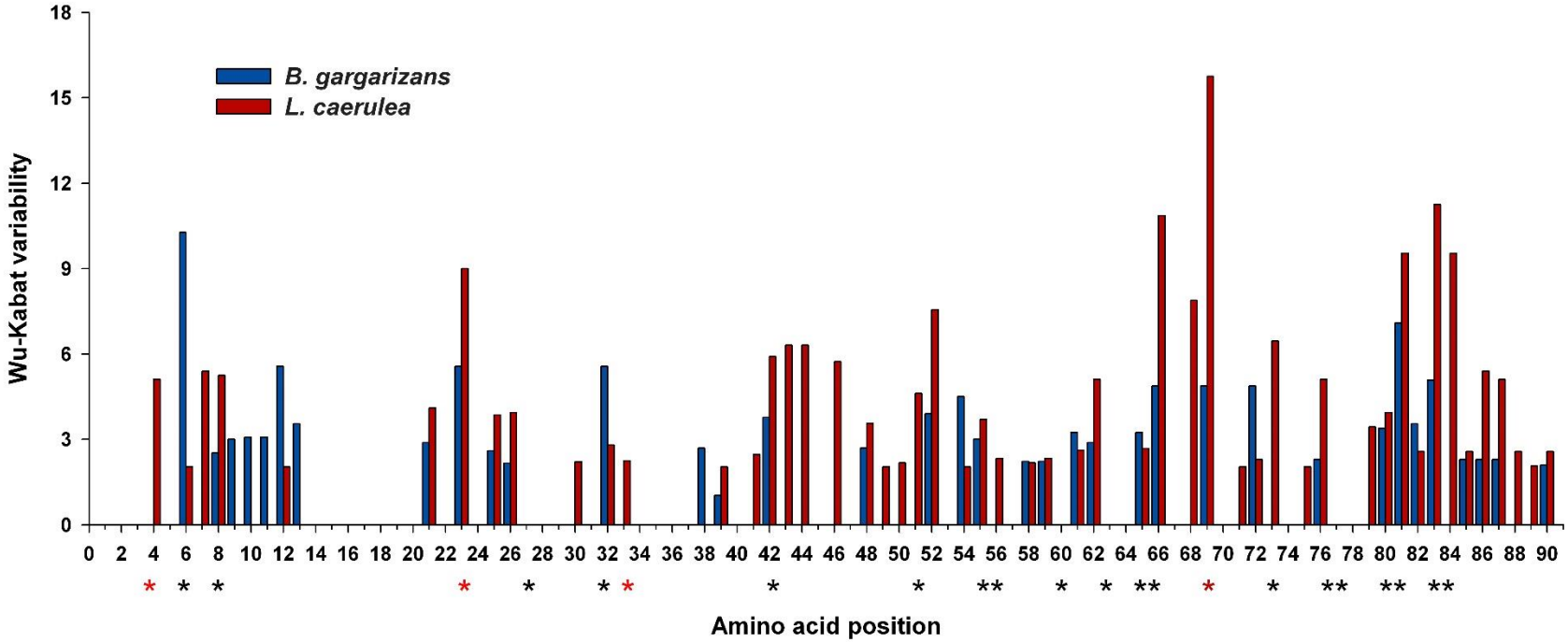

Figure 4.5 Wu-Kabat plot of amino acid sequences of MHC II ßI chain in *B. gargarizans* and *L. caerulea*. Asterisks represent annotated PBR according to human HLA. Red Asterisks stand for sites under positive selection identified using SLAC analysis in HyPhy. PBR sites: 4, 6, 8, 23, 27, 33, 32, 42, 51, 55, 56, 60, 63, 65, 69, 73, 76, 77, 80, 81, 83, 84. PBR positive selection sites: 4, 23, 33, 69.

Table 4.12 Statistics of selection tests for both species.

			Z test	Results From Tajima's Neutrality Test						HyPhy		
		Length (bp)	P	*m*	*S*	*Ps*	*Θ*	*π*	*D*	dN/dS	*Negative selection*	Positive selection
L. caerulea	NonPBR	225	0.35	63	70	0.304	0.065	0.085	1.074	0.436	10	3
	PBR	69	<0.001	63	41	0.586	0.124	0.189	1.715	3.23	0	4
	NonWuKa	243	0.02	63	72	0.296	0.063	0.071	0.432	0.376	8	1
	WuKa	58	<0.001	63	37	0.638	0.135	0.254	2.876	8.24	0	5
	ER	300	0.54	63	122	0.415	0.088	0.127	1.544	0.908	9	8
B. gargarizans	NonPBR	204	0.57	39	35	0.172	0.041	0.051	0.886	1.72	1	0
	PBR	69	0.7	39	33	0.478	0.113	0.188	2.320	1.01	1	0
	NonWuka	225	0.18	39	37	0.164	0.039	0.041	0.171	1.01	1	0
	Wuka	49	0.63	39	32	0.653	0.154	0.287	2.982	3.1	0	0
	ER	273	0.52	39	70	0.256	0.061	0.086	1.545	1.07	4	0

ER, entire region. Neutrality test, HA: dN=dS. *m* = number of sequences, *n* = total number of sites, *S* = Number of segregating sites, $p_s = S/n$, $\Theta = p_s/a_1$, π = nucleotide diversity, and *D* is the Tajima test statistic.

Table 4.13 MHC II ß1 amino acid sites under positive selection by SLAC analysis via HyPhy in *L. caerulea.*

Site1	Site2	dS	dN	dN-dS	P [dN/dS > 1]	P [dN/dS < 1]
3	-3	0	4.494	3.111	0.026	1
10	4	0	6.872	4.757	0.006	1
13	7	0	6.000	4.154	0.008	1
29	23	0	6.341	4.389	0.065	1
39	33	0	3.000	2.077	0.088	1
50	44	0	3.000	2.077	0.088	1
58	52	0	5.963	4.128	0.067	1
72	69	0	4.513	3.124	0.088	1

Site1, the order number represents the entire amplified and translated MHC II amino acid sequences in *L. caerulea* before trimming to the same length with *B. gargarizans*. Site2, the order number represents the amino acids position after trimming to the same length with *B. gargarizans* as shown in Fig 4.3. dN, non-synonymous substitution rate. dS, synonymous substitution rate.

4.3.5 Phylogenetic analysis

Three phylogenetic trees were constructed using entire fragment (Fig 4.6), PBR (Fig 4.7), Wuka sites (Fig 4.8) of MHC IIß1 in both *L. caerulea* and *B. gargarizans*. All MHC II alleles from each species were clustered by species, with no mixed clusters observed. Another tree was constructed which included additional taxa: a Korean species (*Bombina orientalis*), two Australian species (the cane toad - *Rhinella marina* and Alpine tree frog - *Litoria verreauxii alpina*) to the MHC II evolutionary tree, MHC II alleles still clustered by each species and no trans-species polymorphism was observed (Fig 4.9).

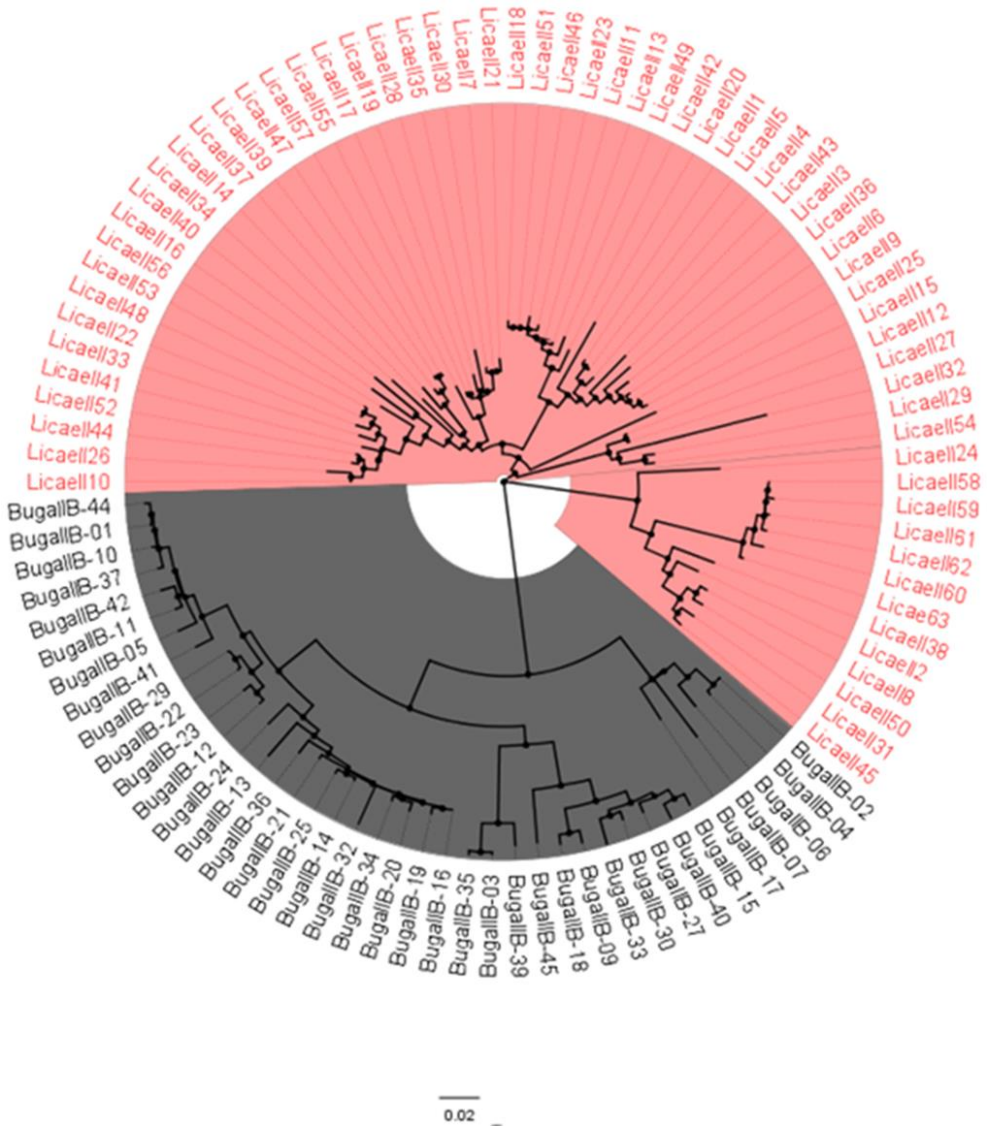

Figure 4.6 Evolutionary relationships of *L. caerulea* and *B. gargarizans* MHC II ß1 alleles using entire DNA sequences. Color indicates species. Red color marked are all alleles from *L. caerulea*. Grey indicates allelels for *B. gargarizans*.

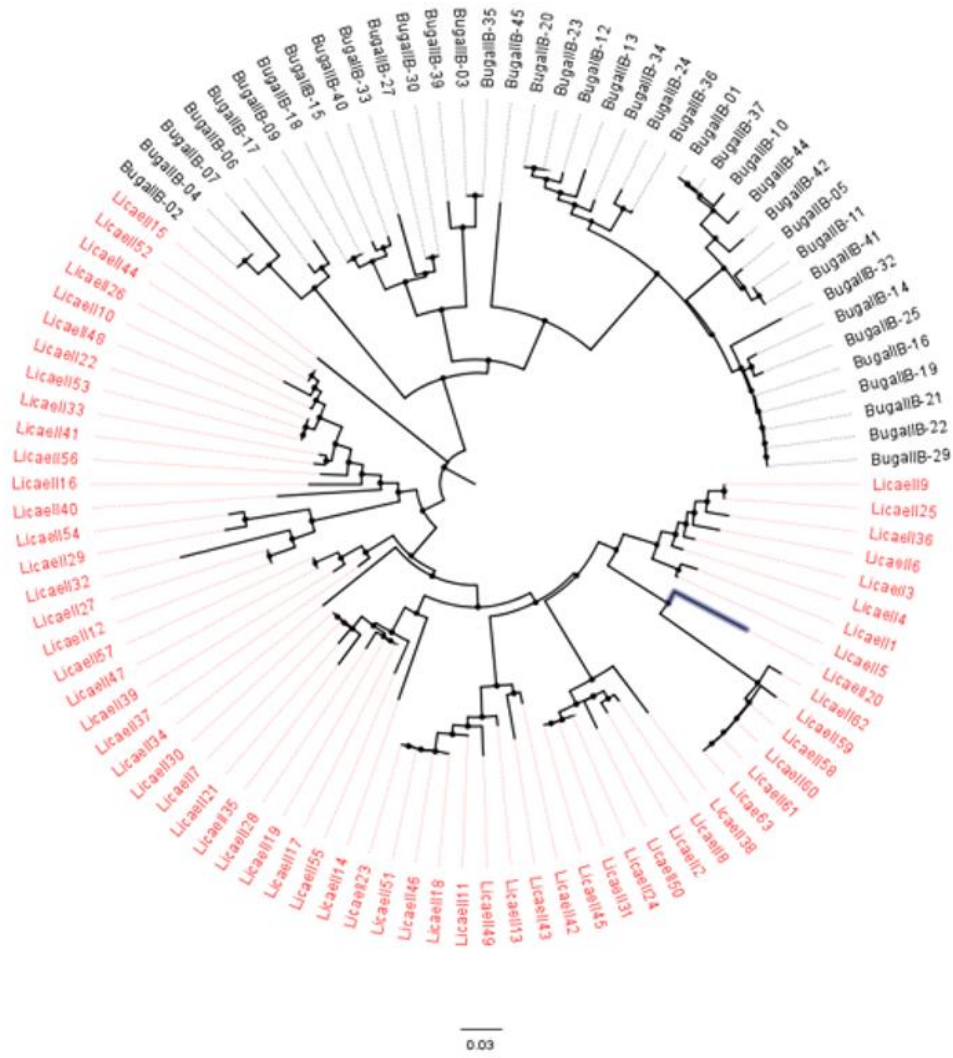

Figure 4.7 Evolutionary relationships of MHC II PBR in *L. caerulea* and *B. gargarizans.*

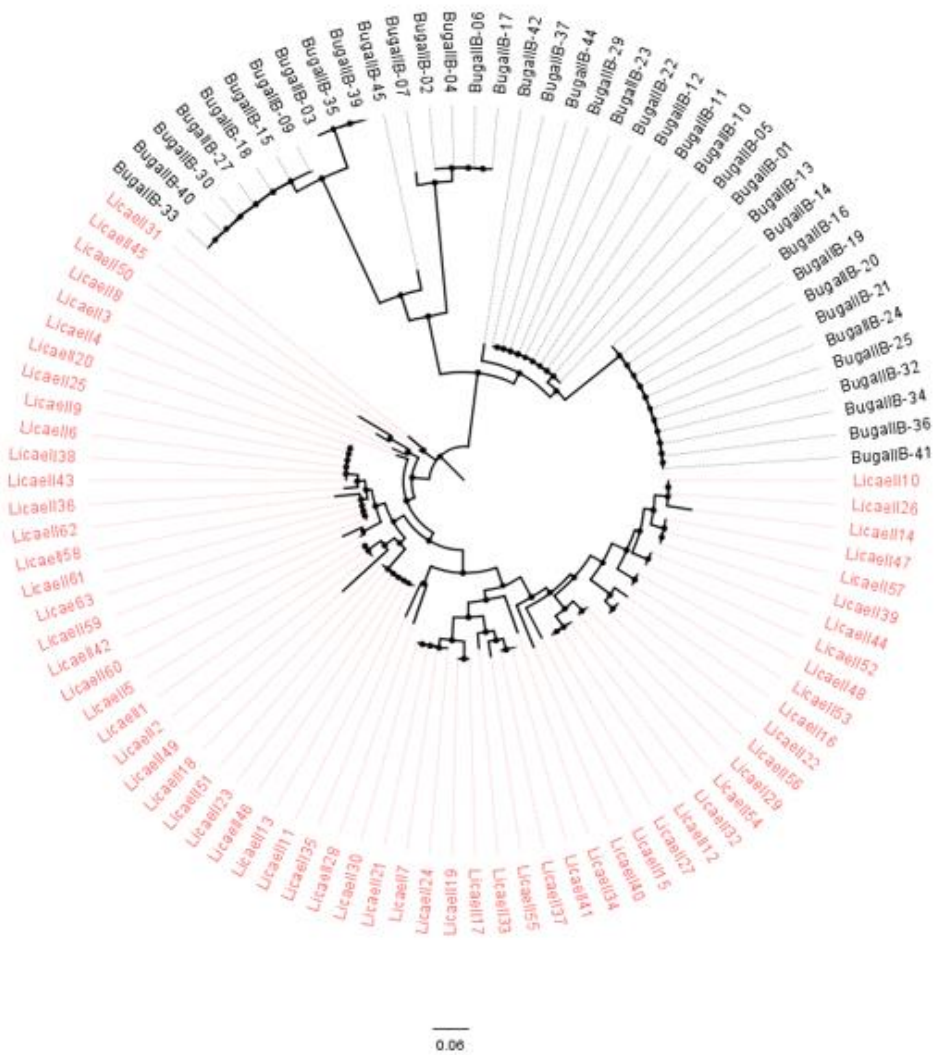

Figure 4.8 Evolutionary relationships of WuKa sites in *L. caerulea* and *B. gargarizans*.

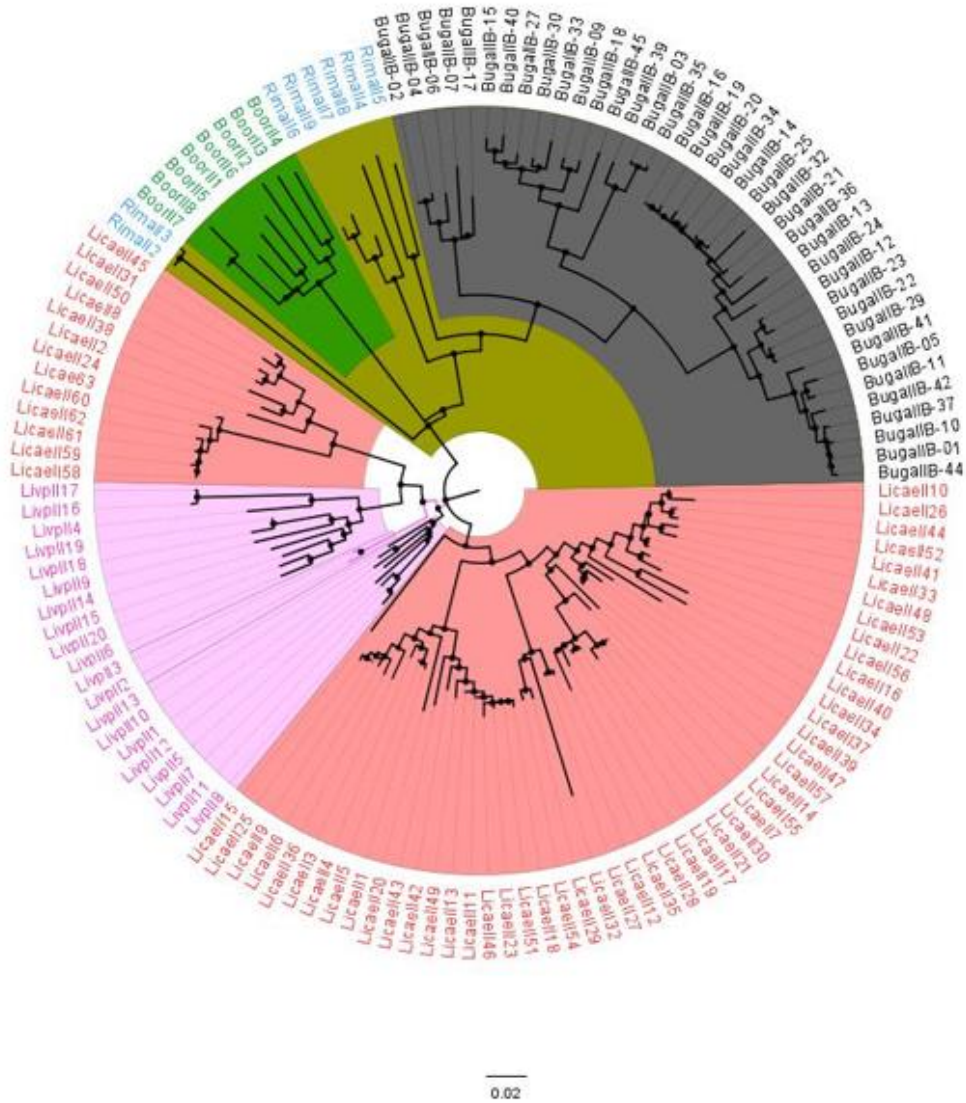

Figure 4.9 Evolutionary relationships of partial amphibian MHC II ß1. The DNA sequences were downloaded from NCBI. Boor, *Bombina orientalis*. Buga, *B. gargarizans*. Rima, *Rhinella marina*. Livp, *Litoria verreauxii alpina.* Licae, *L. caerulea.* Different colors were used for clarify different species clearly.

4.4 Discussion

4.4.1 High MHC II allele variability found in both *L. caerulea* and *B. gargarizans*

This is the first study to genotype MHC IIß1 in *L. caerulea* and apply NGS to compare genetic variation at this locus between resistant and susceptible frog species. In total, 57 MHC IIß1 alleles in *L. caerulea* and 39 alleles in *B. gargarizans* from at least two independent PCR amplicons were observed, and individual MHC polymorphism was much higher than observed in previous studies that used traditional cloning of single amplicons in similar species. NGS based MHC II genotyping in *L. caeruea* identified identical alleles consistently across duplicates and even triplicates, proving that this method is more reliable for genotyping duplicated (CNV) genes such as the MHC.

Each individual expressed 3-12 alleles in both species. Alleles had different relative abundance in both species: some alleles appeared in almost all individuals, and some rare allele appeared only in few individuals. However, it is difficult to establish the number of MHC II loci in both species because both species may exhibit polyploidy, as has been observed in several other amphibian species (Bogart, 1980).

4.4.2 Differential MHC IIß1 expression

This is also the first study using RNA based MHC genotyping with large sample sizes (>30). Therefore, I could compare relative gene expression of each allele at the individual-level and population-level. The results indicated 1) each frog had 1-2 dominant alleles and two or more alleles with inhibited gene expression in

both species, 2) even the most abundant alleles (across all samples) were often found at very low read counts in some individuals, which could indicate inhibited transcriptional levels, 3) no clear expression pattern was found between Bd treated or not-treated frogs in *L. caerulea*, 4) different organs in the same individual appeared to express different alleles, and spleen seemed to express more alleles than liver in *L. caerulea*, and 5) mating preference in *B. gargarizans* might disassortative in order to increase MHC diversity in offspring since all the three pairs possessed different alleles. Even when alleles were shared between mates, the expression patterns of these alleles were often vastly different (e.g. three reads in male, 2000 reads in female). This result is not surprising as it has been observed in other vertebrates such as mice (reviewed by Milinski, 2006). However, the relative gene expression level can only be an indicator of the specific time when their tissue was harvested for NGS, but not represent expression levels when they were mating.

Gene and protein expressions are known to be up or down regulated under different situations during the lifetimes of animals (Oh et al., 2016) and plants (Fu et al., 2014). In the amphibian model, *Xenopus laevis*, MHC Ia gene expression was detected in all developmental stages of tadpoles in lung, gill, and intestine, skin, and then increased dramatically after metamorphosis and were detected in most adult tissues, including stomach, lung, liver, skin, intestine, spleen, and thymus, but not in colon; while MHC Ib gene expression was only detected in adults and at relatively lower and more restricted levels compared to class Ia (Salter-Cid et al., 1998). MHC II molecules were detected only in the surface of B cells, macrophages, spleen reticulum, thymus epithelium, the pharyngobuccal cavity in tadpoles, but were

detected in all T cells of adult Xenopus (Du Pasquier and Flajnik, 1990). However, allelic-level based gene expression of MHC I and MHC II in amphibian is not well studied, especially regarding susceptibility to chytridiomycosis.

In human, transcriptional differentially regulated protective, neutral and suppressive MHC II alleles have been identified and suggested to be associated with disease resistance (Baumgart et al., 1998; Guardiola et al., 1996; Mitchison and Brunner, 1995; Reich et al., 1989; Thomson et al., 1988). Allelic-level gene expression of MHC might be associated with suppressive and protective functions of MHC in amphibian diseases as well.

With regard to the association of MHC variants and Bd resistance, differential gene expression of MHC II alleles was observed in Bd treated and untreated individuals in both *L. caerulea* and *B. gargarizans*, as well as confirmed susceptible and resistant individuals in *L. caerulea*. However, there was no obvious trend regarding Bd resistance and MHC genotype, likely due to low sample sizes. Transcriptional expression of immunes genes other than MHC in each individual is unknown; therefore, it is difficult to assess the role MHC II plays in resistance to chytridiomycosis. Moreover, it is impossible to compare MHC II expression levels in living and dead frogs at likely sites of action, such as the liver and spleen. In addition, gene expression levels likely change over the course of infection, which is also impossible to test in living frogs, and thus any expression data probably reflect transient gene expression.

Fortunately, I succeeding in isolating total RNA from a buccal swab of one frog, a resistant *L. cearulea*, Lit40, from which I genotyped MHC II by TCBS. Unique

MHC II alleles specific to this frog were identified, and at least one allele, LicaeII58 was confirmed by duplicates. Therefore, buccal swab could provide a valuable, non-invasive method for MHC genotyping as well as monitoring MHC expression in future diseases studies.

4.4.3 Does variation of MHC II alleles expression in different organs occur by selection?

Comparison of MHC II genotypes and expression in liver and spleen from three same *L. caerulea* revealed that spleen tends to express more MHC II alleles and identical alleles in the same frog could also be differentially expressed in different organs. The spleen is the largest secondary immune organ (Cesta, 2006) and therefore likely expresses more immune genes, such as MHC alleles. Although this phenomenon in amphibian should be further tested later with increased samples sizes, this phenomenon has been observed in other vertebrates, even at the cell type level. For example, different MHC II haplotypes (I-A^d, I-A^k, and I-A^q) were found to be expressed differentially in mouse bone marrow-derived macrophages but not in splenic B cells (Baumgart et al., 1998). MHC I alleles gene expression varies among distinct leukocyte subsets in macaques, but not significantly in humans (Greene et al., 2011). Specific MHC II variants were expressed at higher levels than others in mice lung than that in heart, kidney, skin, and almost not detected in brain and liver, which might be associated with high immunogenicity in lung allografts (Alwayn et al., 1994). Therefore, it is likely that different MHC alleles that express differentially in different cells and organs, and also varies in species, were also under selection, probably in a similar way as DNA

sequences. Understanding the evolution of amphibian immunity might be important to understand the evolution basis for human diseases since amphibians have much older evolutionary history.

Since different organs could have various MHC genotypes in one individual, then how many alleles can we describe per individual? And how does this relate to disease resistance? The answer depends on specific research goals, yet we should at the very least be aware that genotypes, especially those based on genomic DNA, cannot be the only criteria used to establish the genetic basis of disease resistance, or for that matter behavioral tests, like mate choice. We should be aware of three functional possibilities of MHC II, protective, suppressive and neutral roles (Baumgart et al., 1998). This study suggests that it is essential to compare both gene expression and allele types in the same organ or cell level, especially in studies of disease resistance.

4.4.4 MHC II ß1 selection in *L. caerulea* and *B. gargarizans*

Neutrality test on MHC II ß1 indicated that MHC II ß1 in both species were under balancing selection. This result was especially within expectation for *B. gargarizans* if MHC II was really under selection for chytridiomycosis resistance because Bd originated from Asia and Bd was under balancing selection (O'Hanlon et al., 2018), so that Korean amphibians, such as *B. gargarizans*, have already completed selection, thus they are now under balancing selection, rather than any kind of selective sweep.

Although the entire MHC II allelic DNA sequences of *L. caerulea* were also under balancing selection, eight sites (four PBR and six WuKa sites) were under

positive selection. *L. caerulea* also has significant higher Wu-Kabat variability than that in *B. gargarizans*. Among the positively selected PBR and Wuka sites, four sites were completed conserved in *B. gargarizans*. Therefore, these sites might be associated with Bd resistance, which requires further investigation. Besides, it seems WuKa sites reflect the selection better than PBR sites which were denoted by comparing with human HLA. Amphibian may have different peptide binding sites compared to other vertebrates.

Nevertheless, the different selection patterns of both species suggests that *L. caerulea* in New Guinea is under strong positive selection due to Bd and other emerging infectious diseases. Indeed, it is difficult for Bd to survive in tropical areas. The different selection patterns of MHC II in the two species may simply reflect the difference in pathogen abundance between tropical and subtropical species. On the other hand, strong positive selection on the adaptive immune system (i.e. MHC) might be due to weaker innate immunity in *L. caerulea*. If individuals already possess strong innate immunity against pathogens, adaptive immune system development may be less important, since there are costs for improved immunity (Lochmiller and Deerenberg, 2000; Petit et al., 1999).

Amphibian skin contains many mucous and granular glands, which secrete functional mucins and antimicrobial peptides (AMPs) as first defense line (Rollins-Smith et al., 2011). Various skin peptides have been identified in *B. orientalis* (Chen and Zhao, 1987; Gibson et al., 1991; Hou et al., 2015; Nagalla et al., 1996; Yasuhara et al., 1973; Zhou et al., 2018), *B. gargarizans* (Lee et al., 1998; Park et al., 1996), *H. japonica* (Kawasaki et al., 2008; Zhu et al., 2014c), and *L. caerulea* (Stone et al.,

1993a). Antimicrobial ability of different peptides varies within and between species. It seems peptides in the skin of *B. orientalis* and *B. gargarizans* have higher antimicrobial ability than those in *L. caerulea* (Apponyi et al., 2004; Park et al., 1996; Simmaco et al., 2009; Stone et al., 1993), which supports my second hypothesis that *B. gargarizans* does not need to have strong positive selection in MHC II since it already has a strong innate immunity compared to *L. caerulea*.

4.4.5 Perspective of MHC II evolution in amphibian

Phylogenetic trees of all MHC II alleles identified in both species showed species-dependent clustering. Trans-species polymorphism (TSP) of MHC II was not observed in either species, but has been observed in mammals, birds, fish and other vertebrates (Eimes et al., 2015; Garrigan and Hedrick, 2003; Graser et al., 1996; Lenz et al., 2013). Although I compared one more Korean species (*B. orientalis*) and two more Australian species (*R. marina*, *L. verreauxii alpina*), no TSP was observed. Evolution of MHC II in amphibian might be subject to the concerted evolution model proposed for many bird species (Zelano and Edwards, 2002). This should be further investigated in the future by comparing more tropical and subtropical species.

4.4.6 Conclusion

In summary, I applied NGS to MHC II ß1 genotyping in a Bd-resistant (*B. gargarizans*) and Bd-susceptible species (*L. caerulea*). A variety of MHC II alleles were found in both species, and they were differentially expressed in different organs and individuals. Mating preferences in *B. gargarizans* are likely to be disassortative. No clear pattern of gene expression of different MHC II alleles was found regarding to Bd treatment and resistance. However, PBR of MHC II was under

strong positive selection in *L. caerulea* while not in *B. gargarizans*. MHC II in both species exhibited concerted evolution other than parallel evolution. There were four sites that were under selection in *L. caerulea* but completely conserved in *B. gargarizans*, which might be associated with Bd resistance but not necessarily. For future studies to investigate about MHC II association with chytridiomycosis susceptibilities in specific positively selected sites, buccal swab based MHC II monitoring could be a new and effective direction. Crystal structure of true MHC II PBR in amphibian should be investigated to validate all the associations. Moreover, binding affinity of different MHC II alleles to Bd derived peptide need to be further investigated.

Chapter 5 General Conclusion

5.1 Evolutionary dynamics between hosts and pathogens

This study clearly demonstrates, for the first time, that virulence of Asian Bd (BdAsia-1) has not attenuated over a long period of co-evolution with Korean amphibians. Indeed, BdAsia-1 exhibited hypervirulence compared with BdGPL. Meanwhile, all Korean amphibian survived after Bd infection and proved to be either tolerant or resistant in laboratory for the first time. These results could explain why Bd could cause amphibian decline in other areas but not in Asia.

MHC II ß1 genotyping results indicated that all the alleles were under balancing selection in *B. gargarizans*, implicating that there might be no new emerged disease virulent enough to drive MHC evolution. Although it is technically difficult to know how susceptible Asian amphibians were to Bd infection and the initial virulence of BdAsia, coexistence of the pathogen and host benefited both to become competitive compared to alien lineage and imported susceptible hosts. Since BdAsia was suggested as ancestral lineage among all the Bd lineages isolated until now in the world (O'Hanlon et al., 2018), this implies long term evolution of BdAsia and Asian amphibians could result in higher virulence of pathogen and stronger resistance of hosts no matter how the evolving dynamics of the virulence in BdAsia and the resistance of Asian amphibian were in the past. This phenomenon is not a rare case in regard to pathogen-host coevolution.

One of the best described case for pathogen-host coevolution is myxoma virus and rabbit coevolution system. The myxoma virus was used to control the population of wild rabbit in Australia and became a good system to monitor

evolutionary dynamics of host and pathogen (Di Giallonardo and Holmes, 2015; Fenner and Fantini, 1999; Ratcliffe et al., 1952). The virulence of myxoma virus has become attenuated in 1950s and then increased again since 1970s (Fenner and Fantini, 1999), which might associate with rising viral genes between 1975 and 1985 approximately (Kerr et al., 2017). After a dramatic decrease of rabbit population after applying myxoma virus, the wild rabbits became more resistant since then, which was suggested to be associated with increased virulence of myxoma virus (Elsworth et al., 2014). In a similar pattern, the West Nile virus has been evolving to be more virulent as its main reservoir house sparrows became more resistant (Duggal et al., 2014).

The reciprocally interaction between host and pathogen above matched to Red Queen hypothesis ("running as fast as you can to stay in the same place"). Hypervirulence of pathogen could cause host to evolve a higher resistance, while a higher resistance in host could lead pathogen to evolve a better strategy to invade (Kerr et al., 2017; Laine, 2016; Lively et al., 1990). Red Queen hypothesis also implied the advantages of sexual selection in evolving defense mechanisms for offspring against faster evolving pathogens (Decaestecker et al., 2007; Lively et al., 1990; Morran et al., 2011). Selection of resistance genes in these resistant amphibian hosts in Asia regarding to Bd infection might be already in a stable status since genetic marker, such as MHC II has been under balancing selection in *B. gargarizans*, and no signs for new emerging disease affecting their lives. The coevolution between Asian amphibian and Bd may have been completed at least hundred years ago. However, the selection could be taking place in susceptible

species, such as *L. caerulea*. The positively selected site in MHC II in *L. caerulea* might be signs for better defense mechanisms. Therefore, more studies should be performed to investigate and monitor the evolutionary dynamic of MHC or other immunogenetic markers (such as toll like receptors) in susceptible species in regarding to how hosts evolved resistance to Bd infection.

5.2 Trade-off of evolving a superior immunity

Although developing a better defense mechanism is beneficial against pathogens, there might be cost there, such as growth, reproduction (Sanz et al., 2004; Sheldon and Verhulst, 1996). As tree frogs, *H. japonica* is similar to *L. caerulea* in appearance, but much smaller in body size compared to *L. caerulea*, implying the resistance in *H. japonica* might have cost in body size. Although *B. orientalis* has many toxins in their skin, several individuals without Bd infection died occasionally without any obvious sign of morbidity. Although the size of *H. japonica* and *B. orientalis* are much smaller compared to *L. caerulea* and *B. gargarizans*, they have much longer breeding season compared to *B. gargarizans*, implying there might be reproduction cost in *B. gargarizans* while possessing both good body size and better resistance. Maybe these are the cost of resistance, which may vary according to species. However, in plants, it was suggested that there might be no huge cost while evolving better resistance (Laine, 2016). Nevertheless, the possible fitness costs should be further tested to understand more about the trade-offs of gaining resistance.

5.3 MHC genotyping

NGS is highly recommended to be widely applied to amphibian MHC genotyping research. Genotyping merely based on genomic DNA is not enough to associate with disease susceptibilities or mate choice. Assessment of transcriptional expression level meanwhile is highly recommended as well. Due to the fact that different organs could express different MHC alleles, the same organ or tissue should be used for comparisons. Buccal swab based on MHC genotyping is highly recommended since it could be monitored and noninvasive to animal lives.

5.4 More future directions and applications

In the future, I will compare the difference of the immune system among different hosts in transcriptional level to investigate what contributes to host resistance. Comparison in phenotype, growth and transmission rate of different Bd lineages might help understand the fitness cost of higher virulence. These approaches should assist in the development of management plans for the disease. Moreover, chytridiomycosis as an epidemic disease in amphibians also acts as an important model to study the evolutionary dynamics between pathogens and hosts. This should help researchers to understand more about how different hosts respond to pathogens in order to protect biodiversity as well as improving human living quality.

Besides, Asian amphibians are traded internationally and carry endemic lineages, including BdAsia, with them that may threaten amphibian biodiversity. Therefore, a more deliberate measures should be taken for importation and exportation.

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초록

전염병은 생물 다양성, 세계 보건 및 경제에 대한 거대한 위협으로 알려져 있다. 인류의 새로운 전염병의 약 60% 는 인체 감염 동물 병원체에서 유래되었으며 약 70% 는 야생 동물 병원소를 보유하고 있다. 그러므로 야생 생물 질병 역학을 연구하는 것은 전파 패턴과 병원체와 숙주의 상호 작용 역학을 이해하여 전 세계적으로 제어하는 데 중요합니다. Chytridiomycosis 는 곰팡이 병원균 인 *Batrachochytrium dendrobatidis* (Bd)에 기인하여 전 세계적으로 양서류의 개체수 감소와 종의 멸종과 관련이 있지만 아시아에서는 그렇지 않다. 아시아에서는 Bd 병원체와 양서류 숙주가 100 년 이상 공동 진화했다. 따라서 아시아 양서류 개체군의 감염에 대한 회복력은 풍토병 Bd 계통의 감소한 병독성이나 병원균에 대한 향상된 내성 또는 둘 모두로 인해 생길 수 있다. 한국 양서류 (*Bombina orientalis*)에서 분리 된 BdAsia-1 은 전 세계적으로 양서류 개체수 감소와 관련된 재조합 글로벌 유행병 계통 (BdGPL)의 진화적 기초가 될 것으로 제안되었다. 그러나 BdAsia-1 의 병독성은 여전히 명확하지 않았다. 이전 논문들은 아시아 양서류가 더 저항력이 있다고 주장했지만, 그것을 증명하기 위해 수행 된 감염 실험은 없었다. 이 프로젝트에서 필자는 오스트레일리아의 한 감수성

종인 *Litoria caerulea* 를 사용하여 BdAsia-1 과 BdGPL 간의 병독성을 비교 한 다음 두 Bd lineages 을 사용하여 한국 양서류 (*Bufo gargarizans*, *B. orientalis* 및 *Hyla japonica*)의 감수성을 테스트했다. 피험자는 모두 감염되었지만 한국 양서류는 Bd lineages 과는 상관없이 신속하게 감염을 치유하였다. 한국의 양서류들은 명백한 증상 없이 생존했다. 대조적으로, *L. caerulea* 는 BdAsia-1 또는 BdGPL 에 의한 감염 후 신체 상태의 악화를 겪었으며 시간이 지남에 따라 점진적으로 더 높은 Bd 부하를 나타냈다. 그 후, 대부분의 피험체는 사망했다. *L. caerulea* 에 대한 효과를 비교하면, BdAsia-1 은 BdGPL 보다 더 빠른 질병 진행을 유도했다. 결과는 BdAsia-1 혈통에 속하는 두 개의 한국 Bd 균주 (KBO327 및 KBO347)가 모두 *L. caerulea* 에 과민성이었으며 BdGPL 에 비해 약한 증상이 없음을 분명하게 나타냈다. 테스트 한 모든 한국 양서류는 Bd 계통에 저항성 또는 내성을 지녔다. 병원체의 병독성은 풍토성 아시아 양서류 숙주 종에서 적응 면역 반응에 대한 강력한 선택을 유도했을지도 모른다.

질병에 대한 감수성은 종내 및 종간 다양하며, 상속 가능한 면역 유전 학적 변이에 기인 한 것으로 보인다. 주요 조직 적합성 복합체 (major histocompatibility complex, MHC)는 선택적 질병 저항성 메커니즘을 연구하기위한 우선 순위 중 하나이다. 이전의 연구에서, 일부 감수성 종의 MHC I 및 MHC II 유전자는 Bd 에 의한 숙주 감염에 의해 상향 조절되는 것으로 나타났으나 저항성 종은 전사 발현에 있어

비교할 만한 변화를 나타내지 않았다. Bd 내성 종은 MHC-II 항원 결합 홈 내에서 비슷한 포켓 구조를 공유한다. 감수성이 있는 종들 중에, 전염병의 생존자들은 이러한 형태를 암호화하는 대립 유전자를 가지고있다. 동형 접합 저항성 유전자를 가진 개체는 강화된 저항성으로 인해 병원체의 병독성을 촉진시키는 환경 조건에서 이익을 얻는 것으로 보인다.

반복적으로 감염되고 이어서 Bd 를 제거한 실험체는 병원체에 대해 후천 면역 반응을 일으킬 수 있다. Bd 에 대한 내성을 나타내는 MHC 대립 형질에 대한 강한 방향성도태는 다른 병원체에 반응하는데 필요한 유전적 다양성을 고갈시킬 수 있다. Chytridiomycosis 에 대한 내성은 생활사 비용을 초래하며, 이에 대한 추가 연구가 필요하다..

그러나 이전의 양서류 연구는 거의 모든 MHC genotyping 은 모든 대립 유전자를 효율적으로 식별하고 신뢰할 만한 결과를 제공하는 데 한계가 있는 전통적인 단일 PCR 기반 시퀀싱을 사용하였다. 이것은 MHC II 와 Bd 저항성의 연관성에 대한 이전 연구 결과의 신뢰성을 크게 떨어트렸다. 따라서, 나는 각 샘플에 대해 독립적으로 3 개의 amplicon 을 증폭시킨 후, *L. caerulea* 및 *B. gargarizans* 의 MHC II 유전자형에 대해 next generation sequencing 을 적용하였다. 두 종 모두에서 많은 MHC II 대립 유전자가 동정 되었다. 각 대립 유전자는 개인 및 기관 수준에서 차등적이고 상대적인 유전자 발현을 보였다. MHC II 는 *L. caerulea* 와 *B. gargarizans* 에서 균형 잡힌 선택을 하고

있었지만 MHC II 대립 유전자의 펩타이드 결합 부위와 WuKa 부위의 일부 코돈은 *L. caerulea* 에서 강한 양성 선택을 하고 있었다. 또한 Wu-Kabat 의 다양성은 *L. caerulea* 에서 *B. gargarizans* 의 다양성보다 유의하게 높았으며, *L. caeurlea* 에서 더 높은 단백질 다양성을 나타냈다. 더욱이 *L. caerulea* 에서 양성으로 선별 된 PBR 과 WuKa 부위의 대부분은 *B. gargarizans* 에서 완전히 보존되어 Bd 저항성과 관련이 있음을 시사한다. 그러나, Bd 처리 및 감수성과 관련하여 상이한 MHC II 대립 유전자의 명확한 유전자 발현 패턴은 관찰되지 않았다. 두 개의 저항성 *L. caerulea* 개체에서 5 개의 고유 한 MHC II 대립 유전자가 발견되었지만 *L. caerulea* 의 저항성 표본 크기가 작아 저항성과 관련하여 이 대립 유전자의 명확한 패턴을 확인할 수는 없었다. 후속연구가 *L. caerulea* 에서 양성적으로 선택된 부위의 역할을 입증하기 위해 수행되어야 한다.

주요어 : 전염병, 원헬스, *Batrachochytrium dendrobatidis*, 아시아 양서류, 공진화, 병원체 병독성, 숙주 내성, MHC II 대립 유전자 변이, 양성 선별, Wu-Kabat 다양성, 협조진화

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