CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

FACULTY OF ENVIRONMENTAL SCIENCES

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Diploma Thesis

Application of a molecular diagnostic tool Genie II for monitoring of *Batrachochytrium dendrobatidis* in amphibians in Kyrgyzstan

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DIPLOMA THESIS ASSIGNMENT

Emmanuel Kojo Essel

Nature Conservation

Thesis title

Application of a molecular diagnostic tool Genie II for monitoring of Batrachochytrium dendrobatidis in amphibians in Kyrgyzstan.

Objectives of thesis

The aim of this thesis is to examine the presence of chytrid fungus Batrachochytrium dendrobatidis (Bd) in Kyrgyzstan amphibians through the use of diagnostic field tool Genie II and to create methodology for using the tool for Bd detection in amphibians. The specific objectives are: (i) to describe how DNA samples of amphibians are prepared for the Genie II analysis; (ii) to describe how to operate the Genie II and (iii) how to read results from the machine. The main outputs of this diploma thesis will be results of Bd surveilance and also the protocol for using diagnostic tool Genie II for field detection of Bd presence in amphibians.

Methodology

(i) Literature study as the base for writing literature review – topics: the causes of amphibian decline including the effect of Bd, description of chytridiomycosis, tools and methods for the disease detection including diagnostic machine GenieII.

(ii) Learn how to work with the tool GenieII and conduct Bd detection by the diagnostic tool GenieII in 60 samples of Rana amurensis from Kyrgyzstan.

The proposed extent of the thesis
ca 30–40 pages

Recommended information sources

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Declaration

I hereby declare that work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

In Prague, April 22, 2015 _____

Emmanuel Kojo Essel

Acknowledgement

Abstract

Batrachochytrium dendrobatidis (*Bd*) is a disease-causing fungus, responsible for the rapid amphibian population declines and their extinctions worldwide. Diagnosis of this pathogen is standardly making with quantitative polymerase chain reaction (qPCR) assay, which is time-consuming, expert diagnostician and laboratory demanding. In this research, Genie II assay was applied to monitor the presence of *Bd* in sampled amphibians. Genie II is a modern molecular diagnostic tool which is made simple, sensitive, and easy to operate on field and in laboratory conditions. By the use of Genie II assay, *Bd* presence was detected in 1 out of 60 sampled amphibians from Kyrgyzstan, even when clinical signs of chytridiomycosis were not visible. Hence, usage of this tool will enable rapid detection of *Bd* presence in amphibians, thus enhancing possibilities of treatment and protection of endangered populations, monitoring of pristine environments and prevention of further global spread of this disease through amphibian trade.

Key words: Chytrid fungus, *Rana amurensis*, DNA examination, LAMP, qPCR assay

Abstrakt

Batrachochytrium dendrobatidis (*Bd*) je houba odpovědná za onemocnění způsobující rychlý pokles populace obojživelníků a jejich celosvětové vymření. Rozpoznání přítomnosti tohoto patogenu se standardně provádí pomocí metody kvantitativní polymerázové řetězové reakce (qPCR), která je časově náročná, vyžaduje odborného diagnostika a laboratoř. V tomto výzkumu byl použit přístroj Genie II ke sledování přítomnosti *Bd* u testovaných obojživelníků. Genie II je moderní molekulární diagnostický nástroj, který je jednoduchý, citlivý, snadno ovladatelný jak ve venkovních podmínkách, tak v laboratoři. Pomocí Genie II byla potvrzena přítomnost *Bd* u 1 ze 60 testovaných obojživelníků z Kyrgyzstánu, přestože klinické příznaky nemoci u tohoto jedince nebyly viditelné. Použití tohoto nástroje umožní rychlou detekci *Bd* u obojživelníků, což zvýší možnosti léčby a ochrany ohrožených populací, sledování nedotčeného prostředí a zabrání dalšímu globálnímu šíření nemoci prostřednictvím obchodu s obojživelníky.

Klíčová slova: Houbové onemocnění, *Rana amurensis*, test DNA, LAMP, qPCR test

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1 Introduction

Amphibians, a unique group of vertebrates, are globally facing rapid declines and extinctions: with at least 2,469 (43%) of 7,405 species described as declining and nearly, 168 species described to have gone extinct (http://amphibiaweb.org/; accessed: 04/19/2015). Threats to amphibians result from land-use change, commercial overexploitation and the emerging infectious disease chytridiomycosis, caused by the aquatic fungus pathogen, *Batrachochytrium dendrobatidis* (Dodd and Smith 2003; Daszak et al. 2000). *Batrachochytrium dendrobatidis*, hereafter referred as *Bd*, currently believed as the biggest threats to biodiversity of vertebrates worldwide (Skerratt et al. 2007; Zippel and Mendelson 2008). The status of the infection, whether it persists and develops into disease is modulated by both external and intrinsic factors, including climatic conditions and immunity of host (Fisher et al. 2007; Baláž et al. 2013).

According to Fisher et al. (2009), *Bd* has been found on all continents where amphibians occur except Antarctica. It is believed that *Bd* originated from Africa and was repeatedly introduced as a novel pathogen to new geographic locations (Morehouse et al. 2003; Weldon et al. 2004; Fisher and Garner 2007). Whereas Goka et al. (2009), in contrast postulate that *Bd* may have arisen in Asia since 1902. The emergence of chytridiomycosis has contributed to amphibian population declines in other continents within the last 30 years (Berger et al. 1998; Bosch and Martínez-Solano 2006; Fischer et al. 2009; Kilpatrick et al. 2009). However, the paradigm of *Bd* prevalence in Asia appears drastically different to that in the Americas, Africa, Australia, and Europe, with isolated cases, low infection prevalence and or apparent absence at most sites (Swei et al. 2011b). Despite the low infection prevalence throughout Asia, *Bd* has been detected in Kyrgyzstan (Swei et al. 2011a).

Through infected amphibians, *Bd* is easily dispersed, often by human activities (Spitzen-van der Sluijs and Zollinger 2010); and therefore, requires rapid execution of preventative measures in the field to prevent its spread as well as to control the spread of other diseases. In captive populations, formalin/malachite green solution administered was effective on adult western clawed frog (*Xenopus tropicalis*) (Parker et al. 2002). In this stem, if contamination of water, soil and animals are sensitively detected, appropriate quarantine and disinfection strategies could be implemented to prevent further spread of the pathogen and

disease at large (Boyle et al. 2004). Observing multiple dead frogs in the same body of water could be caused by pollution, for example DDT intoxication (Berger et al. 1998; Boyle et al. 2004). Hence the best method employed for *Bd* detection is by analyzing DNA samples of infected amphibians using quantitative polymerase chain reaction (qPCR) to determine the intensity of infection (Boyle et al. 2004; Hyatt et al. 2007). Recent advances in molecular technologies have seen the development of Genie II, a molecular diagnostic made-simple tool which detects both *Bd* and *Batrachochytrium salamandrivorans* in a fast, easier and efficient way on the field of study (www.optigene.co.uk; 20/11/2014). For the first time in Kyrgyzstan, *Bd* was detected in nine samples of amphibians when the qPCR method was used (Swei et al. 2011a). Hence for the first time in this research, Genie II was used to monitor the presence of *Bd* in Kyrgyzstan.

1.1   Aim and Objectives of the Study

The aim of this thesis is to examine the presence of chytrid fungus, *Bd*, in Kyrgyzstan amphibians through the use of diagnostic field tool Genie II and to create methodology for using the tool for *Bd* detection in amphibians. The specific objectives are:

➢ To describe how DNA samples of amphibians are prepared for the Genie II analysis;
➢ To describe how to operate the Genie II;
➢ Last but not least to describe how to read results from the machine.

2 Literature Review

2.1 Status and Threats to Amphibians

Despite the significance of amphibian species to biodiversity, rapid global declines and extinctions threat their population; 32.5% of 5,743 species as described by Stuart et al. (2004) are threatened, with at least 9 to 122 becoming extinct since 1980. While updates to the amphibian database have been incremental, there has not been a major comprehensive amphibian assessment since 2004. However, according to the assessment of the Red List of Threatened Species of IUCN (2008), nearly one-third (30%) representing 1,905 of 6,260 evaluated extant species are globally threatened. Amphibians have existed on earth for over 300 million years, yet in just the last two decades there have been an alarming number of extinctions, nearly 168 species are believed to have gone extinct and at least 43% of 7,405 known species have populations that are declining (Fig. 1) (http://amphibiaweb.org/; accessed: 04/11/2015).

Figure 1: Maps of Global Amphibian Declines. 1) In red: number of extinct, extinct in the wild or critically endangered species; 2) In orange, number of endangered or vulnerable species; 3) In white, total number of species for biome (http://amphibiaweb.org/; accessed: 04/11/2015).
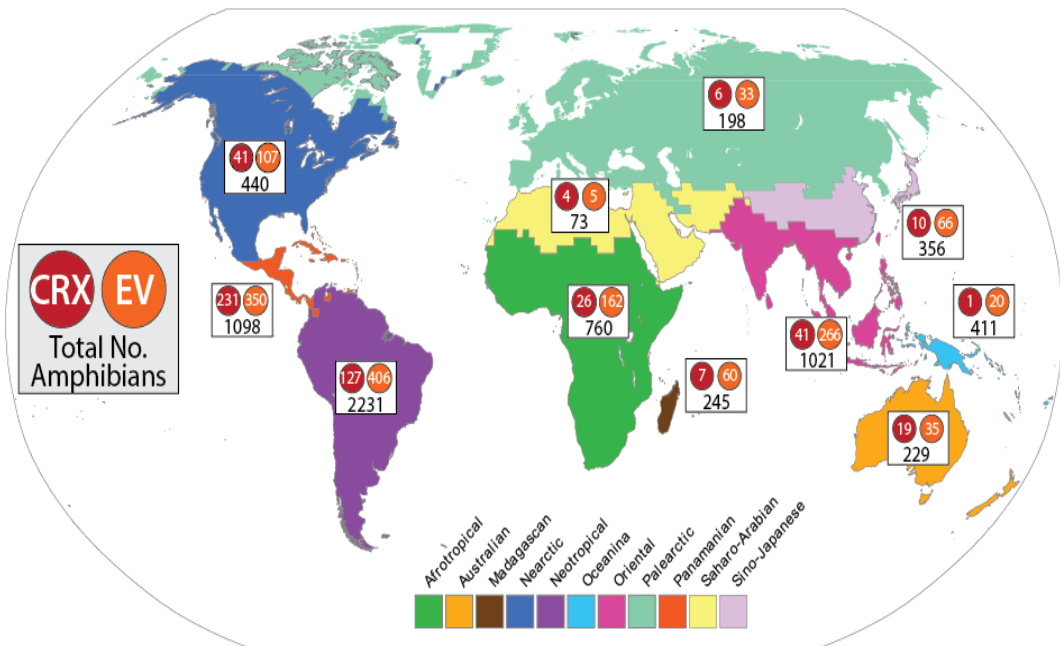
Collins and Storfer (2003), classified amphibian declines into two broad categories; as Class I and Class II factors. Where Class I factors includes habitat alteration, over-exploitation and invasive species; and Class II factors comprising global climate change, pollution, and emerging infectious diseases. Many of these threats to biodiversity are synergistic, where total impact is greater than what we would expect from their independent impacts (Groom et al. 2006). Humans currently appropriately influence more than one-third of the production of terrestrial ecosystem and about half of the usable fresh water on earth (Tilman et al. 2001); and since the human population is on the increase it is not a surprise that biodiversity is threaten (Mittermeier et al. 1998; Pimm and Raven 2000; Brooks et al. 2002).

Humans have been transporting animals from one part of the world to another, sometimes deliberately or accidentally (Bailie et al. 2004). Though the mechanisms by which invasive species cause declines are well understood, the problem is not easily resolved; it has often proven impossible to eradicate invasive species once it has become established. For instance, the roles of introduced species (e.g. *Lithobates catesbeianus*, *Rhinella marina* and *Xenopus laevis*) are potential vectors for transporting chytrid fungal disease (Daszak et al. 2004; Weldon et al. 2004). Because these amphibian species can withstand the disease, and when they are being introduced to many regions around the world, they are likely to cause rapid outbreak of this disease in native amphibian populations in various part of the world (Daszak et al. 2004; Weldon et al. 2004).

Overexploitation such as hunting, collecting, fishing; and the impact of the trade in species and species' parts have obviously; a direct impact is the global or local extinction of species. The total number of amphibians harvested yearly for consumption is unknown. However, in developing countries like Thailand, the unregulated harvesting of amphibians for food or pet trade is likely a primary contributor to amphibian declines (Lau et al. 1999).

The primary causes of amphibian declines and species extinction worldwide are more likely as a result of habitat loss caused by habitat alteration and fragmentation (Dodd and Smith 2003). Habitat alterations such as agricultural intensification, road construction and other varieties of actions directly remove amphibian breeding and feeding areas, or block access to them (Hazell et al. 2003). According to Hero and Morrison (2004), habitat loss is the primary cause of population declines in lowland Australian frogs; negatively affecting 11 of the 12 (91.7%) threatened lowland species.

In recent decades, the increased manufacture and usage of toxic chemicals globally are alarming, and have the ability to pollute geographically disparate regions through windborne transport (Datta et al. 1998). Pesticides are dispersed globally and have both lethal and sub-lethal effects on terrestrial fauna (Sala et al. 2000); and even, low levels of pesticides can cause fatal immune suppression in frogs (Taylor et al. 1999). In the United States, Hayes et al. (2002) exhibited how doses of atrazine could ecologically render male *Xenopus laevis* hermaphrodite. And since atrazine is one of the most commonly used herbicides in the United States, these negative effects are clearly a threat to amphibians and biodiversity at large.

Anthropogenic climate change is perhaps the most unfavorable threat to biodiversity of the present era. Global climate change caused by human-induced alteration of the earth's atmosphere affects conditions of biodiversity. Williams et al. (2003) suggest that amphibian populations are affected by global warming. Also increased UV-B exposure causes mortality in salamander eggs (Blaustein et al. 1995).

Diseases can cause chronic population declines, dramatic die-offs or reductions in the reproductive success and survival of individual species. According to Bailie et al. (2004), invasive diseases have already been implicated in the extinction of some species. An infectious disease such as chytridiomycosis is identified as the largest threat to amphibian populations; 30 out of 113 species of *Atelopus* harlequin toads have gone on extinction (Mendelson et al. 2006). Chytridiomycosis (hereafter CH) is an emerging infectious disease of amphibians known for mass mortalities, population declines, and species extinctions globally (Berger et al. 1998; Bosch et al. 2001; Bosch and Martinez-Solano 2006; Weldon and du Preez 2004). According to Skerratt et al. (2007), CH is the greatest disease-caused loss to biodiversity and to such effect has caused decline or extinction of at least 200 species of frogs. This disease is believed to have caused amphibian population declines in Australia, South America, North America, Central America, New Zealand, Europe, and Africa (Fisher et al. 2009; Van Sluys and Hero 2010), and has been implicated in the extinction of some species of frogs.

2.2   Basic description of *Batrachochytrium dendrobatidis*

Bd is an aquatic fungal pathogen (chytrid fungus), causing emerging infectious skin disease in many populations of amphibians (Berger et al. 1998; Daszak et al. 1999); with over 350 amphibian species infected (Fisher et al. 2009). Through anthropogenic means the fungus is believed to have been recently introduced to many regions (Mazzoni et al. 2003; Morehouse et al. 2003; Rachowicz et al. 2005; Lips et al. 2006). Infection intensity with *Bd* appears to be a key factor; death ensues in adult frogs and salamanders once the individual has reached an infection load of about 10,000 fungal zoospores (Vredenburg et al. 2010; Cheng et al. 2011)

The chytrid fungus is from the kingdom fungi; phylum chytridiomycota; class chytridiomycetes; and included in the largest order chytridiales (Berger et al. 1999; Longcore et al. 1999). The chytrid fungus lacks hyphae and is present in aquatic habitats and moist soil, where it breaks down cellulose, chitin, and keratin (Berger et al. 1999). It thrives well in the superficial keratinized layers; *stratus corneum* and *stratum granulosum* of the epidermis of its host (Daszak et al. 1999). *Bd* may be detected in keratinized cells because these cells are dead and invaded easily (Piotrowski et al. 2004). It occurs in two forms, as a roughly spherical, smooth-walled zoosporangium with diameter of 10–40 μm and mobile zoospores with a size of about two μm in diameter (Berger et al. 2005). Usually within four days, the mobile zoospore attaches to the substrate, grows rhizoids and becomes a zoosporangium which forms drain tube (discharge tube) for release of new zoospores (Fig. 2) (Rosenblum et al. 2010).

Figure 2: Life cycle of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (Rosenblum et al. (2010)

aquatic, motile zoospore initiates colonization of host skin cells

zoospores are released and enter water column or reinfect host

zoospore embeds in host cell and develops into a thallus

thallus matures and new zoospores begin to develop in zoosporangia
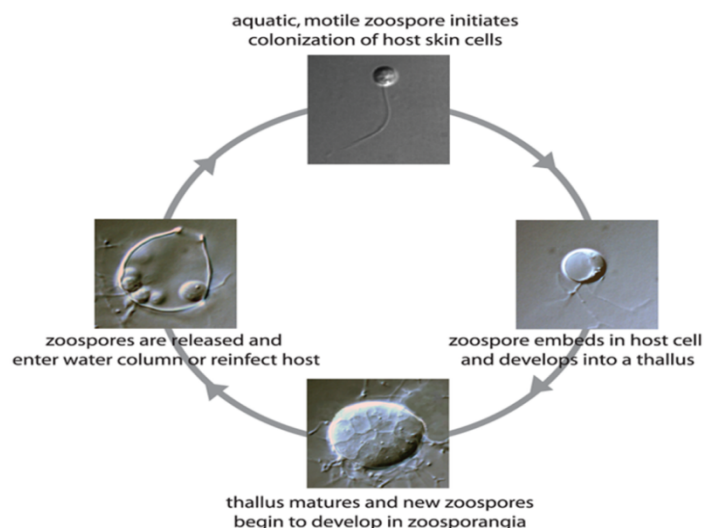
Bd thrives within a wide temperature range of 4–25 °C and over a range of pH, from 4–8 (Piotrowski et al. 2004). Thus, at very low or high temperatures the chytrid fungus becomes dormant and its infection is slow in its host. According to Longcore et al. (1999), Johnson et al. (2003) and Piotrowski et al. (2004), the fungus may fails to make substantial growth above 28 °C, and can be killed after 96 hours at 32 °C. This is because the fungus may lack the time to complete its life cycle before being shed with the epidermal layer (Berger et al. 2005). The chytrid zoospores appear to be elongate to ovoid in shape and ranges from 0.7 to 6 μm in diameter and propelled by a single posterior flagellum with movement, allowing it to swim in water (Berger et al. 2005).
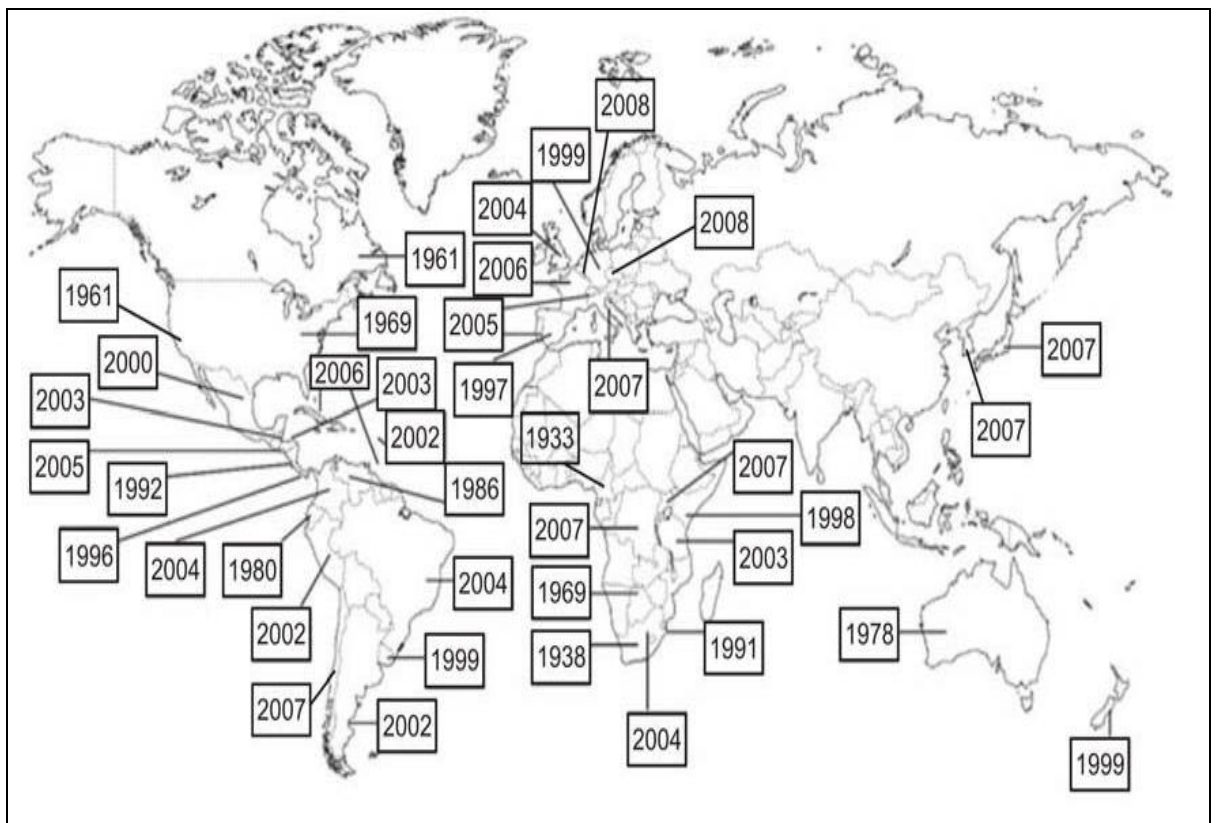
2.3   Distribution of *Batrachochytrium dendrobatidis* in the World

The rapid biodiversity loss worldwide in recent decades is illustrated by the current status, one-third of the world's amphibians species are threatened with extinction (Stuart et al. 2004). It is complex and not known with certainty where *Bd* originated. Nonetheless, the earliest known cases of *Bd* infection date back to 1933 in a museum specimens of Fraser's clawed frogs (*Xenopus fraseri*) in Cameroon, Africa (Soto-Azat et al. 2010). After 1934, large numbers of African clawed frogs (*Xenopus laevis*) from South Africa were shipped throughout the world for use in scientific studies of immunology, later embryology and molecular biology (Weldon et al. 2004); also for pregnancy testing (Gurdon and Hopwood 2000). Thus due to these importance, frogs became a popular pet and laboratory rearing and often were, and still are, used in research and medicine (Stanley 1949; Asashima et al. 2009). Therefore, it is believed that *Xenopus* species moved the disease out of Africa and from there began to infect native amphibian species. Chytridiomycosis was prevalent in South Africa for 23 years since the 1938 detection and consequently, the chytrid fungi proliferated as a result of human activities while breeding those species which had been in contact with wild amphibians. And after those years the first positive specimen was found outside of Africa to other part of the world (Weldon et al. 2004)

Secondary spread of chytrid fungi likely occurred in the United States of America in 1961 due to human-mediated movement of the American bullfrog (*Lithobates catesbeianus*), which acquired the disease, but did not show any clinical pathology and consequently spread

the infection (Garner et al. 2006). Spreading of the disease between the continents continued on to Australia (1978), Central America (1983), South America (1986), Europe (1997) and Oceania (1999) (Weldon et al. 2004), as illustrated below in figure 3. The recent spread of *Bd* is therefore a global problem which is dangerous, especially in humid climatic areas where are favorable conditions for the survival of fungal pathogen (Berger et al. 1998). As reported by Fischer et al. (2009), *Bd* has caused the extinction of 34 species of amphibians and its incidence has been demonstrated in more than 350 species throughout the world.

Figure 3: History of the distribution of *Bd* in the world. In the map is the description of first *Bd* detection on various parts of the world (Kriger and Hero 2009; Padgett-Flohr and Hopkins 2009; Solís et al. 2009; Yang et al. 2009).

2.3.1   Distribution of *Batrachochytrium dendrobatidis* in Asia

Bd in amphibians was recently reported in Asia (Ron 2005), compared to its detection and prevalence over the last two decades in other continents (Berger et al. 1998; Bosch and Martínez-Solano 2006; Fischer et al. 2009; Kilpatrick et al. 2009) and consequently, only few details are known from Asia, especially with regards to Kyrgyzstan. Research conducted on genetic diversity in 35 strains of an amphibian *Bd* from Africa, Australia and North America found an extremely low level of sequence difference between strains (Morehouse et al. 2003). Nonetheless, the combination of high diversity and apparent long-standing asymptomatic *Bd* infection of native Japanese amphibian species, Japanese giant salamander (*Andrias japonicus*) and sword-tail newt (*Cynops ensicauda*) has led Goka et al. (2009) to hypothesize that *Bd* may have arisen in Asia since 1902.

Chytridiomycosis was first discovered and reported in Japan (Goka et al. 2009), and followed by cases in South Korea (Yang et al. 2009); China (Bai et al. 2010); Indonesia (Swei et al. 2011a); Malaysia (Swei et al. 2011a); and Cambodia (Mendoza et al. 2011). Hence recently, the distribution of the *Bd* has extended to other areas of Asia; Kyrgyzstan, Laos, India, Philippines, Thailand, Sri Lanka and Vietnam (Swei et al. 2011a; Vörös et al. 2012).

Research carried out in 15 countries of Asia with 3,363 samples over the period of nine years resulted in the detection of 2.35% *Bd* in six of those countries (Swei et al. 2011a). In Thailand, one out of six sampled individuals tested positive for *Bd* (Vörös et al. 2012). In Cambodia, *Bd* was detected on 59 out of 144 samples representing 41% from four sites in the southwestern part of Cambodia (Mendoza et al. 2011). According to Yang et al. (2009), three of seven species of wild frogs surveyed in Korea were detected of *Bd*. In Japan, *Bd* has been found on wild and captive salamanders, as well as wild frogs (Goka et al. 2009). According to Swei et al. (2011a), the highest prevalence of *Bd* infection in Asia was found in Kyrgyzstan (100%, n = 9). In Indonesia, four species of wild frogs were also recorded of low prevalence of *Bd* (Kusrini et al. 2008).

According to Rowley et al. (2010), there has not been any report of amphibian mortality or decline by *Bd* in Asia, and most of research conducted reported low prevalence or absence. The reason for that is still unknown; perhaps there is the need for more intensive research to assess the threat of *Bd* to Amphibians in Asia.

2.3.2 Distribution of *Batrachochytrium dendrobatidis* in Europe

In the European Union, the incidence of *Bd* has been broadly distributed; Great Britain, France, Italy, Spain, Germany, Belgium, Switzerland, Austria, Hungary, Denmark, Czech Republic, Estonia, Slovakia, Slovenia, Latvia and in Portugal (Garner et al. 2005; Baláž et al. 2014a). Through the introduction of infected species into native populations, the world trade in amphibians has been implicated in the emergence and distribution of chytridiomycosis (Fisher and Garner 2007). To this effect, *Bd* has been introduced in most of the European populations with either the African clawed frogs or the American bullfrogs.

The presence of *Bd* in Europe was recorded for the first time in Spain samples in 1999 (Bosch et al. 2001; Garner 2005). The occurrence of *Bd* was reported in Germany in 2000, where the pathogen was detected on amphibians imported from South America. The same year was the presence of *Bd* reported on specimens bred in captivity, again from Germany and also from Belgium (Mutschmann et al. 2000). In Great Britain, the American bullfrog carrying the fungus occurred in ponds used for breeding of common frog (*Rana temporaria*), common toad (*Bufo bufo*), northern crested newt (*Triturus cristatus*) and other amphibian species (Cunningham et al. 2005). Also in France, the chytrid fungus was detected in American bullfrogs (Garner et al. 2006). The first recorded death of individuals due to chytrid fungus occurred in Spain in the National Park Peñalara and at least three species of amphibians; common toad, fire salamander (*Salamandra salamandra*) and common midwife toad (*Alytes obstetricans*) have currently undergone mass mortality (Bosch et al. 2001). In 2001, it was also recorded the incidence of chytridiomycosis in Italian wild population, appenine yellow-bellied toad (*Bombina pachypus*) (Stagni et al. 2004). The result of these and other cases observed in 2001 has indicated a rapid decline in the abundance of local amphibian populations in Europe (Bosch and Martínez-Solano 2001). Currently, the incidence of chytrid fungus is recorded in most localities in Europe. However, due to the lack of attention its presence in some other localities is yet undetected (Civiš et al. 2010).

2.3.3 Distribution of *Batrachochytrium dendrobatidis* in the Czech Republic

The monitoring of the chytrid fungus in the Czech Republic is highly significant since it lies in the center of Europe, sharing borders with Germany which is known for the rapid decline of some amphibian species as a result of the *Bd* proliferation (Mutschmann et al.

2000). So in the Czech Republic, the country has begun carrying out regular monitoring, which involves Civiš, Vojar, and Baláž in various distinguished institutions such as CULS, Prague and UVP, Brno (Civiš et al. 2010). Zavadil and Moravec (2003); suggest that many amphibian populations are vulnerable to declines or might have declined. The presence of *Bd* was first detected in 2008 (Civiš et al. 2012), with infected species including: common toad, alpine newt (*Ichthyosaura alpestris*), fire-bellied toad (*Bombina bombina*), yellow-bellied toad (*Bombina variegata*) and entire genus of *Pelophylax* (green frogs). In a recent study by (Baláž et al. 2014b), prevalence of *Bd* is increasing based on a large dataset; 206 out of 1,562 samples tested *Bd* positive from 9 out of 15 tested species in all 9 areas. Thus it has been detected that the species mostly infected were most commonly found in sub-adults of green frogs and in yellow-bellied toad (Baláž et al. 2014b). Although amphibians in the Czech Republic are legally protected and under conservation effort by Nature Conservation Agency of the Czech Republic and the government body (Jeřábková et al. 2013), there is still the needs for further research

2.4   General Overview of Methods for *Batrachochytrium dendrobatidis* Diagnosis

Techniques to identify *Bd* include electronic microscopy (Berger et al. 2002). Histochemistry is sometimes used to detect *Bd* infection from archived amphibians (Olsen et al. 2004) and also histopathology of the toe clips as a technique (Briggs and Burgin 2004). It can also be detected by immunohistochemistry of the skin (Berger et al. 2002; Van Ells et al. 2003), and real-time TaqMan quantitative polymerase chain reaction (hereafter qPCR) assay (Hyatt et al. 2007; Boyle et al. 2004). Among these techniques, histopathology (Fig. 4) and electronic microscopic examination of epidermis of amphibians are common methods used to identify chytrid disease. However, qPCR is internationally accepted as compared to other methods because it is the diagnostic tool which is sensitive, objective and produce accurate quantifications with respect to assessing the presence of *Bd* (Boyle et al. 2004; Kriger et al. 2006; Smith 2007). Usually examination of animals suspected to be infected with the disease is conducted by skin swabbing and then DNA extraction as sample for use in the qPCR reaction (Boyle et al. 2004; Briggs 2004; Hyatt et al. 2007; Smith 2007).

Figure 4: Histopathology of the skin section of a tree frog (*Litoria caerulea*) showing chytridiomycosis case. D: indicates matured zoosporangium containing zoospores; I: indicating immature stage of zoosporangium; E: the epidermis and the arrow sign on the top left is an empty zoosporangium after zoospores are discharged (Berger et al. 1999).
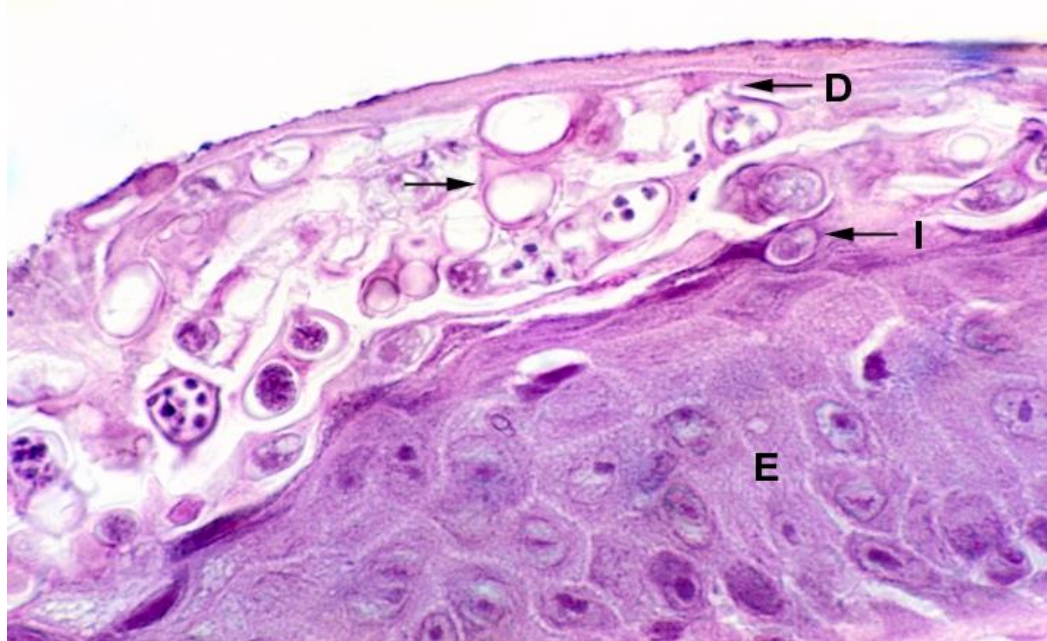
The clinical signs of an amphibian infected with *Bd* includes general lethargy, dehydration, loss of appetite, uneven skin shedding, occasional ulceration or necrosis of digits and neurological defects (Duffus and Cunningham 2010; Baláž et al. 2013). In some cases abnormal epidermal sloughing, epidermal ulcer occurring most rarely hemorrhages in the skin, muscle, or eye, hyperemia (inflammation) of digital and ventral skin, and congestion of viscera may also result (Daszak et al. 1999). Usually observing multiple dead frogs (Fig. 5) in the same body of water may also be an indication of the chytrid fungus but proper examination must be ensured because their demise could also result from other factors (Berger et al. 1998); for example pollution.

Figure 5: Mortality incidence on mountain yellow-legged frogs (*Rana muscosa*) as a result of chytridiomycosis infection. It occurred in August, 2008 at Sixty Lake Basin in the Sierra Nevada mountain; California, USA (http://www.amphibiaweb.org/chytrid/chytridiomycosis.html; 15/10/2014)

2.4.1 Description of Genie II and its Functions

The internationally accepted and widely assay method used for detecting *Bd* is the real-time quantitative polymerase chain reaction (qPCR) (Hyatt et al. 2007), but it still has some limitations. For instance, qPCR is susceptible to phenolic compound inhibitions (Hyatt et al. 2007; Baláž et al. 2014b), and it was claimed that a number of *Bd* lineages cannot be detected when the standard methodology is used (Goka et al. 2009). Studies into molecular biology have recently led to the production of Genie II, a molecular diagnostic made-simple tool which detects both *Bd* and *Batrachochytrium salamandrivorans* (hereafter *Bs*) on the site of study. Genie II (Fig. 6) is more advantageous than the qPCR because it is more convenient, less technical and thus helps to reduce DNA degradation (www.optigene.co.uk; 20/11/2014).

Figure 6: Genie II instrument used for the detection of *Bd* (http://www.optigene.co.uk/instruments; 21/11/2014)
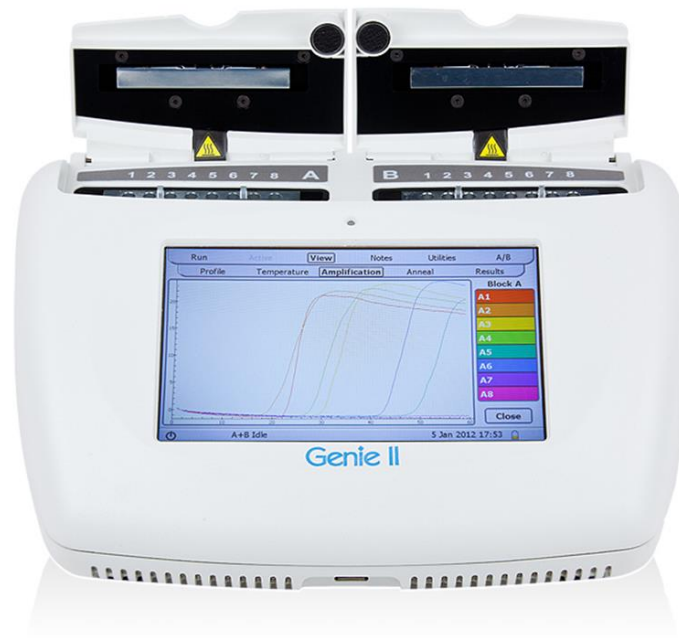
It has become apparent with the use of loop-mediated isothermal amplification (LAMP) for the molecular detection of DNA or RNA (Notomi et al. 2000) and for this purpose, the Genie II as a product of OptiGene Limited, UK serves as a sophisticated instrument that enhance the sensitive detection of bacteria, viruses and fungi at a molecular level (www.optigene.co.uk; 20/11/2014). By the forestry commission in England, Genie II was used to detect ash-die back disease in a suspected forest within an hour. Also the Swiss plant product service at the border controls, Zurich airport ensures that no plant product enters their country without being tested for pest infestation like the *Thrips Palmi*; through the use of the Genie II assay method which takes them usually approximately 40 minutes. Last but not least, in the Czech Republic, a Genie II machine which is owned by the Czech University of Life Sciences, Prague was used to carry out a field study by researchers from the university and University of Veterinary and Pharmaceutical Sciences, Brno in collaboration with Institute of Zoology, London; in a known positive *Bd* site in south-eastern part of the Czech Republic; with amphibian samples composed of yellow-bellied toad and green frogs. The

result from the samples used revealed that all 20 individuals of yellow-bellied toad practically carried *Bd* with no visible signs of infection; and a small number of the 25 individuals of green frogs also produced a positive result. These research findings represent the first application of the Genie II assay to detect *Bd* presence in the Czech Republic and the world at large (www.optigene.co.uk; 20/11/2014; Jiří Vojar, 4/2015, personal communication). It is therefore evident that application of the Genie II assay method is important and thus an effective diagnostic approach in the detection of *Bd* in less time.

2.5   Control and Treatment Strategies of *Batrachochytrium dendrobatidis*

Due to the proliferation of the disease chytridiomycosis, conservation and research programs are embarked to determine mechanisms and evolution of chytrid resistance, control and treatment of amphibians which are chytrid fungal-infected (Mendelson et al. 2006); even though there is currently no method or vaccine known for preventing or eradicating *Bd* in already established areas (Kriger and Hero 2007). However, *Bd* is susceptible to a range of antifungal agents and low levels of heat (>30 °C) when tested in vitro, but there are few proven methods for clearing amphibians from *Bd* infection (Berger et al. 2010). Heating (32 °C for 5 days and 37 °C for two periods of 8 hours, 24 hours apart) has been demonstrated as effective against *Bd* in two amphibian species (Phillott et al. 2010; Woodhams et al. 2003).

Itraconazole baths have been widely used in amphibian rescue and conservation programs and anecdotal evidence suggests that it is effective for adults and sub adults. Successful treatment of infected tadpoles of one species has been reported in a controlled trial using low dose itraconazole (1.5 mg litre^{-1}), but may have been associated with depigmentation (Garner et al. 2009). Benzalkonium chloride which is expensive and only 80% successful has been used for treatment of superficial fungal skin inflammation in *Xenopus laevis* and this caused probably chytridiomycosis sponge. Although there was a prolongation of infected survival juveniles, Australian green tree frog (*Litoria caerulea*) in laboratory conditions, hence benzalkonium chloride (1 mg/l) or fluconazole (25 mg/l) failed to avert the subsequent 100% mortality. Reasons for failure may lie on defense in response to the modification of skin cells (Berger et al. 2005).

Safe and effective treatment against *Bd* infections (adults and tadpoles) has been reported for voriconazole. This treatment consists of spraying once daily for 7 days at 1.25 mg

litre[-1] (Martel et al. 2010). Furthermore, a summary of disinfection protocols were summarized in a paper by (Retallick et al. 2004) where protocols are detailed in respect to capture, handling and holding of wild amphibians; skin disinfection before and after invasive procedures; marking of frogs; disinfection of skin, sealing of wounds and treatment of accessory equipment (Table 1). These protocols are designed to provide within-site hygiene measures to minimize risk of *Bd* transmission among individuals. The paper also details protocols for entry, exit and between-site hygiene measures to prevent increased risk of *Bd* spread above background levels.

Table 1: Disinfection strategies suitable for treating *Bd* infections: according to Retallick et al. (2004).

Application	Disinfectant	Concentration	Time
Disinfecting surgical equipment and other Instruments (e.g. scales, calipers)	Benzalkonium chloride	2 mg ml^{-1}	1 minute
	Ethanol	70%	1 minute
Disinfecting collection, equipment and containers	Sodium hypochlorite	1%	1 minute
	Path X or Quaternary ammonium compound 128	1 in 500 dilution	0.5 minute
	Trigene	1 in 5000 dilution	1 minute
	F10	1 in 5000 dilution	1 minute
	Virkon	2 mg ml^{-1}	1 minute
	Potassium permanganate	1%	10 minutes
	Complete drying		>3 hours
	Heat	60 °C	30 minutes
	Heat	37 °C	8 hours
Disinfecting footwear	Sodium hypochlorite	1%	1 minute
	Path X or Quaternary ammonium compound 128	1 in 500 dilution	0.5 minute
	Trigene	1 in 5000 dilution	1 minute
	F10	1 in 5000 dilution	1 minute
	Complete drying		>3 hours
Disinfecting cloth	Hot wash	60 °C or greater	30 minutes

3 Methodology

3.1 Description of Study Area

The sampling of amphibians was conducted in the Issyk-Kul region of northern part of Kyrgyzstan. The country Kyrgyzstan is located in Central Asia bordering Kazakhstan, China, Tajikistan and Uzbekistan (Fig. 7). Kyrgystan lies between latitude 39° and 44° N; and longitudes 69° and 81° E; and an average elevation of 2,750 m above sea level. It has a continental climate with cold winters and warm summers: in the highlands, the temperatures range between −20 to 12 °C and −6 °C to 24 °C on lowlands.

Figure 7: Map of the three study sites (Balykchy; Dong-Talaa; and Tupka) in Kyrgyzstan. On the bottom left is a map of Central Asia which depicts Kyrgyzstan.

(Source: http://upload.wikimedia.org/wikipedia/commons/6/68/Map_of_Central_Asia.png).

In the Issyk-Kul region, the three sampled localities (Balykchy; Dong-Talaa; and surroundings of river Tupka) (Fig. 7) were near the Lake Issyk-Kul, an endorheic mountain lake located between steppic and high mountain environments at 1,608 m above sea level in

the northern part of Kyrgystan. The Lake Issyk-Kul is one of the most important nature sites in Kyrgystan with an area of 6,236 km^2; a length of 250 km; 60 km of width; and a maximum depth of 668 m (Sevastyanov et al. 1991). About 118 rivers and streams flow into the lake; the largest are the Djyrgalan and Tyup. The average annual water temperature is 12.1 °C whereas the average annual air temperature is 8.3 °C. The Issyk-Kul region is located in an arid-zone: from west to east is a desert followed by semi-desert and then steppe. Also, the region is dominated by xerophytes except in areas where more water is available; and mesophytic or wetland vegetation occurs as well (Sevastyanov and Smirnova 1986). The average altitude is 1,800 m above sea level; average annual rainfall ranging from 160−180 mm; average min temperature of −5 ℃ and average maximum temperature at 25 ℃.

The first sampled location was around the river Tupka of geographic coordinate 42°38'02.4"N 78°57'46.8"E and east to Lake Issyk-Kul. The river Tupka is located at an elevation of 2,200 m above sea level and surrounded by some sparse spruce forest. It was also observed some growth of willows, steppes and meadows. The second visit in the study area was Dong-Talaa, 42°07'19.2"N 76°33'18.0"E and south-west to the Issyk-Kul Lake. At this locality is a floodplain of a river used as grassland. There was no tree in this vegetation rather shallow marsh and densely overgrown clumps of sedge. The last visited area: Balykchy is located at the western part of Lake Issyk-Kul, at 42°28′N 76°11′E, an elevation of about 1,900m above sea level and composed of rocky deserts with sparse and saline vegetation.

The fauna of the land vertebrates is represented by 335 species. There are 3 species of amphibians; 11 species of reptiles; 54 species of mammals; and 267 species of birds. Among the non-vertebrates the most common animal groups are molluscs, spiders, and insects. Between 60 and 80 thousand water birds (16 species) gather around Lake Issyk Kul for wintering. Nine species of mammals, 18 species of birds, 12 species of insects that are found within the Biosphere Territories of Issyk-Kul region had been included in the Red Book of the Kyrgyz Republic, and three bird species had been included in the International Red Book (source: http://www.unesco.org/mab/doc/mys/2008/FinalRep_Kyrgyzstan.pdf, accessed on 1/04/2015).

3.2 Description of Sampled Species

Three species of amphibians including Siberian wood frog (*Rana amurensis*), marsh frog (*Pelophylax ridibundus*) and green toad (*Pseudepidalea viridis*) were examined in the three above described localities.

3.2.1 Siberian wood frog (*Rana amurensis*)

Morphology

It has a vomerine teeth present with the posterior part of the tongue free and forked. Its toes are webbed. Pupil of the eye is usually horizontal. Shins (knee to ankle) shorter than body by 1.75−2.4 times; when the shins are positioned perpendicularly to the body axis, the heels contact or slightly overlap. When the hind leg is stretched along the body, the tibio-tarsal articulation does not usually reach the eye. The inner metatarsal tubercle is small, 2.3−5.6 times shorter than first toe. Dorsal coloration is greyish or grey-brown with small dark spots. Light middorsal band with distinct edges extends from eye to cloaca. It has flank and thigh skin granular; granulae often red. Its belly is white or white-yellowish with large, irregular, partially fused blood-red spots. The red spots may alternate with dark spots, and the red pattern on the belly starts to form in about the second year of life. Males differ from females by having dark nuptial pads on the first finger (Fig. 8) (Kuzmin 1999; http://amphibiaweb.org/., accessed: 23/03/2015).

Figure 8: Siberian wood frog captured in the Balykchy, Issyk-Kul. Photo by captured by Iva Ulbrichová, 2014.

<u>Distribution, Habitat, Abundance and Special Behaviors</u>

Rana amurensis commonly called Siberian wood frog; Khabarovsk frog; Heilongjiang brown frog; or Amur brown frog' is specie of true frog which lives in a wide range; West and East Siberia, the Russian Far East, Korea, Northern and Central Mongolia, and Northeastern China (Kuzmin, 1999). Distribution of this frog is in countries like: China, Kazakhstan, Korea, Democratic People's Republic of, Korea, Republic of, Kyrgyzstan, Mongolia, and Russian Federation (http://amphibiaweb.org., accessed: 23/03/2015).

In terms of habitat and abundance; according to Kuzmin (1999), the species is present in coniferous (fir, spruce, larch etc.); mixed and deciduous forests (through which it penetrates the tundra and forest steppe zones); shrub lands and grasslands. It is found most frequently in open, wet places such as wet meadows and forest glades, swamps, overgrown lakeshores, riverbanks, and floodplains. On Sakhalin Island, the species is present in tussock tundra like bogs. Reproduction and larval development takes place in shallow lakes, ponds, ditches, large puddles and marshes with stagnant water. Large numbers of this frog may be found hibernating in the bottom mud of ponds and pools. It may be found in slightly modified habitats. Hibernation occurs from early September–early November (usually October) to March–early June (usually April–May), depending on latitude. The frog hibernates in holes at the bottom of lakes and rivers, and in wells, usually in groups up to a few thousand individuals. Terrestrial hibernation seems to be more typical for southern regions.

Reproduction takes place from March–April (usually May elsewhere), whereas in cold northern areas the breeding season may extend until the first half of July. Breeding choruses are absent; the species belongs to the group of "mute" brown frogs. Amplexus is pectoral (axial). The clutch contains 250−4,000 eggs deposited in 1−2 clumps. Metamorphosis occurs from June−August. The maximum age was determined as 5−11 years old in different regions (Kuzmin 1999).

With respect to feeding, the larvae of *R. amurensis* consume mainly algae growing on underwater substrates (Phaeophyta, Zygnemales and Bacillariophyta), as well as higher plants, detritus and small aquatic invertebrates. Juveniles consume mainly terrestrial insects, but sometimes also aquatic arthropods. Adults consume mainly terrestrial invertebrates and the diet varies by season and habitat. The frog sometimes eats aquatic prey. The latter (Mollusca, Gerridae, Dytiscidae, Haliplidae and larval Odonata) are especially important in

the northern part of the frog's range. Small amounts of aquatic organisms have been found in the stomachs of frogs caught at breeding ponds (Kuzmin 1999).

3.2.2   Green toad (*Pseudepidalea viridis*)

Morphology

The green toad ranges from 48−99 mm snout-vent length. The following characteristics are used to describe the green toad: the parotoid glands behind the eyes are prominent; the pupil of the eye is horizontal; the tympanic membrane and male guttural resonator are present; it has a diploid set of chromosomes $2n = 22$; the internal edge of the tarsus contains a longitudinal skin fold; the 3rd toe has a singular subarticular tubercles; the tip of 4th finger exceeds the 1st articulation of the 3rd finger; the dorsal skin is tuberculate, greyish or olive with green or olive spots and red or red-orange points on the flanks and greyish belly (Fig. 9). Male differs from the female by having nuptial pads on the first finger (in breeding season on the 1st, 2nd and 3rd fingers), smaller body size, and sometimes more greenish dorsal background coloration (greyish in females) during the breeding season (Kuzmin 1999).

Figure 9: Green toad captured during field sampling in Balykchy, Issyk-Kul. Photo captured by Iva Ulbrichová, 2014.

Distribution, Habitat, Abundance and Special Behaviors

The species inhabits a large area from north-western Africa through Europe to Siberia and Middle Asia. Green toad is one of the most polytopic amphibians of the Palearctic. It lives in the zones of forests, forest steppes, steppes, semi-deserts and deserts. It is more tolerant to dry conditions than many other amphibians. It inhabits both wet swampy areas as well as dry deserts of different types. In the forest zone, the species tends to live in open areas and bushlands, often far away from water bodies, whereas in the southern dry parts of the range it primarily inhabits moist sites such as oases, the shores of irrigation ditches and lakes. There it uses irrigation ditches and channels as corridors for dispersal. Spawning occurs in a diverse range of water bodies including ponds, swamps, lakes, stream- and river pools, reservoirs, ditches and puddles, as a rule not deeper than 50 cm. Both fresh and saline waters are used for spawning. Green toad is a common species throughout a large part of its distribution. It is generally rare at the north of its distribution, but in some places it forms dense populations in anthropogenic areas. This trend is very typical for green toads, and in some regions their abundance in anthropogenic habitats is much higher than in adjacent natural habitats. The use of burrows sometimes increases green toad density in the colonies of some burrowing rodents. In suitable habitats, its abundance reaches more than 100 individuals per 100 m^2. The toadlet population density during metamorphosis reaches several dozens of individuals per 1 m^2. In arid areas, the toad seems to be distributed more unevenly, forming dense populations in oases separated by vast dry areas unavailable to the toads (Kuzmin 1999).

Hibernation occurs on land, but sometimes it occurs in water such as streams, ditches and wells. Toads hibernate singly or in groups. The timing of hibernation varies significantly through the range, in dependence on altitude and latitude. In southern parts of distribution, the hibernation often is absent, and the toads are active throughout the year. On the other hand, in southern deserts, aestivation supposedly occurs. Reproductive period is also quite variable, from February to July in different parts of the range. In the southern areas, the reproductive period is the longest (ca. 170 days), whereas the duration of development prior to metamorphosis is shortest (ca. 21−25 days) (Kuzmin 1999).

Spawning occurs in a diverse range of water bodies including ponds, swamps, lakes, stream- and river pools, reservoirs, ditches and puddles, as a rule not deeper than 50 cm. Both fresh and saline waters are used for spawning. The Green Toad uses two mating strategies:

active female choice by the competing males and active male choice by the females. Assortative mating has been recorded. The clutch contains 2,000−30,000 eggs arranged in 1−2 rows. The spawn is deposited in two strings of 2−7 m length. Metamorphosis occurs from spring through the summer, in dependence on the latitude and altitude. Mass appearance of newly metamorphosed juveniles is typical for the Green Toad. In such cases pond shores may be covered with thousands of toadlets which disperse from the pond soon after their metamorphosis. Sometimes migrating toadlets form large groups moving as a large band. Maximum longevity is estimated at 7−10 years in different populations of the Caucasus (Kuzmin, 1999).

Tadpoles consume detritus and algae and move towards the shore in daytime and to greater depths in the evening. Animals (Protozoa, Rotatoria, Microcrustacea) are consumed in smaller amounts. Newly metamorphosed toadlets prey upon Collembola, Coleoptera, Acarina and Diptera. Adults eat mainly crawling invertebrates, including spiders, beetles etc. Small amounts of aquatic invertebrates sometimes occur in stomachs of individuals caught in the spring along pond shores. However, the majority of toads do not feed during their breeding migrations. In similarity to other toad species, the Green Toad displays myrmecophagy. Ants compose a significant component of the adult but not juvenile food. Therefore, this peculiarity develops in the toad's postmetamorphic life and may relate to age changes in its foraging strategy (Kuzmin 1999).

3.2.3   Marsh frog (*Pelophylax ridibundus*)

Morphology

Pelophylax ridibundus formerly known as *Rana ridibunda* is a large frog which ranges from 48–170 mm snout-vent length. It has snout moderately sharp. It has vomerine teeth present and posterior part of the tongue free and forked. Its toes are webbed. The pupil of the eye is horizontal. When the shins are positioned perpendicularly to the body axis, the heels overlap (with the exception of Transcaucasian specimens). Inner metatarsal tubercle is low. Dorsal coloration of different tints is from greyish-green or from entirely grey to green. Large dark dorsal spots vary considerably in size, number and arrangement. Light mid-dorsal line is often present (Fig. 10). No temporal spot. Belly is greyish-white or greyish-yellow with dark spotted or blotched-like pattern, sometimes without this pattern. Males differ from females by

having paired grey vocal sacs behind the mouth angles and nuptial pads on the first finger (Kuzmin 1999).

Figure 10: Marsh frog captured during field sampling in Balykchy, Issyk-Kul. Photo captured by Iva Ulbrichová, 2014.

Distribution, Habitat, Abundance and Special Behaviors

The marsh frog inhibits a wide area from the eastern France to the eastern Kazakhstan. they are present in countries like; Afghanistan, Albania, Armenia, Austria, Azerbaijan, Bahrain, Belarus, Bosnia and Herzegovina, Bulgaria, China, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Georgia, Germany, Greece, Hungary, Iran, Islamic Republic of, Iraq, Israel, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Luxembourg, Macedonia, the Former Yugoslav Republic of, Moldova, Republic of, Montenegro, Netherlands, Poland, Romania, Russian Federation, Saudi Arabia, Serbia, Slovakia, Slovenia, Tajikistan, Turkey, Turkmenistan, Ukraine. Introduced countries includes: Belgium, Spain, Switzerland, United Kingdom (Kuzmin 1999; http://amphibiaweb.org/., accessed: 23/03/2015).

Marsh frog is a highly opportunistic amphibian. It lives in mixed and deciduous forests; forest steppe; steppe; semi-desert; and desert zones. Being a semiaquatic species, the frog inhabits a wide variety of flowing and stagnant water habitats, from shallow puddles and ponds to large lakes and rivers, as well as mountain streams. The Marsh Frog is tolerant of high water salinity (it occurs in waters of salinity 0.9−8.3 parts per thousand), and in some places reproduces near the western shore in the Caspian Sea. In general, the Marsh Frog prefers open, well-warmed areas with abundant herbaceous vegetation. The diversity of inhabited water bodies and the extent of terrestrial migrations are higher in wet than in arid areas. Nevertheless, the latter are also successfully colonized by the frog using river valleys and channels. Marsh frog is generally an abundant amphibian. In the rivers of the southern part of Europe, its abundance may reach more than a thousand individuals per kilometer of the riverbank. However, it is relatively rare in swift mountain streams. In general, the species is most abundant in the southern parts of its range.

Hibernation occurs from September–October (in northern regions) or November–December (in the south) to the beginning of June or January-February, respectively. In southern regions, the hibernation is frequently interrupted by warm weather. In unfrozen water bodies, the frog remains active throughout the winter: this is normal in the southern parts of its distribution. As a rule, hibernation occurs in water, but in some locations it occurs in rodent burrows and holes in river banks and lake shores. Group hibernation is typical, but the groups usually do not exceed several dozen individuals (Kuzmin, 1999).

Breeding starts from several days to one month after the frogs' spring appearance. The males form loud choruses, which are especially intensive at the peak of the breeding period. Amplexus is pectoral (axillary). The clutch contains about 670–13,000 eggs. The time of metamorphosis depends on weather, peculiarities of habitat and latitude but usually falls in April–November. In some places of Europe and Asia regular hibernation of tadpoles (up to a few thousand individuals in one pond) has been recorded. Such tadpoles attain sometimes exceptionally large size (total length to 186 mm) and undergo metamorphosis in the next spring. Sexual maturity is attained in the 1st–4th year of life, and the maximum life span has been recorded as 5–12 years (Kuzmin 1999; http://amphibiaweb.org/., accessed: 23/03/2015).

Tadpoles consume detritus, algae, and higher plants in addition to animals (mainly invertebrates) and their corpses. Benthic objects remain the most important component of their diet. Adults consume mainly terrestrial and aquatic insects. Feeding does not cease

during the breeding season. Marsh frog is quite voracious and sometimes attacks not only animals but even the branches of riparian vegetation moving in the wind. In fish ponds, it eats small fishes, but the intensity of predation is too low for any significant influence on the fish crop. Marsh Frog adults, being the largest frogs in Europe, often eat conspecific and other amphibians, as well as reptiles and even small birds and rodents. Cannibalism becomes especially severe during periods of low humidity and precipitation, as well as high temperature (Kuzmin 1999; http://amphibiaweb.org/., accessed: 23/03/2015).

3.3   Data Collection

According to Berger et al. (1998), a disease may present chytrid fungi specific effects on the body and influence behavior of infected amphibians. But caution must be ensured because this may coincide with the symptoms of other diseases or factors. However, *Bd* infected amphibians show symptoms such as red inguinal area (red leg) and general lethargy but to be sure the reliable method employed for the detection of *Bd* was by DNA examination (Hyatt et al. 2007). Thus collection of samples from live amphibians were performed by swabbing of their skin using tubed sterile dry swab tip MW100 (Medical Wire and Equipment Co., UK) as shown in figure 11. Sampling of amphibians on my behalf was conducted by a colleague from the forestry faculty of Czech University of Life Sciences in a person Ing. Iva Ulbrichová, Ph.D.

Figure 11 : Tubed sterile dry swab tip MW100 used for the abstraction of genetic material from the skin of amphibians. Photo derived from Petr Civiš, 2010.

The collection of samples and records were gathered from the study area during field work in July and August, 20. During the field operations, hygiene protocols for disease control were respected (Speare 2001; Boyle et al. 2004). For this reason, handling of each animal was done always with new surgical gloves and other disposal materials. Animals were captured individually and kept in plastic containers with little water, swab-examined in few minutes and immediately released back to their habitats. For every catch, each individual was also thoroughly inspected for visual signs seeming like chytridiomycosis, particularly lethargy or other skin changes. Places providing the highest possible signal in genetic evaluation were the abdomen, hips, back, inner thighs and between toes of hind legs. Hence swabbing of each region of the body was severally done in a standardized approach (Hyatt et al. 2007) (Fig. 12). After completion of the sampling swabs, each sample was provided a specific mark and kept in an impervious container (Fig. 12). Hence such operative samples were stored in a cooling device at about 4 °C for further diagnosis. Samplings were conducted at high daytime temperatures, which are favorable for the chytrid pathogens and thus a greater chance of detecting infections. It was ensured that all swabs taken were free from soil and dirt by rinsing off excessive dirty where necessary. Because soil and dirt could make a *Bd* positive site appear to be negative after analysis. A total of 60 swab samples from three species of amphibians were finally obtained from the three sites visited.

Figure 12: Example of photos showing swabbing of a common toad (left) and on right, labelling of the swab tip with respect to the amphibian examined. Photos were taken in Czech University of Life Sciences, Prague by Emmanuel Essel, 2014.

3.4 Data Analysis

The molecular diagnoses of the samples were conducted under the supervision of my consultant; Vojtech Baláž, Msc., Ph.D. at the laboratory of University of Veterinary and Pharmaceutical Sciences, Brno; and also in the presence of my thesis supervisor, Ing. Jiří Vojar, Ph.D.

3.4.1 DNA Extraction

Following the techniques of Boyle et al. (2004); the DNA of samples were extracted. In the following parts are the list of apparatuses and procedures that were employed:

Apparatus

- The consumables used includes: scalpel blades, petri dishes, 2 ml screw top centrifuge tubes, 0.5 mm silica beads, PrepMan Ultra, 0.5 ml eppendorfs, and pipette tips of various sizes (10, 200 and 1,000).
- The equipments used includes: analytical scales, pipette, tube racks, centrifuge (to reach a speed of 14,500 rpm), MagNA lyser homogenisator, and thermoblock (to reach 100 °C).

Procedure
- Firstly, it was weighted out 0.04−0.05g 0.5 mm silica beads into a 2 ml screw top centrifuge tubes.
- Then followed by pipetting Prepman ultra into each of the same 60 centrifuge tubes. This was done by pipetting 60 μl per each sample and then labelling all tubes appropriately.
- Followed by placing the tip of the swab in a sterile petri dish and using a sterile scalpel blade to slice off each tip. Each sliced tip was placed in the corresponding centrifuge tube. Tips were cut approximately 3−4 mm with new blade for each sample. The petri dishes were turned around four times to enhance different edge for cutting and then changed for a new dish.
- Homogenization of samples using a MagNA lyser for 45 seconds was done.
- Samples were centrifuged for 30 seconds (14,500 rpm).

- Homogenization of samples using a MagNA lyser for 45 seconds was repeated.
- Centrifugation of samples for 30 seconds (14,500 rpm) was repeated.
- Samples were placed in a thermoblock and set on 100 °C for 10 minutes.
- Removed and allowed to cool for 2 minutes.
- Centrifuged at 14,500 rpm for 3 minutes.
- Lastly, pipette of 20 µl tip was used to collect each supernatant and stored them in a sterile 0.5 ml Eppendorf. For every pipetting, new pipette tips were used.

3.4.2 Genie LAMP Reaction

The aim of the Genie II LAMP reaction was to determine the presence of *Bd* from the prepared DNA of amphibians. LAMP reaction was carried out with a total volume of 25 µl of the reagents mixture. The Genie II machine with its complementary isothermal mastermix: 0.6 M KOH; *Bd*/*Bs* Lamp reaction mix; negative and positive controls provided by the OptiGene Limited were used for the experiment using the steps below:

- Using the pipette, 20.0 µl of reaction mix (ISO−004 LNL) was distributed into each of the 8 wells (Block A) of the Genie II machine.
- 2.5 µl of 0.6 M KOH was pipetted into each of those wells.
- 2.5 µl of 6 undiluted DNA samples of amphibians were added again to the first 6 wells.
- The last 2 wells were added also 2.5 µl of negative and positive controls for the purpose of checking respectively contamination and functionality of these solutions.
- The wells were ensured locked properly.
- The software program displayed on the TFT LCD was set to run; amplification of 65 ºC annealed of 98−80 ºC at 0.05 °C/s and loaded for 30 minutes.
- Each well was named according to the labels on each of the samples distributed.
- Last but not least the program was consequently set to start the analysis on block A

Since each block of the Genie II machine can run independently, the same steps were repeated for the other 8 wells (Block B) to analyze 8 different samples with the exception of the positive and negative controls. Every pipetting was done with new pipette tips throughout the experiment. Hence the total 60 samples were finally analyzed. And the results from the

analysis were transferred from the machine's internal memory to personal computer for secondary storage.

4 Results

4.1 Presence of *Batrachochytrium dendrobatidis* infection

Primary data for 60 individuals from three amphibian species were generated from three sites in Issyk-Kul region of Kyrgyzstan from July to August, 2014 (Table 2). Only 1 out of the 60 sampled individuals analyzed by Genie II was positive for *Bd* (Table 2). All other samples analyzed were negative. The infected amphibian was an adult Siberian wood frog (*Rana amurensis*) of 40 mm body length in the Balykchy locality (Fig. 13). Positive isothermal amplification as a result of fluorescence detection was revealed by the end of the 30 minutes of run from the DNA sample of the Siberian wood frog (Fig. 14); with an annealing temperature peak of 82.78 °C. During all field surveillances, the infected frog and all other individuals did not show any clinical signs of chytrid fungi infection nor mass mortalities encountered in all the three localities.

Table 2: Presence of *B. dendrobatidis* analyses by Genie II, indicating the level of infection to species samples generated from Kyrgyzstan.

Date of sampling	Site	Species	Sample Size	Examined	Positive *Bd*
7/27/2014	Tupka River	*Rana amurensis*	10	10	-
8/22/2014	Dong-talaa	*Rana amurensis*	7	7	-
8/23/2014	Balykchy	*Rana amurensis*	13	13	1
8/23/2014	Balykchy	*Pelophylax ridibundus*	10	10	-
8/23/2014	Balykchy	*Pseudepidalea viridis*	20	20	-

Figure 13: Length of all Siberian wood frogs sampled from the locality Balykchy. In red color, it is the height of the frog which was detected *Bd* positive. While blues colors are the negative sampled frogs.
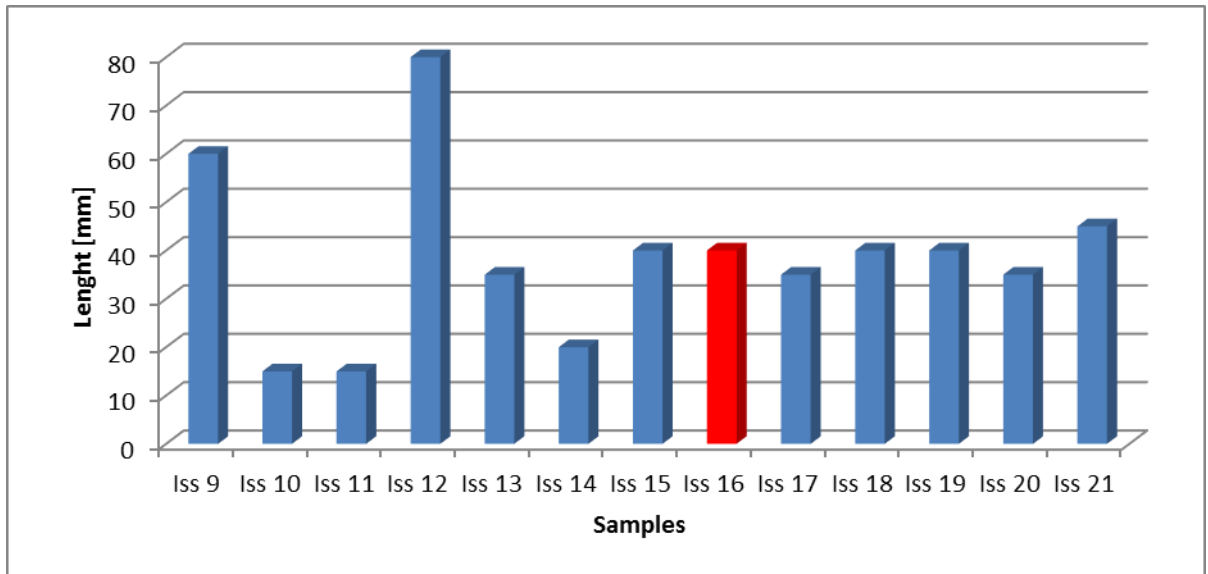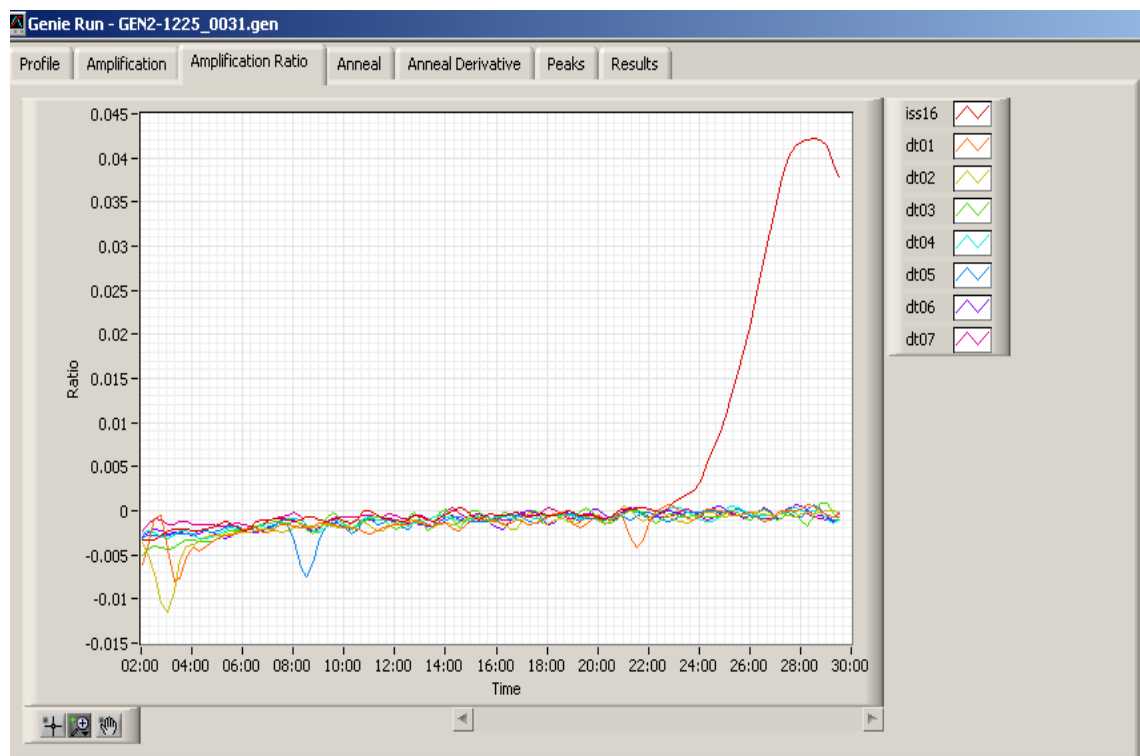

Figure 14: Positive amplification of the infected Siberian wood frog by Genie II.

4.2   Methodological Guide for Genie II Operations

Operations with the Genie II machine were completely safe and easy to use; thanks to OptiGene Limited, UK for this molecular diagnostic made-simple tool. However to avoid risk to the safety of the equipment, operator and or anybody in the vicinity, the following principles must be observed to achieve a successful result. In the following parts will be described, in accordance with the user manual provided by the OptiGene Limited, UK on how the Genie machine is set up to run (hardware and software) in the detection of positive or negative *Bd*.

4.2.1   Setting up the Genie Machine

Laboratory Preparation

The desk or table in the laboratory was ensured to be levelled and stable. The machine was then placed on the center of the prepared table and cleared of obstructions at all times. Restricting airflow may impede operation and could affect performance: therefore, the air at the front of the machine and the outlet vents at the rear were ensured not restricted. Electrical source was ensured close to the instrument to avoid injury from trailing wires. Because Genie II is an electrical instrument, it was kept away from sinks and other wet areas.

Connections

It was not the first time using the machine so contents in the box included Genie II instrument; power supply; power lead; USB connection lead; USB memory stick containing Genie II software; and user manual. Genie II has an internal rechargeable battery but for this experiment, the power supply plug was connected into the back of the instrument and then attached to the power supply. Located to the rear of the instrument is an on/off power button, therefore it was switched to on and powered up to progress the experiment.

4.2.2 Starting Analyses by the Genie II Machine

The main menu popped up after switching the machine on and since Genie II uses a touchscreen for viewing and inputting data; 'Run' was selected to start the analyses. On the status bar the time and date were set. Pressing 'Run' proceeded to profile screen. Hence by selecting 'New' a profile was created. Adjustments were made on the profile with respect to the amplification and anneal temperature as well as time for the run (Fig. 15); and followed by 'enter'. Hence the profile was named and saved.

Figure 15: Adjustment of amplification, anneal and time for running of samples.

As described in the previous chapter: Methodology (3.4.2), Genie II LAMP reaction mix was prepared for 'Run'. After this preparation, the saved profile was reloaded. By selecting 'Start' to continue the 'Run', a prompt asked whether the experiment is to be run on block 'A' or block 'B', or both. Each block runs independently, but for this experiment it was begun with block 'A'. Thus each of the eight wells was assigned a name according to the mark or label on the tube that contained the extracted DNA.

4.2.3   Reading Results from the Genie II Machine

Once the analysis was completed, the machine displayed 'A Finished' on the status bar. This meant that the analysis has been completed with Block 'A' and the result was ready for display.

<u>Amplification</u>

This shows the fluorescence data that is being acquired during the amplification phase of the experiment. Usually positive amplification is shown by the expansion of the fluorescence intensity per time ratio which gives an indication of positive *Bd* (Fig. 16). Whereas negative *Bd*, does not show any expansion of fluorescence.

Figure 16: Example of a graph showing positive amplification with respect to the intensity of fluorescence per time.
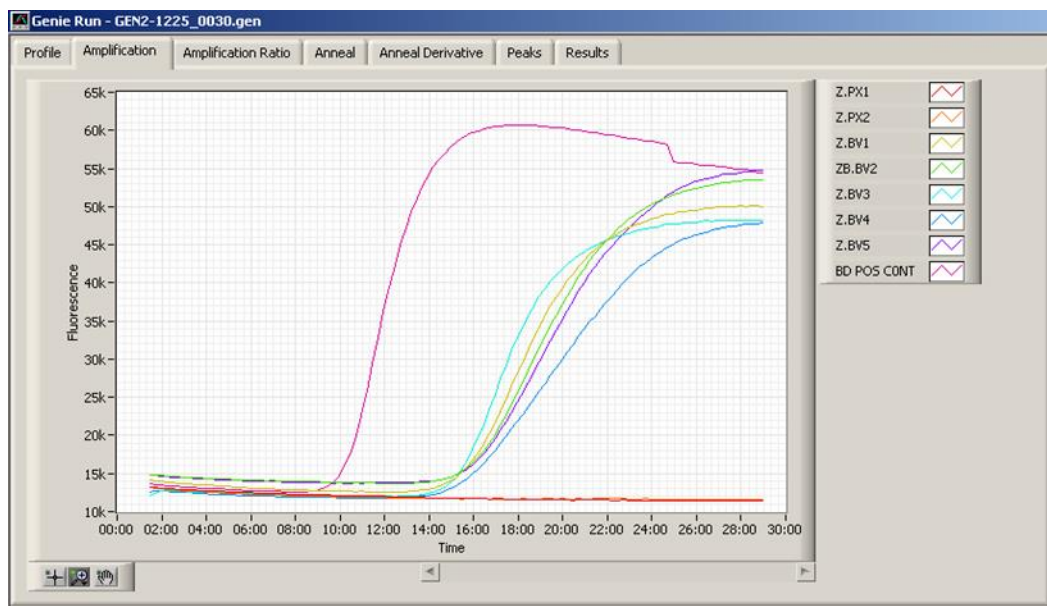
<u>Anneal</u>

This shows the fluorescence derivative data that is being acquired during the anneal phase of the experiment. Usually anneal is shown by the expansion of the fluorescence intensity per temperature ratio which gives an indication of positive *Bd* (Fig. 17).

Figure 17: Example of a graph showing anneal with respect to the intensity of fluorescence per temperature.
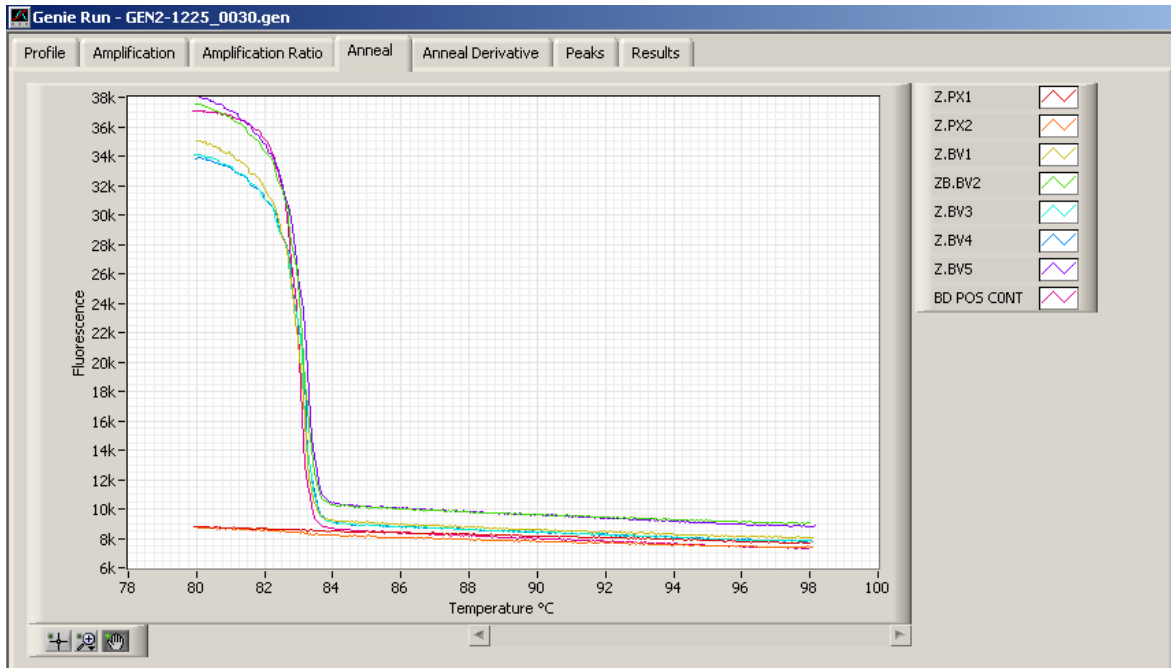
Result

The outcome of the experiment could also be explored by selecting result on the screen. After clicking on the result, the sample name and its respective amplification time and anneal temperature will display in a table format. Whilst Genie II is running, the display can be switched to view 'Amplification', 'Anneal' and 'Results' between block 'A' and block 'B' by pressing the 'A/B' button on the top menu bar. Data for both blocks together is displayed by cycling through the 'A/B' button. When displaying both graphs together, block 'A' data is shown with solid lines, whilst block B data is shown with dashed lines.

5 Discussion

5.1 Presence of *Bd* in Kyrgyzstan

During the field work, all the amphibian species sampled in the three studied areas did not show any clinical signs of *Bd* infection such as discolored skin, shedding of skin, abnormal postures, unnatural behavior or seizures. And even the positive amplification of the *Bd* positive Siberian wood frog came at the end of the 30 minutes of analysis by the Genie II machine. All these conditions give an indication of low level of *Bd* presence compared to usual situations where positive amplification is shown within the first 15 minutes of analysis. Although only 1 out of the 60 samples representing 1.67 % was *Bd* positive, this finding is in accordance with the trend that *Bd* has not yet caused epidemic-level of declines in Kyrgyzstan and Asia at large (Swei et al. 2011b). The survey by Swei et al. (2011a), revealed positive *Bd* in only 2.35 % of 3,363 amphibian samples gathered from 15 countries over a period of nine years (Table 3). From their results, the highest prevalence was found in Kyrgyzstan with 100 % (n = 9) infection prevalence; however nine samples cannot form a significant predictive value (Digiacomo and Koepsell 1986). Furthermore, all these samples were taken from one region in Kyrgyzstan.

Table 3: Status of *Batrachochytrium dendrobatidis* infection in Asia. (Analysis according to Swei et al. 2011a).

Country	Samples	High	Bd Status Infection positive	Low	Bd Status including low infection positives Infection positive
	No.	No.	High positive (95% Credible Interval)	No.	% Low positive (95% Credible Interval)
Cambodia	384	0	0 (0-0.95)	0	no change
China	256	0	0 (0-1.43)	0	no change
Indonesia	797	3	0.25 (0.14-1.09)	8	1.38 (0.72-2.36)
Kazakhstan	4	0	0 (0-45.7)	0	no change
Kyrgystan	9	9	100 (69.15-99.75)	0	no change
Laos	551	4	0.73(0.29-1.84)	0	no change
Malaysia	111	1	0.90 (0.21-4.87)	1	1.80(0.56-6.30)
Mongolia	23	0	0 (0.11-14.25)	0	no change
Myanmar	62	0	0 (0,04-5.69)	0	no change
Pakistan	5	0	0 (0.42-45.93)	0	no change
Papua New Guinea	73	0	0 (0.03-4.86)	0	no change
Philippines	412	33	8.01 (5.77-11.04)	1	8.25(5.96-11.28)
South Korea	29	1	3.45 (0.82-17.22)	1	6.9(2.11-22.07)
Sri Lanka	117	0	0 (0.02-3.10)	10	8.55 (4.38-14.51)
Vietnam	530	0	0 (0-0.69)	7	1.32 (0.65-2.70)
Total	**3363**	**51**	**1.52 (1.15-1.98)**	**28**	**2.35 (1.89-2.92)**

All countries sampled in this study and status of amphibian infection with *Batrachochytrium dendrobatidis*. Positives samples were divided into "low infection" and "high infection" positives with low infection defined as samples with corrected genomic equivalent (Z_{swab}) values <1 but >0. Percent positives are given in the in the columns along with the 95% Bayesian credible intervals.

Despite less research of *Bd* in Asia, the few studies that have examined *Bd* in Asia have either failed to find it or found low prevalence (Kusrini et al. 2008; McLeod et al. 2008; Swei et al. 2011b; Vörös et al. 2012). Thus, there are currently no reports of major population declines as a result of *Bd* in Asia (Swei et al. 2011b); compared to the growing evidences of mass mortalities and declines in Central America, California, Europe and Australia (Van Sluys and Hero 2010; Vredenburg et al. 2010). Swei et al. (2011b) postulated that in Asia, the epidemiology of *Bd* infection dynamics on amphibian hosts, were usually on average 300 zoospores which was less than the documented mortality threshold of 10,000 zoospores. This perhaps means that the distribution of the chytrid fungus in Asia does not follow a clear geographic pattern that explains the linear, wave-like spread of the pathogen across the entire continent. Hence, the emergence and spread of *Bd* in Asia is relatively low (Fig. 18) (Swei et al. 2011a).

Figure 18: Map of predicted and observed *Batrachochytrium dendrobatidis* distribution in Asia. Map of Asia and Papua New Guinea showing Maxent predicted probability of *Bd* from low to high environmental suitability. Black and white shows the sample localities from field surveys; red with black dots indicate low positive *Bd* records and red circles indicating high positive *Bd* records (Swei et al. 2011a).
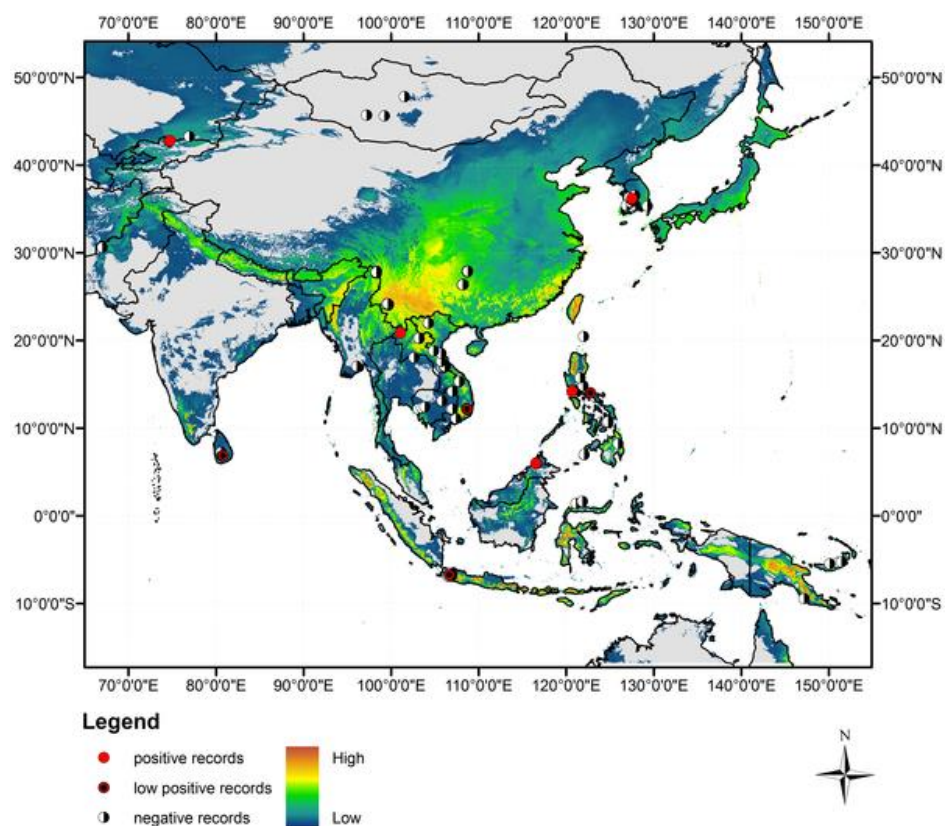
Though the qPCR method of diagnosis has gained much recognition worldwide, it usually takes much time for diagnosis, and requires an expert diagnostician to carry the analysis. Sometimes this takes months or years and thereby causes DNA deterioration of samples stored as well as slowing actions that could be taken to reduce the spread of the pathogen. On the other hand, working with Genie needs less expertise and works very fast. The positive and negative controls used proved the sensitivity and functionality of the reaction mixture, and thereby boost the authenticity of the achieved results. Hence results from this study can be trusted even though samples analyzed were not large and taken from one region in Kyrgyzstan. However, the Genie II LAMP technique still remains an excellent tool for rapid diagnosis of *Bd* infection on the field or in laboratory, and should greatly improve our ability to acquire more data necessary for thoroughly understanding the dynamics of *Bd* so as to conserve remaining amphibian populations

6   Conclusions

This thesis has presented useful information on human influences that leads to global decline or extinction of amphibians. Thus with much emphasis on the *Batrachochytrium dendrobatidis* (*Bd*) which causes the emerging infectious disease to amphibians. Hence the best and easy-to-use tool: Genie II has been used to monitor the low prevalence of *Bd* in Kyrgyzstan. The vivid methodological guide for working with Genie II has also been described in this thesis for future use. An attempt has been made to capture the extent or distribution of the *Bd* in Asia and the world as a whole.

The most useful response to a newly emerging pathogen is to predict locally its routes of spread, its impact and risk to populations that will be affected. In this stem, this thesis has provided information on geographical distribution of *Bd* across the globe, and the effect of the fungus on amphibian populations. The ability of an assay to detect low levels of organisms at an early stage of infection is vital to prevent the spread of *Bd* infections between habitats and populations. It would also allow early treatment and protection of endangered species. This thesis demonstrated that the Genie II as a molecular diagnostic tool can successfully detect *Bd* on wild or captive amphibians, even when they exhibit no clinical signs of disease, and that swabs can be successfully stored for long period of time at low temperature, with no apparent decrease in sensitivity. It also explained its simplicity, convenience, less expertise and rapid detection of chytrid fungus and other pathogens. Thus serves as a motivating and powerful tool to help prevent the spread of *Bd*, particularly to areas not already infected.

This finding suggests an early stage of the *Bd* infection in Kyrgyzstan and Asia as a whole. Therefore, conducting *Bd* infection surveys in a systematic fashion, I increased considerably the usefulness of the information presented within this thesis. This survey's methodology and approach to understanding the ecology can serve as a model not only for future *Bd* and *Batrachochytrium salamadrivorans* research, but also for research into others, perhaps as yet unidentified, wildlife diseases-causing organisms. The global distribution and prevalence of *Bd* remains far from clarified and recent evidence suggesting that *Bd* may have evolved from or at least have a long history in Asia. Hence this thesis suggests intensive survey and molecular analysis of *Bd* throughout Asia, to assess infection trials and to also acquire knowledge about the susceptibility of Asian amphibians to *Bd* infection.

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Figures

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Tables

Tab 1: Retallick, R. W. R., Mccallum, H., and Speare, R., 2004: Endemic infection of the amphibian chytrid fungus in a frog community post decline. *PLOS Biology*, 2: 1965–1971.

Tab 3: Swei, A., Rowley, J. J., Rödder, D., Diesmos, M. L., Diesmos, A. C., Briggs, C. J., Brown, R., Cao, T. T., Cheng, T. L., Chong, R. A., Han, B., Hero, J. M., Hoang, H. D., Kusrini, M. D., Le, D. T., McGuire, J. A., Meegaskumbura, M., Min, M. S., Mulcahy, D. G., Neang, T., Phimmachak, S., Rao, D. Q., Reeder, N. M., Schoville, S. D., Sivongxay, N., Srei, N., Stöck, M., Stuart, B. L., Torres, L. S., Tran, D. T., Tunstall, T. S., Vieites, D., and Vredenburg, V.T., 2011a: Is chytridiomycosis an emerging infectious disease in Asia? *PLoS One*, 6(8): e23179.

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