A genetic survey of the amphibian pathogen *Batrachochytrium dendrobatidi*s collected in British Columbia, Canada and Peninsular Malaysia

by

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BSc, Brock University, 2004
BA, Brock University, 2005

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in the Department of Biology

Supervisory Committee

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Abstract

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The amphibian pathogen, *Batrachochytrium dendrobatidis* (*Bd*), has been the cause of mass declines of amphibian populations worldwide (Berger et al. 1998). This pathogen has been shown to infect approximately 387 different amphibian species and causes declines in approximately 200 species (Skerratt et al. 2009). The total impact on amphibian biodiversity as well as their ecosystems has yet to be determined but it has already been suspected in some species extinctions (Schloegel et al. 2006). The distribution of this amphibian pathogen has been described by two competing hypotheses, the novel and endemic pathogen hypotheses. The endemic pathogen hypothesis states that the pathogen has always been a part of the ecosystem and has only recently become pathogenic due to environmental factors. The novel pathogen hypothesis states that the pathogen has just recently been introduced and has encountered a naïve host which has resulted in population declines (Rachowicz et al. 2005). Research into these two hypotheses has been very active yet the results have still been conflicted (Pounds et al. 2006; James et al. 2009). In our study we assess two relatively under surveyed locations for the presence of *Bd*, both in Peninsular Malaysia and British Columbia (BC). The results of the amphibian survey showed that *Bd* was currently ubiquitous throughout the province of BC. This was coupled with a population genetic evaluation of two *Bd* strains in British Columbia which led us to conclude that they were a part of a novel pathogen which may have been introduced through the amphibian trade possibly from the east coast of Canada. During the first two years of surveying for the presence of *Bd* in Peninsular Malaysia we found no evidence of the pathogen. In the third and final year of the survey we did discover low prevalence of the pathogen, which was supported by a

recently published report of initial *Bd* detection in Peninsular Malaysia (Savage et al. 2011). We were not able to definitively state which of the competing hypotheses (NPH vs EPH) was correct for either collection region. Our population genetic results for two isolates collected from Bullfrogs on Vancouver Island suggest that *Bd* may have been introduced via the animal trade however the endemicity for the rest of the province remains unresolved. In peninsular Malaysia *Bd* may represent a novel pathogen or it could exist as an endemic pathogen with a low prevalence.

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Thank You

Live Believe Love Laugh Prioritize

Chapter 1

General Introduction

Up to one-third of all amphibians are at risk of extinction (Wake & Vredenburg 2008). In the 1980's, scientists began noticing significant declines among amphibian populations worldwide (Wake, 1991). Early work focused on habitat destruction as the leading cause of population declines (Lehtinen et al. 1999; Petranka et al. 1993). More recently scientists have detected amphibian declines in pristine, montane regions which had no direct link to human interactions (Blaustein & Wake 1990). This initiated debate regarding whether amphibian decline was directly attributable to human activities such as habitat destruction and fragmentation, indirectly due to human activities resulting from the amphibian trade or capture for food use, or whether there might be some other unknown factors coming into play (Collins & Storfer 2003). Global climate change resulting in increased UV-B radiation could reduce amphibian embryo survival (Blaustein et al. 2003). Certain chemical pollutants are endocrine disruptors which through failed metamorphosis or reproduction may lead to amphibian population declines (Hayes et al., 2006). Another cause for this decline could be attributable to invasive species on native populations either through direct competition or through the transfer of disease (Fisher & Shaffer 1996). Infectious diseases have been shown to adversely affect amphibian populations, especially in pristine locations. One of the more recently characterized amphibian pathogens is *Batrachochytrium dendrobatidis* (*Bd*), which has been associated with population declines in many parts of the world (Berger et al. 1998; Bradley et al. 2002; Bosch 2001; Lötters et al. 2009; Ron et al. 2003).

The abundance and ubiquity of amphibians in many ecosystems make them an important class of vertebrate animals due to their biodiversity. In some instances amphibians can be the most abundant vertebrate in their ecosystem (Beard et al. 2002). Their bi-phasic life-history makes them important in both aquatic and terrestrial food webs. Most studies have shown the importance of amphibians on nutrient cycling or energy flow (Regester et al. 2006). The greater threat of this recently discovered pathogen is much greater than its affect on local amphibian biodiversity. The

ramifications of amphibian species loss will have a large impact on other organisms as well as the ecosystems that they belong to (Whiles et al., 2006).

***Batrachochytrium dendrobatidis* (*Bd*)** (Longcore et al. 1999)

A recent threat to amphibian populations is the waterborne fungal pathogen, *Bd* which was named by Longcore et al. (1999). This aquatic fungal pathogen is the causal agent of chytridiomycosis, first associated with amphibian declines in 1997 (Berger et al. 1998). This relatively recently described fungus is a pathogen which had not been known to infect amphibian hosts. *Bd* has now been implicated in many enigmatic declines of amphibian populations worldwide (Lötters et al., 2009) and has been found to infect approximately 387 amphibian species, of which 200 have shown population declines (Skerratt et al. 2007). *Bd* has been discovered in 45 of 78 countries tested so far (Rosenblum et al. 2009) and is found on every continent that has amphibian hosts.

Bd is part of the Kingdom Fungi; division Chytridiomycota,class Chytridiomycetes, and order Chytridiales. The previous classification of the Chytridiomycota was based on the features of their aquatic zoospore stage that has a flagellum. The incorporation of a flagellum and early fossil records suggest that Chytridiomycota are among the earliest representatives of the Kingdom Fungi (Ibelings et al., 2004). Recent phylogenies developed using rRNA sequences and sections of RNA polymerase II subunits among other genes using specific gene sequences has confirmed *Bd*'s basal location in the Fungi (Bowman et al. 1992; James et al. 2006). Work combining the analysis of the zoospore ultra-structure information along with molecular data has supported this conclusion (James et al. 2000; Letcher et al. 2005).

The Chytridiomycota are ubiquitous aquatic fungi that acquire nutrients by breaking down chitin, keratin and cellulose in decaying matter. Some chytrid fungi are facultative and obligate pathogens parasitizing algae, invertebrates and plants (Gleason, Kagami, Lefevre, & Simengando, 2008). Two pathogens of vertebrates are known, the

little studied fish pathogen *Ichthyochytrium vulgare* (Svobodova & Kolarova, 2004) and *Bd*, which infects the skin of amphibian hosts sometimes fatally (Berger et al. 1998).

Bd's life cycle is similar to other chytrid fungi. It consists of two stages, a motile flagellated zoospore stage and a stationary thallus stage. The brief zoospore stage is motile in water due to use of its flagellum, until it attaches to a susceptible host and encysts under the skin. During the thallus stage, the flagellum is lost and the thalli grow and produce multinucleated sporangia over four to five days. A discharge tube is then produced by the sporangia as they develop many individual zoospores ready to be released (Berger et al. 2005). The presence of a sexual phase, though not directly observed in nature or in culture, has been inferred from genetic evidence for sexual recombination (Morgan et al. 2007; James et al. 2009). A possible resting stage has been described by Di Rosa and coworkers (Di Rosa et al. 2007).

Bd inhabits aquatic ecosystems as well as moist soil. *Bd* is able to survive temperatures between 4°C and 25°C with optimal growth between 17°C and 25°C. Temperatures above 28°C stopped growth and sometimes killed the fungus (Piotrowski et al. 2004). Although it has a small optimal temperature range, *Bd* is able to survive from 4°C and 25°C by shifting from growth to reproduction and virulence (Woodhams et al. 2008).The optimal pH range for the fungus is between 6 and 7 typically found in freshwater ecosystems (Piotrowski et al., 2004). The ability to continue to grow, despite unfavourable environmental conditions could make *Bd* a resilient pathogen despite not having a proven resting sexual stage.

Host - Pathogen Interactions

The mode of infection of *Bd* on the amphibian host is not completely understood. It is believed that the zoospore encysts on the skin surface where upon germination it generates a germ tube which penetrates the host (Longcore et al. 1999). Once the integument has been breached the fungus grows in epidermal cells a few layers deep. The fungus continues its growth and development in keratinized cells near the skin surface of

the amphibian (Pessier et al. 1999). The fungus produces discharge tubes which merge with and dissolve the epidermal cell membrane (Berger et al. 2005). The fungus continues to grow, and produce zoospores that are released both inside the host and the surrounding aquatic environment. The fungus is able to transmit infective propagules by direct host to host contact or through the water (Rachowicz et al. 2007).

Pathogenesis is not completely understood. It has been shown that disruption of the epidermis leads to electrolyte imbalances and eventually cardiac arrest (Voyles et al. 2007). The growth and proliferation of the pathogen in the host can lead to death of the host under certain conditions (Vredenburg et al. 2010). Virulence factors which permit this pathogen to evade amphibian host defences can include secreted enzymes for growth in skin as well as toxins which may act specifically on the host. The sequencing of two *Bd* genomes has recently revealed potential virulence factor genes. Rosenblum et al. (2008) identified serine-type peptidases which may help the fungus circumvent the antimicrobial peptides that are part of the host defence system. They also showed many fungalysin like metallopeptidases which are important virulence factors for dermatophytes (Rosenblum et al. 2008). These complex and specific amphibian pathogenicity factors may suggest that *Bd* and amphibians have co-existed for many years.

Host defence in amphibians consists of both innate and acquired immune responses. One such innate response is the production of antimicrobial peptides which have been shown to inhibit the growth of *Bd in vitro* (Rollins-Smith et al. 2003). These antimicrobial peptides vary by species and their efficacy against *Bd* needs to be field-tested. There is also an acquired immune response that is mounted by the amphibian after infection. Typically asymptomatic, *Xenopus laevis*, with reduced lymphocyte function were more susceptible to *Bd* infection despite being asymptomatic under normal conditions (Ramsey et al. 2010). Some amphibians harbour bacteria which can serve to inhibit *Bd* (Harris et al. 2006). Symbiotic bacteria are thought to work synergistically with the amphibians' innate immune defence to protect it from chytrid infection (Woodhams et al. 2007).

Novel and Endemic Pathogen Hypotheses

The recent characterization of this pathogen and its widespread movement and impact has led scientists to classify *Bd* as an emerging infectious disease (EID) (Daszak et al. 2003). The earliest known sample of *Bd* comes from an African museum specimen of *X. laevis* from 1938 (Weldon et al. 2004) and the first population declines associated with the presence of *Bd* were reported in 1997 (Berger et al. 1998). The fungus has been discovered on every continent home to an amphibian host. *Bd* has also been shown to infect many amphibian hosts and cause declines in approximately 200 species (Skerratt et al. 2007).

The emergence of new infectious diseases can be explained by two competing hypotheses (Rachowicz et al. 2005). The novel pathogen hypothesis (NPH) states that the pathogen has just recently been introduced and is being vectored by a newly colonized host. This usually involves a host vector that introduces the pathogen to a vulnerable species. The endemic pathogen hypothesis (EPH) states that the pathogen is endemic to the region and is becoming pathogenic due to changes in biotic or abiotic factors in the environment. These factors can either act to lower the immune response of the host or increase the virulence of the pre-existing pathogen.

The introduction of a novel pathogen into a new region where it can encounter naïve hosts is reliant on an appropriate vector. To be most effective this vector should harbour the pathogen but be asymptomatic. The African clawed frog, *Xenopus laevis,* is considered to be a potential vector for *Bd* due to its ability to tolerate infection by this pathogen (Weldon et al. 2004). These frogs were also widely traded because they were used in research and for pregnancy tests worldwide hence the frog trade may have contributed to the world-wide dissemination of the pathogen. A second potential vector for *Bd* is the American bullfrog *Lithobates catesbeianus*. Like the African clawed frog the American bullfrog can be heavily infected with the chytrid fungus but not succumb to the pathogen (Daszak et al. 2004; Schloegel et al. 2010). The American bullfrog has been widely distributed through the trade of live animals for food purposes and upon escape is

able to thrive in many non-native environments (Fisher & Garner 2007; Schloegel et al. 2009). The bullfrog has tested positive for *Bd* in many areas around the world including Vancouver Island where it is considered an invasive species (Garner et al. 2006; Schloegel et al. 2010; Hanselmann et al. 2004; Bai et al. 2010). The same genotype of *Bd* has also been shown to be associated with both farmed and wild bullfrogs in close proximity suggesting that the pathogen, in some cases, has been introduced through the bullfrog trade (Schloegel et al. 2010).

The introduction of a novel pathogen would be expected to result in a wave-like pattern of occurrence radiating from the initial point of introduction. This wave-like pattern would result in a distinct front which would be spatially and temporally dependant on the pathogen in a newly inhabited region. Occurrences of disease fronts associated with mass die-offs of animals have been seen in both Australia (Laurance et al. 1996) and Central and South America (Berger et al. 1998; Lips et al. 2008). There has also been correlation of the presence of *Bd* at these disease fronts and a high prevalence of the pathogen in native frog populations (Lips et al. 2006). These observations could be interpreted as a novel introduction of the pathogen at these locations. Early population genetic work using multi locus typing (MLST) has shown *Bd* to be highly uniform (Morehouse et al., 2003), suggesting that *Bd* reproduced clonally. A follow-up MLST study by Morgan and coworkers (Morgan et al. 2007) suggested that *Bd* had probably been relatively recently been introduced to North America, specifically in California, as a novel pathogen. These pioneering population genetic analyses suggest that *Bd* could be a novel pathogen which has been introduced globally to naïve amphibian populations though the actual place of origin for this pathogen remains unresolved.

There have been three separate arguments for the possible geographic origin of *Bd*. The first proposed origin is Africa. This has received support due to an endemic vector, *X. laevis*, as well as an early known detection of the pathogen (Weldon et al. 2004). Another possible origin or source of increased distribution is North America. Genetic surveys show increased diversity among several loci compared to African isolates. One would expect the highest genetic diversity in the originating site and with

less diverse populations being found at secondary sites due to founder effects. The North American strains also showed similarities to many European strains which may indicate movement of the pathogen from North America (James et al. 2009). The North American hypothesis is also supported by the American bullfrog which is one of the most frequently traded vectors (Fisher & Garner 2007; Schloegel et al. 2009). The most recently proposed origin for *Bd* is Asia. This is supported by a possible endemic haplotype using ITS sequencing as well as an early detection on an endemic *Andrias japonicus* museum sample from 1902 (Goka et al. 2009). This was recently coraborated by a separate finding of this same haplotype in China as well as lower levels of prevalence (Bai et al. 2012).

The EPH explains the emergence of disease using biotic and abiotic environmental factors which change the interaction between the host and the pathogen. The main argument supporting the EPH is the global distribution of *Bd* (www.bd-maps.net). There is also evidence of the pathogen in museum samples that predate noticeable declines in amphibian populations (Weldon et al. 2004; Ouellet et al. 2005). There is also no conclusive indication of a resting stage which would make the fungus able to withstand transport worldwide, despite its documented fragile life stages (Piotrowski et al., 2004). All of these factors suggest that *Bd* is an endemic fungus which may have recently become virulent in certain regions due to changes in environmental factors. Climate and other stresses which lead to population declines can reduce amphibian immune responses (Carey, 1993; Reading, 2007). There is also evidence linking temperature changes to ideal *Bd* growth conditions which could make it a more potent pathogen. It was shown that a rise in average temperature at night due to climate change resulted in an optimal growth temperature for *Bd* (Pounds et al., 2006). The influence of these abiotic variables on the pathogenicity of *Bd* may indicate that the pathogen has always been in the environment and is only now becoming virulent.

Despite *Bd* isolates being genetically highly conserved even small changes could be significant to pathogenicity. Different isolates of *Bd* have varying levels of virulence towards the same amphibian host (Berger et al. 2005; Retallick & Miera 2007). There are

also distinct phenotypic differences, zoospore densities, among isolates (Voyles 2011). Genotypic differences are associated with differences in virulence and phenotypes as well as distinct proteomic profiles (Fisher et al. 2009). Hence despite *Bd* being genetically conserved, there are sufficient differences which can lead to significant phenotypic differences among isolates.

Population Genetics of *Bd*

The first survey of the *Bd* genome used MLST. A total of 10 loci were examined of which 5 were found to be variable. The low genetic variability among genomes tested suggested that *Bd* was typical of a recently emerging clone and that sexual reproduction was rare (Morehouse et al., 2003). Morgan et al. (2007) surveyed *Bd* populations for 15 variable loci. This increase in the number of loci allowed for better resolution of population structure and showed that, although highly conserved, *Bd* did show signs of possible sexual reproduction. The results of these surveys showed that *Bd* was highly similar but population structure could be resolved with these improved markers.

The ability to sequence greater numbers of loci in shorter time with less expense led to more robust surveys involving more *Bd* strains collected globally. Consequently, the population genetic study examined 17 loci and, more importantly, surveyed 59 isolates from all over the world to determine if any similarity patterns could be detected. This survey confirmed that *Bd* was diploid and, although still highly conserved, there was clustering of similar *Bd* isolates according to their geographic distribution (James et al. 2009). The authors proposed that a single hybrid ancestor, which may have undergone meiosis, led to this newly distributed highly similar strain. Whole genome sequencing of 20 different worldwide strains of *Bd* found that 16 of the 20 genomes showed 99.9% homology and were labelled by the research group as a global panzootic lineage (GPL) (Farrer et al., 2011). The remaining strains grouped together as a common lineage which was named the Cape lineage due to their association with Africa as well as a Swiss lineage which seemed distinct. The GPL lineage was also shown to be hypervirulent

when compared to the Cape lineage of *Bd*. The Swiss lineage was not tested for virulence due to its late addition to the study (Farrer et al., 2011). These recent findings confirmed the highly conserved nature of *Bd* in many areas of the world and suggest that the GPL is a novel pathogen which has possibly been spread worldwide. The GPL could be replacing endemic strains of *Bd* in certain regions of the world and it is possible that there have historically been many epidemics.

The overall goal of this study was to better understand this emerging infectious disease which is adversely affecting amphibian biodiversity worldwide. One of the objectives of this study was to survey for the presence of this pathogen in British Columbia, Canada and Peninsular Malaysia and, if present, to ascertain the origin of *Bd* in these locales. As an experimental framework we chose to test the Novel and Endemic Pathogen Hypotheses to determine which best represented the surveyed locations. The next step was to use MLST markers to describe the population genetic structure of select BC isolates in relation to other isolates collected worldwide and place the Canadian isolates in an international context. It was anticipated that this might help to determine whether *Bd* is a novel pathogen which has recently been introduced or if it is an endemic pathogen which is only now becoming virulent in many areas throughout the world.

Chapter 2

Testing for the Novel and Endemic Pathogen Hypothesis in British Columbia

Introduction

With over 6000 documented species, and more being discovered every year, amphibians are an important part of ecosystem biodiversity (Wake & Vredenburg 2008). There has been a marked decline in amphibian populations observed by scientists since the 1980's (Wake, 1991). Most explanations of amphibian declines have been linked to anthropogenic causes with the greatest impact being from habitat destruction (Lehtinen et al., 1999; Petranka et al., 1993). Recently, however, amphibian declines have been occurring in pristine, montane areas far from obvious human interactions. These declines are referred to as enigmatic declines due to their mysterious nature (Blaustein & Wake, 1990; Stuart et al., 2004). One possible cause for these enigmatic declines was postulated to be the pathogen *Batrachochytrium dendrobatidis* (*Bd*), which causes chytridiomycosis in amphibians and can lead to death (Lötters et al., 2009). The amphibian pathogen, *Bd*, was first implicated in amphibian declines in 1998 in both Panama and Australia (Berger et al. 1998). The impact of *Bd* on amphibians can be seen in its range of hosts as *Bd* is documented to infect over 300 species of amphibians (Skerratt et al. 2007). The large impact on amphibians and its recent discovery as a pathogen has led to *Bd* being classified as causing an emerging infectious disease (EID) (Daszak et al. 1999; Daszak et al. 2003).

Two competing hypotheses have been put forward to explain EIDs. The novel pathogen hypothesis states that it is novel and has only recently been introduced to an area with a susceptible host (Rachowicz et al. 2005). The wave like introductions of the pathogen which have been noticed in many different areas is consistent with a novel pathogen (Laurance et al. 1996; Berger et al. 1998; Lips et al. 2006; Lips et al. 2008). The recent sequencing of 20 *Bd* genomes has also led researchers to propose that there are

three clades of *Bd* which include a hypervirulent global panzootic lineage (GPL), a hypovirulent Cape lineage, of African origin, as well as a Swiss lineage (Farrer et al., 2011).Two potential vectors of the pathogen have been identified as the African clawed frog *Xenopus laevis* (Weldon et al. 2004) and the American Bullfrog *Lithobates catesbeianus* (Daszak et al. 2004; Schloegel et al. 2010) both of which are mainly asymptomatic carriers of the pathogen. *X. laevis* is used extensively as a laboratory research animal model and was being used for early pregnancy tests (Weldon et al. 2004). *L. catesbeianus* was farmed for frog legs around the world and has escaped or been released from these farms to establish stable or expanding populations in wetlands around the world (Fisher & Garner 2007; Schloegel et al. 2009; Hanselmann et al. 2004).

The alternative hypothesis is that the pathogen is endemic and has recently changed into a virulent strain, or that its host has become susceptible due to biotic or abiotic factors. In support of this endemic pathogen hypothesis, *Bd* has been found on every continent which has amphibians (Fisher et al. 2009) and continues to be found in previously untested countries (Bai et al. 2010; Savage et al. 2011). It has also been shown that *Bd* has been found in locations with few or no amphibian mass mortalities (Weldon et al. 2004; Ouellet et al. 2005). There is also some evidence that climate variations are allowing the pathogen to persist and thrive in new environments (Pounds et al., 2006). Changes in climate may also make the amphibian host more susceptible to this endemic pathogen (Carey, 1993; Reading, 2007). Another reason to believe that *Bd* may be an endemic pathogen is that despite being genetically highly uniform, most strains differ in both phenotype and pathogenicity (Berger et al. 2005; Retallick & Miera 2007; Voyles 2011; Fisher et al. 2009). These factors suggest that *Bd* may have been present in many ecosystems prior to these amphibian declines.

In British Columbia, bullfrogs which are one of the proposed vectors for *Bd* spread, have been introduced in some southern regions while much of the rest of the province has had no history of bullfrog introductions. In the 1930's the bullfrog was introduced into BC as a food source (Green, 1978) and currently has a range which is restricted to south eastern Vancouver Island and south western Lower Mainland BC

(Govindarajulu, 2004). The first indication of an amphibian testing positive for *Bd* in British Columbia was from a museum sample which was collected in the 1970's (Ouellet et al., 2005). There have also been reports of *Bd* infecting Western Toads in BC although only a small sample size has been screened (Deguise & Richardson, 2009; Raverty & Reynolds, 2001). BC northern leopard frogs have also been shown to harbour the pathogen and die from chytridiomycosis (Voordouw, Adama, Houston, Govindarjulu, & Robinson, 2010). It is believed that *Bd* may be responsible for the population declines and range contraction of this species in BC (COSEWIC, 2009) *Bd* has also been detected with high prevalence in Vancouver Island populations of bullfrogs (Garner et al. 2006). Knowledge of the historic and current range for the bullfrog in British Columbia allows us to test for the presence of *Bd* amongst amphibians inside and outside this range to determine whether the bullfrog might have served as a vector for *Bd*. It would be anticipated that if *Bd* were recently introduced to BC via the invasive bullfrog species that there would be a wave of *Bd* infections emanating from the vector's range. The finding of ubiquitous distribution could therefore indicate that the pathogen was either endemic or had already spread throughout the province prior to the introduction of the bullfrog to southern BC. Widespread occurrence of *Bd* in the province could also be interpreted as there being other types of vectors.

Genetic variation of *Bd* isolates collected from within and outside of the bullfrog range could also be used to assess endemnicity. These genotypes could also be compared to worldwide samples to see if there were any genetic differences. To test this possibility we examined possible variation in the internal transcribed spacer region of the ribosomal DNA repeat (ITS-rDNA) of the *Bd* genome, *Bd* samples collected from bullfrogs on Vancouver Island were compared to *Bd* samples collected from other amphibians in the Yukon and Northern BC, outside the bullfrog's range. The use of ITS sequencing has provided a useful tool for identification of *Bd,* due to the high degree of conservation of ITS region and reliable identification to the genus level. There is a very low degree of variability in the ITS-rDNA of *Bd* and only two research groups (Bai et al., 2012; Goka et al., 2009) demonstrated significant differences in the ITS of *Bd* isolates from Japan and China. The samples from BC would also be compared to worldwide profiles to see if

there were similarities or differences among global strains. The presence of unique differences at the ITS region would provide supporting evidence for the EPH. The lack of genetic differences at this ITS region would suggest support for the NPH.

Because sequence analysis of the ITS region provides information on a single highly conserved region of the genome there is a risk that there is not sufficient information to resolve a higher population structure. A recent approach to differentiate between highly similar strains relied upon the sequencing and typing of multiple loci (MLST) in order to establish population information (Urwin & Maiden 2003; Taylor & Fisher 2003). Despite there being only a few studies using MLST to examine *Bd* population genetics, questions regarding the genetic origins of *Bd* strains found worldwide are now beginning to be resolved (Morehouse et al. 2003; Morgan et al. 2007; James et al. 2009; Walker et al. 2010; Schloegel et al. 2010). These studies have uncovered subtle differences between strains when sequence types obtained from various locations around the world were compared. One of the advantages of ITS screening is that skin swabs generally provide sufficient DNA for testing for presence or absence of the pathogen. MLST relies on gathering sequence information for several single-locus markers and generally requires more DNA than can be found in a single skin swab and relies on DNA extracted from live *Bd* cultures. Only two live cultures were available which limited this analysis to two viable *Bd* cultures obtained from bullfrogs from Vancouver Island. The MLST analysis, using the two bullfrog strains, can be used to distinguish between the NPH and EPH and help to better understand the origins of the bullfrog stains in BC. The MLST data may show that the BC strains are different than the GPL strain, suggesting that they are endemic. The data may also show that the strains found in BC are similar to the GPL thus making the case for the NPH.

The objective of the current study was to determine whether *Bd* was a novel or endemic pathogen in the province of British Columbia. Once the range of the prevalence was established, the next goal was to look at the population genetics of select *Bd* samples from BC to determine whether there was support for the novel or endemic hypotheses.

The combined survey and population genetic information should reveal insights as to the origins of *Bd* in British Columbia.

Materials and Methods

Sampling Protocol

Given the large area of British Columbia, amphibian samples were collected as part of a volunteer sample collection effort coordinated by the BC Ministry of Environment. The volunteer nature of the project essentially constrained the effort to a haphazard sampling design. The samples were taken by 22 different field ecologists from all over BC. These researchers were given protocols to collect samples which included tadpole mouthparts, toe clips and cotton swabs. There were 956 samples collected from all over the province and from a diversity of species and life-stages (Table 2.1) (Table A5).

DNA extraction from amphibian swabs, tadpole mouthparts and amphibian toe clips

The DNA extraction protocol for swabs, tadpole mouthparts and toe clips were all variations of the methodology in Boyle et al. (2004). Tadpole mouthparts, toe clips and the tip of a sample swab were put into sterilized 1.5ml micro centrifuge tubes. An aliquot of 40µl or 60µl of the PrepMan Ultra was added to the respective sample (tadpole mouthpart, toe clip or swab) as well as 40mg of Zirconium/Silica beads (0.5mm). The sample was then homogenized for 45 seconds in the Minibeadbeater (Biospec, Bartlesville,OK) and centrifuged for 30 seconds at 13000 rpm. This was repeated after which the samples were placed in a 100°C heating block for 10 minutes. The tubes were then left at room temperature for 2 minutes and then centrifuged for 3 minutes at 13000 rpm. A 20µl aliquot of supernatant was then removed and placed in a sterile 1.5ml micro centrifuge tube. This sample was then diluted 1:10 with RNase/DNase free H_20 to make a working solution of DNA for PCR both of these tubes were then stored at -20°C.

Surveillance protocol using Taqman probe based qPCR

A TaqMan based qPCR based assay was employed to test for presence or absence of *Bd*-specific DNA from amphibian samples. The TaqMan quantitative PCR protocol

adapted from (Boyle et al. 2004) was performed on a Stratagene Mx4000 qPCR machine (Stratagene/Agilent, Santa Clara, CA). The qPCR thermocycle conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 95°C for 15 seconds 60°C for 1 minute and this was repeated for 50 cycles. The primers that were used were developed by Boyle et al 2004 based on the ITS region of the *Bd* genome. The primers sequences were ITS1-3 Chytr (5' - CCT TGA TAT AAT ACA GTG TGC CAT ATG TC - 3') and 5.8S Chytr (5' – AGC CAA GAG ATC CGT TGT CAA A – 3'). The probe used for the TaqMan assay was a Minor Groove Binding Probe (MGB) Chytr MGB2 (5' – 6FAM CGA GTC GAA CAA AAT MGBNFQ – 3') (Applied Biosystems, Carlsbad, CA). The qPCR reaction consisted of 12.5µl of 2x TaqMan Reagent Master Mix (Applied Biosystems, Carlsbad, CA), 0.45µl of both ITS1-3 Chytr and 5.8S Chytr, 0.625µl of Chytr MGB2 Probe, 5.975µl of RNase/DNase free H_20 and 5µl of DNA sample. Each of the qPCR reactions was performed with positive and negative controls as well as a blank sample to ensure quality assurance.

Culturing and DNA extraction of isolated *Bd* samples

Cultures of *Bd* were isolated from skin of amphibians and grown on 1% Tryptone agar and broth as well as TgHL and mTgHL agar (Boyle et al. 2004). Isolates were grown in an 18°C incubator for seven days prior to DNA extraction. A 20 ml liquid culture of *Bd* was concentrated by centrifugation for 1 minute at 10,000 rpms and the supernatant was removed. The *Bd* cells were then suspended in 200µl of PrepMan Ultra (Applied Biosystems, Carlsbad, CA), placed in a 100°C heating block for 10 minutes, cooled for 2 minutes and centrifuged at 10,000 rpm for 3 minutes. A 150µl aliquot of total DNA supernatant was removed and diluted in RNase/DNase free H_20 in a 1 : 10 dilution and stored at -20°C. The two *Bd* isolates from BC which were used in this study were cultured by Finn Hamilton. PTH001 was isolated in the spring of 2009 from a bullfrog tadpole that was originally from Nanaimo, BC area and was housed in the University of Victoria Aquatics Centre. PTH002 was isolated from a bullfrog tadpole which was caught in Beaver Pond near Elk Lake on Vancouver Island also in the spring of 2009.

Population genetics survey using ITS region of the *Bd* genome

In order to determine the genetic population structure of *Bd* we first examined the ITS region of the genome which has previously been used in many fungal population genetic surveys. This was accomplished using a nested PCR protocol developed by Goka and coworkers (2009) which required two sets of PCR primers. The first round of PCR was performed with forward primer Bd18SF1 (5′-TTTGTACACACCGCCCGTCGC-3′) and reverse primer Bd28SR1 (5′-ATATGCTTAAGTTCAGCGGG-3′) from Goka et al. 2009. The PCR reaction consisted of 5µl of 10x Buffer, 1µl of 10mM dNTPs, 1µl of 50mM of forward and reverse primer, 0.5µl taq polymerase (Dreamtaq, Thermo Fisher, Waltham, MA), 39.5µl RNase/DNase free H_20 and 2µl of sample DNA. The PCR thermocycler protocol for these primers were as follows: 95°C for 9 minutes; 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes which was repeated for 29 cycles followed by a final extension of 72°C for 7 minutes.

The second set of primers in the nested PCR protocol was adapted from a previous study (S. Annis, Dastoor, & Ziel, 2004). The forward primer was Bd1a (5′-CAGTGTGCCATATGTCACG-3′) and reverse primer was Bd2a (5′-CATGGTTCATATCTGTCCAG-3′). The same PCR reaction formula was used with the exception that 2µl of PCR product from the first PCR was used as the target DNA. The PCR thermocyler conditions for these primers were as follows: 95°C for 9 minutes; 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds which was repeated for 29 cycles followed by a final extension of 72°C for 7 minutes. The PCR products from both reactions were separated by eletrophoresis on a 1.5% agarose gel at 100 Volts for 1 hour. The gel was then visualized by staining in a GelRed solution (Cedarlane, Burlington, ON). The PCR products were purified using the Qiagen PCR purification kit (Qiagen, Germantown, MD). The PCR purified samples were then cloned using pGem-T cloning vector (Promega, Madison, WI) and transformed into E. Cloni 10G electrocompetent cells (Lucigen, Middleton, WI). The subsequent transformants were purified using the Qiagen Plasmid Mini Prep Kit (Qiagen, Germantown, MD). The

purified plasmid products were then sent to Operon (Eurofins MWG Operon, Huntsville, AL) for sequence determination using the T7 and SP6 primer sites found on the pGem-T vector.

The samples used in the ITS assay included samples in the bullfrog range and samples outside of this range. The two bullfrog strains were from Vancouver Island strains PTH 001 and PTH 002. These two strains were sampled with two replicates by both cloned PCR products and direct sequencing. The other six samples were all PCR products which were directly sequenced by Operon (Eurofins MWG Operon, Huntsville, AL). These 6 samples were all located in Northern BC and the Yukon Territory, far from the bullfrog range. The global samples which were compared with the BC and Yukon samples were obtained from the National Center for Biotechnology Information (NCBI) and were selected by their availability with approximately five samples (except USA) from each of the following countries Ecuador, Italy, USA and Japan.

ITS Sequence analysis using bioinformatics tools

The primary sequence data were combined using the forward and reverse sequences, looking closely at the chromatogram, to create a single consensus sequence for each strain which was also trimmed to the same length. This sequence data were then analyzed and edited using sequence analysis tools BioEdit (Hall, 1999) and Mega 5.0 (Tamura et al., 2011). Other *Bd* ITS sequences were obtained from the NCBI database to be used to compare and create a phylogenetic tree. The trees were created using Mega 5.0 (Tamura et al. 2011) and using the algorithm UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

Higher Resolution Population Genetic survey using MLST markers

To better define the origin of *Bd* isolates from bullfrogs in British Columbia it was preferable to examine variation in genetic loci in addition to the ITS region. A series of multi locus sequence type (MLST) primers were developed by Morehouse et al. 2003, Morgan et al. 2007, James et al. 2009 and Walker et al. 2010 (Appendix). The optimal

amplification by these primers was determined using a gradient PCR program on the Eppendorf mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). The thermocycle information for all the primers used in this experiment were as follows: 95°C for 2 minutes; 95°C for 30 seconds, varying annealing temperature see Table 2.1 for 30 seconds, 72°C for 1 minute and 30 seconds this repeated for 29 cycles followed by a final elongation step of 72°C for 5 minutes. The PCR reaction formula consisted of 5µl of 10x Buffer, 1µl of 10mM dNTPs, 1µl of 50mM of forward and reverse primer, 0.5µl taq polymerase (Dreamtaq, Fermentas, Burlington, ON), 39.5µl RNase/DNase free H_20 and 2µl of sample DNA. The PCR products were separated by electrophoresis and visualized as previously described. The PCR products were purified using the Qiagen PCR purification kit (Qiagen, Germantown, MD). The PCR purified samples were then cloned using pGEM - T cloning vector (Promega, Madison, WI) which was used with E. Cloni 10G electrocompotent cells (Lucigen, Middleton, WI). The subsequent transformants were purified using the Qiagen Plasmid Mini Prep Kit (Qiagen, Germantown, MD). The purified plasmid products were then sent to Operon (Eurofins MWG Operon, Huntsville, AL) for sequence determination using the T7 and SP6 primer sites found on the PGem – T vector.

The MLST portion of this study was completed with the two cultured samples obtained from bullfrogs on Vancouver Island. These two strains were PTH001 and PTH002, the samples from the province wide survey could not be used due to the inability to have cultured samples with which to sequence at multiple loci. These two BC bullfrog samples were compared with MLST data which was published by James et al. 2009.

MLST analysis using bioinformatic tools

The MLST data were analysed and scored using the protocol outlined by James et al. (2009). The loci were scored as strain type a, b or c and were compared to other sequences generated from samples throughout the world (James et al. 2009). The data were analysed using the Mesquite bioinformatics tool which was able to look at

evolutionary analysis this was accomplished by making a phylogenetic tree from cluster analysis which looked at the source of distance for cluster analysis. The distance from the character matrix was uncorrected. (W. P. Maddison & Maddison, 2011). The phylogenetic tree cluster analysis was done using UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm to generate a phylogenetic tree.

Results

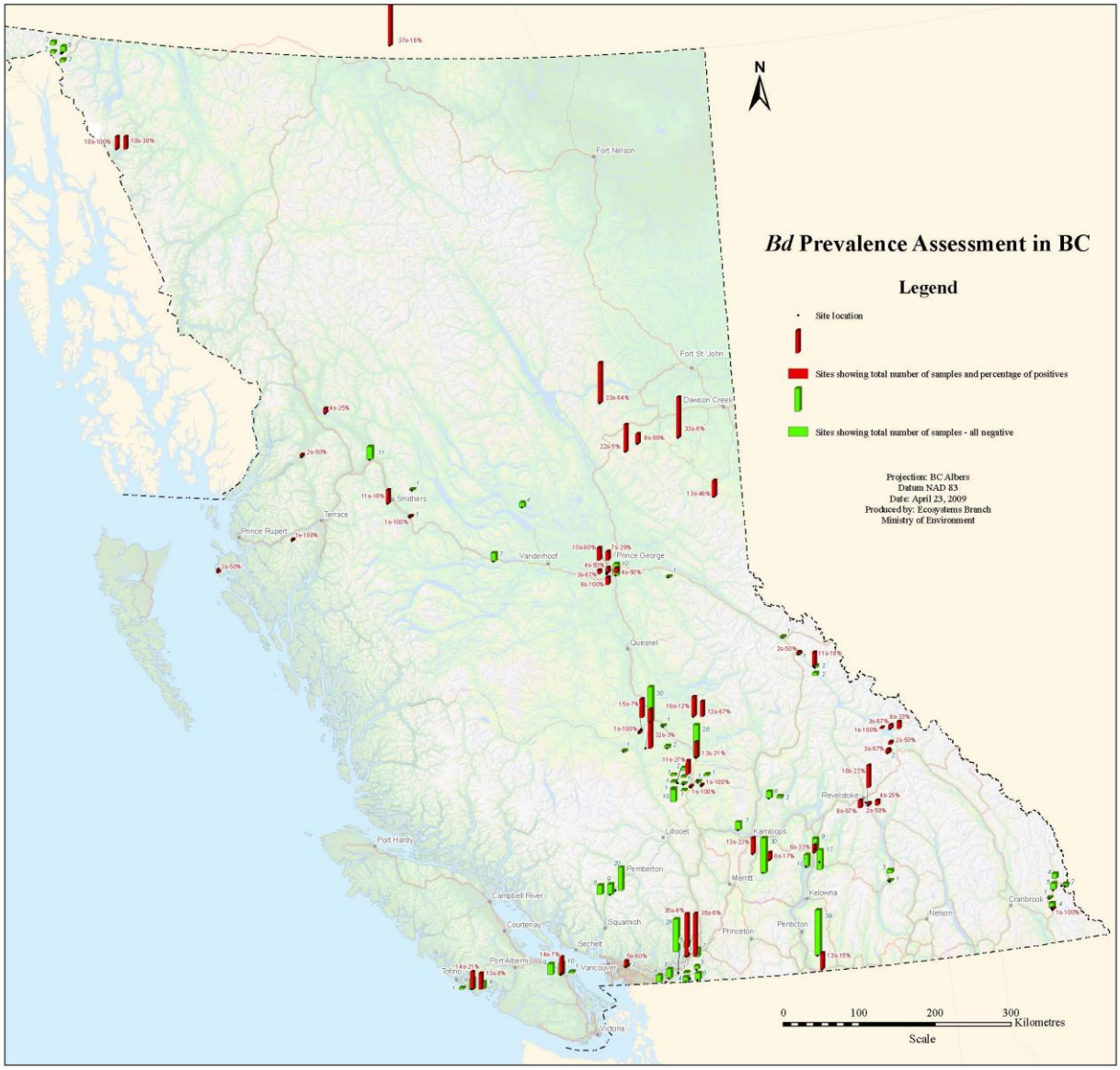

Figure 2.1: Prevalence of *Bd* among amphibians sampled across British Columbia. Green bars are the total number of negative samples at a given site. The red bars indicate the total sample size at a given site. The percentage of positive samples is written beside the red bars. The larger the bar the greater the total sample size for both positive and negative samples. This figure was produced by BC Ministry of Environment (Govindarajulu et al. 2009)**.**

Table 2.1: The sample size and percent prevalence of *Bd* among amphibians tested in British Columbia. This includes amphibian life stage as well as their status as introduced or native species. These prevalence values are followed by the 95% Confidence Interval for each Sample. This table was produced by BC Ministry of Environment (Govindarajulu et al. 2009)**.**

Species	Life-stage/sample	Sample size	Prevalence %	95% Confidence Interval	
				Lower	Upper
Native Anurans					
Western Toad *Anaxyrus boreas*	Metamorph	28	0	0%	12%
	Post-metamorphic	238	20%	16%	26%
	Tadpoles	217	2%	1%	5%
Great Basin Spadefoot *Spea intermontanus*	Post-metamorphic	19	0%	0%	16%
	Tadpoles	35	14%	6%	30%
Pacific Chorus Frog *Pseudacris regilla*	Post-metamorphic	24	4%	1%	20%
	Tadpoles	53	4%	1%	13%
Redlegged Frog *Rana aurora*	Post-metamorphic	46	4%	1%	14%
	Tadpoles	8	0%	0%	32%
Oregon Spotted Frog *Rana pretiosa*	Post-metamorphic	5	20%	4%	62%
Columbia Spotted Frog *Rana lutieventris*	Post-metamorphic	130	41%	33%	50%
	Tadpoles	17	6%	1%	27%
Wood Frog *Lithobates sylvaticus*	Post-metamorphic	28	71%	53%	85%
Introduced Anurans					
Bullfrog *Lithobates catesbeianus*	Post-metamorphic	1	0%	0%	79%
Green Frog *Lithobates clamitans*	Post-metamorphic	21	14%	5%	35%
	Tadpoles	38	8%	3%	21%
Salamanders					
Roughskin Newt *Taricha granulosa*	Post-metamorphic	20	1%	3%	30%
Pacific Giant Salamander *Dicamptodon spp.*	Larval	27	0%	0%	12%

Results of Amphibian Survey

The BC wide survey indicated that there was near ubiquity of *Bd* in all locations sampled throughout the province of British Columbia (Figure 2.1) (Table A5). Locations with *Bd* positive animals were found both within and outside of the known bullfrog (suspected vector) range. During our sampling we placed a higher emphasis on collecting from mainland BC in this survey because Vancouver Island had been thoroughly sampled previous to this study by Garner et al. (2006). Because of the nature of the sampling techniques the samples were collected by many different scientists as a supplement to their own research hence some areas of the province were well-covered and other areas were sparsely sampled. Despite this unevenness in sampling density it was clear that almost all frog species tested positive (Table 2.1) and *Bd* was prevalent in all regions tested (Figure 2.1) (Table A5).

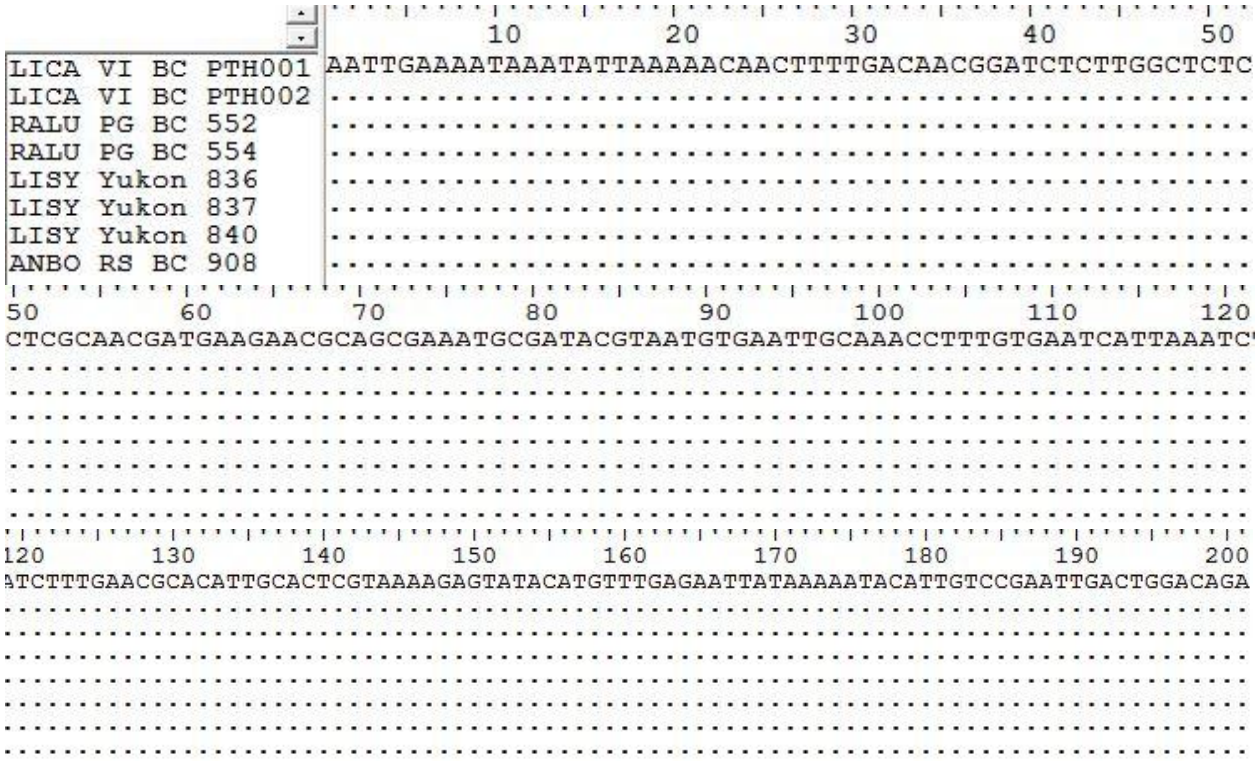

Figure 2.2 The local British Columbia and Yukon ITS sequence homology visualized using bioinformatics software Bioedit. The top sequence is the actual sequenced ITS region and the dots below represent total homology to the original sample. The location code are as follows PG = Prince George, BC; VI = Vancouver Island, BC; RS = Revelstoke, BC. Also indicated are the corresponding sample numbers and strain delineations.

Assesment of Population Genetic Structure from Inside and Outside Bullfrog range Using ITS markers

Each ITS amplicon was sequenced in both directions and the sequence for the forward read was compared to the reverse complement of the reverse read to crosscheck the sequence accuracy. The sequence of ITS region was determined for a total of eight isolates which were collected from within and outside of the bullfrog range in BC. The ITS sequence was determined for isolates PTH001 and PTH002 which were cultured from bullfrogs on Vancouver Island. The ITS sequences from outside the bullfrog range corresponded to three isolates collected the Yukon, two from Prince George, BC and one from Revelstoke, BC. All of the ITS sequences, when compared using bioinformatic software, showed complete identity at all overlapping regions (Figure 2.2). While the rRNA region for these *Bd* strains was highly conserved, the ITS regions in many other fungi was found to be highly variable and was used to generate population genetic information. The ITS region of the BC samples were also compared with other *Bd* ITS regions from many different countries and isolated from many different amphibian species as well as one *Rhizophydium littoreum* out group (Figure 2.3). Most of the global ITS sequences clustered together with the BC strains; the only exceptions were two isolates from Japan. These were shown to have distinct haplotypes when compared to other *Bd* ITS sequences. The isolate from the salamandar *Andrias japonicas* is thought to be endemic Japan and it was separate from the other global strains (Goka et al., 2009). There was insufficient variation in ITS region of the BC isolates to infer any substructuring of the BC population.

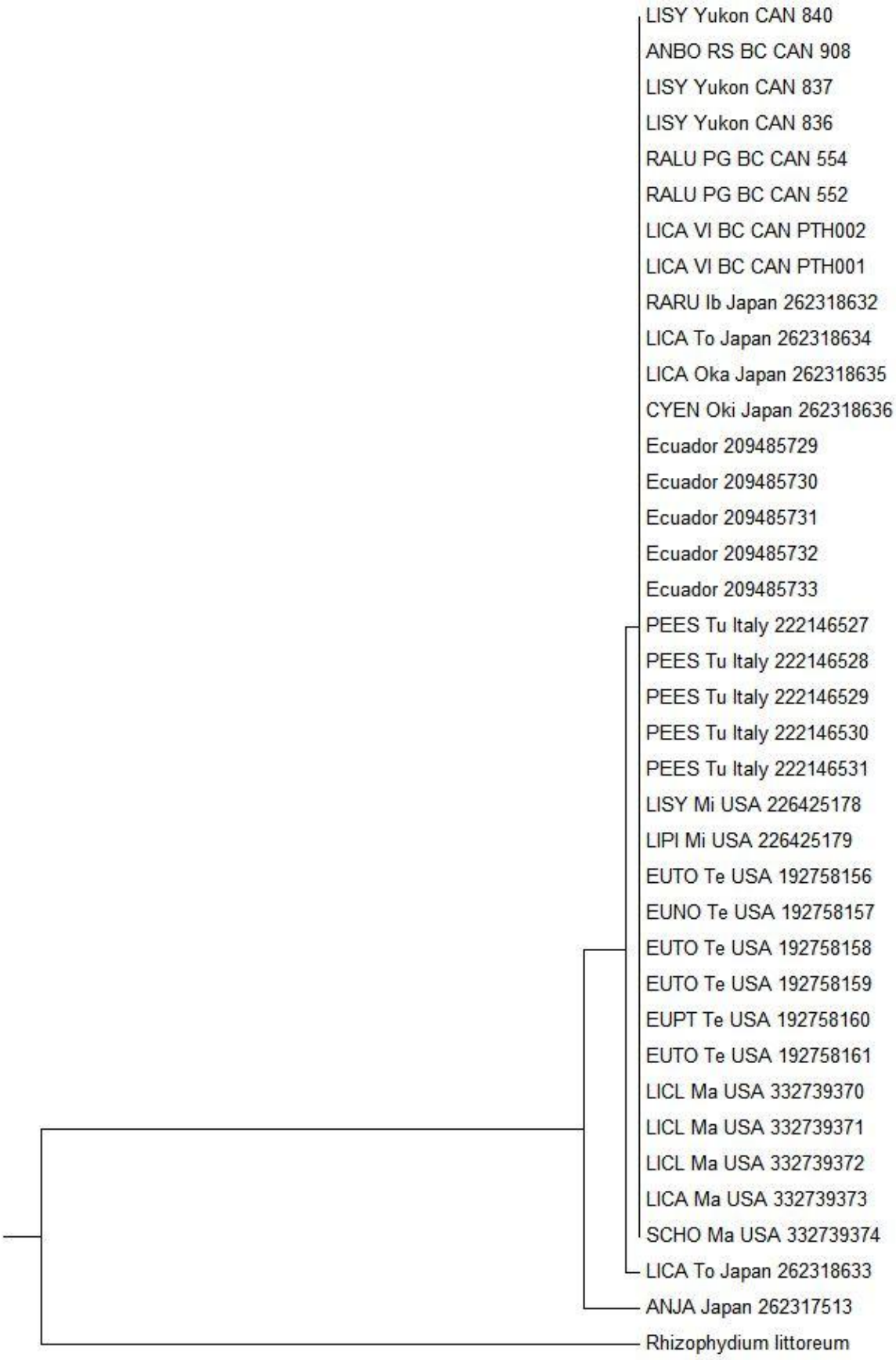
LISY Yukon CAN 840
ANBO RS BC CAN 908
LISY Yukon CAN 837
LISY Yukon CAN 836
RALU PG BC CAN 554
RALU PG BC CAN 552
LICA VI BC CAN PTH002
LICA VI BC CAN PTH001
RARU Ib Japan 262318632
LICA To Japan 262318634
LICA Oka Japan 262318635
CYEN Oki Japan 262318636
Ecuador 209485729
Ecuador 209485730
Ecuador 209485731
Ecuador 209485732
Ecuador 209485733
PEES Tu Italy 222146527
PEES Tu Italy 222146528
PEES Tu Italy 222146529
PEES Tu Italy 222146530
PEES Tu Italy 222146531
LISY Mi USA 226425178
LIPI Mi USA 226425179
EUTO Te USA 192758156
EUNO Te USA 192758157
EUTO Te USA 192758158
EUTO Te USA 192758159
EUPT Te USA 192758160
EUTO Te USA 192758161
LICL Ma USA 332739370
LICL Ma USA 332739371
LICL Ma USA 332739372
LICA Ma USA 332739373
SCHO Ma USA 332739374
LICA To Japan 262318633
ANJA Japan 262317513
Rhizophydium littoreum

Figure 2.3:Comparison of the ITS region of global isolates of *Bd* to those of BC. Location information is given in the form of State/Province Country where provided. Species are represented by species codes where provided in the legend below. The GenInfo Identifier number from GenBank is also included for sequences attained from NCBI.

Species Code Legend

Species Code	Species
ANBO	*Anaxyrus boreas* formally *Bufo boreas*
LISY	*Lithobates sylvaticus* formally *Rana sylvaticus*
RALU	*Rana luteiventris*
LICA	*Lithobates catesbeianus* formally *Rana catesbeina*
RARU	*Rana rugosa*
CYEN	*Cynops ensicauda*
PEES	*Pelophylax esculentus*
ANJA	*Andrias japonicus*
LIPI	*Lithobates pipiens*
EUTO	*Eurycea tonkawae*
EUNO	*Eurycea noetenes*
EUPT	*Eurycea pterophila*
LICL	*Lithobates clamitans* formally *Rana clamitans*
SCHO	*Scaphiopus holbrookii*

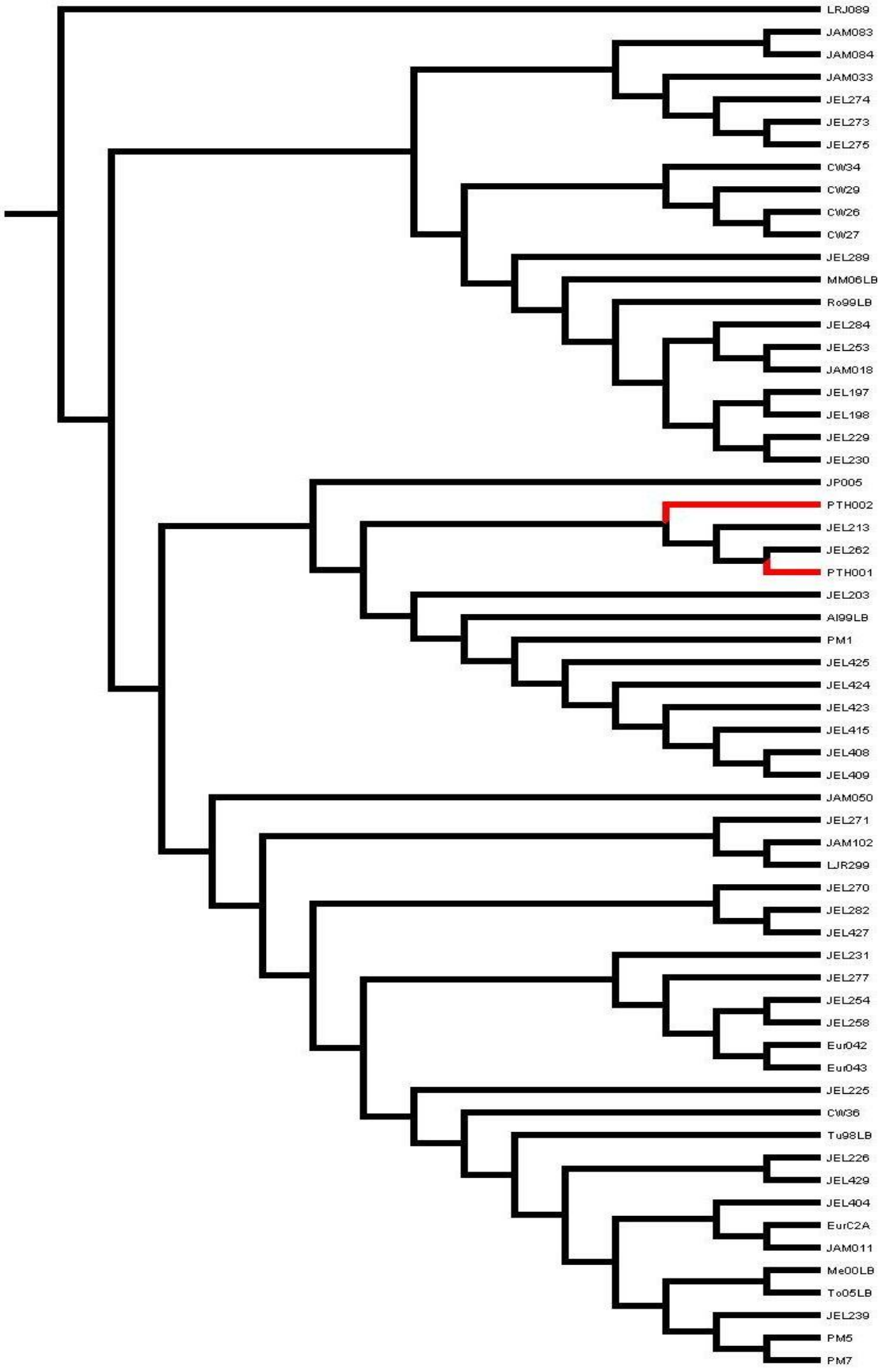
LRJ089
JAM083
JAM084
JAM033
JEL274
JEL273
JEL275
CW34
CW29
CW26
CW27
JEL289
MM06LB
Ro99LB
JEL284
JEL253
JAM018
JEL197
JEL198
JEL229
JEL230
JP005
PTH002
JEL213
JEL262
PTH001
JEL203
Al99LB
PM1
JEL425
JEL424
JEL423
JEL415
JEL408
JEL409
JAM050
JEL271
JAM102
LJR299
JEL270
JEL282
JEL427
JEL231
JEL277
JEL254
JEL258
Eur042
Eur043
JEL225
CW36
Tu98LB
JEL226
JEL429
JEL404
EurC2A
JAM011
Me00LB
To05LB
JEL239
PM5
PM7

Figure 2.4: This phylogeny was created using Mesquite bioinformatics software and the UPGMA method of analysis. BC samples, labelled in red, represent PTH001 and PTH002 and all other samples are from James et al. 2009 representing a global collection of *Bd* isolates. The detailed information on these strains is included in Appendix A.

The assessment of MLST on two BC samples compared to global MLST profiles

The two BC samples, PTH001 and PTH002, were sequenced at multiple loci and scored according to primers designed by James et al. (2009). PTH001 and PTH002 were both isolated from bullfrogs on Vancouver Island in the spring of 2009. The sequence of 7 of the 17 loci was determined for PTH001 while the sequence of 13 of the 17 published loci were determined for PTH002. The seven loci common to PTH001 and PTH002 isolates were identical. The PTH001 strain was also identical, at these 7 loci, to isolate JEL262 which was isolated from a bullfrog specimen in Quebec, Canada (Figure 2.4). PTH002, while identical to JEL262 at 7 loci, was identical to the Quebec isolate at 10 of the 13 sequenced loci. The isolate PTH002 and PTH001 were also highly similar to strain JEL213 which was isolated from a *Rana muscosa* in Mono Co. California, USA (Figure 2.4). The isolate PTH001 shared 6 of the 7 loci with JEL213 and PTH002 shared 9 out of 13 loci with JEL213. The two *Bd* strains isolated from Vancouver Island, BC formed a clade with the bullfrog strain from Quebec, Canada and the *R. muscosa* strain from California. The complete sequence of PTH002 has since been completed by another research group, led by Trenton Garner and Matt Fisher, and it was determined to belong to the global panzootic lineage (GPL) as described by (Farrer et al., 2011). It was also found to be most similar to a bullfrog strain JEL261 which was also isolated from Quebec, Canada much like JEL262 (Personal Communication Rhys Farrer). The strain JEL262 was not used in their whole genome analysis of *Bd*.

Discussion

The role of bullfrogs as a probable vector for the spread of *Bd* is well established with many studies confirming their suitability for pathogen transfer and their wide distribution resulting from the amphibian trade (Weldon et al. 2004; Daszak et al. 2004; Schloegel et al. 2010). In British Columbia, *Bd* was first reported to infect amphibian populations on Vancouver Island where bullfrogs are considered to be an invasive species, (Garner et al. 2006) and it was suggested that bullfrogs might be responsible for the spread of this pathogen to naïve populations of amphibians. The range of the bullfrog in British Columbia is limited to the southern regions by natural geographic barriers or this invasive species may not yet had sufficient time to spread further (Govindarajulu, 2004). The well-defined range of the bullfrog led us to expect that if *Bd* was associated with the presence of bullfrogs that there would be a close association between the presence of bullfrog and incidence of *Bd* in British Columbia. It was also considered that *Bd* could radiate out of the bullfrog range and that the initiation of *Bd* vectoring could coincide with the beginning of the frog trade in southern BC. We found instead that *Bd* was found distributed all over mainland BC and was not simply limited to the range of the bullfrog.

These results suggest that either bullfrogs are not the only vector of *Bd* or that the pathogen was endemic to BC prior to the arrival of the bullfrog. There may be other vectors for this pathogen, such as native amphibian species. There has been some speculation that there are other possible vectors for transmission linked to human activities like tourism travel and trade. Most of the samples were collected in easily accessed waterways and locations. More interestingly, the possibility has been raised that *Bd* might be transferred by water fowl (Johnson & Speare 2005). This could explain how *Bd* could spread through water systems which flow in opposite directions without a direct connection between headwaters. The widespread distribution of *Bd* throughout the province and its relatively high prevalence suggests that *Bd* is now endemic to BC though the exact history of introduction is unclear. There are reports of positive *Bd* samples

found in interior BC which date back to the 1970's (Ouellet et al., 2005) which, if taken alone, may have suggested an isolated area of infection. There is also an early case from the year 2000 of *Bd* infecting a Western Toad (*Anaxyrus boreas*) in the Peace River District which is approximately 700 kilometres from the northernmost bullfrog site in Powell River BC. At the time this was thought to be the northernmost incidence of *Bd* in North America (Raverty & Reynolds, 2001). More recently studies have shown *Bd* to have high prevalence in Western Toads, and Northern Leopard Frogs (*Lithobates pipiens*) as well as bullfrog populations in Southern British Columbia (Garner et al. 2006; Deguise & Richardson 2009; Voordouw et al. 2010). With the current study the range of *Bd* incidence has now been extended as far north as the Yukon. This northern range has also been confirmed in another study that found *Bd* in the North West Territories (Schock et al., 2009). The data from our study and results from both early and recent surveys suggest that if *Bd* was a novel introduction that it has spread rapidly and is now found throughout British Columbia.

Analysis of the population structure of *Bd* through molecular techniques can help us to better understand how recently *Bd* entered BC and even perhaps how it has become so widespread. The usual approach for assessing the population structure among fungal individuals is by examination of ITS rDNA region. The success in finding different haplotypes using this method in Japanese strains of *Bd* was encouraging and we sought to determine whether there might be similar variation of types in Canada (Goka et al., 2009). We examined the ITS rDNA sequence of *Bd* samples collected from within the bullfrog range and compared them to samples from outside the range. There was no variation at the ITS region and it was determined to be insufficient to discriminate specific haplotypes. There is also insufficient resolution to show distinct groupings when comparing the samples to other samples taken from across the world. The only samples which demonstrated significant differences at the ITS region were two Japanese samples including a suspected endemic strain belonging to *Andrias japonicas* (Goka et al., 2009). This result corresponds to early work done by Morehouse et al. (2003) which demonstrated very few differences in the genome overall leading researchers to surmise that *Bd* in North America may have resulted from the recent introduction of a limited

number of clones. Based on the ITS results alone we could not resolve whether *Bd* represented a novel or endemic pathogen in BC due to the high degree of similarity between all strains. It was therefore important to examine the genome with genetic markers having a higher resolving power and multiple variable loci, in order to determine whether the population structure could be further dissected. This type of study can help determine not only whether *Bd* was novel or endemic but also establish a time of introduction.

The use of MLST to determine differences between *Bd* strains has been used by many different research groups and generally yields genotypic resolution for even recently introduced fungal pathogens. This technique was used to illustrate geographical groupings within the *Bd* species sampled (James et al., 2009). In the current study we compared two BC bullfrog isolates to other previously published global isolates. The two BC isolates proved to be most similar to a bullfrog strain from Quebec, JEL262, and also a California strain JEL213. The similarity to a bullfrog strain from Quebec was confirmed by whole genome sequencing of PTH002 which was most similar to JEL261 which was another bullfrog isolate from Quebec. The strain PTH002 was also shown to be a part of the hypervirulent global panzootic lineage (GPL) (Personal Communication Rhys Farrer). It was suggested by Farrer et al. (2011) based on statistical analysis of SNP locations in 20 sequenced genomes, that the GPL isolates were probably the result of a meiotic event of a single hybrid clone of a previous strain (Farrer et al., 2011). This would imply that isolates, belonging to the GPL, are a part of a newly developed lineage of *Bd*. The similarity between the two BC strains and the Quebec strain may indicate that this is a relatively recently introduced strain of *Bd* which may have been transported across North America by the bullfrog trade. The first bullfrogs may have been introduced as early as the 1930's in BC (Green 1978). The differences in BC strain, PTH002, could represent small genetic changes that accumulated after *Bd* was introduced to British Columbia. The MLST data suggests that *Bd*, from these two isolates, has only been recently introduced to BC, perhaps as early as the first bullfrog introductions in the 1930's, and may be linked to the amphibian trade due to the high degree of similarity to

the bullfrog strain from Quebec. The genetic structure of *Bd* isolates from the remainder of the province remains to be determined.

Conclusions

The combined results of the *Bd* survey demonstrate that this fungal pathogen is now ubiquitous throughout all sampled areas in the province of BC. This augments results from previous studies which found *Bd* in BC as early as the 1970's (Ouellet et al. 2005) as well as far north as Yukon and the North West Territories (Schock et al. 2009). This is far outside the range of the assumed vector, the bullfrog and may suggest other possible vectors. The distribution of *Bd* also suggests that the pathogen is now endemic to the region but does not indicate the origins. The ITS and MLST analysis support a recent introduction of the fungal pathogen to the bullfrogs on Vancouver Island possibly from an east coast bullfrog. The two bullfrog isolates have been shown to be similar to these strains as well as being part of the hypervirulent global panzootic lineage of *Bd* which is suspected to be a novel pathogen. The genetic profiles of *Bd* isolates from other locations and hosts in BC should be analysed in order to determine whether these strains come from the same origin as the Vancouver Island bullfrog strains. This can be done through MLST work, which has proven to be very effective, or the more recent trend of complete genome sequencing. The evidence from our research shows that the two *Bd* isolates are a part of the novel GPL which may have entered through the amphibian trade in British Columbia.

Chapter 3

Batrachochytrium dendrobatidis (*Bd*) Surveillance in Malaysia

Chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is a new threat to amphibian biodiversity which is causing population declines in many areas throughout the world. The pathogen was first identified in 1997 associated with amphibian declines in Panama and Australia (Berger et al. 1998). The fungal pathogen grows on the keratinized amphibian skin and is thought to cause an electrolyte imbalance which can lead to death in susceptible species (Voyles et al. 2007). This newly discovered pathogen has been found on every continent where amphibians are present and currently has a world-wide distribution (Fisher et al. 2009). This fungal pathogen is able to infect as many as 387 different amphibian species and has been shown to cause population declines in approximately 200 of these (Skerratt et al. 2007). Amphibians face a number of anthropogenic threats to their survival and it is hypothesized that as many as 100 species have gone extinct since the 1980's (Stuart et al., 2004). The cause of these extinctions is not readily known but in some cases, such as the Sharp-snouted Day Frog *Taudactylus acutirostris*, chytridiomycosis caused by *Bd* has been implicated (Schloegel et al. 2006). The recent discovery of this pathogen and its ability to cause declines in overall amphibian biodiversity has led to it being classified as an emerging infectious disease (EID) (Daszak et al. 2003).

The recent emergence of *Bd* as an infectious pathogen has been explained by two competing hypotheses (Rachowicz et al. 2005). These two hypotheses are the novel pathogen hypothesis (NPH) and the endemic pathogen hypothesis (EPH). The NPH states that the pathogen has recently been introduced, usually through an asymptomatic vector, to a new area having susceptible hosts. The expectation is that the introduced pathogen would have limited genetic variability. There have been many studies which examine the vectoring of *Bd* by both the African clawed-frog *Xenopus laevis* and the American Bullfrog *Lithobates catesbeianus*. These two frog species are mainly asymptomatic to the pathogen and have been widely traded throughout the world (Weldon et al. 2004;

Daszak et al. 2004; Fisher & Garner 2007; Schloegel et al. 2010). The novel pathogen hypothesis is supported by a pattern of disease fronts radiating from presumed initial infection events in certain regions of the world, as exemplified by the spread of chytridiomycosis in Central America (Lips et al. 2008). The competing hypothesis, EPH, states that *Bd* has always been present in most regions of the world but is only now becoming virulent due to changed in the environmental which may affect both the host and the pathogen. The most convincing evidence for the EPH is the fact that *Bd* has been shown to be ubiquitous in areas for which it has been tested (Kilburn et al., 2011; Lannoo et al., 2011). These two competing hypotheses can only be resolved by testing for the presence of the pathogen on a global scale, and not just sampling areas where obvious amphibian declines have been observed.

Early research on the incidence of *Bd* in the wild had been limited to those areas associated with amphibian declines. One of the first areas where amphibian population declines were noted was in Panama, Central America, which was also where *Bd* was first described as an amphibian pathogen (Berger et al. 1998). Since this pioneering study several other surveys have examined the spread of *Bd* from Panama and Central America using genetic markers to assess whether the new epidemics may have originated from this region of the world or whether there has been a longer presence of *Bd* (Lips et al. 2006; Lips et al. 2008; Woodhams et al. 2008; Crawford et al. 2010). Some of the first declines attributed to chytridiomycosis were also noted in Australia (Berger et al. 1998) and more recently these have received increased scientific attention as the enormity of the problem became apparent (Drew et al. 2006; Kriger et al. 2007; Retallick et al. 2004). In North America there have been declines in amphibian populations observed in California that were attributable to *Bd* (Fellers et al. 2001). Several studies of the Sierra Nevada area of California were consequently undertaken to assess the spread of this pathogen and understand the epidemiology of this disease (Fellers et al. 2007; Morgan et al. 2007; Rachowicz et al. 2006). The over arching theme for all of these amphibian declines has been that *Bd* has been primarily found in pristine montane areas.

Because our current knowledge of *Bd* distribution has come from testing in regions demonstrating obvious amphibian declines, other areas around the world, which have not yet demonstrated these types of amphibian declines, are under sampled (Bai et al., 2010; Yang et al., 2009). Until very recently there have been relatively few studies that survey areas in Asia for the presence of *Bd* despite the incredible biodiversity of amphibians in those regions. Recent surveys have shown that *Bd* occurs in the Peoples Republic of China with an average prevalence of 15% (Bai et al., 2010) and 7.6% (Bai et al. 2012), Japan with an average prevalence of 27% (Goka et al., 2009), South Korea with an average prevalence of 38% (Yang et al., 2009), and there have been sporadic reports of incidence in Indonesia (Kusrini, Skerratt, Garland, Berger, & Endarwin, 2008). The lack of historical surveillance information in these countries limits our knowledge of the spread of *Bd* and its geographic distribution.

Malaysia, due to its climate and geography, has high amphibian biodiversity. There are 218 known amphibian species in Malaysia with 63 of them being endemic (iucn.org). The pristine montane rain forested habitat of Malaysia is similar to areas in other parts of the world that have experienced amphibian declines (Berger et al. 1998; Lips et al. 2008; Pounds et al. 2006). The ideal habitat for amphibians and the limited testing in the vicinity make Malaysia an important area of the world to study. Because there had been no published results of *Bd* testing in Peninsular Malaysia we undertook a survey for the presence of *Bd*. We reasoned that the presence of *Bd* throughout all of Peninsular Malaysia then would lend support to the EPH. Conversely the lack of evidence of *Bd* would in turn support the recent introduction of the pathogen, NPH. The ultimate goal was to try and differentiate between the novel and endemic pathogen hypothesis as it relates to *Bd* in Peninsular Malaysia.

Materials and Methods

Collection of DNA samples

Amphibian swab samples were collected over a three year period from 2008 through to 2010. The samples were collected by members of the University of Victoria and members of the Malaysia Field School program with identifications coming from Evan Quah and Mohd Abdul Muin at the Universiti Sains Malaysia. The protocol for obtaining the samples was adapted from the BC Ministry of the Environment *Bd* surveillance protocol. Swab samples which were collected from individual amphibians and place in isolated sample containers. There were only two juveniles sampled, in 2010, all of the other samples were adult amphibians. There was a single sampling site on May 28 2008 where 18 samples of 6 different species were taken. In 2009, 2 different locations were sampled with 47 samples taken from 9 different species these were taken on May 23 and June 2. In 2010 the sampling area increased to 10 different sites spread across Peninsular Malaysia (Figure 3.1) with 99 samples from 22 different species (Table 3.1). These samples were taken from May 17 to June 15 and November 4 to 26 2010. There were in total 24 different species tested in Peninsular Malaysia and a total of 164 samples taken over the three year period (Table 3.1).

DNA extraction from amphibian swabs

The DNA extraction protocol for processing amphibian swabs were variations of the methodology in Boyle et al. (2004). The tip of the amphibian swab sample was placed in a sterilized 1.5ml Starstedt microfuge tube. A 60µl aliquot of the PrepMan Ultra (Applied Biosystems, Carlsbad, CA) was added to the amphibian swab sample as well as 40mg of Zirconium/Silica beads (0.5mm). The sample was then homogenized for 45 seconds in the Minibeadbeater (Biospec, Bartlesville,OK) and centrifuged for 30 seconds at 13000 rpm. This was repeated after which the samples were placed in 100°C heating block for 10 minutes. The tubes were then cooled to room temperature for 2 minutes then centrifuged for 3 minutes at 13000 rpm. Twenty µl of supernatant was then transferred to

a sterile 1.5ml micro centrifuge tube and diluted 1 in 10 with RNase/DNase free H_20 to make a working solution of DNA. All sample tubes were then stored at -20°C.

Surveillance protocol using Taqman probe based qPCR

The DNA from the amphibian samples were then used in a TaqMan based qPCR assay in order to test for presence or absence of *Bd*. The TaqMan quantitative PCR protocol was adapted from a previous study (Boyle et al. 2004) and was performed on a Stratagene Mx4000 qPCR machine (Stratagene, Santa Clara,CA). The qPCR thermocycling conditions were as follows 50°C for 2 minutes, 95°C for 10 minutes followed by 95°C for 15 seconds 60°C for 1 minute and this was repeated for 50 cycles. The primers that were used were developed by Boyle et al 2004 based on the ITS region of the *Bd* genome. They were ITS1-3 Chytr (5' - CCT TGA TAT AAT ACA GTG TGC CAT ATG TC - 3') and 5.8S Chytr (5' – AGC CAA GAG ATC CGT TGT CAA A – 3'). The probe used for the TaqMan assay was a Minor Groove Binding Probe (MGB) Chytr MGB2 (5' – 6FAM CGA GTC GAA CAA AAT MGBNFQ – 3') (Applied Biosystems, Carlsbad, CA). The qPCR reaction consisted of 12.5µl of 2x TaqMan Reagent Master Mix (Applied Biosystems, Carlsbad, CA), 0.45µl of both ITS1-3 Chytr and 5.8S Chytr, 0.625µl of Chytr MGB2 Probe, 5.975µl of RNase/DNase free H_20 and 5µl of DNA sample. Each of the qPCR reactions was performed with positive and negative controls as well as a blank sample to ensure quality assurance.

Results

Table 3.1: Amphibian species sampled in Peninsular Malaysia from 2008 – 2010. All amphibian species were swabbed and tested for the presence of *Bd*. The species are listed in order of prevalence with the species with the most frequently sampled at the top and the least frequently sampled species at the bottom. The last column indicates the ratio of positive and negative samples for the amphibian pathogen *Bd*. The asterix indicates species and year for the sole sample that tested positive for *Bd*.

Species	2008	2009	2010	Totals	Positive/Negative
Hylarana erytraea	0	29	12	41	0/41
Fejervarya limnocharis	1	4	14	19	0/19
Leptobrachium hendricksoni	0	0	18*	18	1/18
Amolops larutensis	0	0	9	9	0/9
Limnonectes blythii	5	1	3	9	0/9
Duttaphrynus melanostictus	0	1	7	8	0/8
Microhyla butleri	0	6	2	8	0/8
Phrynoidis aspera	2	1	5	8	0/8
Hylarana glandulosa	0	1	6	7	0/7
Hylarana labialis	2	0	5	7	0/7
Odorrana hosii	6	0	1	7	0/7
Kaloula pulchra	0	0	4	4	0/4
Polypedates leucomystax	0	1	3	4	0/4
Occidozyga lima	0	3	0	3	0/3
Limnonectes laticeps	2	0	0	2	0/2
Lithobates catesbeianus	0	0	2	2	0/2
Hylarana picturata	0	0	1	1	0/1
Fejervarya cancrivora	0	0	1	1	0/1
Humerana miopus	0	0	1	1	0/1
Hylarana nigrovittata	0	0	1	1	0/1
Ingerophrynus parvus	0	0	1	1	0/1
Microhyla heymonsi	0	0	1	1	0/1
Limnonectes hascheanus	0	0	1	1	0/1
Xenophrys aceras	0	0	1	1	0/1
24	18	47	99	164	1/164

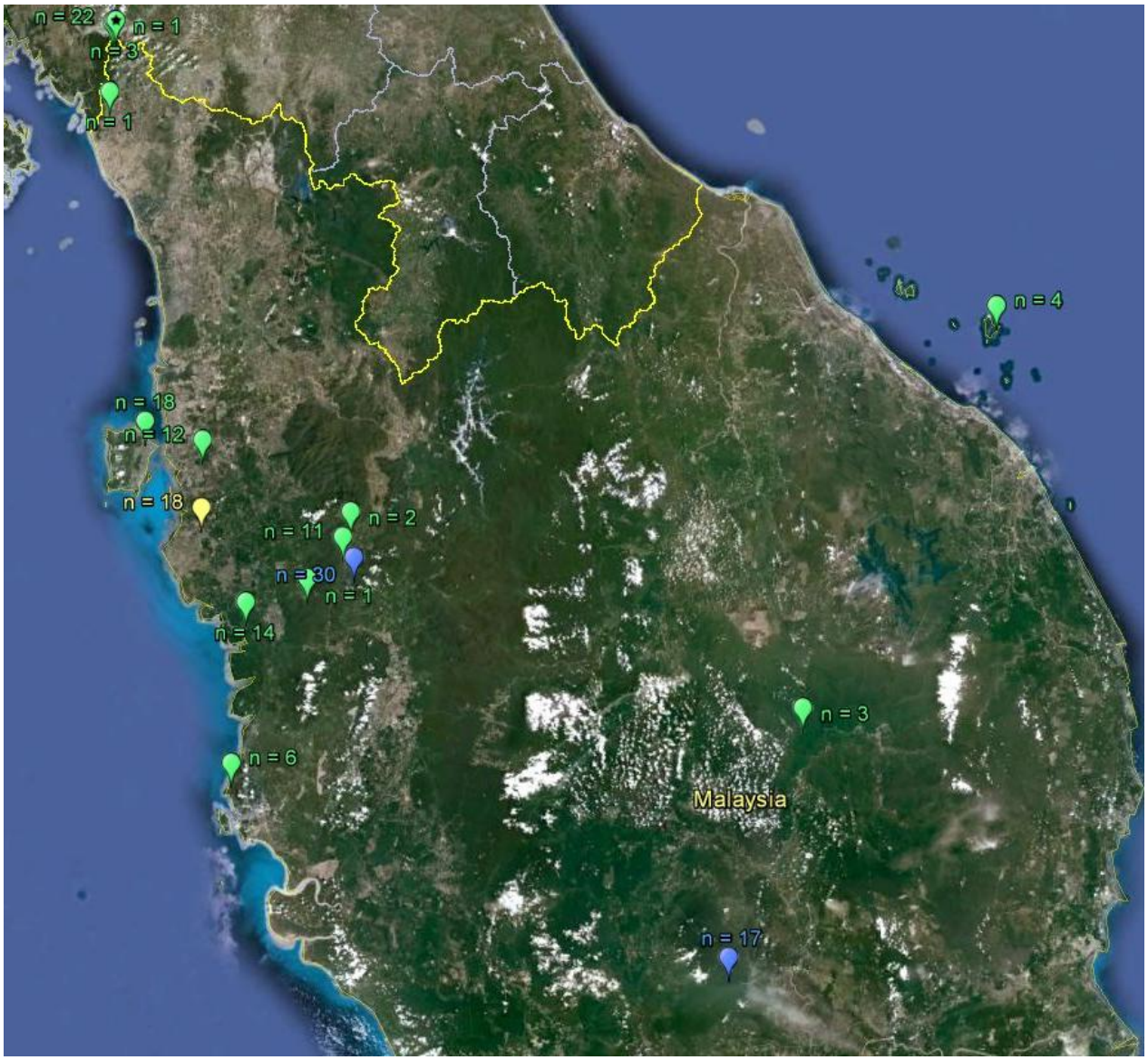

Figure 3.1: A map of Peninsular Malaysia with sample locations and number of samples indicated by coloured markers. The sample size is indicated by "n" and the colour markers indicate the year samples were taken. Yellow = 2008, blue = 2009 and green = 2010. The star on the green marker (upper left) indicates the site of the positive sample.

Table 3.2: *Bd* testing grouped by sampling study site and date. Included are the GPS coordinates and approximate elevations, in meters, at each site. The sampling size is indicated by n and positive samples by "+". The prevalence values for *Bd* are also given with 95% confidence intervals calculated using the published methodology of Robert Newcombe, 1998.

Location	GPS Location	Collection Dates	Elev. (m)	+	n	Prevalence (%) by site (95% CI)
Nibong Tebal, Pulau Pinang	N5 08 36 E100 29 24	May 28 2008	8	0	18	0 (0-21.88)
Batu Ring, Lenggong Perak	N4 59 04 E100 58 52	May 23 2009	178	0	30	0 (0-14.13)
Bukit Rengit, Langchang, Pahang	N3 42 53 E102 10 14	June 2 2009	120	0	17	0 (0-22.92)
Wang Kelian, Perlis	N6 41 51 E100 11 28	May 17 - 23 2010	272	1	26	3.85 (0.2-21.59)
Ujung Bukit, Perlis	N6 28 24 E100 11 07	May 27 2010	36	0	1	0 (0-94.54)
Matang Mangrove Forest, Perak	N4 50 36 E100 38 00	May 31 2010 - June 1 2010	4	0	14	0 (0-26.76)
Batu Kurau River, Perak	N4 55 05 E100 49 53	June 1 2010	4	0	1	0 (0-94.54)
Kuala Tahan, Pahang	N4 30 18 E102 24 32	June 4 2010	128	0	3	0 (0-69)
Redang Island, Terengganu	N5 46 37 E103 01 57	June 13 2010	11	0	4	0 (0-60.42)
Muka Head, CEMACS, Penang	N5 25 08 E100 18 28	June 15 2010	13	0	18	0 (0-21.88)
Telak Senangin, Perak	N4 19 54 E100 35 04	Nov. 4 2010	14	0	6	0 (0-48.32)
Gua Asar, Perak	N5 07 44 E100 58 14	Nov. 5 2010	105	0	2	0 (0-80.21)
Lata Kekabu, Perak	N5 02 59 E100 56 44	Nov. 5 2010	110	0	11	0 (0-32.14)
Bukit Mertajam, Penang	N5 21 29 E100 29 36	Nov. 26 2010	53	0	12	0 (0-30.13)

Surveillance results of a multiyear amphibian assessment of Peninsular Malaysia

There were a total of 164 samples taken from 24 different species collected from 14 different study sites from all over Peninsular Malaysia over the three year period. The only sample which tested positive for *Bd* was collected during the 2010 survey from an adult *Leptobrachium hendricksoni* which was sampled in the far North West of Peninsular Malaysia near the Thailand border (Figure 3.1). This positive sample was collected near a small creek next to heavily forested area in a mountainous region. Of all the 24 species sampled only the two *L. catesbeianus* samples, from 2010, were introduced species to Peninsular Malaysia. These samples were taken from the Bukit Mertajam region of Penang (Table 3.2) located near urban areas on the North West Coast of Peninsular Malaysia (Figure 3.1). The only species which appeared on IUCN's (International Union for Conservation of Nature) red-list for endangered species was the near threatened (NT) species *Limnonectes blythii*. All of the other species tested belonged in a least concern (LC) designation from the IUCN. The survey also included 6 species which are believed to be decreasing in population size and many others which had insufficient data according to the IUCN website (www.iucnredlist.org). Figure 3.1 indicates that samples were taken from many different locations in Peninsular Malaysia. The majority of samples were taken from the North Western Part of the country with only two sample sites in the South. The samples were taken from a wide range of elevations from 4 meters to 272 meters. The sample sites also encompassed many different habitats with some being close to urban areas while others were in remote and heavily forested regions.

Discussion

The results from our multiyear survey show that *Bd* has only recently been detected in Peninsular Malaysia with the first incidence being recorded in 2010. Our surveys prior to 2010, although limited in scope, resulted in no detection *Bd* presence despite sample sizes of 17 or greater at some sites tested. The prevalence was also low in this 2010 sampling with only 1 of 99 total samples testing positive for *Bd*. Since our multiyear survey of Peninsular Malaysia, a survey published by Savage et al. (2011) demonstrated the presence of *Bd* in samples collected in March 2010. In our study from 2008 through to 2010 we found a single positive sample in May 2010 in northern Peninsular Malaysia. We did not sample the same high elevation sites which were utilized by Savage et al. (2011), but our low and mid level elevation survey and sample sizes compare to similar sites explored in their study. Although we do not have exact coordinates from their study we did cover many of the same states with the exception of Kedah where we tested a few amphibians that were not included in the published survey (Savage et al., 2011). Our single *Bd* positive amphibian was collected in the Perlis area of Peninsular Malaysia and this site had a low prevalence level of 3.85%. This was the only region which did not test positive in the study by Savage et al. (2011), although this was also their smallest sample size with only 4 amphibians tested. The other published prevalence values were low at the different sampling sites with the highest being 16% in Perak which also had their smallest sampling of all positive regions. The conclusion from the Savage survey suggests that *Bd* is endemic to Peninsular Malaysia, and based on their results this is quite convincing. With our findings at the same locations and done 2 years prior to their survey, as well as their low 7.8% prevalence value, we suggest that *Bd* may not be endemic to Malaysia but may be a novel pathogen which has just recently entered this ecosystem.

In order to explain how *Bd* could go from being undetected in a country to being present in many locations within a short period of time, the rate of pathogen spread needs to be examined. There have been relatively few studies which have been able to look at this question. One study, recently completed in South America, estimated the highest rate of spread at 282 km per year (Lips et al. 2008). This is quite high and is not seen in

every location that has been monitored for the rate of spread (Lips et al. 2006). The detection of *Bd* throughout Peninsular Malaysia by Savage et al. (2011) after our study failed to detect it raises questions as to how *Bd* went from undetected to present at most sample sites. If the initial infections were on the central coastal regions it would be possible for the pathogen to spread both north and south within a short period of time. There is also the possibility that due to low prevalence values and smaller sampling size *Bd* may have been in the environment prior to our detection of it.

Another possible reason for this discrepancy could be seen in differences in elevation and seasonal sampling of the pathogen in the respective studies. Our sampling occurred mainly in May with Savage et al. (2011) sampling in March which is typically cooler and is more suitable for *Bd* growth (Piotrowski et al. 2004). The warmer temperatures may have contributed to our inability to detect *Bd*. The other factor which may have led to our lower prevalence values is the elevation discrepancies in the study sites. In tropical regions *Bd* has been shown to be found at higher elevations due to ideal temperature ranges (Berger et al. 1998; Lips et al. 2008; Pounds et al. 2006). The survey by Savage et al. (2011) resulted in 8 of their 10 positives being above 100 meters. The sampling that was conducted by our group focused mainly on lower elevation sites and our only positive occurred at our highest elevation site (272 meters). These contributing factors may have hindered our ability to detect *Bd* at similar prevalence values as the other research group. Asia has been identified as having a possible endemic population of *Bd* (Goka et al. 2009; Bai et al. 2012). The research by Bai et al. (2012) suggests that endemic *Bd* may have a prevalence value as low as 7.6% in China. If this low prevalence value is indicative of endemic *Bd* in Asia, our sample size would need to increase to 38 to detect the pathogen.

The vector that is most likely propagating *Bd* spread worldwide is the bullfrog, which is mostly asymptomatic to the disease (Daszak et al. 2004; Schloegel et al. 2010) as well as being heavily traded in the amphibian market (Fisher & Garner 2007; Schloegel et al. 2009). This potential vector is heavily traded in Asia, including Malaysia, and many of these frogs are able to escape into local ecosystems. In our study two

bullfrogs were collected from the wild, which were both from the Penang region of Peninsular Malaysia. This would suggest that the bullfrog has likely been introduced into the wild in Peninsular Malaysia, at least at this one observed location. This area also tested positive for *Bd* in the study done by Savage et al. (2011). The observation of the potential bullfrog vector in the wild in Peninsular Malaysia suggests that *Bd* could have been introduced fairly recently in this country. The combined information from these two studies in Peninsular Malaysia, both our repeat sampling and one positive being found in the same year that low prevalence levels were found in the Savage survey suggests to us that this may be a recently introduced pathogen.

The recent trend to test for *Bd* around the world has led to the pathogen being found in more and more countries every year (Bai et al., 2010; Savage et al., 2011; Yang et al., 2009). When *Bd* is first detected in a regional survey it is important to determine whether the pathogen is endemic to that area or if it is a novel pathogen which has just entered a new region. One way of monitoring for a novel or endemic pathogen is to conduct multiyear surveys. This is not easy to accomplish without sustained research programs or a commitment by local governments to continue these types of surveys even past the first stages of detection. Another way to assess whether the pathogen has long been present in a particular system is to sample historical specimens which may have been preserved during earlier amphibian surveys in order to try to determine earlier times and places of introduction. This approach has been successful in establishing a baseline for *Bd* introduction and can help pinpoint the exact location of introduction and rate of spread of the pathogen (Lips et al. 2008; Lips 2011; Weldon et al. 2004; Ouellet et al. 2005; Soto-Azat et al. 2009). For well-established and persistent infections the origins of a pathogen can be surmised by examining the population genetic structure of the pathogen. This has also been done recently with some success, and will only continue to gain refinement as more is discovered about the *Bd* genome (James et al. 2009; Goka et al. 2009; Morgan et al. 2007; Farrer et al. 2011). This would definitely aid in the determination of the NPH versus EPH in Peninsular Malaysia, considering two different surveys (Savage et al. 2011) may suggest alternate hypotheses for the introduction of this pathogen.

Chapter 4

General Conclusions

The emerging infectious pathogen, *Bd*, has been directly linked to declines to amphibian populations around the world (Daszak et al., 2003). This latest threat to amphibian biodiversity must be carefully monitored in order to mitigate the possible mass extinctions of amphibians. The first steps in combating this new threat are to monitor the incidence of this pathogen, determine its geographic range, and also to assess any changes to virulence of this fungus. Effective monitoring has been facilitated by utilizing Genus-specific DNA probes and accurate qPCR testing of amphibian samples to positively identify infected individuals (Boyle et al. 2004). The increased surveillance for *Bd* has led to it being recovered in almost every country surveyed and pathogen free regions are becoming rare (Fisher et al. 2009). In our study we were able to examine two previously underrepresented regions, the large province of British Columbia and Peninsular Malaysia. Both of these study sites showed differences in the prevalence of the pathogen. In BC we found a near ubiquitous distribution throughout the entire province, where *Bd* was sampled, which could suggest that the pathogen is endemic to the region. In Peninsular Malaysia we only found very low prevalence and detected the pathogen in only the third year of our three year assessment. Another survey, conducted by Savage et al. (2011) also found the presence of *Bd* in Peninsular Malaysia in March of 2010. Because of the distribution throughout the peninsula they concluded that the fungus was endemic (Savage et al., 2011). These surveys add incrementally to our knowledge about the world-wide distribution of *Bd*. If we are to protect our amphibian biodiversity we must first have a better understanding of this pathogen's distribution and the movement of virulent types.

One of the most important questions for any emerging pathogen is the question of its origin. Since the emergence of *Bd* as a new pathogen there have been two hypotheses which have attempted to resolve this question. There is the possibility that *Bd* is an endemic pathogen which, due to environmental factors, has recently become more

virulent. The alternative hypothesis posits that *Bd* is a newly arrived pathogen which is adversely impacting a naïve amphibian population thus reducing its biodiversity (Rachowicz et al., 2005). Between these two hypotheses is the possibility of different strains of *Bd* are present in lower levels but successful virulent strains can arise through sexual recombination and recent introductions and cause amphibian declines. In order to better elucidate between these hypotheses in BC we examined the population genetic structure of selected *Bd* isolates. We began with an examination of the ITS region of the rDNA repeat in the genome. There were no ITS sequence differences between any of the isolates collected in BC hence this marker could not be used to resolve populations but did indicate a very low genetic variability within the BC isolates. This is consistent with reports from other regions of the world and points to the extremely high conservation of *Bd* genomes worldwide (Morehouse et al. 2003; Morgan et al. 2007; James et al. 2009; Walker et al. 2010; Schloegel et al. 2010). Two *Bd* strains isolated from bullfrogs on Vancouver Island were used in a higher resolution MLST genome survey. These samples were compared to other MLST profiles from worldwide *Bd* strains (James et al. 2009) and showed that our two samples most resembled strain JEL262 which was isolated from a bullfrog in Quebec, Canada. This was subsequently confirmed by whole genome sequencing which placed our strain PTH002 closest to another bullfrog strain, JEL261, isolated from Quebec, Canada in the same survey (Farrer et al., 2011). The whole genome sequencing also grouped the BC strain PTH002 to the hypervirulent global panzootic lineage (Personal Communication Rhys Farrer). These results suggest that our two strains of *Bd* may have originated from the east coast bullfrogs, perhaps after being introduced through the amphibian trade. This does not indicate the origin of the other *Bd* infected areas in BC and additional research needs to be done to determine the genetic diversity, or lack thereof, for the rest of the province.

This study showed support for elements of both the NPH and EPH in BC and Peninsular Malaysia and it is likely that the pathogen distribution and disease incidence is more complex than originally anticipated. In BC both surveillance and genetic data were analyzed. The near ubiquity of *Bd* in all areas tested in BC tended to favour the EPH (Figure 2.1). There was no discernible disease front for the pathogen in the province and

it may be that the pathogen is endemic though the time of the original introduction is unknown. The limited genetic diversity of the pathogen, based on the ITS sequence analysis, would tend to support the recent introduction of a limited number of genotypes (NPH) but without further analysis of many more gene loci, having greater variability, it is currently impossible to establish a date for the first arrival of the pathogen. The results of the MLST analysis of *Bd* from two bullfrogs on Vancouver Island did link *Bd* populations on Vancouver Island to Quebec thus supporting the NPH. This linkage was also confirmed with whole genome sequencing, which was completed by another research group. Most tellingly the genome analysis placed the Vancouver Island PTH002 isolate within the hypervirulent GPL. During three years of screening in Peninsular Malaysia we detected a single sample in the third year of sampling. This very low prevalence in the last year of testing may indicate the very early stages of an emerging pathogen. Another survey by Savage et al. (2011) found higher prevalence values at many different locations indicating that *Bd* may be endemic. The results of both surveys show support for both of the competing hypotheses.

It is important to further monitor both locations to gain a better understanding of this pathogen which has the potential to devastate amphibians in both temperate and tropical regions of the world. In BC more work needs to be done utilizing MLST or other genomic methods in areas other than Vancouver Island and hosts other than bullfrogs to see which hypothesis is better supported. It would also be useful to test museum samples to see if there is a definitive time frame of *Bd* introduction in the province, this has been done with some success in Central America (Lips et al., 2008). In Peninsular Malaysia similar methods should be utilized. There also needs to be a more thorough survey which encompasses a greater area of the country at multiple elevations and during multiple seasons. There are two important research objectives which will help increase our knowledge of this recently discovered pathogen. The first involves more comprehensive surveillance in under represented regions so we can better assess *Bd*'s distribution worldwide. The second area of research which will better elucidate between the NPH and EPH are thorough genetic analyses using MLST profiles or whole genome sequencing with an emphasis on potential origins of the pathogen or suspected endemic locations

(Weldon et al. 2004; Bai et al. 2012). This relies on the culturing of living *Bd* samples and determining their genome sequences to map the worldwide distribution of the GPL. These approaches will hopefully resolve the debate of *Bd* being a novel or endemic pathogen. The results to date suggest that if *Bd* was a novel pathogen, globally, it may now be considered endemic in many regions due to its ubiquitous distribution. It is anticipated that increased knowledge of the *Bd* genome and surveillance will lead to tools and approaches to help ameliorate the loss of amphibian biodiversity, by hopefully finding improved ways to counteract this deadly pathogen.

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Appendix

Appendix A
Supplemental Information for Chapter 2

MLST Expanded Methodology

Table A1: The primers used in the MLST analysis of the two BC isolates of *Bd*. Included are the primer sequences the optimal annealing temperatures and their target amplicon size.

Primer	Primer Sequence	Annealing Temp.	Amplicon Size	Source
8702X2 F	5′-GGATCTGCCAGTTTCGATCTACTCG-3′	53	245bp	Morgan et al. 2007
8702X2 R	5′-GAATATGGCATGGGAGAAGTAGCC-3′	53		
CTSYN1 F	5′-ACCAACTATAACATCATCAAG-3′	50	553bp	Morehouse et al. 2003
CTSYN1 R	5′-CGAATATCAGTCAACGCAAGC-3′	50		
BdC5 F	5′-TAATAGCGCCGACCGAACTA-3′	56	747bp	James et al. 2009
BdC5 R	5′-ATGCCAAACCATGAGCAAAT-3′	56		
BdC18 F	5′-GCGAATACGACTGCAAATGA-3′	65	885bp	James et al. 2009
BdC18 R	5′-TGAGCTCTAGCCGACATTGA-3′	65		
BdC24 F	5′-GACAATGTGCTCACGGCTTA-3′	57	726bp	James et al. 2009
BdC24 R	5′-CTCTCCAAGGCTGAATCTGG-3′	57		
9893X2 F	5'-GTTGGTATTACTCAACGTCCATACAC-3'	51	199bp	Morgan et al. 2007
9893X2 R	5'-ACTCAGTCGTACGTAGCTAGTTTG-3'	51		
Mb-b13-8b F	5'-GAAAACATGGCATGCAGTGG-3'	52	219bp	Morgan et al. 2007
Mb-b13-8b R	5'-CGGCGAAGCTCTCGCTAC-3'	52		
b7-10c F	5'-TGTCTGAATGATTTTCCCTCGG-3'	50	314bp	Morgan et al. 2007
b7-10c R	5'-GGTAGCTCAGTAGTTCCATGC-3'	50		
9908X2 F	5'-TGCTGACAATGGTGCCAGCTAT-3'	55	395bp	Morgan et al. 2007
9908X2 R	5'-TAGCCGTTTCGACAGTGGTGGC-3'	55		
6677X2 F	5'-CACCAACGGAGGATGATCGCACA-3'	55	190bp	Morgan et al. 2007
6677X2 R	5'-CTTGAAAAACCAAGCCACAGTCCTAG-3'	55		
6873X2 F	5'-TCGTCCTGATGAGATGCAAACCAG-3'	54	337bp	Morgan et al. 2007
6873X2 R	5'-GAGTTTCCAGGCAAGTGTTTTGCT-3'	54		
8329X2 F	5'-CTGAATCTTGCCTCGTCTAGTAGC-3'	52	336bp	Morgan et al. 2007
8329X2 R	5'-TATCAAGGTCTTTTGGCAAGACCG-3'	52		
8009X2 F	5'-TCGTGAAGAGCTTGGAAAGTCG-3'	53	342bp	Morgan et al. 2007
8009X2 R	5'-AGTTCTGTCGTCAATGCTGTAGGG-3'	53		
8667Y2 F	5'-AAGTGTAGACATGGCACCCGAGTT-3'	53	289bp	Morgan et al. 2007
8667Y2 R	5'-ATACATGTACAAGACCAAGAAGGTCG3'	53		

Primer	Primer Sequence	Annealing Temp.	Amplicon Size	Source
lsu35 F	5'-ATCCCTGTGGTAACTTTTCTG-3'	50	688bp	Morgan et al. 2007
lsu35 R	5'-ACGGACATGGGGAATCTGACT-3'	50		
r6046 F	5'-CTATCTGCGCTCCCGTGTCAA-3'	53	496bp	Morgan et al. 2007
r6046 R	5'-AGGGCTGCAACAACTGGATTT-3'	53		

MLST Results including James et al. 2009 Sequences

Table A2: List of MLST loci from worldwide survey by James et al. 2009, included are two BC isolates PTH001 and PTH002. The letters correspond to listed nucleotides for each loci: CTSYN1 a=G, b=G/A, c=A;; R6046 a=--, b=CA/--, c=CA; BdC5 a=C, b=C/T, c=T; BdC24 a=A, b=A/T, c=T; BdC18.1 a=C, b=C/T, c=T; 8702x2 a=T, b=T/C, c=C; 8009x2 a=G, b=T/G, c=T; 8329x2 a=A, b=A/G, c=G; mb-b13 a=--, b=AT/--, c=AT; 9893x2 a=--, b=TA/--, c=TA; b7-10c a=G, b=G/C, c=C; 6873x2 a=A, b=A/G, c=G; 6677x2 a=A, b=A/G, c=G

Strain	CTSYN1	R6046	BDC5	BDC24	BDC18.1	8702X2	8009X2	8329X2	MB-B13
JEL197	b	b	a	a	b	a	a	a	b
JEL198	b	b	a	a	b	a	a	a	b
JEL213	b	b	a	b	b	b	c	a	b
JEL229	b	b	a	b	b	b	a	a	b
JEL230	b	b	a	b	b	b	a	a	b
JEL262	b	b	a	b	b	b	c	c	b
JEL226	b	b	b	a	b	b	b	b	b
JEL277	b	b	a	b	c	b	b	b	b
JEL203	b	a	c	a	a	b	c	c	b
JEL270	b	a	b	b	c	c	b	b	b
JEL271	b	a	b	b	b	b	a	b	b
JEL282	b	a	b	a	c	b	b	b	b
JEL225	a	a	b	a	b	a	b	a	b
JEL231	b	c	a	b	b	c	b	b	b
JEL239	b	a	b	a	b	b	b	b	b
JEL273	b	a	b	a	a	b	a	b	a
JEL275	b	a	b	a	a	b	a	b	a
JEL253	a	a	a	a	b	b	a	a	b
JEL254	b	b	a	b	b	b	b	b	b
JEL258	b	b	a	b	b	b	b	b	b
JEL274	c	a	b	a	a	b	a	b	a
JEL284	b	c	a	b	b	b	a	a	b
JEL289	b	c	a	b	c	b	a	c	b
PM5	b	a	b	a	b	b	b	b	b
PM7	b	a	b	a	b	b	b	b	b
PM1	b	a	a	a	b	b	c	c	b
JEL404	b	b	b	b	b	b	b	b	b
CW26	c	a	b	a	b	c	a	b	b
CW27	c	a	b	a	b	c	a	b	b
CW29	c	a	b	a	b	c	a	b	b
CW34	c	a	b	a	b	c	a	b	b

Strain	CTSYN1	R6046	BDC5	BDC24	BDC18.1	8702X2	8009X2	8329X2	MB-B13
CW36	b	a	b	a	b	b	b	b	b
JEL408	b	a	c	a	b	b	c	c	b
JEL409	b	a	c	a	b	b	c	c	b
JEL415	b	a	c	a	b	b	c	c	b
JEL423	b	a	c	a	b	b	c	c	b
JEL424	b	a	c	a	b	b	c	c	b
JEL425	b	a	c	a	b	b	c	c	b
JEL427	b	a	c	a	c	a	b	b	b
JEL429	b	a	b	a	b	b	b	b	c
Me00LB	b	a	b	a	b	c	b	b	b
Tu98LB	b	a	b	a	b	b	c	b	b
MM06LB	b	a	a	a	b	c	a	a	c
To05LB	b	a	b	a	b	c	b	b	b
Ro99LB	b	a	a	a	c	b	a	a	b
Al99LB	b	a	c	a	b	c	c	c	b
Eur042	b	b	a	b	b	c	b	b	b
Eur043	b	b	a	b	b	c	b	b	b
EurC2A	b	a	b	b	b	b	b	b	b
JAM018	b	a	a	b	b	b	a	a	b
JP005	b	a	a	a	b	b	c	a	b
JAM011	b	a	b	b	b	b	b	b	b
JAM050	b	b	b	b	a	b	b	b	a
JAM102	b	a	b	b	b	b	c	b	b
LJR299	b	c	b	b	b	b	b	b	b
JAM033	b	c	c	a	a	b	a	b	a
JAM083	c	a	c	b	a	b	a	c	b
JAM084	c	a	c	b	a	b	a	c	a
LRJ089	a	b	c	c	a	b	c	c	c
PTH001	?	?	a	?	b	b	c	c	b
PTH002	a	b	a	c	b	b	c	c	b

Table A3: This is a continuation of the Appendix table A2.

Strain	9893X2	B7-10C	6873X2	6677X2
JEL197	a	a	a	a
JEL198	a	a	a	a
JEL213	a	c	c	c
JEL229	a	c	a	a
JEL230	a	c	a	a
JEL262	b	c	c	c
JEL226	a	b	b	b
JEL277	a	b	b	b
JEL203	a	c	c	c
JEL270	c	b	b	b
JEL271	c	b	a	b
JEL282	a	b	?	b
JEL225	a	b	b	b
JEL231	?	b	b	a
JEL239	a	b	a	b
JEL273	a	a	a	a
JEL275	a	a	a	a
JEL253	a	a	a	a
JEL254	?	b	b	b
JEL258	a	b	b	b
JEL274	a	a	a	a
JEL284	b	a	a	a
JEL289	a	a	a	a
PM5	a	b	b	b
PM7	a	b	b	b
PM1	a	c	c	?
JEL404	b	b	b	b
CW26	a	a	a	a
CW27	a	a	a	a
CW29	a	a	a	a
CW34	a	a	a	a
CW36	a	b	a	b
JEL408	a	c	c	c
JEL409	a	c	c	c
JEL415	a	c	c	c
JEL423	a	c	c	c
JEL424	a	c	c	c
JEL425	a	c	c	c

Strain	9893X2	B7-10C	6873X2	6677X2
JEL427	a	b	b	b
JEL429	a	b	b	b
Me00LB	a	b	b	b
Tu98LB	a	c	b	b
MM06LB	a	a	a	c
To05LB	a	b	b	b
Ro99LB	a	a	a	a
Al99LB	a	c	c	c
Eur042	a	b	b	b
Eur043	a	b	b	b
EurC2A	c	b	b	b
JAM018	a	a	a	c
JP005	a	c	c	a
JAM011	a	b	b	b
JAM050	b	b	c	b
JAM102	c	b	c	b
LJR299	b	b	c	b
JAM033	c	a	a	c
JAM083	b	a	a	c
JAM084	b	a	a	c
LRJ089	c	c	a	a
PTH001	?	?	?	c
PTH002	c	c	c	c

Table A4: Strain information for MLST work completed in Figure 2.4. Most of these strains come from work done by James et al. 2009. Included are the two BC isolates which were cultured by Finn Hamilton.

Sample Name	Geographic Region	Species
Al99LB	Altonville, New South Wales, Australia	*Litoria caerulea*
CW-026	Namaqualand, South Africa	*Amietia fuscigula*
CW-027	Namaqualand, South Africa	*Amietia fuscigula*
CW-029	Namaqualand, South Africa	*Xenopus laevis*
CW-034	Namaqualand, South Africa	*Xenopus laevis*
CW-036	Port Elizabeth, South Africa	*Amietia fuscigula*
EUR042	Pyrenees, Spain	*Alytes obstetricians*
EUR043	Pyrenees, Spain	*Alytes obstetricians*
EURC2A	Sierra de Guadarrama, Spain	*Alytes obstetricians*
JAM011	Mono Pass, California, USA	*Rana muscosa*
JAM018	Mono Pass, California, USA	*Rana muscosa*
JAM033	Summit Meadow, California, USA	*Rana muscosa*
JAM050	Hitchcock Lakes, California, USA	*Rana muscosa*
JAM083	Little Indian Valley, Califronia, USA	*Rana muscosa*
JAM084	Little Indian Valley, Califronia, USA	*Rana muscosa*
JAM102	Woods Lake, California, USA	*Rana muscosa*
JEL197	National Zoological Park, DC, USA	*Dendrobates azureus*
JEL198	National Zoological Park, DC, USA	*Dendrobates azureus*
JEL203	Bronx Zoo, New York, USA	*Dyscophus guineti*
JEL213	Mono Co., California, USA	*Rana muscosa*
JEL225	Africa (from captive population in Wisconsin, USA)	*Silurana tropicalis*
JEL226	Yavapai Co., Arizona, USA	*Rana yavapaiensis*
JEL229	Montrose Canyon, Arizona, USA	*Hyla arenicolor*
JEL230	Montrose Canyon, Arizona, USA	*Rana yavapaiensis*
JEL231	Mesquite Wash, Arizona, USA	*Rana yavapaiensis*
JEL239	Ghana (Imported)	*Silurana tropicalis*
JEL253	Melbourne, Victoria, Australia (captive)	*Limnodynastes dumerilii*
JEL254	Orono, Maine, USA	*Rana pipiens*
JEL258	Orono, Maine, USA	*Rana sylvatica*
JEL262	Quebec, Canada	*Rana catesbeiana*
JEL270	Point Reyes, California, USA	*Rana catesbeiana*
JEL271	Point Reyes, California, USA	*Rana catesbeiana*
JEL273	Clear Creek Co., Colorado, USA	*Bufo boreas*
JEL274	Clear Creek Co., Colorado, USA	*Bufo boreas*
JEL275	Clear Creek Co., Colorado, USA	*Bufo boreas*
JEL277	Arizona, USA	*Ambystoma tigrinum*
JEL282	Toledo Zoo, Ohio, USA	*Bufo americana*

Sample Name	Geographic Region	Species
JEL284	Wisconsin, USA (captive)	*Rana pipiens*
JEL289	Milford, Maine, USA	*Rana pipiens*
JEL404	Crocker Pond, Oxford County, Maine	*Rana catesbeiana*
JEL408	El Cope, Panama	*Colostethus inguinalis*
JEL409	Silenciosa, Panama	*Eleutherodactylus talamance*
JEL415	between Loop and Silenciosa, Panama	*Eleutherodactylus podi-noblei*
JEL423	Guabal, Panama	*Phyllomedusa lemur*
JEL424	Loop trail, Panama	*Cochranella euknemos*
JEL425	El Cope, Panama	*Bufo haematiticus*
JEL427	Puerto Rico	*Eleutherodactylus coqui*
JEL429	Venezuela	*Rana catesbeiana*
JP005	Berkeley, California, USA	*Xenopus laevis*
LJR089	Laurel Creek, California, USA	*Rana muscosa*
LJR299	Point Reyes, California, USA	*Rana aurora draytonii*
Me00LB	Melbourne, Victoria, Australia (captive)	*Litoria lesueuri*
MM06LB	Mt. Misery, Queensland, Australia	*Litoria rheocola*
PM-01	Panama	*Eleuthodactylus caryophyllaceum*
PM-05	Panama	*Smilisca phaeota*
PM-07	Panama	*Smilisca phaeota*
Ro99LB	Rockhampton, Queensland, Australia	*Litoria caerulea*
To05LB	James Cook University, Queensland, Australia (captive)	*Litoria caerulea*
Tu98LB	Tully, Queensland, Australia	*Nyctimystes dayi*
PTH001	Nanaimo, Vancouver Island, BC, Canada	*Lithobates catesbeianus*
PTH002	Beaver Pond, vancouver Island, BC, Canada	*Lithobates catesbeianus*

Table A5: Data collected from an amphibian survey of British Columbia and the Yukon. The nearest town, region and date collected are indicated along with species code and *Bd* status indicated by 0=negative and 1=positive testing.

Town	Date Collected	Sample ID	Spp. Code	Region	Bd Status
100 Mile House	09/07/2008	3	BUBO	Cariboo	0
100 Mile House	09/07/2008	4	BUBO	Cariboo	0
100 Mile House	09/07/2008	5	BUBO	Cariboo	0
100 Mile House	09/07/2008	6	BUBO	Cariboo	0
100 Mile House	09/07/2008	7	BUBO	Cariboo	0
100 Mile House	09/07/2008	8	BUBO	Cariboo	0
100 Mile House	09/07/2008	9	BUBO	Cariboo	0
100 Mile House	09/07/2008	10	BUBO	Cariboo	1
100 Mile House	09/07/2008	11	BUBO	Cariboo	0
100 Mile House	09/07/2008	12	BUBO	Cariboo	0
100 Mile House	09/07/2008	13	BUBO	Cariboo	0
100 Mile House	09/07/2008	14	BUBO	Cariboo	0
100 Mile House	09/07/2008	15	BUBO	Cariboo	0
100 Mile House	09/07/2008	16	BUBO	Cariboo	0
100 Mile House	09/07/2008	17	BUBO	Cariboo	0
100 Mile House	09/07/2008	18	BUBO	Cariboo	0
100 Mile House	09/07/2008	19	BUBO	Cariboo	0
100 Mile House	09/07/2008	20	BUBO	Cariboo	0
100 Mile House	09/07/2008	21	BUBO	Cariboo	0
100 Mile House	09/07/2008	22	BUBO	Cariboo	0
100 Mile House	09/07/2008	23	BUBO	Cariboo	0
100 Mile House	09/07/2008	24	BUBO	Cariboo	0
100 Mile House	09/07/2008	25	BUBO	Cariboo	0
100 Mile House	09/07/2008	26	BUBO	Cariboo	0
100 Mile House	09/07/2008	27	BUBO	Cariboo	0
100 Mile House	09/07/2008	28	BUBO	Cariboo	0
100 Mile House	09/07/2008	29	BUBO	Cariboo	0
100 Mile House	09/07/2008	30	BUBO	Cariboo	0
100 Mile House	09/07/2008	31	BUBO	Cariboo	0
100 Mile House	09/07/2008	32	BUBO	Cariboo	0
100 Mile House	09/07/2008	33	BUBO	Cariboo	0
100 Mile House	09/07/2008	34	BUBO	Cariboo	0
70 Mile House	10/07/2008	35	SPIN	Cariboo	0
70 Mile House	10/07/2008	36	SPIN	Cariboo	0
70 Mile House	10/07/2008	37	SPIN	Cariboo	0
70 Mile House	10/07/2008	38	SPIN	Cariboo	0
70 Mile House	10/07/2008	39	SPIN	Cariboo	0
70 Mile House	10/07/2008	40	SPIN	Cariboo	0
70 Mile House	10/07/2008	41	SPIN	Cariboo	0
70 Mile House	10/07/2008	42	SPIN	Cariboo	0
70 Mile House	10/07/2008	43	SPIN	Cariboo	0
70 Mile House	10/07/2008	44	SPIN	Cariboo	0
100 Mile House	08/07/2008	45	PSRE	Cariboo	0

100 Mile House	08/07/2008	46	RALU	Cariboo	0
100 Mile House	08/07/2008	47	RAAU	Cariboo	0
100 Mile House	08/07/2008	48	BUBO	Cariboo	1
100 Mile House	08/07/2008	49	BUBO	Cariboo	0
100 Mile House	08/07/2008	50	BUBO	Cariboo	0
100 Mile House	08/07/2008	51	BUBO	Cariboo	0
100 Mile House	08/07/2008	52	BUBO	Cariboo	0
100 Mile House	08/07/2008	53	BUBO	Cariboo	0
100 Mile House	08/07/2008	54	BUBO	Cariboo	0
100 Mile House	08/07/2008	55	BUBO	Cariboo	0
100 Mile House	08/07/2008	56	BUBO	Cariboo	0
100 Mile House	08/07/2008	57	BUBO	Cariboo	0
100 Mile House	08/07/2008	58	BUBO	Cariboo	0
100 Mile House	08/07/2008	59	BUBO	Cariboo	0
70 Mile House	10/07/2008	60	SPIN	Cariboo	0
70 Mile House	10/07/2008	61	SPIN	Cariboo	0
70 Mile House	10/07/2008	62	SPIN	Cariboo	1
70 Mile House	10/07/2008	63	SPIN	Cariboo	0
70 Mile House	10/07/2008	64	SPIN	Cariboo	0
70 Mile House	10/07/2008	65	SPIN	Cariboo	0
100 Mile House	11/07/2008	66	RALU	Cariboo	0
100 Mile House	11/07/2008	67	RALU	Cariboo	0
100 Mile House	11/07/2008	68	RALU	Cariboo	0
100 Mile House	11/07/2008	69	RALU	Cariboo	0
100 Mile House	11/07/2008	70	RALU	Cariboo	0
100 Mile House	11/07/2008	71	RALU	Cariboo	0
100 Mile House	11/07/2008	72	RALU	Cariboo	1
100 Mile House	11/07/2008	73	RALU	Cariboo	0
100 Mile House	11/07/2008	74	RALU	Cariboo	0
100 Mile House	10/07/2008	75	BUBO	Cariboo	0
100 Mile House	10/07/2008	76	BUBO	Cariboo	0
100 Mile House	10/07/2008	77	BUBO	Cariboo	0
100 Mile House	10/07/2008	78	BUBO	Cariboo	0
100 Mile House	10/07/2008	79	BUBO	Cariboo	0
100 Mile House	10/07/2008	80	BUBO	Cariboo	0
100 Mile House	10/07/2008	81	BUBO	Cariboo	0
100 Mile House	10/07/2008	82	BUBO	Cariboo	0
100 Mile House	10/07/2008	83	BUBO	Cariboo	0
100 Mile House	10/07/2008	84	BUBO	Cariboo	0
100 Mile House	10/07/2008	85	BUBO	Cariboo	0
100 Mile House	10/07/2008	86	BUBO	Cariboo	0
100 Mile House	10/07/2008	87	BUBO	Cariboo	0
100 Mile House	10/07/2008	88	BUBO	Cariboo	0
100 Mile House	10/07/2008	89	BUBO	Cariboo	0
100 Mile House	10/07/2008	90	BUBO	Cariboo	0
100 Mile House	10/07/2008	91	BUBO	Cariboo	0
100 Mile House	10/07/2008	92	BUBO	Cariboo	0
100 Mile House	10/07/2008	93	BUBO	Cariboo	0

100 Mile House	10/07/2008	94	BUBO	Cariboo	0
100 Mile House	10/07/2008	95	BUBO	Cariboo	0
100 Mile House	10/07/2008	96	BUBO	Cariboo	0
100 Mile House	10/07/2008	97	BUBO	Cariboo	0
100 Mile House	10/07/2008	98	BUBO	Cariboo	0
100 Mile House	10/07/2008	99	BUBO	Cariboo	0
100 Mile House	10/07/2008	100	BUBO	Cariboo	0
100 Mile House	10/07/2008	101	BUBO	Cariboo	0
100 Mile House	10/07/2008	102	BUBO	Cariboo	0
Williams Lake	12/07/2008	103	BUBO	Cariboo	0
Williams Lake	12/07/2008	104	BUBO	Cariboo	0
Williams Lake	12/07/2008	105	BUBO	Cariboo	0
Williams Lake	12/07/2008	106	BUBO	Cariboo	0
Williams Lake	12/07/2008	107	BUBO	Cariboo	0
Williams Lake	12/07/2008	108	BUBO	Cariboo	0
Williams Lake	12/07/2008	109	BUBO	Cariboo	0
Williams Lake	12/07/2008	110	BUBO	Cariboo	0
Williams Lake	12/07/2008	111	BUBO	Cariboo	0
Williams Lake	12/07/2008	112	BUBO	Cariboo	0
Williams Lake	12/07/2008	113	BUBO	Cariboo	0
Williams Lake	12/07/2008	114	BUBO	Cariboo	0
Williams Lake	12/07/2008	115	BUBO	Cariboo	0
Williams Lake	12/07/2008	116	BUBO	Cariboo	0
Williams Lake	12/07/2008	117	BUBO	Cariboo	0
Williams Lake	12/07/2008	118	BUBO	Cariboo	0
Williams Lake	12/07/2008	119	BUBO	Cariboo	0
Williams Lake	12/07/2008	120	BUBO	Cariboo	0
Williams Lake	12/07/2008	121	BUBO	Cariboo	0
Williams Lake	12/07/2008	122	BUBO	Cariboo	0
Williams Lake	12/07/2008	123	BUBO	Cariboo	0
Williams Lake	12/07/2008	124	BUBO	Cariboo	0
Williams Lake	12/07/2008	125	BUBO	Cariboo	0
Williams Lake	12/07/2008	126	BUBO	Cariboo	0
Williams Lake	12/07/2008	127	BUBO	Cariboo	0
Williams Lake	12/07/2008	128	BUBO	Cariboo	0
Williams Lake	12/07/2008	129	BUBO	Cariboo	0
Williams Lake	12/07/2008	130	BUBO	Cariboo	0
Williams Lake	12/07/2008	131	BUBO	Cariboo	0
Williams Lake	12/07/2008	132	BUBO	Cariboo	0
Kamloops	19/07/2008	133	SPIN	Thompson	1
Kamloops	19/07/2008	134	SPIN	Thompson	1
Kamloops	19/07/2008	135	SPIN	Thompson	0
Kamloops	19/07/2008	136	SPIN	Thompson	0
Kamloops	19/07/2008	137	SPIN	Thompson	0
Kamloops	19/07/2008	138	SPIN	Thompson	0
Kamloops	19/07/2008	139	SPIN	Thompson	0
Kamloops	19/07/2008	140	SPIN	Thompson	0
Kamloops	19/07/2008	141	SPIN	Thompson	0

Kamloops	19/07/2008	142	SPIN	Thompson	0
Kamloops	19/07/2008	143	SPIN	Thompson	1
Kamloops	19/07/2008	144	SPIN	Thompson	0
Kamloops	19/07/2008	145	SPIN	Thompson	0
Kamloops	19/07/2008	146	SPIN	Thompson	1
Kamloops	19/07/2008	147	SPIN	Thompson	0
Kamloops	19/07/2008	148	SPIN	Thompson	0
Kamloops	19/07/2008	149	SPIN	Thompson	0
Kamloops	19/07/2008	150	SPIN	Thompson	0
Kamloops	19/07/2008	151	SPIN	Thompson	0
Kamloops	19/07/2008	152	BUBO	Thompson	0
Kamloops	19/07/2008	153	BUBO	Thompson	0
Kamloops	19/07/2008	154	BUBO	Thompson	0
Kamloops	19/07/2008	155	BUBO	Thompson	0
Kamloops	19/07/2008	156	BUBO	Thompson	0
Kamloops	19/07/2008	157	BUBO	Thompson	0
Kamloops	19/07/2008	158	BUBO	Thompson	0
Kamloops	19/07/2008	159	BUBO	Thompson	0
Kamloops	19/07/2008	160	BUBO	Thompson	0
Kamloops	19/07/2008	161	BUBO	Thompson	0
Kamloops	19/07/2008	162	BUBO	Thompson	0
Kamloops	19/07/2008	163	BUBO	Thompson	0
Kamloops	19/07/2008	164	BUBO	Thompson	0
Kamloops	19/07/2008	165	BUBO	Thompson	0
Kamloops	19/07/2008	166	BUBO	Thompson	0
Kamloops	19/07/2008	167	BUBO	Thompson	0
Kamloops	19/07/2008	168	BUBO	Thompson	0
Kamloops	19/07/2008	169	BUBO	Thompson	0
Kamloops	19/07/2008	170	BUBO	Thompson	0
Kamloops	19/07/2008	171	BUBO	Thompson	0
Kamloops	19/07/2008	172	BUBO	Thompson	0
Kamloops	19/07/2008	173	BUBO	Thompson	0
Kamloops	19/07/2008	174	BUBO	Thompson	0
Kamloops	19/07/2008	175	BUBO	Thompson	0
Kamloops	19/07/2008	176	BUBO	Thompson	0
Kamloops	19/07/2008	177	BUBO	Thompson	0
Kamloops	19/07/2008	178	BUBO	Thompson	0
Kamloops	19/07/2008	179	BUBO	Thompson	0
Kamloops	19/07/2008	180	BUBO	Thompson	0
Kamloops	19/07/2008	181	BUBO	Thompson	0
Harrison Hot Springs	21/07/2008	182	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	183	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	184	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	185	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	186	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	187	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	188	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	189	PSRE	Lower Mainland	0

Harrison Hot Springs	21/07/2008	190	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	191	PSRE	Lower Mainland	0
Harrison Hot Springs	21/07/2008	192	RAAU	Lower Mainland	0
Harrison Hot Springs	21/07/2008	193	RAAU	Lower Mainland	0
Harrison Hot Springs	21/07/2008	194	RAAU	Lower Mainland	0
Harrison Hot Springs	21/07/2008	195	RAAU	Lower Mainland	0
Harrison Hot Springs	21/07/2008	196	RAAU	Lower Mainland	0
Harrison Hot Springs	21/07/2008	197	RAAU	Lower Mainland	0
Harrison Hot Springs	21/07/2008	198	RAAU	Lower Mainland	0
Harrison Hot Springs	21/07/2008	199	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	200	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	201	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	202	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	203	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	204	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	205	RACL	Lower Mainland	1
Harrison Hot Springs	21/07/2008	206	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	207	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	208	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	209	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	210	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	211	RACL	Lower Mainland	1
Harrison Hot Springs	21/07/2008	212	RACL	Lower Mainland	0
Burns Lake	14/07/2008	213	RALU	Skeena	0
Burns Lake	14/07/2008	214	RALU	Skeena	0
Burns Lake	14/07/2008	215	RALU	Skeena	0
Burns Lake	14/07/2008	216	RALU	Skeena	0
Burns Lake	14/07/2008	217	RALU	Skeena	0
Burns Lake	14/07/2008	218	RALU	Skeena	0
Burns Lake	14/07/2008	219	RALU	Skeena	0
Penticton	20/07/2008	249	PSRE	Okanagan	1
Penticton	20/07/2008	250	PSRE	Okanagan	0
Penticton	20/07/2008	251	PSRE	Okanagan	0
Penticton	20/07/2008	252	PSRE	Okanagan	0
Penticton	20/07/2008	253	PSRE	Okanagan	0
Penticton	20/07/2008	254	PSRE	Okanagan	0
Penticton	20/07/2008	255	PSRE	Okanagan	0
Penticton	20/07/2008	256	PSRE	Okanagan	0
Penticton	20/07/2008	257	PSRE	Okanagan	0
Penticton	20/07/2008	258	PSRE	Okanagan	1
Penticton	20/07/2008	259	PSRE	Okanagan	0
Penticton	20/07/2008	260	PSRE	Okanagan	0
Penticton	20/07/2008	261	PSRE	Okanagan	0
Fort St John	15/07/2008	262	BUBO	Peace	0
Fort St John	15/07/2008	263	BUBO	Peace	0
Fort St John	15/07/2008	264	BUBO	Peace	0
Fort St John	15/07/2008	265	BUBO	Peace	0
Fort St John	15/07/2008	266	BUBO	Peace	0

Fort St John	15/07/2008	267	BUBO	Peace	0
Fort St John	15/07/2008	268	BUBO	Peace	0
Fort St John	15/07/2008	269	BUBO	Peace	0
Fort St John	15/07/2008	270	BUBO	Peace	0
Fort St John	15/07/2008	271	BUBO	Peace	0
Fort St John	15/07/2008	272	BUBO	Peace	1
Fort St John	15/07/2008	305	BUBO	Peace	0
Fort St John	15/07/2008	306	BUBO	Peace	0
Fort St John	15/07/2008	307	BUBO	Peace	0
Fort St John	15/07/2008	308	BUBO	Peace	0
Fort St John	15/07/2008	309	BUBO	Peace	0
Fort St John	15/07/2008	310	BUBO	Peace	0
Fort St John	15/07/2008	311	BUBO	Peace	0
Fort St John	15/07/2008	312	BUBO	Peace	0
Fort St John	15/07/2008	313	BUBO	Peace	0
Fort St John	15/07/2008	314	BUBO	Peace	0
Fort St John	15/07/2008	315	BUBO	Peace	0
Fort St John	15/07/2008	316	BUBO	Peace	0
Fort St John	15/07/2008	317	BUBO	Peace	0
Fort St John	15/07/2008	318	BUBO	Peace	0
Fort St John	15/07/2008	319	BUBO	Peace	0
Fort St John	15/07/2008	320	BUBO	Peace	0
Fort St John	15/07/2008	321	BUBO	Peace	0
Fort St John	15/07/2008	322	BUBO	Peace	0
Fort St John	15/07/2008	323	BUBO	Peace	0
Fort St John	15/07/2008	324	BUBO	Peace	0
Fort St John	15/07/2008	325	BUBO	Peace	1
Fort St John	15/07/2008	326	BUBO	Peace	0
Fort St John	15/07/2008	327	BUBO	Peace	0
Fort St John	15/07/2008	328	BUBO	Peace	0
Fort St John	15/07/2008	329	BUBO	Peace	0
Fort St John	15/07/2008	330	BUBO	Peace	0
Fort St John	15/07/2008	331	BUBO	Peace	0
Fort St John	15/07/2008	332	BUBO	Peace	0
Fort St John	15/07/2008	333	BUBO	Peace	0
Fort St John	15/07/2008	334	BUBO	Peace	0
Fort St John	15/07/2008	335	BUBO	Peace	0
Fort St John	15/07/2008	336	BUBO	Peace	0
Fort St John	15/07/2008	337	BUBO	Peace	0
Fort St John	15/07/2008	338	BUBO	Peace	0
Fort St John	15/07/2008	339	BUBO	Peace	0
Fort St John	15/07/2008	340	BUBO	Peace	0
Fort St John	15/07/2008	341	BUBO	Peace	0
Fort St John	15/07/2008	342	BUBO	Peace	0
Fort St John	15/07/2008	343	BUBO	Peace	0
Fort St John	15/07/2008	344	BUBO	Peace	0
Fort St John	15/07/2008	345	BUBO	Peace	0
Fort St John	15/07/2008	346	BUBO	Peace	1

Fort St John	15/07/2008	347	BUBO	Peace	0
Fort St John	15/07/2008	348	BUBO	Peace	0
Harrison Hot Springs	21/07/2008	350	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	351	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	352	RACL	Lower Mainland	0
Harrison Hot Springs	21/07/2008	353	RACL	Lower Mainland	0
Kamloops	18/07/2008	354	BUBO	Thompson	0
Kamloops	18/07/2008	355	BUBO	Thompson	0
Kamloops	18/07/2008	356	BUBO	Thompson	0
Kamloops	18/07/2008	357	BUBO	Thompson	0
Kamloops	18/07/2008	358	BUBO	Thompson	0
Kamloops	18/07/2008	359	BUBO	Thompson	0
Kamloops	18/07/2008	360	BUBO	Thompson	0
Horsefly	12/07/2008	378	RALU	Cariboo	1
Horsefly	12/07/2008	379	RALU	Cariboo	0
Horsefly	12/07/2008	380	RALU	Cariboo	1
Horsefly	12/07/2008	381	RALU	Cariboo	1
Horsefly	12/07/2008	382	RALU	Cariboo	1
Horsefly	12/07/2008	383	RALU	Cariboo	1
Horsefly	12/07/2008	384	RALU	Cariboo	1
Horsefly	12/07/2008	385	RALU	Cariboo	0
Horsefly	12/07/2008	386	RALU	Cariboo	0
Horsefly	12/07/2008	387	RALU	Cariboo	0
Horsefly	12/07/2008	388	RALU	Cariboo	1
Horsefly	12/07/2008	389	RALU	Cariboo	1
Horsefly	11/07/2008	391	PSRE	Cariboo	0
Horsefly	11/07/2008	392	BUBO	Cariboo	0
Horsefly	11/07/2008	393	BUBO	Cariboo	0
Horsefly	11/07/2008	394	BUBO	Cariboo	1
Horsefly	11/07/2008	395	BUBO	Cariboo	0
Horsefly	11/07/2008	396	BUBO	Cariboo	0
Horsefly	11/07/2008	397	BUBO	Cariboo	0
Horsefly	11/07/2008	398	BUBO	Cariboo	0
Horsefly	11/07/2008	399	BUBO	Cariboo	0
Horsefly	11/07/2008	400	BUBO	Cariboo	0
Horsefly	11/07/2008	401	BUBO	Cariboo	1
Horsefly	11/07/2008	402	BUBO	Cariboo	0
Horsefly	11/07/2008	403	BUBO	Cariboo	0
Horsefly	11/07/2008	404	BUBO	Cariboo	0
Horsefly	11/07/2008	405	BUBO	Cariboo	0
Horsefly	11/07/2008	406	BUBO	Cariboo	0
Fort St John	15/07/2008	429	RASY	Peace	1
Fort St John	15/07/2008	430	RASY	Peace	1
Fort St John	15/07/2008	431	RASY	Peace	1
Fort St John	15/07/2008	432	RASY	Peace	0
Fort St John	15/07/2008	433	RASY	Peace	1
Fort St John	15/07/2008	434	RASY	Peace	1
Fort St John	15/07/2008	435	RASY	Peace	1

Fort St John	15/07/2008	436	RASY	Peace	1
100 Mile House	11/07/2008	437	RALU	Cariboo	1
100 Mile House	11/07/2008	438	RALU	Cariboo	0
100 Mile House	11/07/2008	439	RALU	Cariboo	1
100 Mile House	11/07/2008	440	RALU	Cariboo	1
70 Mile House	10/07/2008	441	BUBO	Cariboo	1
70 Mile House	10/07/2008	442	BUBO	Cariboo	0
70 Mile House	10/07/2008	443	BUBO	Cariboo	0
70 Mile House	10/07/2008	444	BUBO	Cariboo	0
70 Mile House	10/07/2008	445	BUBO	Cariboo	1
100 Mile House	08/07/2008	446	BUBO	Cariboo	1
Agassiz	04/09/2008	474	RACL	Lower Mainland	0
Agassiz	04/09/2008	475	RACL	Lower Mainland	0
Agassiz	04/09/2008	476	RACL	Lower Mainland	0
Agassiz	04/09/2008	477	RACL	Lower Mainland	0
Agassiz	04/09/2008	478	RACL	Lower Mainland	0
Agassiz	04/09/2008	479	RACL	Lower Mainland	0
Agassiz	04/09/2008	480	RACL	Lower Mainland	0
Agassiz	01/04/2008	481	TAGR	Lower Mainland	0
Agassiz	01/04/2008	482	TAGR	Lower Mainland	0
Agassiz	01/04/2008	483	TAGR	Lower Mainland	0
Agassiz	01/04/2008	484	RAPR	Lower Mainland	1
Agassiz	01/04/2008	485	RAAU	Lower Mainland	0
Agassiz	01/04/2008	486	RAAU	Lower Mainland	0
Nanaimo	19/04/2008	487	TAGR	Vancouver Island	0
Nanaimo	19/04/2008	488	TAGR	Vancouver Island	0
Nanaimo	19/04/2008	489	TAGR	Vancouver Island	0
Nanaimo	19/04/2008	490	TAGR	Vancouver Island	0
Nanaimo	19/04/2008	491	TAGR	Vancouver Island	0
Nanaimo	19/04/2008	492	TAGR	Vancouver Island	0
Nanaimo	19/04/2008	493	TAGR	Vancouver Island	0
Nanaimo	19/04/2008	494	TAGR	Vancouver Island	0
Nanaimo	19/04/2008	495	RAAU	Vancouver Island	0
Agassiz	01/04/2008	496	RACL	Lower Mainland	0
Agassiz	01/04/2008	497	RACL	Lower Mainland	0
Agassiz	01/04/2008	498	RACL	Lower Mainland	0
Agassiz	01/04/2008	499	RACL	Lower Mainland	0
Agassiz	01/04/2008	500	RACL	Lower Mainland	0
Agassiz	01/04/2008	501	RACL	Lower Mainland	0
Agassiz	01/04/2008	502	RACL	Lower Mainland	0
Agassiz	01/04/2008	503	RACL	Lower Mainland	1
Agassiz	01/04/2008	504	RACL	Lower Mainland	0
Agassiz	01/04/2008	505	RACL	Lower Mainland	0
Agassiz	01/04/2008	506	RACL	Lower Mainland	0
Agassiz	01/04/2008	507	RACL	Lower Mainland	0
Agassiz	01/04/2008	508	RACL	Lower Mainland	0
Agassiz	01/04/2008	509	RACL	Lower Mainland	0
Agassiz	01/04/2008	510	RACL	Lower Mainland	0

Agassiz	01/04/2008	511	RACL	Lower Mainland	0
Agassiz	01/04/2008	512	RACL	Lower Mainland	0
Agassiz	01/04/2008	513	RACL	Lower Mainland	0
Agassiz	01/04/2008	514	RACL	Lower Mainland	0
Agassiz	01/04/2008	515	RACL	Lower Mainland	0
Agassiz	01/04/2008	516	RACL	Lower Mainland	0
Agassiz	01/04/2008	517	RACL	Lower Mainland	0
Agassiz	01/04/2008	518	RACL	Lower Mainland	0
Agassiz	01/04/2008	519	RACL	Lower Mainland	0
Agassiz	01/04/2008	520	TAGR	Lower Mainland	0
Agassiz	01/04/2008	521	TAGR	Lower Mainland	0
Agassiz	01/04/2008	522	RAPR	Lower Mainland	0
Agassiz	01/04/2008	523	RAPR	Lower Mainland	0
Agassiz	01/04/2008	524	TAGR	Lower Mainland	0
Prince George	23/06/2008	525	RALU	Cariboo	0
Prince George	23/06/2008	526	RALU	Cariboo	1
Prince George	23/06/2008	527	RALU	Cariboo	1
Prince George	23/06/2008	528	RALU	Cariboo	1
Prince George	23/06/2008	529	RALU	Cariboo	0
Prince George	23/06/2008	530	RALU	Cariboo	0
Prince George	23/06/2008	531	RALU	Cariboo	1
Prince George	23/06/2008	532	RALU	Cariboo	0
Prince George	23/06/2008	533	RALU	Cariboo	1
Prince George	23/06/2008	534	RALU	Cariboo	1
Prince George	23/06/2008	535	RALU	Cariboo	1
Prince George	23/06/2008	536	RALU	Cariboo	1
Prince George	23/06/2008	537	RALU	Cariboo	1
Prince George	23/06/2008	538	RALU	Cariboo	1
Prince George	23/06/2008	539	RALU	Cariboo	0
Prince George	23/06/2008	540	RALU	Cariboo	0
Prince George	23/06/2008	541	RALU	Cariboo	1
Prince George	23/06/2008	542	RALU	Cariboo	0
Prince George	23/06/2008	543	RALU	Cariboo	1
Prince George	23/06/2008	544	RALU	Cariboo	1
Prince George	23/06/2008	545	RALU	Cariboo	1
Prince George	23/06/2008	546	RALU	Cariboo	0
Prince George	23/06/2008	547	RALU	Cariboo	0
Prince George	23/06/2008	548	RALU	Cariboo	1
Prince George	23/06/2008	549	RALU	Cariboo	0
Prince George	23/06/2008	550	RALU	Cariboo	1
Prince George	23/06/2008	551	RALU	Cariboo	0
Prince George	23/06/2008	552	RALU	Cariboo	1
Prince George	23/06/2008	553	RALU	Cariboo	1
Prince George	23/06/2008	554	RALU	Cariboo	1
Prince George	23/06/2008	555	RALU	Cariboo	1
Prince George	23/06/2008	556	RASY	Cariboo	1
Prince George	23/06/2008	557	BUBO	Cariboo	0
Nanaimo	30/06/2008	558	RACA	Vancouver Island	0

70 Mile House	12/06/2008	559	SPIN	Cariboo	0
70 Mile House	14/05/2008	560	SPIN	Cariboo	0
70 Mile House	14/05/2008	561	SPIN	Cariboo	0
70 Mile House	03/07/2008	562	SPIN	Cariboo	0
70 Mile House	03/05/2008	563	SPIN	Cariboo	0
70 Mile House	17/06/2008	565	SPIN	Cariboo	0
Whistler	10/08/2008	566	BUBO	Lower Mainland	0
Whistler	10/08/2008	567	BUBO	Lower Mainland	0
Whistler	10/08/2008	568	BUBO	Lower Mainland	0
Whistler	10/08/2008	569	BUBO	Lower Mainland	0
Whistler	10/08/2008	570	BUBO	Lower Mainland	0
Whistler	10/08/2008	571	BUBO	Lower Mainland	0
Whistler	10/08/2008	572	BUBO	Lower Mainland	0
Whistler	10/08/2008	573	BUBO	Lower Mainland	0
Whistler	10/08/2008	574	BUBO	Lower Mainland	0
Whistler	10/08/2008	575	BUBO	Lower Mainland	0
Whistler	10/08/2008	576	BUBO	Lower Mainland	0
Whistler	10/08/2008	577	BUBO	Lower Mainland	0
Whistler	10/08/2008	578	BUBO	Lower Mainland	0
Whistler	10/08/2008	579	BUBO	Lower Mainland	0
Whistler	10/08/2008	580	BUBO	Lower Mainland	0
Whistler	10/08/2008	581	BUBO	Lower Mainland	0
Whistler	10/08/2008	582	BUBO	Lower Mainland	0
Whistler	10/08/2008	583	BUBO	Lower Mainland	0
Whistler	10/08/2008	584	BUBO	Lower Mainland	0
Whistler	10/08/2008	585	BUBO	Lower Mainland	0
Riske Creek	27/05/2008	586	BUBO	Cariboo	0
70 Mile House	20/05/2008	587	BUBO	Cariboo	0
70 Mile House	14/05/2008	588	BUBO	Cariboo	0
70 Mile House	08/05/2008	589	BUBO	Cariboo	0
70 Mile House	26/05/2008	590	BUBO	Cariboo	1
70 Mile House	18/06/2008	591	BUBO	Cariboo	1
70 Mile House	30/07/2008	592	RALU	Cariboo	0
Whistler	10/08/2008	593	BUBO	Lower Mainland	0
Whistler	10/08/2008	594	BUBO	Lower Mainland	0
Whistler	10/08/2008	595	BUBO	Lower Mainland	0
Whistler	10/08/2008	596	BUBO	Lower Mainland	0
Whistler	10/08/2008	597	BUBO	Lower Mainland	0
Whistler	10/08/2008	598	BUBO	Lower Mainland	0
Whistler	10/08/2008	599	BUBO	Lower Mainland	0
Whistler	10/08/2008	600	BUBO	Lower Mainland	0
Whistler	01/10/2008	601	RAAU	Lower Mainland	0
Whistler	01/10/2008	602	RAAU	Lower Mainland	0
Whistler	01/10/2008	603	RAAU	Lower Mainland	0
Whistler	01/10/2008	604	RAAU	Lower Mainland	0
Whistler	01/10/2008	605	RAAU	Lower Mainland	0
Whistler	01/10/2008	606	RAAU	Lower Mainland	0
Whistler	01/10/2008	607	RAAU	Lower Mainland	0

Whistler	01/10/2008	608	RAAU	Lower Mainland	0
Whistler	01/10/2008	609	RAAU	Lower Mainland	0
Chilliwack	01/04/2008	610	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	611	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	612	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	613	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	614	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	615	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	616	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	617	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	618	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	619	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	620	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	621	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	622	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	623	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	624	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	625	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	626	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	627	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	628	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	629	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	630	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	631	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	632	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	633	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	634	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	635	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	636	BUBO	Lower Mainland	0
Chilliwack	01/04/2008	637	BUBO	Lower Mainland	0
Tumbler Ridge	09/07/2008	648	RALU	Peace	1
Tumbler Ridge	09/07/2008	649	RASY	Peace	1
Tumbler Ridge	09/07/2008	650	RALU	Peace	0
Tumbler Ridge	09/07/2008	651	RASY	Peace	0
Tumbler Ridge	09/07/2008	652	RASY	Peace	1
Tumbler Ridge	09/07/2008	653	RASY	Peace	1
Tumbler Ridge	09/07/2008	654	BUBO	Peace	0
Tumbler Ridge	09/07/2008	655	RASY	Peace	1
Tumbler Ridge	09/07/2008	656	RASY	Peace	0
Tumbler Ridge	09/07/2008	657	RALU	Peace	0
Tumbler Ridge	09/07/2008	658	RASY	Peace	1
Tumbler Ridge	09/07/2008	659	RALU	Peace	0
Tumbler Ridge	09/07/2008	660	BUBO	Peace	0
Chilliwack	04/08/2008	672	DITE	Lower Mainland	0
Chilliwack	04/08/2008	673	DITE	Lower Mainland	0
Chilliwack	04/08/2008	674	DITE	Lower Mainland	0
Chilliwack	04/08/2008	675	DITE	Lower Mainland	0
Chilliwack	04/08/2008	676	DITE	Lower Mainland	0

Chilliwack	04/08/2008	677	DITE	Lower Mainland	0
Chilliwack	06/08/2008	678	DITE	Lower Mainland	0
Chilliwack	06/08/2008	679	DITE	Lower Mainland	0
Chilliwack	06/08/2008	681	DITE	Lower Mainland	0
Chilliwack	23/07/2008	682	DITE	Lower Mainland	0
Chilliwack	23/07/2008	683	DITE	Lower Mainland	0
Chilliwack	23/07/2008	684	DITE	Lower Mainland	0
Chilliwack	23/07/2008	685	DITE	Lower Mainland	0
Chilliwack	23/07/2008	686	DITE	Lower Mainland	0
Chilliwack	23/07/2008	687	DITE	Lower Mainland	0
Chilliwack	13/08/2008	689	DITE	Lower Mainland	0
Chilliwack	21/07/2008	690	DITE	Lower Mainland	0
Chilliwack	21/07/2008	691	DITE	Lower Mainland	0
Chilliwack	21/07/2008	692	DITE	Lower Mainland	0
Chilliwack	21/07/2008	693	DITE	Lower Mainland	0
Chilliwack	28/07/2008	694	DITE	Lower Mainland	0
Chilliwack	28/07/2008	695	DITE	Lower Mainland	0
Chilliwack	28/07/2008	696	DITE	Lower Mainland	0
Chilliwack	28/07/2008	697	DITE	Lower Mainland	0
Chilliwack	28/07/2008	699	DITE	Lower Mainland	0
Chilliwack	28/07/2008	700	DITE	Lower Mainland	0
Chilliwack	28/07/2008	701	DITE	Lower Mainland	0
Prince George	08/09/2008	702	BUBO	Omineca	0
Prince George	08/09/2008	703	BUBO	Omineca	0
Prince George	08/09/2008	704	BUBO	Omineca	0
Prince George	08/09/2008	705	BUBO	Omineca	0
Prince George	08/09/2008	706	BUBO	Omineca	0
Prince George	08/09/2008	707	BUBO	Omineca	0
Prince George	08/09/2008	708	BUBO	Omineca	0
Prince George	08/09/2008	709	BUBO	Omineca	0
Prince George	08/09/2008	710	BUBO	Omineca	0
Prince George	08/09/2008	711	BUBO	Omineca	0
Ucluelet	06/06/2008	751	AMGR	Vancouver Island	0
Ucluelet	25/09/2008	752	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	753	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	754	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	755	RAAU	Vancouver Island	1
Ucluelet	25/09/2008	756	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	757	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	758	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	759	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	760	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	761	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	762	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	763	RAAU	Vancouver Island	0
Ucluelet	25/09/2008	764	RAAU	Vancouver Island	0
Ucluelet	29/07/2008	765	PSRE	Vancouver Island	0
Ucluelet	29/07/2008	766	PSRE	Vancouver Island	0

Ucluelet	29/07/2008	767	RAAU	Vancouver Island	0
Ucluelet	29/07/2008	768	RAAU	Vancouver Island	0
Ucluelet	29/07/2008	769	RAAU	Vancouver Island	0
Ucluelet	29/07/2008	770	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	771	TAGR	Vancouver Island	0
Ucluelet	17/10/2008	772	TAGR	Vancouver Island	0
Ucluelet	17/10/2008	773	TAGR	Vancouver Island	1
Ucluelet	17/10/2008	774	TAGR	Vancouver Island	1
Ucluelet	17/10/2008	775	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	776	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	777	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	778	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	779	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	780	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	781	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	782	PSRE	Vancouver Island	0
Ucluelet	17/10/2008	783	RAAU	Vancouver Island	0
Ucluelet	17/10/2008	784	RAAU	Vancouver Island	1
Ucluelet	01/11/2008	785	RAAU	Vancouver Island	0
Ucluelet	01/11/2008	786	RAAU	Vancouver Island	0
Ucluelet	01/11/2008	787	RAAU	Vancouver Island	0
Ucluelet	01/11/2008	788	RAAU	Vancouver Island	0
Ucluelet	01/11/2008	789	RAAU	Vancouver Island	0
Ucluelet	01/11/2008	790	RAAU	Vancouver Island	0
Ucluelet	01/11/2008	791	RAAU	Vancouver Island	0
Ucluelet	01/11/2008	792	TAGR	Vancouver Island	0
Ucluelet	01/11/2008	793	TAGR	Vancouver Island	0
Surrey	02/10/2008	816	RACL	Lower Mainland	1
Surrey	02/10/2008	817	RACL	Lower Mainland	0
Surrey	02/10/2008	818	RACL	Lower Mainland	1
Surrey	02/10/2008	819	RACL	Lower Mainland	0
Surrey	02/10/2008	820	RACL	Lower Mainland	1
Nanaimo	14/04/2008	860	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	861	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	862	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	863	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	864	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	865	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	866	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	867	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	868	BUBO	Vancouver Island	0
Nanaimo	14/04/2008	869	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	870	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	871	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	872	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	873	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	874	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	875	BUBO	Vancouver Island	0

Nanaimo	15/04/2008	876	BUBO	Vancouver Island	1
Nanaimo	15/04/2008	877	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	878	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	879	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	880	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	881	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	882	BUBO	Vancouver Island	0
Nanaimo	15/04/2008	883	BUBO	Vancouver Island	0
Agassiz	25/09/2008	972	RAPR	Lower Mainland	0
Agassiz	25/09/2008	973	RAAU	Lower Mainland	0
Agassiz	25/09/2008	974	RAPR	Lower Mainland	0
Agassiz	25/09/2008	975	RACL	Lower Mainland	0
Agassiz	25/09/2008	976	RACL	Lower Mainland	0
Agassiz	25/09/2008	977	RACL	Lower Mainland	0
Prince George	04/06/2008	1009	BUBO	Omineca	0
Prince George	04/06/2008	1010	BUBO	Omineca	0
Prince George	04/06/2008	1011	BUBO	Omineca	0
Prince George	04/06/2008	1012	BUBO	Omineca	0
Prince George	05/06/2008	1013	RALU	Omineca	1
Prince George	05/06/2008	1014	RALU	Omineca	1
Prince George	05/06/2008	1015	RALU	Omineca	0
Prince George	05/06/2008	1016	RALU	Omineca	1
Prince George	05/06/2008	1017	RALU	Omineca	0
Prince George	05/06/2008	1018	RALU	Omineca	0
Prince George	05/06/2008	1019	RALU	Omineca	1
Prince George	05/06/2008	1020	RALU	Omineca	1
Prince George	05/06/2008	1021	RALU	Omineca	0
Prince George	05/06/2008	1022	RALU	Omineca	1
Prince George	03/07/2008	1023	BUBO	Omineca	0
Prince George	03/07/2008	1024	BUBO	Omineca	0
Prince George	03/07/2008	1025	BUBO	Omineca	0
Prince George	03/07/2008	1026	BUBO	Omineca	1
Prince George	03/07/2008	1027	BUBO	Omineca	0
Prince George	03/07/2008	1028	BUBO	Omineca	1
Prince George	03/07/2008	1029	RALU	Omineca	0
Prince George	15/07/2008	1030	BUBO	Omineca	1
Prince George	15/07/2008	1031	BUBO	Omineca	1
Prince George	15/07/2008	1032	BUBO	Omineca	1
Prince George	15/07/2008	1033	BUBO	Omineca	1
Prince George	15/07/2008	1034	BUBO	Omineca	1
Prince George	15/07/2008	1035	BUBO	Omineca	1
Prince George	22/07/2008	1036	RASY	Omineca	0
Prince George	22/07/2008	1037	BUBO	Omineca	0
Prince George	22/07/2008	1038	RALU	Omineca	1
Prince George	22/07/2008	1039	BUBO	Omineca	1
Prince George	05/08/2008	1040	RASY	Omineca	0
Prince George	05/08/2008	1041	RASY	Omineca	1
Prince George	05/08/2008	1042	BUBO	Omineca	1

Prince George	13/08/2008	1043	BUBO	Omineca	0
Prince George	13/08/2008	1044	BUBO	Omineca	1
Prince George	13/08/2008	1045	BUBO	Omineca	1
Prince George	13/08/2008	1046	BUBO	Omineca	0
Surrey	15/10/2008	1047	RACL	Lower Mainland	0
Surrey	15/10/2008	1048	RACL	Lower Mainland	0
Vancouver	04/02/2009	1049	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1050	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1051	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1052	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1053	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1054	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1055	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1056	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1057	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1058	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1059	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1060	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1061	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1062	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1063	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1064	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1065	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1066	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1067	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1068	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1069	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1070	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1071	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1072	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1073	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1074	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1075	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1076	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1077	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1078	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1079	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1080	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1081	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1082	RAPR	Lower Mainland	0
Vancouver	04/02/2009	1083	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1108	RAAU	Lower Mainland	0
Agassiz	25/03/2009	1109	RAAU	Lower Mainland	0
Agassiz	25/03/2009	1110	RAAU	Lower Mainland	0
Agassiz	25/03/2009	1111	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1112	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1113	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1114	TAGR	Lower Mainland	0

Agassiz	25/03/2009	1115	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1116	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1117	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1118	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1119	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1120	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1121	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1122	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1123	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1124	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1125	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1126	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1127	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1128	TAGR	Lower Mainland	0
Agassiz	25/03/2009	1129	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1130	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1131	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1132	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1133	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1134	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1135	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1136	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1137	RAPR	Lower Mainland	1
Agassiz	25/03/2009	1138	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1139	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1140	RAPR	Lower Mainland	0
Agassiz	25/03/2009	1141	RAAU	Lower Mainland	0
Agassiz	25/03/2009	1142	RAAU	Lower Mainland	0
Agassiz	25/03/2009	1143	RAAU	Lower Mainland	0
Agassiz	25/03/2009	1144	RAAU	Lower Mainland	0
Aggassiz	11/08/2009	1193	RACL	Lower Mainland	0
Aggassiz	11/08/2009	1194	RACL	Lower Mainland	0
Aggassiz	11/08/2009	1195	RACL	Lower Mainland	0
Chilliwack	30/06/2009	1196	ASTR	Lower Mainland	0
Chilliwack	30/06/2009	1197	DITE	Lower Mainland	0
Chilliwack	02/07/2009	1200	DITE	Lower Mainland	0
Chilliwack	07/07/2009	1202	DITE	Lower Mainland	0
Chilliwack	07/07/2009	1203	TAGR	Lower Mainland	0
Chilliwack	09/07/2009	1204	RAAU	Lower Mainland	0
Chilliwack	10/07/2009	1208	RAAU	Lower Mainland	0
Chilliwack	13/07/2009	1209	RAAU	Lower Mainland	0
Chilliwack	14/07/2009	1211	DITE	Lower Mainland	0
Chilliwack	15/07/2009	1212	DITE	Lower Mainland	0
Chilliwack	15/07/2009	1213	ASMO	Lower Mainland	0
Chilliwack	16/07/2009	1214	DITE	Lower Mainland	0
Chilliwack	16/07/2009	1215	DITE	Lower Mainland	0
Chilliwack	20/07/2009	1216	BUBO	Lower Mainland	0
Chilliwack	29/07/2009	1217	ASTR	Lower Mainland	0

Chilliwack	31/07/2009	1218	RAAU	Lower Mainland	1
Chilliwack	04/08/2009	1219	DITE	Lower Mainland	0
Chilliwack	08/08/2009	1220	ASTR	Lower Mainland	0
Chilliwack	10/08/2009	1221	DITE	Lower Mainland	0
Chilliwack	10/08/2009	1222	DITE	Lower Mainland	0
Chilliwack	10/08/2009	1223	DITE	Lower Mainland	0
Cranbrook	19/08/2009	1232	ASMO	Kootenay	0
Cranbrook	19/08/2009	1233	ASMO	Kootenay	0
Cranbrook	19/08/2009	1234	ASMO	Kootenay	0
Cranbrook	19/08/2009	1235	ASMO	Kootenay	0
Cranbrook	19/08/2009	1236	ASMO	Kootenay	0
Cranbrook	19/08/2009	1237	ASMO	Kootenay	0
Cranbrook	19/08/2009	1238	ASMO	Kootenay	0
Cranbrook	19/08/2009	1239	ASMO	Kootenay	0
Cranbrook	19/08/2009	1240	ASMO	Kootenay	0
Cranbrook	19/08/2009	1241	ASMO	Kootenay	0
Cranbrook	19/08/2009	1242	ASMO	Kootenay	0
Cranbrook	19/08/2009	1243	ASMO	Kootenay	0
Aldergrove	24/04/2009	1259	TAGR	Lower Mainland	0
Aldergrove	24/04/2009	1260	PSRE	Lower Mainland	0
Aldergrove	24/04/2009	1276	PSRE	Lower Mainland	1
Aldergrove	24/04/2009	1277	PSRE	Lower Mainland	1
Aldergrove	24/04/2009	1278	PSRE	Lower Mainland	0
Aldergrove	24/04/2009	1279	TAGR	Lower Mainland	0
Aldergrove	24/04/2009	1280	PSRE	Lower Mainland	0
Aldergrove	24/04/2009	1281	PSRE	Lower Mainland	0
Aldergrove	24/04/2009	1282	PSRE	Lower Mainland	0
Aldergrove	24/04/2009	1283	PSRE	Lower Mainland	0
Aldergrove	24/04/2009	1284	PSRE	Lower Mainland	0
Aldergrove	24/04/2009	1285	PSRE	Lower Mainland	0
Cranbrook	24/04/2009	1286	RAAU	Kootenay	0
Cranbrook	24/04/2009	1287	TAGR	Kootenay	0
Cranbrook	24/04/2009	1288	TAGR	Kootenay	0
Cranbrook	24/04/2009	1289	RAAU	Kootenay	0
Cranbrook	24/04/2009	1290	TAGR	Kootenay	0
Cranbrook	24/04/2009	1291	TAGR	Kootenay	0
Cranbrook	24/04/2009	1292	TAGR	Kootenay	0
Cranbrook	24/04/2009	1293	RAAU	Kootenay	0
Cranbrook	24/04/2009	1294	RAAU	Kootenay	0
Cranbrook	24/04/2009	1295	RAPR	Kootenay	0
Penticton	20/07/2008	220	PSRE	Okanagan	0
Penticton	20/07/2008	222	PSRE	Okanagan	0
Penticton	20/07/2008	223	PSRE	Okanagan	0
Penticton	20/07/2008	224	PSRE	Okanagan	0
Penticton	20/07/2008	225	PSRE	Okanagan	0
Penticton	20/07/2008	226	PSRE	Okanagan	0
Penticton	20/07/2008	227	PSRE	Okanagan	0
Penticton	20/07/2008	228	PSRE	Okanagan	0

Penticton	20/07/2008	229	PSRE	Okanagan	0
Penticton	20/07/2008	230	PSRE	Okanagan	0
Penticton	20/07/2008	231	PSRE	Okanagan	0
Penticton	20/07/2008	232	PSRE	Okanagan	0
Penticton	20/07/2008	233	PSRE	Okanagan	0
Penticton	20/07/2008	234	PSRE	Okanagan	0
Penticton	20/07/2008	235	PSRE	Okanagan	0
Penticton	20/07/2008	236	PSRE	Okanagan	0
Penticton	20/07/2008	237	PSRE	Okanagan	0
Penticton	20/07/2008	238	PSRE	Okanagan	0
Penticton	20/07/2008	239	PSRE	Okanagan	0
Penticton	20/07/2008	240	PSRE	Okanagan	0
Penticton	20/07/2008	241	PSRE	Okanagan	0
Penticton	20/07/2008	242	PSRE	Okanagan	0
Penticton	20/07/2008	243	PSRE	Okanagan	0
Penticton	20/07/2008	244	PSRE	Okanagan	0
Penticton	20/07/2008	245	PSRE	Okanagan	0
Penticton	20/07/2008	246	PSRE	Okanagan	0
Penticton	20/07/2008	247	PSRE	Okanagan	0
Penticton	20/07/2008	248	PSRE	Okanagan	0
Penticton	20/07/2008	349	PSRE	Okanagan	0
Armstrong	18/07/2008	361	BUBO	Thompson	0
Armstrong	18/07/2008	362	BUBO	Thompson	0
Armstrong	18/07/2008	363	BUBO	Thompson	0
Armstrong	18/07/2008	364	BUBO	Thompson	0
Armstrong	18/07/2008	365	BUBO	Thompson	0
Armstrong	18/07/2008	366	BUBO	Thompson	0
Armstrong	18/07/2008	367	BUBO	Thompson	0
Armstrong	18/07/2008	368	BUBO	Thompson	0
Armstrong	18/07/2008	369	BUBO	Thompson	0
Armstrong	18/07/2008	370	BUBO	Thompson	0
Armstrong	18/07/2008	371	PSRE	Thompson	0
Armstrong	18/07/2008	372	PSRE	Thompson	0
Armstrong	18/07/2008	373	PSRE	Thompson	0
Armstrong	18/07/2008	374	PSRE	Thompson	0
Armstrong	18/07/2008	375	PSRE	Thompson	0
Armstrong	18/07/2008	376	PSRE	Thompson	0
Armstrong	18/07/2008	377	PSRE	Thompson	0
Penticton	20/07/2008	419	PSRE	Okanagan	0
Penticton	20/07/2008	420	PSRE	Okanagan	0
Penticton	20/07/2008	421	PSRE	Okanagan	0
Penticton	20/07/2008	422	PSRE	Okanagan	0
Penticton	20/07/2008	423	PSRE	Okanagan	0
Penticton	20/07/2008	424	PSRE	Okanagan	0
Penticton	20/07/2008	425	PSRE	Okanagan	0
Penticton	20/07/2008	426	PSRE	Okanagan	0
Penticton	20/07/2008	427	PSRE	Okanagan	0
Penticton	20/07/2008	428	PSRE	Okanagan	0

NA	16/05/2008	449	SPIN	Okanagan	0
NA	16/05/2008	450	SPIN	Okanagan	0
NA	16/05/2008	451	SPIN	Okanagan	0
NA	16/05/2008	452	SPIN	Okanagan	0
NA	16/05/2008	453	SPIN	Okanagan	0
NA	16/05/2008	454	SPIN	Okanagan	0
NA	16/05/2008	455	SPIN	Okanagan	0
NA	16/05/2008	456	SPIN	Okanagan	0
NA	16/05/2008	457	SPIN	Okanagan	0
NA	16/05/2008	458	SPIN	Okanagan	0
Armstrong	12/05/2008	459	BUBO	Okanagan	0
Armstrong	12/05/2008	460	BUBO	Okanagan	0
Armstrong	12/05/2008	461	BUBO	Okanagan	1
Armstrong	12/05/2008	462	BUBO	Okanagan	1
Armstrong	12/05/2008	463	BUBO	Okanagan	0
Armstrong	12/05/2008	464	BUBO	Okanagan	0
Armstrong	11/04/2008	465	BUBO	Okanagan	0
Armstrong	11/04/2008	466	BUBO	Okanagan	0
Armstrong	11/04/2008	467	BUBO	Okanagan	0
Armstrong	11/04/2008	468	BUBO	Okanagan	0
Armstrong	11/04/2008	469	BUBO	Okanagan	0
Armstrong	11/04/2008	470	SPIN	Okanagan	0
Armstrong	11/04/2008	471	SPIN	Okanagan	0
Armstrong	11/04/2008	472	SPIN	Okanagan	0
Armstrong	11/04/2008	473	BUBO	Okanagan	0
Fernie	06/08/2008	884	BUBO	Kootenay	0
Fernie	06/08/2008	885	BUBO	Kootenay	0
Fernie	06/08/2008	886	BUBO	Kootenay	0
Fernie	06/08/2008	887	BUBO	Kootenay	0
Fernie	07/08/2008	888	RALU	Kootenay	0
Fernie	07/08/2008	889	RALU	Kootenay	0
Fernie	08/08/2008	890	RALU	Kootenay	0
Fernie	08/08/2008	891	RALU	Kootenay	0
Fernie	08/08/2008	892	RALU	Kootenay	0
Fernie	08/08/2008	893	RALU	Kootenay	0
Fernie	08/08/2008	894	RALU	Kootenay	0
Fernie	10/08/2008	895	RALU	Kootenay	0
Fernie	10/08/2008	896	RALU	Kootenay	0
Fernie	10/08/2008	897	RALU	Kootenay	0
Fernie	10/08/2008	898	RALU	Kootenay	0
Fernie	11/08/2008	899	RALU	Kootenay	0
Fernie	09/08/2008	900	BUBO	Kootenay	1
Golden	26/08/2008	901	RALU	Kootenay	1
Golden	26/08/2008	902	RALU	Kootenay	0
Golden	27/08/2008	903	RALU	Kootenay	0
Golden	27/08/2008	904	RALU	Kootenay	0
NA	14/05/2008	905	BUBO	Kootenay	0
NA	14/05/2008	906	BUBO	Kootenay	0

NA	14/05/2008	907	BUBO	Kootenay	0
Revelstoke	15/05/2008	908	BUBO	Kootenay	1
Revelstoke	15/05/2008	909	BUBO	Kootenay	1
Revelstoke	15/05/2008	910	BUBO	Kootenay	1
Revelstoke	15/05/2008	911	BUBO	Kootenay	1
Revelstoke	15/05/2008	912	BUBO	Kootenay	0
Revelstoke	15/05/2008	913	BUBO	Kootenay	0
Revelstoke	16/05/2008	914	BUBO	Kootenay	0
Revelstoke	16/05/2008	915	BUBO	Kootenay	0
Revelstoke	16/05/2008	916	BUBO	Kootenay	0
Revelstoke	16/05/2008	917	BUBO	Kootenay	1
Revelstoke	16/05/2008	918	BUBO	Kootenay	0
Revelstoke	16/05/2008	919	BUBO	Kootenay	0
Revelstoke	16/05/2008	920	BUBO	Kootenay	0
Revelstoke	16/05/2008	921	BUBO	Kootenay	0
Revelstoke	16/05/2008	922	BUBO	Kootenay	0
Revelstoke	16/05/2008	923	BUBO	Kootenay	0
Revelstoke	16/05/2008	924	BUBO	Kootenay	0
Revelstoke	16/05/2008	925	BUBO	Kootenay	1
Revelstoke	16/05/2008	926	BUBO	Kootenay	0
Revelstoke	16/05/2008	927	BUBO	Kootenay	0
Revelstoke	16/05/2008	928	BUBO	Kootenay	1
Revelstoke	25/06/2008	929	BUBO	Kootenay	0
Revelstoke	25/06/2008	930	BUBO	Kootenay	0
Revelstoke	25/06/2008	931	BUBO	Kootenay	0
Revelstoke	25/06/2008	932	BUBO	Kootenay	1
Revelstoke	19/09/2008	933	BUBO	Kootenay	0
NA	16/06/2008	934	BUBO	Omineca	1
NA	17/06/2008	935	BUBO	Omineca	0
NA	17/06/2008	936	BUBO	Omineca	0
NA	17/06/2008	937	BUBO	Omineca	0
NA	17/06/2008	938	BUBO	Omineca	0
NA	17/06/2008	939	BUBO	Omineca	0
NA	17/06/2008	940	BUBO	Omineca	0
NA	17/06/2008	941	BUBO	Omineca	0
NA	16/09/2008	942	BUBO	Omineca	0
NA	16/09/2008	943	BUBO	Omineca	0
NA	18/09/2008	944	BUBO	Peace	0
NA	18/09/2008	945	BUBO	Peace	1
NA	16/06/2008	946	RALU	Omineca	1
NA	16/06/2008	947	RALU	Omineca	0
NA	17/06/2008	948	RALU	Omineca	0
NA	17/06/2008	949	RALU	Omineca	0
NA	17/06/2008	950	RALU	Omineca	1
NA	17/06/2008	951	RALU	Omineca	1
NA	21/06/2008	952	RALU	Peace	1
NA	29/06/2008	953	RALU	Peace	1
NA	29/06/2008	954	RALU	Peace	1

NA	29/06/2008	955	RALU	Peace	0
NA	01/07/2008	956	RALU	Omineca	0
NA	01/07/2008	957	RALU	Omineca	0
NA	18/07/2008	958	RALU	Omineca	0
NA	18/07/2008	959	RALU	Omineca	1
NA	16/09/2008	960	RALU	Omineca	0
NA	18/09/2008	961	RALU	Peace	0
NA	18/09/2008	962	RALU	Peace	0
NA	18/09/2008	963	RALU	Peace	0
NA	18/09/2008	964	RALU	Peace	1
Revelstoke	16/05/2008	965	RALU	Kootenay	1
NA	20/09/2008	966	RALU	Kootenay	0
Revelstoke	16/05/2008	967	PSRE	Kootenay	0
Revelstoke	16/05/2008	968	PSRE	Kootenay	0
Revelstoke	19/09/2008	969	PSRE	Kootenay	1
Tetejaune	25/06/2008	978	BUBO	Omineca	0
Moyie	06/08/2009	1159	ASMO	Kootenay	0
Moyie	06/08/2009	1160	ASMO	Kootenay	0
Moyie	06/08/2009	1161	ASMO	Kootenay	0
Moyie	07/08/2009	1162	RALU	Kootenay	0
Moyie	07/08/2009	1163	ASMO	Kootenay	0
Moyie	07/08/2009	1164	RALU	Kootenay	0
Moyie	07/08/2009	1165	ASMO	Kootenay	0
Moyie	07/08/2009	1166	ASMO	Kootenay	0
Moyie	08/08/2009	1167	ASMO	Kootenay	0
Moyie	08/08/2009	1168	ASMO	Kootenay	0
Moyie	08/08/2009	1169	ASMO	Kootenay	0
Moyie	08/08/2009	1170	ASMO	Kootenay	0
Moyie	08/08/2009	1171	ASMO	Kootenay	0
Moyie	08/08/2009	1172	ASMO	Kootenay	0
Moyie	08/08/2009	1173	ASMO	Kootenay	0
Moyie	08/08/2009	1174	ASMO	Kootenay	0
Moyie	08/08/2009	1175	ASMO	Kootenay	0
Moyie	08/08/2009	1176	ASMO	Kootenay	0
Moyie	08/08/2009	1177	ASMO	Kootenay	0
Moyie	09/08/2009	1178	ASMO	Kootenay	0
Moyie	09/08/2009	1179	ASMO	Kootenay	0
Moyie	09/08/2009	1180	ASMO	Kootenay	0
Moyie	10/08/2009	1182	RALU	Kootenay	0
Moyie	10/08/2009	1183	RALU	Kootenay	0
Moyie	10/08/2009	1184	ASMO	Kootenay	0
Moyie	10/08/2009	1185	ASMO	Kootenay	0
Moyie	10/08/2009	1186	ASMO	Kootenay	0
Moyie	11/08/2009	1187	ASMO	Kootenay	0
Moyie	11/08/2009	1188	ASMO	Kootenay	0
Moyie	11/08/2009	1189	ASMO	Kootenay	0
Moyie	11/08/2009	1190	ASMO	Kootenay	0
Moyie	11/08/2009	1191	ASMO	Kootenay	0

Moyie	13/08/2009	1192	RALU	Kootenay	0
Cranbrook	18/08/2009	1226	ASMO	Kootenay	0
Cranbrook	18/08/2009	1227	ASMO	Kootenay	0
Cranbrook	18/08/2009	1228	ASMO	Kootenay	0
Cranbrook	18/08/2009	1229	ASMO	Kootenay	0
Cranbrook	18/08/2009	1230	ASMO	Kootenay	0
Cranbrook	18/08/2009	1231	ASMO	Kootenay	0
Cranbrook	19/09/2009	1244	ASMO	Kootenay	0
Cranbrook	19/09/2009	1245	ASMO	Kootenay	0
Cranbrook	19/09/2009	1246	ASMO	Kootenay	0
Cranbrook	19/09/2009	1247	ASMO	Kootenay	0
Cranbrook	20/09/2009	1248	ASMO	Kootenay	0
Cranbrook	20/09/2009	1249	ASMO	Kootenay	0
Cranbrook	20/09/2009	1250	ASMO	Kootenay	0
Cranbrook	22/08/2009	1251	ASMO	Kootenay	0
Cranbrook	22/08/2009	1252	ASMO	Kootenay	0
Cranbrook	08/08/2009	1253	ASMO	Kootenay	0
Cranbrook	08/08/2009	1254	ASMO	Kootenay	0
Cranbrook	08/08/2009	1255	ASMO	Kootenay	0
Cranbrook	08/08/2009	1256	ASMO	Kootenay	0
Cranbrook	08/08/2009	1257	ASMO	Kootenay	0
Cranbrook	19/08/2009	1258	RALU	Kootenay	0
Atlin	20/07/2008	638	RALU	Skeena	1
Atlin	20/07/2008	639	RALU	Skeena	0
Atlin	20/07/2008	640	BUBO	Skeena	0
Atlin	20/07/2008	641	RALU	Skeena	0
Atlin	20/07/2008	642	RALU	Skeena	0
Atlin	20/07/2008	643	BUBO	Skeena	0
Atlin	20/07/2008	644	BUBO	Skeena	0
Atlin	20/07/2008	645	RALU	Skeena	0
Atlin	20/07/2008	646	RALU	Skeena	1
Atlin	20/07/2008	647	RALU	Skeena	1
Lindeman City	26/07/2008	794	RALU	Yukon	0
Lindeman City	26/07/2008	795	RALU	Yukon	0
Lindeman City	26/07/2008	796	RALU	Yukon	0
NA	26/07/2008	797	BUBO	Yukon	0
NA	26/07/2008	798	BUBO	Yukon	0
Lindeman City	26/07/2008	799	RALU	Yukon	0
Lindeman City	26/07/2008	800	RALU	Yukon	0
Lindeman City	10/08/2008	801	BUBO	Yukon	0
Lindeman City	10/08/2008	802	BUBO	Yukon	0
Lindeman City	10/08/2008	803	BUBO	Yukon	0
Lindeman City	10/08/2008	804	BUBO	Yukon	0
Lindeman City	10/08/2008	805	BUBO	Yukon	0
Atlin	10/05/2008	842	BUBO	Skeena	1
Atlin	10/05/2008	843	BUBO	Skeena	1
Atlin	10/05/2008	844	BUBO	Skeena	1
Atlin	10/05/2008	845	BUBO	Skeena	1

Atlin	10/05/2008	846	BUBO	Skeena	1
Atlin	10/05/2008	847	BUBO	Skeena	1
Atlin	10/05/2008	848	BUBO	Skeena	1
Atlin	10/05/2008	849	BUBO	Skeena	1
Atlin	10/05/2008	850	BUBO	Skeena	1
Atlin	10/05/2008	851	BUBO	Skeena	1
Hazelton	13/07/2008	407	BUBO	Skeena	0
Hazelton	13/07/2008	408	BUBO	Skeena	0
Hazelton	13/07/2008	409	BUBO	Skeena	0
Hazelton	13/07/2008	410	BUBO	Skeena	0
Hazelton	13/07/2008	411	BUBO	Skeena	0
Hazelton	13/07/2008	412	BUBO	Skeena	0
Hazelton	13/07/2008	413	BUBO	Skeena	0
Hazelton	13/07/2008	414	BUBO	Skeena	0
Hazelton	13/07/2008	415	BUBO	Skeena	0
Hazelton	13/07/2008	416	BUBO	Skeena	0
Hazelton	13/07/2008	417	BUBO	Skeena	0
Hazelton	13/07/2008	418	BUBO	Skeena	0
Smithers	24/07/2008	661	RALU	Skeena	0
Smithers	24/07/2008	662	RALU	Skeena	0
Smithers	24/07/2008	663	BUBO	Skeena	0
Smithers	24/07/2008	664	BUBO	Skeena	0
Smithers	24/07/2008	665	RALU	Skeena	0
Smithers	24/07/2008	666	RALU	Skeena	0
Smithers	24/07/2008	667	RALU	Skeena	0
Smithers	24/07/2008	668	RALU	Skeena	1
Smithers	24/07/2008	669	RALU	Skeena	1
Smithers	24/07/2008	670	RALU	Skeena	0
Smithers	24/07/2008	671	RALU	Skeena	0
NA	01/08/2008	806	BUBO	Skeena	1
NA	23/08/2008	807	BUBO	Skeena	0
NA	02/08/2008	808	BUBO	Skeena	0
NA	02/08/2008	809	BUBO	Skeena	0
NA	02/08/2008	810	BUBO	Skeena	0
NA	02/08/2008	811	RASY	Skeena	1
Prince George	24/07/2008	812	BUBO	Omineca	1
Porcher Island	25/09/2008	813	BUBO	Skeena	0
Porcher Island	25/09/2008	814	BUBO	Skeena	1
Smithers	15/08/2008	815	BUBO	Skeena	0
Whitehorse	23/07/2008	835	RASY	Yukon	1
Whitehorse	23/07/2008	836	RASY	Yukon	0
Whitehorse	23/07/2008	837	RASY	Yukon	1
Whitehorse	23/07/2008	838	RASY	Yukon	1
Whitehorse	23/07/2008	839	RASY	Yukon	1
Whitehorse	23/07/2008	840	RASY	Yukon	1
Whitehorse	23/07/2008	841	BUBO	Yukon	1
NA	22/05/2008	970	BUBO	Skeena	1
NA	22/05/2008	971	BUBO	Skeena	0

Whitehorse	23/07/2008	979	BUBO	Yukon	0
Whitehorse	23/07/2008	980	BUBO	Yukon	0
Whitehorse	23/07/2008	981	BUBO	Yukon	0
Whitehorse	23/07/2008	982	BUBO	Yukon	0
Whitehorse	23/07/2008	983	BUBO	Yukon	0
Whitehorse	23/07/2008	984	BUBO	Yukon	0
Whitehorse	23/07/2008	985	BUBO	Yukon	0
Whitehorse	23/07/2008	986	BUBO	Yukon	0
Whitehorse	23/07/2008	987	BUBO	Yukon	0
Whitehorse	23/07/2008	988	BUBO	Yukon	0
Whitehorse	23/07/2008	989	BUBO	Yukon	0
Whitehorse	23/07/2008	990	BUBO	Yukon	0
Whitehorse	23/07/2008	991	BUBO	Yukon	0
Whitehorse	23/07/2008	992	BUBO	Yukon	0
Whitehorse	23/07/2008	993	BUBO	Yukon	0
Whitehorse	23/07/2008	994	BUBO	Yukon	0
Whitehorse	23/07/2008	995	BUBO	Yukon	0
Whitehorse	23/07/2008	996	BUBO	Yukon	0
Whitehorse	23/07/2008	997	BUBO	Yukon	0
Whitehorse	23/07/2008	998	BUBO	Yukon	0
Whitehorse	23/07/2008	999	BUBO	Yukon	0
Whitehorse	23/07/2008	1000	BUBO	Yukon	0
Whitehorse	23/07/2008	1001	BUBO	Yukon	0
Whitehorse	23/07/2008	1002	BUBO	Yukon	0
Whitehorse	23/07/2008	1003	BUBO	Yukon	0
Whitehorse	23/07/2008	1004	BUBO	Yukon	0
Whitehorse	23/07/2008	1005	BUBO	Yukon	0
Whitehorse	23/07/2008	1006	BUBO	Yukon	0
Whitehorse	23/07/2008	1007	BUBO	Yukon	0
Whitehorse	23/07/2008	1008	BUBO	Yukon	0

Appendix B

Supplemental Information for Chapter 3

Table A6: Peninsular Malaysia location and amphibian species indication with GPS co-ordinates, approximate elvation, *Bd* positives and sample size delineated by "n".

Location/Species	GPS Location	Dates of Collection	Elevation (m)	Positives	n
Nibong Tebal, Pulau Pinang	**N5 08 36 E100 29 24**	**May 28 2008**	**8**	**0**	**18**
Hylarana labialis				0	2
Fejervarya limnocharis				0	1
Limnonectes laticeps				0	2
Limnonectes blythii				0	5
Odorrana hosii				0	6
Phrynoidis aspera				0	2
Batu Ring, Lenggong Perak	**N4 59 04 E100 58 52**	**May 23 2009**	**178**	**0**	**30**
Microhyla butleri				0	6
Fejervarya limnocharis				0	4
Hylarana erytraea				0	12
Occidozyga lima				0	3
Hylarana glandulosa				0	1
Phrynoidis aspera				0	1
Polypedates leucomystax				0	1
Limnonectes blythii				0	1
Duttaphrynus melanostictus				0	1
Bukit Rengit, Langchang, Pahang	**N3 42 53 E102 10 14**	**June 2 2009**	**120**	**0**	**17**
Hylarana erytraea					17
Wang Kelian, Perlis	**N6 41 51 E100 11 28**	**May 17 - 23 2010**	**272**	**1**	**26**
Leptobrachium hendricksoni				1	11
Hylarana glandulosa				0	2
Xenophrys aceras				0	1
Limnonectes hascheanus				0	1
Kaloula pulchra				0	1
Limnonectes blythii				0	3
Hylarana nigrovittata				0	1
Fejervarya limnocharis				0	1
Polypedates leucomystax				0	1
Microhyla heymonsi				0	1
Humerana miopus				0	1

Location/Species	GPS Location	Dates of Collection	Elevation (m)	Positives	n
Microhyla butleri				0	2
Phrynoidis aspera				0	1
Ujung Bukit, Perlis	**N6 28 24 E100 11 07**	**May 27 2010**	**36**	**0**	**1**
Phrynoidis aspera				0	1
Matang Mangrove Forest, Perak	**N4 50 36 E100 38 00**	**May 31 2010 - June 1 2010**	**4**	**0**	**14**
Fejervarya limnocharis				0	13
Fejervarya cancrivora				0	1
Batu Kurau River, Perak	**N4 55 05 E100 49 53**	**June 1 2010**	**4**	**0**	**1**
Phrynoidis aspera				0	1
Kuala Tahan, Pahang	**N4 30 18 E102 24 32**	**June 4 2010**	**128**	**0**	**3**
Kaloula pulchra				0	3
Redang Island, Terengganu	**N5 46 37 E103 01 57**	**June 13 2010**	**11**	**0**	**4**
Duttaphrynus melanostictus				0	4
Muka Head, CEMACS, Penang	**N5 25 08 E100 18 28**	**June 15 2010**	**13**	**0**	**18**
Hylarana erytraea				0	12
Hylarana glandulosa				0	2
Duttaphrynus melanostictus				0	2
Polypedates leucomystax				0	2
Telak Senangin, Perak	**N4 19 54 E100 35 04**	**November 4 2010**	**14**	**0**	**6**
Hylarana labialis				0	5
Ingerophrynus parvus				0	1
Gua Asar, Perak	**N5 07 44 E100 58 14**	**November 5 2010**	**105**	**0**	**2**
Phrynoidis aspera				0	2
Lata Kekabu, Perak	**N5 02 59 E100 56 44**	**November 5 2010**	**110**	**0**	**11**
Amolops larutensis					9
Hylarana picturata					1
Odorrana hosii					1
Bukit Mertajam, Penang	**N5 21 29 E100 29 36**	**November 26 2010**	**53**	**0**	**12**
Duttaphrynus melanostictus					1
Leptobrachium hendricksoni					7
Lithobates Catesbeianus					2
Hylarana glandulosa					2